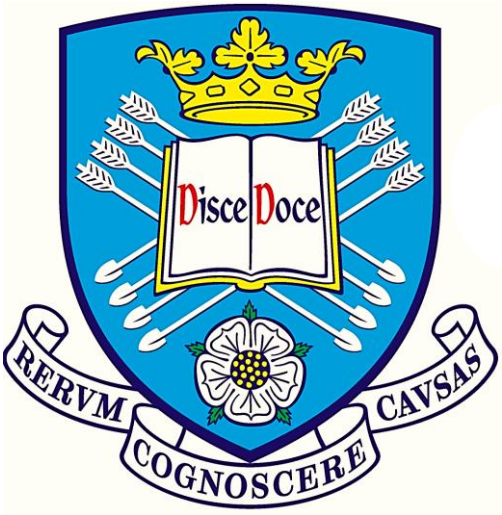
Radiation Effects on Mesenchymal Stem Cells in a Model of Fibrosarcoma



Tariq Ahmad Aabed

The University of Sheffield

School of Medicine

Department of Oncology and Metabolism

Thesis submitted for the degree of Doctor of Philosophy

November 2018

In memory of my brother "Muhammad Aabed"

To my brother

With love and eternal appreciation

"Tariq, I will miss you for 4 years" ... April 2014

"Muhammad, I missed you for ever"... August 2014

Abstract

Background: Radiotherapy is a mainstay of sarcoma treatment, but can cause fibrosis, characterized by production of extra-cellular matrix proteins such as collagen by cancer-associated fibroblasts (CAFs) in the cancer stroma and surrounding normal tissues, which makes tumours more aggressive and resistant to further treatment. Mesenchymal stem cells (MSCs) can be recruited to irradiated tumours and can differentiate into CAF-like cells but the mechanisms of these effects remain unclear.

Aim: Determine the mechanisms of radiation effects on the recruitment of MSCs to tumours and their differentiation into CAF-like cells.

Methods: Mouse MSCs were irradiated directly or exposed to irradiated mouse fibrosarcoma cells (FS120 or FS188) or their conditioned media (CM) and/or irradiated endothelial cells. Expression of CAF/fibrosis markers (collagen, fibronectin, PDGF receptor-β and α-SMA) by MSCs was assessed 3-4 days' post radiation. Trans-well migration assays were also performed. Candidate proteins were investigated for their ability to stimulate migration and maturation of MSCs to CAF-like cells and for the ability of radiation to stimulate their production in fibrosarcoma cells. Irradiated FS120 and FS188 solid tumours were analysed for collagen, using Masson's trichrome staining, and α-SMA using IHC and immunofluorescence.

Results: Direct irradiation of MSCs had limited effects on their expression of CAF markers and migration, but exposure to irradiated tumour cells or CM and/or endothelial cells increased these effects. Candidate proteins TGF-β1, MCP-1, and SDF-1α all significantly enhanced the migration of MSCs, and radiation increased their production in fibrosarcoma cells. FS188 cells produced more MCP-1 than FS120 cells and FS188 and FS120 cells and their CM increased MSC migration in a radiation-dependent manner. Migration could be at least partially blocked by an MCP-1 blocking antibody. MSC expression of the MCP-1 receptor, CCR2, was increased after exposure to irradiated FS188 cells or their CM. *In vivo*, irradiated fibrosarcomas showed significant increases in collagen content, with more collagen in FS188 than in FS120 tumours.

Conclusion: Results support the notion that MSCs play an important role in radiation-induced CAF activity and fibrosis in sarcoma. MCP-1 was identified as an important mediator of these effects. Moreover, endothelial cells were shown to play an important role in the recruitment of MSCs in response to radiation. *In vitro* results identifying FS188 cells as being more pro-fibrotic than FS120 cells were consistent with *in vivo* results. Further work to understand these processes should help to develop novel treatment strategies for combination with radiotherapy.

Acknowledgements

This thesis represents not only 4 years of hard work, but it was hard and challenge events that happened during it. I lost my lovely brother (Lieutenant Colonel Muhammad Ahmad Aabed) who had been killed by ISIS (27th August 2014) after occupying my city Mosul in Iraq just 4 months after starting my PhD.

First, I wish to thank my supervisor, Dr. Chryso Kanthou, for her patience, support, teaching, advice, suggestions, corrections and comments during writing of this thesis that helped me bring this work into success. She has been supportive since the days I begun working in this project. She was more than supervisor to me and to my wife, who lost her mother died in cancer at the time where ISIS occupied Mosul city. I remember Chryso`s emotional words to me and to my wife that supported us during our difficult days.

For my second supervisor, Professor Gillian Tozer, I would like to express my deepest appreciation for her kind guidance, suggestion, and help in writing correction and support during my difficult time throughout the course of this study.

I would like also to thank all members of Tumour Microcirculation Group (TMG), especially Matt Fisher, Jack Hurrel, Brenda Aguero and others for their kindness and support that made this project more enjoyable.

It is also my duty to record my sincere thanks to Dr. Rob Ireland, my tutor, for his support always from the first meeting with him until the end.

A special thanks to Dr. Matthew Hatton, consultant clinical oncologist at Weston Park Hospital, and honorary senior lecturer at the University of Sheffield for offering me an observership in radiotherapy and medical oncology units during my PhD. study.

My thanks also to Iraqi Ministry of Higher Education and Scientific Research for funding this study.

This journey would not have been possible without the support of my wife, Dr. Saja, for her kind support and superhuman patience, for keeping me strong and sane through the difficult parts of the project. To my lovely kids, Alaabed, Mumen, Sama and Muhammad, who missed a lot of Daddy time while I was busy in the lab. I thank you all for your patience and love you forever.

Finally, I am indebted to my mother, brothers and sisters for their love, prayer and being continuous source of hope and ambitious.

Lastly and most of all, thank you Allah for your abundant blessing in my life.

List of Abbreviations

Abbreviation	Meaning
2D	Two Dimensional
3D	Three Dimensional
AAs	Anti-angiogenic Agents
ABC	Avidin-Biotin Complex
AIAs	Angiogenesis-Inhibiting Agents
ANOVA	Analysis Of Variance
APS	Ammonium Per-Sulphate
BCA	Bicinchoninic Acid Assay
bFGF	Basic Fibroblast Growth Factor
BM	Bone Marrow
BMP	Bone Morphogenic Protien
BMPR	Bone Morphogenic Protien Receptor
BSA	Bovine Serum Albumin
°C	Celsius
CA4P	Combretastatin A-4 disodium Phosphate
CAF	Cancer-Associated Fibroblast
CAIX	Carbonic Anhydrase IX
CCR	Chemokine Receptor
cm	Centimetre
CM	Conditioned Media
CTGF	Connective Tissue Growth Factor
CTR	Control
Cu^{+1}	Cuprous ion
Cu^{+2}	Cupric ion
DAB	Di-Amino-Benzidine
DDR	DNA Damage Response

DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Di-Methyl-Sulf-Oxide
DMXA	5,6-Dimethyl-Xanthenone-4-acetic Acid
DNA	Deoxyribonucleic Acid
DSBs	Double-Strand Breaks
DPX	Distyrene, Plasticizer, and Xylene
ECL	Enhanced Chemi-Luminescence
ECM	Extra Cellular Matrix
EDTA	Ethylene Diamine Tetra-Acetic acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-Linked Immunosorbent Assay
EMA	European Medicines Agency
EMEM	Eagle's Minimum Essential Medium
EMT	Epithelial-Mesenchymal Transition
En-MT	Endothelial-Mesenchymal Transition
EORTC	European Organisation for Research and Treatment of Cancer
ERK	Extracellular Signal-like Kinases
Exp.	Experiment
FAP-α	Fibroblast Activation Protein α
FCS	Foetal Calf Serum
FDA	Food and Drug Administration
FS	Mouse Fibrosarcoma
FSP	Fibroblast Specific Protein
g	Gram
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GISTs	Gastrointestinal Stromal Tumours
GJIC	Gap Junctional Intercellular Communication

Gy	Gray
H2O2	Hydrogen peroxide
H & E	Haematoxylin And Eosin
HDMEC	Human Dermal Microvascular Endothelial cells
HGF	Hepatocyte Growth Factor
HIF	Hypoxia-inducible factor
HUVEC	Human Umbilical Vascular Endothelial Cell
IF	Immunofluorescence
IgG	Immunoglobulin G
IGF	Insuline-like Growth Factor
IHC	Immunohistochemistry
IL6	Interleukin-6
IL8	Interleukin-8
IM	Imatinib Mesylate
IMRT	Intensity-Modulated Radiation Therapy
IORT	Intra-operative Radiation Therapy
IR	Ionising Radiation
kDa	Kilo Dalton
Kv	Kilo volt
LET	Linear Energy Transfer
LMS	Leiomyosarcoma
LOX	Lysyl Oxidase
LPS	Liposarcoma
LYS	Lysine
M	Molar
mA	milli Ampere
mAb	Monoclonal Antibody

MCP-1	Monocyte Chemoattractant Protein-1
M-CSF	Macrophage Colony Stimulating Factor
MFH	Malignant Fibrous Histiocytomas
mGy	milli Gray
ml	milli Litre
mM	milli Molar
MMP	Matrix Metalloproteinas
MOM	Mouse On Mouse
mRNA	Messenger Ribonucleic Acid
MSC	Mesenchymal Stem cell
MW	Molecular Weight
N	Number
NGS	Next-Generation Sequencing
ng	Nano gram
nm	Nano metre
P	Probability
PAGE	Polyacrylamide Gel Electrophoresis
pg	Pico gram
PBS	Phosphate Buffered Saline
PDGF	Platelet-Derived Growth Factor
PDGFR	Platelet-Derived Growth Factor Receptor
PDGFR-β	Platelet-Derived Growth Factor Receptor Beta
PE	Plating Efficiency
PENTs	Peripheral Primitive Neuroectodermal Tumours
P-gp	Permeability glycoprotein
PMSF	Phenyl Methyl Sulfonyl Fluoride
PVDF	Poly-Vinylidene Di-Fluoride

RGS5	Regulator of G-protein Signalling 5
RIF	Radiation-Induced Fibrosis
RNA	Ribonucleic Acid
ROI	Regions Of Interest
ROS	Reactive Oxygen Species
RPM	Round Per Minute
RT	Radiotherapy
SDF-1α	Stromal Derived Factor-1-alpha
SDS	Sodium Dodecyl Sulphate
SEM	Standard Error of the Mean
SF	Survival Fraction
SM	Sunitinib Malate
SMART	Sensitivity Modulated Advanced Radiotherapy
SRF	Serum Response Factor
STAT3	Signal Transducer and Activator of Transcription 3
STS	Soft Tissue Sarcoma
TAM	Tumour-Associated Macrophage
TEMED	Tetra Methyl Ethylene Diamine
TGF-β	Transforming Growth Factor-beta
TIMP	Tissue Inhibitors of Metalloproteinase
TKIs	Tyrosine Kinase Inhibitors
TME	Tumour Microenvironment
TNF-α	Tumour Necrosis Factor alpha
TMG	Tumour Microcirculation Group
V	Volt
VDAs	Vascular-Disrupting Agents
VEGF	Vascular Endothelial Growth Factor
vs	Versus

VTAs	Vascular Targeting Agents
WHO	World Health Organisation
α-SMA	Alpha Smooth Muscle Actin
µg	Micro gram (unit of mass)
µl	Micro liter (unit of volume)
µm	Micro meter

List of contents

List of figures

List of tables

CHAPTER ONE:

Introduction

1. Introduction

1.1 Clinical aspects of soft tissue sarcoma (STS)

Soft tissue sarcomas (STSs) are a group of rare malignant tumours that arise from mesenchymal cells. Although STSs are relatively rare, they constitute a higher proportion of cancer morbidity and mortality in paediatric and adolescent practice than in adults (Gronchi and Casali, 2013). STSs account for 0.5-1% of all adult cancers (Lahat et al., 2008) and 6-7% in children and young adults which are relatively more common than most other types of solid malignancies (Grimer et al., 2010, Sultan et al., 2010). The most common locations of STS are the extremities (50%), trunk (15%), retroperitoneum (15%) and the abdominal cavity (15%) (Clark et al., 2005, Gronchi and Casali, 2013). According to the World Health Organization (WHO) tumour classification, there are more than 50 subtypes of STS that can present in any age group and any location in the body (Fletcher, 2014, Fletcher et al., 2002). The diversity in sarcoma subtypes and their rarity make sarcomas very difficult to study.

There is a link between the histopathological subtype and the age of the patient; the most common types in adults are liposarcoma and leiomyosarcoma (Fletcher, 2014), while in the elderly, the most common type is undifferentiated pleomorphic sarcoma which was previously known as malignant fibrous histiocytoma (MFH) (Sharon and Weiss, 2008). Out of all STS in children, around 50% are rhabdomyosarcomas (Kapoor and Das, 2012) **(Figure 1.1).**

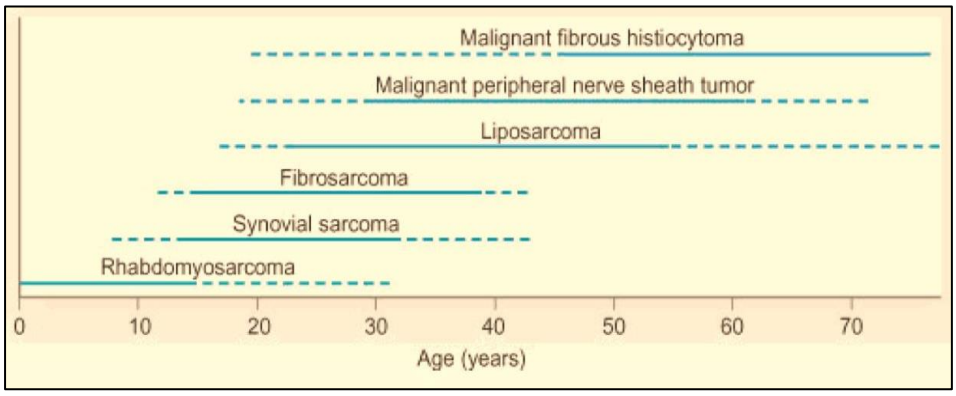


Figure 1.1 The relation between age and sarcoma incidence (Sharon and Weiss, 2008)**.**

1.2 Standard treatment of STS

1.2.1 Surgery

The standard treatment for localized STS is surgical excision, ideally aiming for a radical resection margin and preserving function if possible (Mendenhall et al., 2009). Al-Refaie and colleagues showed that for extremity tumors > 3 cm in largest diameter patients should also be treated with adjuvant radiotherapy (Al-Refaie et al., 2010), while, for larger tumours or small tumours with marginal or involved resection margins, combinations of surgery followed by radiotherapy are preferred (Mendenhall et al., 2009). Surgical treatment of STS entails a fine balance between preserving function and minimizing the risk of local tumour recurrence (Grimer et al., 2010). The type of surgical resection is determine by the tumour size and depth, the anatomical location of the tumour, the performance status of the patient, and the involvement of nearby structures. 10–20% of tumours recur locally, despite improvements in local control but in most cases, can still be controlled.

1.2.2 Radiotherapy

Although various studies have shown a relation between radiotherapy (RT) treatment and an increase in the risk of angiosarcoma and bone sarcoma (Lagrange et al., 2000, Virtanen et al., 2006), RT is still one of the standard effective treatment for intermediate and high grade STS (Grimer et al., 2010).

RT can be given either pre or postoperatively. Some selected patients who have widely negative margins may not require radiation (Pisters et al., 2007b). Advances in RT technology, such as intensity-modulated radiation therapy (IMRT), brachytherapy and intraoperative radiation therapy (IORT), have led to improved treatment outcomes in patients with STS (DeLaney et al., 2005). It has been demonstrated that post-operative or adjuvant RT achieves local control and survival similar to radical resection (Yang et al., 1998). Various studies have shown that neo-adjuvant RT has better long-term functional effects and decreases the risk of joint stiffness (O'Sullivan et al., 2002, Davis et al., 2005, Zagars et al., 2003). However, despite these benefits, Cannon et al and O'Sullivan et al have showed a high wound complication rate (34%-35%) in patients

treated with neo-adjuvant RT compared with patients treated with adjuvant RT (16%-17%) (Cannon et al., 2006, O'Sullivan and Levin, 2003, O'Sullivan et al., 2002).
Furthermore, a recent study by Folkert and colleagues has demonstrated that treatment by IMRT caused less skin toxicity than using conventional RT (Folkert et al., 2014). Despite the effectiveness of RT in the treatment of STS, RT has acute and chronic toxicities (El-Bared et al., 2015). Acute toxicities occur within 120 days and include skin toxcicity and problems with wound healing. Whereas chronic RT toxicities occur after 120 days and include problems such as joint stiffness, oedema and fibrosis (El-Bared et al., 2015). Recently, a novel study by Alqathami and colleagues called SMART (Sensitivity Modulated Advanced Radiotherapy) involving multi-compartment phantom radio-chromic dosimetry, showed that it may be possible to reduce the complications caused by radiotherapy (Alqathami et al., 2012).
Generally, the recommended neo-adjuvant RT dose is 50 Gy with daily 1.8-2 Gy fractions, while for adjuvant RT the dose should be 60-66 Gy in two Gy fractions (Kepka et al., 2005, Zagars and Ballo, 2003).

1.2.3 Chemotherapy

Distant metastases from STSs remain a significant problem with a high mortality (Torres et al., 2007). The importance of systemic chemotherapy for most histological subtypes in adult STS patients remains controversial, except for some rare, predominantly paediatric and young adult types such as rhabdomyosarcoma, osteosarcoma, and Ewing`s sarcoma. Chemotherapy is given either as neo-adjuvant (preoperative) and/or adjuvant (postoperative) for patients with tumours (Grimer et al., 2010) belonging to these sub-types. For other subtypes in the non-metastatic setting, the use of chemotherapy is controversial. One important study was conducted by Penella Woll and colleagues in 2012 (Woll et al., 2012). This study represents the second largest trial for sarcoma adjuvant therapy after the first EORTC (European Organisation for Research and Treatment of Cancer) trial by (Bramwell et al., 1994). The Woll et al study consisted of 351 patients followed up for a long time and divided into control and patients treated by ifosfomide plus high dose doxorubicin after tumour resection (Woll et al., 2012). Radiotherapy given to both groups if the resection margin was positive. The results were in agreement with previous studies in which unselected patients with sarcoma showed no benefit from doxorubicin and ifosfomide (Petrioli et

al., 2002). However, this study has some weakness such as including some patients (24%) with small tumours (<5 cm), which are usually treated by surgery and radiotherapy alone without chemotherapy. Furthermore, the dose of ifosfomide used in the Woll study is lower than that were used in the previous trial by (Frustaci et al., 2001) who used a conventional chemotherapy dose.

Doxorubicin and ifosfomide are the most active chemotherapy agents in the treatment of STSs (Elias et al., 1989). A previous study by Frustaci and colleagues has shown an increase in disease-free survival for sarcoma patients treated by epirubicin and ifosfomide (Frustaci et al., 2001). While other studies have shown improvement in local control and disease free survival but no improvement in overall survival (Bramwell et al., 1994, Petrioli et al., 2002). Different STS histopathological sub-types have shown variation in their chemo-sensitivity. The most chemo-sensitive sarcomas are synovial sarcoma, myxoid/ round cell liposarcoma, extra-skeletal Ewing`s tumour and childhood rhabdomyosarcoma (Grimer et al., 2010), while the most chemo-resistant sarcoma are alveolar soft part sarcoma, dedifferentiated liposarcoma, GIST (gastrointestinal stromal tumours), low grade liposarcoma and clear cell sarcoma (Grimer et al., 2010). Pisters and colleagues showed that there is no need for routine chemotherapy for low-grade resectable tumours or small size high-grade STS (Pisters et al., 2007a). The benefits of preoperative chemo-radiotherapy include early treatment of subclinical tumour spread and assessment of response, but its importance on survival in STS patients remains unknown. For metastatic STS, chemotherapy is used with palliative intent but response rates are generally poor (Clark et al., 2005).

1.3 Angiogenesis and vascular targeted therapies in STS

Angiogenesis can be defined as the growth of new blood vessels from existing vessels (Carmeliet, 2000). It is a physiological and pathological event which occurs in normal development and reproduction in early life (as in organogenesis), or in adulthood (as in wound healing and menstruation) in which vascular endothelial growth factor (VEGF) and its receptors play a central role (Fox et al., 2001). Formation of these new blood vessels is important for most solid tumours (Folkman, 1986), as it provides tumour cells with nutrition and oxygen which help cells to survive and they represent the primary route for metastatic spread.

Moreover, the tumour microenvironment is affected by these new blood vessels, and hence the response of the tumour to the therapy is also affected (Vaupel, 2004). Angiogenesis is an important target in sarcoma because it is responsible for tumour growth and metastasis. Many studies have been focused on anti-angiogenic treatment in sarcoma. Angiogenesis occurs when tumours switch to an angiogenic state during tumour development (Bergers and Benjamin, 2003). Many studies have shown that the level of pro angiogenic factors is high in malignant tumours, and this leads to growth and metastases of tumours (Grunstein et al., 1999, Holash et al., 1999). Using vascular targeting agents (VTAs) for cancer treatment was described by Juliana Denekamp through her work on mouse blood vessels obstruction (Denekamp, 1982, Denekamp, 1993, Denekamp, 1999). There are two types of VTAs; those called antiangiogenic agents (AAs), or angiogenesis-inhibiting agents (AIAs), which inhibit new blood vessels formation, and those that act against already existing tumour vasculature, called vascular-disrupting agents (VDAs).

1.3.1 Anti-angiogenic agents

The action of antiangiogenic agents (AAs) is through the inhibition of new blood vessel formation by affecting the various stages involved in angiogenesis. It had been documented that tumour cells secrete angiogenic factors that are up regulated by different factors like hypoxia, activation of oncogenes, or loss of tumour suppressor gene function (Holash et al., 1999, Denekamp and Hill, 1991). These angiogenic factors were shown to play an important role in neovascularization and are the chief targets for AAs (Ferrara et al., 2003). The most specific and potent angiogenic factor is VEGF,

which stimulates the proliferation and migration of endothelial cells and leads to an increase in vascular permeability (Ferrara et al., 2003, Pugh and Ratcliffe, 2003, Dvorak, 2000). When VEGF ligands bind to their tyrosine kinases receptors (VEGFR1, VEGFR2 and VEGFR3), this leads to activation of downstream signalling. Bevacizumab (Avastin) is the first AA (anti-VEGF) agent approved for clinical uses. It is a humanized anti-VEGF monoclonal antibody that produces angiogenesis inhibition through preventing VEGF from binding to its receptors (Wong et al., 2014). Clinical trials by Hurwitz and his colleagues for patients with metastatic colorectal cancer showed that using bevacizumab combined with fluorouracil chemotherapy significantly improved overall survival when compared with chemotherapy alone (Hurwitz et al., 2004).

This improvement in overall survival after combination therapies was also shown in patients with non-small lung cancer (NSCLC), renal cell carcinoma and metastatic breast cancer (Miller et al., 2007, Sandler et al., 2006). Anti-angiogenic agents are also being tested or used in the treatment of STS. In a prospective study done by (Agulnik et al., 2013) for patients with angiosarcoma (unresectable or metastatic), showed there were responses to bevacizumab as a single agent (Agulnik et al., 2013). In addition, a combination of both bevacizumab with doxorubicin was used in the treatment of 17 patients with metastatic STS as phase II trial by (D'Adamo et al., 2005). However, the response rate was 12% in comparison with doxorubicin alone but the overall survival was promising (16 months) (D'Adamo et al., 2005).

Tyrosine kinase inhibitors (TKIs) are another type of AAs that inhibit the tyrosine kinase activity of VEGF receptors. The approval of using Imatinib mesylate (IM) in the treatment of GISTs in 2002 was the base for using TKIs in the treatment of cancer (Demetri et al., 2002). IM is an oral tyrosine kinase inhibitor (TKI) acts in patients with positive cKit tumours by inhibition of cKit (CD117). Imatinib also used in the treatment of dermatofibrosarcoma protuberance because it also inhibits platelet-derived growth factor receptor beta (PDGFR-β). Some GIST patients who cannot tolerate IM, are treated with another TKI called sunitinib malate (SM) which inhibits VEGF-R2, cKit and PDGFR-β (Demetri et al., 2006).

It was also shown that SM had activity in liposarcomas and leiomyosarcomas according to phase II study was done by Mahmood et al (Mahmood et al., 2011). Interestingly SM also showed anti-tumour effects in advanced alveolar soft part sarcoma (Stacchiotti et

al., 2011). Sorafenib is another TKI which inhibits VEGF-R2, VEGF-R3, PDGFR and cKit, and has effects on advanced angiosarcomas according to a phase II trial (Maki et al., 2009). Pazopanib, an oral AA drug that inhibits multiple tyrosine kinases, was approved for treatment of STS by the FDA (Food and Drug Administration) and EMA (European Medicines Agency). A randomized phase III trial called PALETTE had shown the significant improvement in progression free survival of STS patients excluding liposarcoma (van der Graaf et al., 2012). Moreover, it has been shown that Pazopanib plus bevacizumab and sunitinib was more effective in the treatment of metastatic alveolar soft part sarcoma than bevacizumab and sunitinib alone (Read and Williams, 2016).

1.3.2 Vascular-disrupting agents

Vascular-disrupting agents (VDAs) selectively target already established tumour vasculature leading to the death of cancer cells secondary to an extensive and rapid decrease in tumour blood perfusion. There are two types of VDAs, ligand-directed (biological) VDAs and small molecule VDAs. The work of ligand-directed VDAs is through using peptides, antibodies, or growth factors which bind to tumour endothelium, and death of these endothelial cells by targeting toxins like ricin or pro-coagulants to the tumour endothelium (Thorpe, 2004). Small molecule VDAs include flavonoids and tubulin binding agents (Siemann et al., 2005).

On flavonoid derivative is 5, 6-dimethyl-xanthenone-4-acetic acid (DMXAA). It's action is through the production of vasoactive factors and cytokines like tumour necrosis factor alpha (TNF-α) which causes damage and necrosis of endothelial cells of the tumour vasculature (Baguley, 2003). The combination of DMXAA with radiation had been studied in preclinical mouse models by Murata et al (2001) using the C3H mammary carcinoma and the KHT sarcoma. Although this study showed some improvement of tumour response compared to radiation alone, this was highly dependent on the dose of both DMXAA and radiation gave, and the time interval between the drug and radiation (Murata et al., 2001).

Tubulin-binding agents include colchicine; vinblastine and vincristine, and the newer agent combretastatin A-4 disodium phosphate (CA4P). The active component of CA4P, CA4, binds to the colchicine binding site on tubulin and works by disrupting tumour endothelial cells causing a decrease in blood flow and tumour necrosis (Tozer et al.,

2001). Tozer and her colleagues (2008) tested CA4P in a mouse fibrosarcoma model. They found that mouse fibrosarcomas expressing only the VEGF120 isoform of VEGF (FS120 tumours) responded well to CA4P, whereas those expressing VEGF188 isoform (FS188 tumours) responded less well (Tozer et al., 2008). This was thought to be because FS188 tumours have more stable blood vessels than FS120 tumours (Tozer et al., 2008). Another combretastatin derivative, ombrabulin, is a pro-drug, which showed a synergistic activity with cisplatin through the rapid destruction of tumour blood vessels and subsequent necrosis when tested in animal models (Morinaga et al., 2003). In 2015, a big phase III randomized trial compared ombrabulin plus cisplatin against placebo plus cisplatin in patients having advanced soft-tissue sarcomas who failed to respond to ifosfomide and anthracycline chemotherapy (Blay et al., 2015). The results of this study showed a significant improvement in progression-free survival in patients treated with ombrabulin and cisplatin compared to patients treated with cisplatin alone. However, this drug failed to show meaningful clinical benefit for patients with advanced STS, to support its routine uses in the clinic.

In a subcutaneous Ewing's sarcoma mouse model, it has been shown that treatment of mice with OXi4503/CA1P (second generation tubulin binding agent) caused a shutdown of vasculature completely within 24 hours, and haemorrhage with necrosis after 48 hours (Dalal and Burchill, 2009). Furthermore, the effects of OXi4503/CA1P were enhanced when doxorubicin was used concurrently and tumour growth delay was observed. These studies demonstrate that VDAs have potential therapeutic agents for cancer treatment. Eribulin mesylate (Halaven), a synthetic drug derived from marine sponge *Halichondria okadai*, was approved by the FDA in 2016 for treatment of liposarcoma. Eribulin works via disrupting the microtubule irreversibly by prevent the formation of the mitotic spindle and subsequently cell cycle arrest that cause apoptosis (Smith et al., 2010). In a randomised open-label phase 3 trial, Schffoeffski and his colleagues showed a significant improvement in overall survival (from 8.4 months to 15.6 months) for patients with advanced liposarcoma treated by eribulin versus dacarbazine (Schffoeffski et al., 2016).

1.4 Tumour microenvironment

Stephen Paget's theory in 1889 was to propose the concept of the tumour microenvironment (TME). It suggests that metastases need a suitable tissue environment (soil) to help tumour cells (seeds) to grow (Paget, 1989). Thus the TME is currently considered as a network of tumour cells as seeds, and the stromal tissue (fibroblasts, immune cells, cytokines and vascular tissue) and the extracellular matrix (ECM) as the soil (Balkwill et al., 2012). The TME is produced as a result of the communication between cancer cells and non-malignant cells, the latter having tumour promoting function at all stages of carcinogenesis (Hanahan and Coussens, 2012). The communications between cells are determined by many chemokines and cytokines, inflammatory enzymes, growth factors, and matrix remodeling enzymes. Multiple studies have indicated the similarities between the process of wound healing with the cells of TME in their structure, activities and development (Grivennikov et al., 2010, Hanahan and Weinberg, 2011).

1.4.1 Tumour cells

Tumour cells promote their own survival, proliferation and spread through many mechanisms, including suppression of apoptosis, evasion of the host immune response, deregulated proliferation, induction of neovascularization and mobility (Hanahan and Weinberg, 2000). Cancer cells in the TME suffer from genetic and epigenetic instability making them more aggressive and out of control. Hypoxia and hypoxia/reoxygenation caused by rapid tumour cell proliferation and vascular abnormalities are major drivers of genomic instability (Pires et al., 2010, Harding et al., 2011). Reactive oxygen species (ROS) that are formed due to the hypoxic environment within the TME cause damage to the DNA such as single or double strand breaks, DNA aberrations, and point mutations, and so on. Moreover, hypoxia by itself impairs DNA damage repair (Chang et al., 2002) which contributes to genetic instability.

1.4.2 Stromal tissue

Stroma is the connective and supportive tissue. Balkwill and colleagues (Balkwill et al., 2012) described TME stromal cells as fibroblasts, myofibroblasts, immune cells, pericytes, and vascular endothelial cells. The main cells within cancer stroma are fibroblasts. They are spindle shaped and metabolically active cells that are responsible for the production and turnover of ECM. These cells cause fibrosis through their differentiation into myofibroblasts during tissue injury (Li and Wang, 2011). Fibroblasts within cancer stroma are called cancer-associated fibroblasts (CAFs), activated myofibroblasts, or tumour-associated fibroblasts (Li et al., 2007, Kalluri, 2016). CAFs express α-smooth muscle actin (α-SMA), fibroblast specific protein (FSP)-1, and platelet-derived growth factor receptors-β (PDGFR-β) (Sugimoto et al., 2006, Anderberg et al., 2009). CAFs are a source of matrix metalloproteases (MMPs), plasminogen activators, and cathepsins, which all have protease activity and induce invasiveness (Roy et al., 2009, Boire et al., 2005, Xing et al., 2010). MMPs were shown to be essential in the growth of tumours, angiogenesis and metastasis (Stetlerstevenson et al., 1993, Boire et al., 2005), as MMPs release growth factors such as angiogenic factors and chemokines that bind to the matrix which then stimulate tumour cells to grow and endothelial cells to form new blood vessels (Rundhaug, 2003, Bergers et al., 2000, Iozzo et al., 2009, Ebrahem et al., 2010). Despite the fact that CAFs are thought to be tumour promoting, a study conducted by Oezdemir and his colleagues have shown the opposite. They showed that depletion of CAFs from pancreatic cancer in mice leads to increase tumour invasiveness and hypoxia with a decrease in the immunity and survival (Oezdemir et al., 2014). More information about CAFs will be discussed in detail later in this chapter.

Another important stromal cell in the TME (**Figure 1.2**) is the vascular endothelial cell. During cancer growth certain chemokines and growth factors like VEGFs, fibroblast growth factors (FGFs) and PDGFs stimulate endothelial cells and pericytes to drive the process of neovascularization (Carmeliet and Jain, 2011). Pericytes are another important stromal cells which have stem cell-like properties and may constitute a source of myofibroblasts progenitors in addition to their functions such as induction of angiogenesis, sustaining tumour growth, metastasis and immune-suppression (Armulik et al., 2011). Pericytes are found surrounding blood vessels and express markers like

PDGF receptor-β, NG2 and desmin, and recruitment and differentiation of pericytes depend on signaling pathways of PDGF, transforming growth factor beta (TGF-β) and angiopoietin (Gaengel et al., 2009). Pericytes limit tumour metastasis through their desensitization effects on the endothelium to VEGF and making blood vessels more stable; consequently, tumour cells find it more difficult to get through them to metastasis (Gerhardt and Semb, 2008, Armulik et al., 2011).

Tumour-associated macrophages (TAMs) are important innate immune cells within the TME. Different cytokines, chemokines, and growth factors play a role in the recruitment of macrophages/monocytes cells into the TME such as monocyte chemoattractant protein-1 (MCP-1) (Qian et al., 2011), VEGF (Valkovic et al., 2002), PDGF (Solinas et al., 2009) and plasminogen (Phipps et al., 2011). These recruited monocytes differentiate into M1 macrophages (pro-inflammatory) and M2 macrophages (pro-tumorigenic). In the TME, macrophages that related to tumour development have an M2-like phenotype (Sica and Mantovani, 2012, Mantovani and Locati, 2013). Presence of TAMs in the tumour stroma causes the resistance of a tumour to treatment with anti-angiogenic agents. Firstly, as hypoxia is a major feature of the TME, TAMs secrete multiple pro-angiogenic factors (in response to hypoxia) that activate endothelial cells and enhance angiogenesis (Murdoch et al., 2008, Schmidt and Carmeliet, 2010, Mantovani et al., 2013). Moreover, treatment of tumours with anti-VEGF therapy can cause tumour hypoxia, potentially resulting in recruitment of TAMs to the a tumour, leading to the supply of pro-angiogenic factors to re-start angiogenesis (Tripathi et al., 2014).

TAMs also aid in the degradation of ECM via secretion of different MMPs, which can contribute to metastasis (Huang et al., 2002, Murdoch and Lewis, 2005). The important role of immune system to control or prevent cancer has been studied since years. Nevertheless, cancer immunotherapy has become a reality only within the last two decades. For cancer immunotherapy, two approaches are currently in progress: direct targeting of the tumour and indirect targeting the tumour (via activation of immune cells) (Sathyanarayanan and Neelapu, 2015). The response to immunotherapy depends on the interactions between cancer cells and the immunomodulators within the TME. This means that the key role in the activation or reduction of the immune responses is the TME (Tang et al., 2016).

Understanding the communications between the TME and immunotherapy is critical to provide new methods in improving the effectiveness of current immunotherapies.

1.4.3 Extracellular matrix

ECM is a non-cellular element of tissue that provides physical support and regulates the function and homeostasis of all eukaryotic cells (Lu et al., 2011). The main components of the tumour ECM are elastin, collagen, proteoglycans and other essential proteins, which are synthesized mainly by CAFs. Cell surface receptors called integrins bind components of the ECM and promote cancer angiogenesis, growth and metastasis (Alphonso and Alahari, 2009). The spread and progression of cancers depend mainly on the ECM, as the adhesion of a cell to the ECM is a key to the movement of cancer cells out of and into the TME.

Because of increased ECM deposition and remodeling as a consequence of altered gene expression of CAFs, tumours are stiffer than the surrounding normal tissues. It has been shown that the lysyl oxidase (LOX) enzyme causes cross-linking of collagen fibers and contribute to stiffening of the matrix (Levental et al., 2009). The cells adhere to the matrix through integrins and produce enzymes that can degrade matrix and therefore allow them to move/migrate through it which is an important step in tumour metastasis (Taddei et al., 2013).

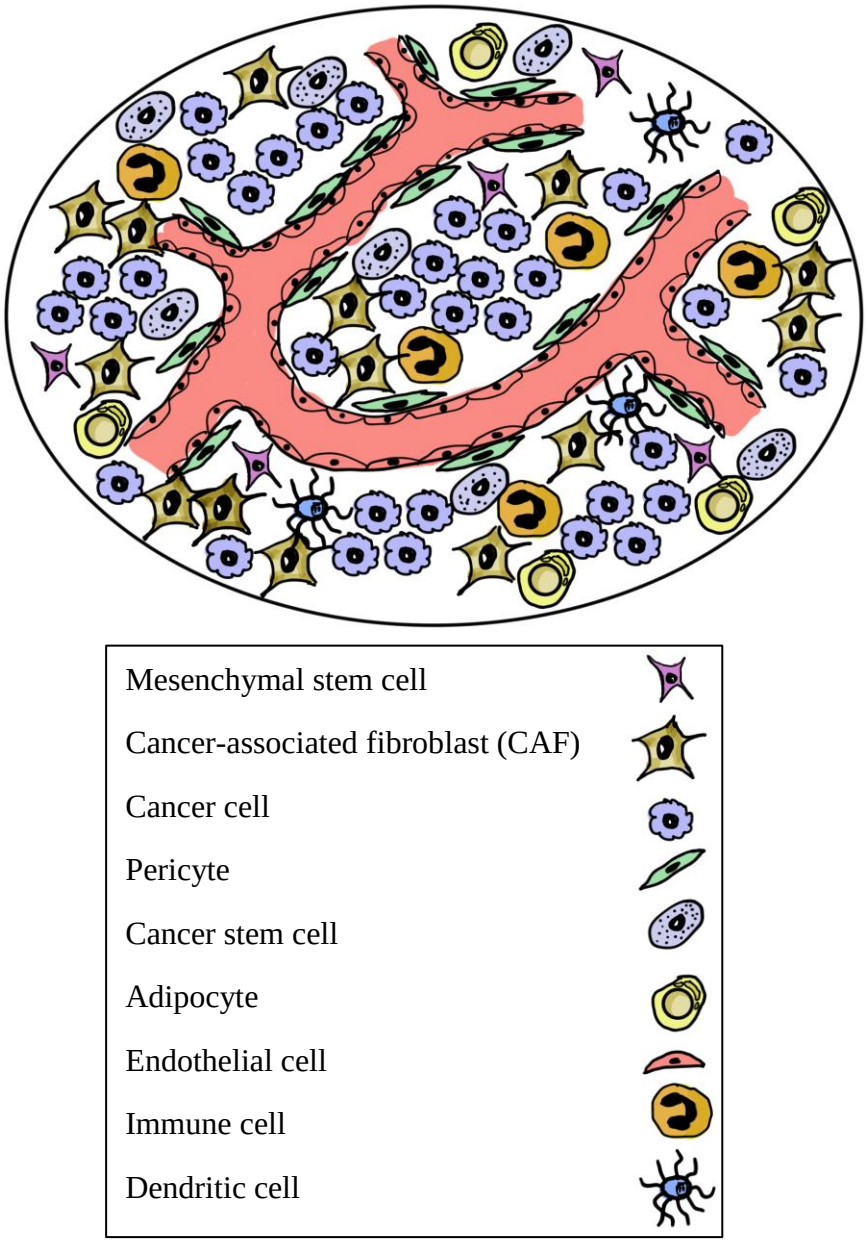


Figure 1.2 Tumor Microenvironment cells: TME consist of the parenchymal cells (malignant cells, and cancer stem cells) which are responsible of tumor recurrence and metastases, and the stromal cells like endothelial cells, mesenchymal stem cells, cancer cells, pericytes, immune cells, inflammatory cells, and fibroblasts. When fibroblasts activated and accumulated in tumor area, they called Cancer-Associated Fibroblasts (CAFs), which cause fibrosis. This Figure based on figure from (Hanahan and Weinberg, 2000).

1.4.4 Hypoxia and TME

Hypoxia is a very common feature in solid tumours and, as mentioned above, it occurs because tumour cells proliferate avidly, with alteration of tumour metabolism and development of abnormal tumour blood vessels that cause a decrease in oxygen and nutrients (Vaupel and Harrison, 2004). Studying tumour hypoxia is very important because is strongly related to many factors that support tumour growth as well as radioresistance in malignant tumours, recurrence of a tumour after radiotherapy and even in determining the prognosis of cancer patients after RT (Brown and William, 2004, Aebersold et al., 2001).

Hypoxia in tumours is classified into two types; acute perfusion-limited hypoxia and chronic diffusion-limited hypoxia, this division was according to the duration of exposure to hypoxia and the causative factors (Brown and William, 2004). In chronic hypoxia, there is a gradient of oxygenation that surrounds the perfused vessels; this means that within this oxygen gradient, there are cells with different oxygen concentrations (normoxic cells near the vessels and cells anoxic in distal areas). In acute perfusion limited hypoxia, the instability in blood flow inside the vessels may cause rapid fluctuations in oxygenation levels in a tumour (Lanzen et al., 2006).

It was establish that cancer cells become more invasive, aggressive and metastasize in hypoxic conditions. For example, culturing of multiple myeloma cells in hypoxic conditions *in vitro* and then injecting them into mice allowed these cells to metastasise into other bone marrow (BM) compared to the same cells cultured within normal oxygen condition (Azab et al., 2012, Muz et al., 2015). Moreover, *in vivo* studies by Cairns et al. showed that mouse sarcoma tumours exposed to acute hypoxia enhanced their metastasis to the lungs (Cairns et al., 2001).

Hypoxia leads to the formation of the transcription factor, hypoxia-inducible factor 1 (HIF-1), which is positively related to the degree of hypoxia inside a tumour (Semenza, 2000). There are three types of HIF with their oxygen-sensitive α subunit, HIF-1-α, HIF-2-α and HIF-3-α (Wang et al., 1995), which are stabilised under hypoxia. It has been shown that HIF-1 plays a role in the regulation of paracrine signalling molecules, such as TGF-β, PDGF-β and basic fibroblast growth factor (bFGF), secreted by hypoxic tumour cells (Caniggia et al., 2000, Moeller et al., 2004, Schito et al., 2012). These paracrine signalling molecules enhance changes of progenitor cells into CAFs (Gilkes et al., 2014).

Moreover, hypoxia was shown to enhance the expression of CXCL12, stromal derived factor 1 alpha (SDF1-α) receptor in many cells (Schioppa et al., 2003), and SDF1-α has been shown to promote the growth of cancer (Orimo et al., 2005).

Regarding effects of hypoxia on ECM, it has been shown that HIF-1 enhanced fibrosis in the liver, kidney and adipose tissue via its remodelling effects on ECM (Moon et al., 2009, Halberg et al., 2009, Higgins et al., 2007). Moreover, HIF-1 and HIF-2 have been shown to enhance breast cancer metastasis through the transcription of integrin subunits α5 and β1 (Ju et al., 2017). In addition to integrins, the synthesis of ECM proteins such as collagen and fibronectin can regulate by hypoxia. It has been shown that secretion of collagen can be regulated by HIF at different stages (Bentovim et al., 2013, Eisinger-Mathason et al., 2013). Beside these effects, hypoxia causes ECM degradation through the activity of MMP2, MMP9 and MMP15 via HIF-1 (Krishnamachary et al., 2003, Choi et al., 2011).

It is well known that endothelial cells, which are crucial for the structure of blood vessels, play a key role in the migration of tumour cells (Franses et al., 2011). It was shown that using endothelial cells depleted of HIF-1α caused decreased migration of cancer cells via endothelial cells. These results were opposite if using HIF-2 α depletion, in which the migration of cancer cells was enhanced. This differences between HIF-1α and HIF-2α could be due to their abilities to differentially regulate nitric oxide, that regulates endothelial cell function (Branco-Price et al., 2012). Many studies showed the involvement of HIF-1α and HIF-2α at all stages of blood vessels formation (Semenza and Wang, 1992, Conway et al., 2001, Carmeliet, 2005).

HIFs are also involved in the recruitment of endothelial progenitor cells from BM into tumour tissue and cause their differentiation into endothelial cells via VEGF, stimulation of VEGF-R2, and PDGF (Conway et al., 2001, de la Puente et al., 2013). Hypoxia also enhances tumour metastasis via epithelial-mesenchymal transition (EMT) (Thiery and Sleeman, 2006). EMT in hypoxia is associated with a decrease of epithelial-associated gene expression (β-catenin and E-cad) and increase in mesenchymal-like gene expression (SMA, vimentin, N-cad, and CXCR4) (Kim et al., 2002, Hsu et al., 2000, Manotham et al., 2004, Muz et al., 2015). Furthermore, hypoxia increased TGF-β that regulates EMT (Azab et al., 2012).

It was demonstrated that inhibition of the hypoxia-inducible enzyme, carbonic anhydrase IX (CAIX), caused decreased growth and metastasis of tumours (Lou et al., 2011).

Although severe hypoxia is ultimately toxic for cells, many tumour cells are adapted for a hypoxic environment, for instance by upregulation of enzymes involved in glycolysis, and hypoxia is even involved in tumour cells becoming resistant to treatment with RT and chemotherapy (Rohwer and Cramer, 2011). The mechanisms of hypoxia-induced resistance of tumour cells to treatment include cell cycle arrest, reduced apoptosis and senescence and, control p53, autophagy and mitochondrial activity (Das et al., 2008, Rohwer and Cramer, 2011). Moreover, hypoxia affects the delivery of drugs and cellular uptake of the chemotherapy via drug efflux pump expression like permeability glycoprotein (P-gp) (Abraham et al., 2015).

1.5 Cancer-Associated Fibroblasts (CAFs)

1.5.1 Characteristics and activation of CAFs

CAFs are large heterogenous spindle-shape cells that represent major components of stromal cells within the TME. The most common origin of CAFs are resident fibroblasts, but it is thought that they can also originate from pericytes, adipocytes, epithelial cells, endothelial cells, and mesenchymal stem cells MSCs (Anderberg and Pietras, 2009). Early studies did not show any effects of CAFs on tumour progression, but later studies have shown that CAFs are important cells in cancer development and progression (Cirri and Chiarugi, 2012, Franco et al., 2010). It has been shown that cancer proliferation and survival in the TME depends on CAFs that maintain the microenvironment (Cirri and Chiarugi, 2012, Marsh et al., 2013). Some studies showed that targeting cancer cells therapeutically using anti-cancer therapies is not enough without targeting CAFs that maintain the TME (Sun, 2015). Although activated CAFs generally express fibroblast activation protein α (FAP-α), PDGF receptor α and β, and vimentin, α-SMA is the most common markers used to recognise CAFs (Sugimoto et al., 2006, Park et al., 1999, Kim et al., 2015).

1.5.2 Role of CAFs in cancer

1.5.2.1 CAFs and tumour growth

It has been shown that CAFs enhance tumour growth and survival, migration, and invasiveness of tumours by secreting paracrine and/or autocrine cytokines, chemokines and growth factors like hepatocyte growth factor (HGF), epidermal growth factor (EGF), insulin-like growth factor (IGF 1 & 2), and the receptor integrin α11 (Oestman and Augsten, 2009). These cytokines and chemokines secreted by CAFs contribute to the recruitment of BM-derived MSCs and immune cells into the TME (Servais and Erez, 2013). It has been shown that CCL5, CCL14, and CCL12 or SDF-1α play roles as pro-metastatic factors (Luker et al., 2012, Augsten et al., 2014, Mi et al., 2011). These factors (especially SDF-1α) and TGF-β cause recruitment of BM-derived CAFs into tumour stroma (Quante et al., 2011).

Mi et al, 2011 showed in oral cancer that chemokine, MCP-1, expression were up regulated in CAFs (Li et al., 2014). This increase in MCP-1 level promoted the secretion of ROS in the cancer cells which later cause proliferation, migration, and invasion of the tumour cells (Li et al., 2014).

CAFs also cause ECM degradation through secretion of MMPs such as MMP3 which enhances tumour invasiveness (Lochter et al., 1997). MMP3 enhanced cleavage of E-cadherin protein of epithelial cells (mammary), which then undergo an epithelial-to-mesenchymal transition that enhances cancer invasiveness.

1.5.2.2 CAFs and tumour angiogenesis

VEGF has been shown to play a crucial role in angiogenesis and lymphangiogenesis and subsequent cancer progression and metastasis (Shibuya and Claesson-Welsh, 2006). In addition to tumour cells, a primary source of VEGF in the TME is CAFs which increase their expression of VEGF due to cancer-stromal cell signalling interactions (Gomes et al., 2013). It has been shown that production of VEGF within TME depends on PDGF derived from CAFs. PDGF activates VEGF via its receptor (PDGFR) that plays an important role in angiogenesis through recruitment of resident fibroblasts and BM stromal cells (Gomes et al., 2013, Ferrara, 2010). BM stromal cells can differentiate into smooth muscle cells and endothelial cells that proliferate and migrate in response

to PDGF (Ferrara, 2010). Endothelial cells produce PDGF-BB that cause recruitment of pericytes to the vessel wall and subsequent angiogenesis (Zhang and Liu, 2013, Bergers and Song, 2005). Another study suggested that colon cancer cells stimulate fibroblasts to secrete IL-6 that then causes angiogenesis (Nagasaki et al., 2014). Moreover, when IL-6 receptor was blocked using a neutralizing antibody, angiogenesis was inhibited.

1.5.2.3 CAFs and tumour metastasis

Many studies showed the role of CAFs in tumour metastasis (Karagiannis et al., 2012, Pavlides et al., 2012). Some studies have suggested that the CAFs have mesenchymal-like phenotype that might cause enhancement of cancer metastasis (Dumont et al., 2013). A recent study by Wang et al, 2017 showed that conditioned media (CM) from CAFs that were isolated from lung cancer increased the migration of lung cancer cells *in vitro* (Wang et al., 2017). Moreover, in this study, CAFs caused EMT (increase vimentin level and decreased E-cadherin level) that promoted lung cancer cells metastasis.

It has been shown in gastric cancer that the expression of SRF (serum response factor) was high in fibroblasts causing cancer cell metastasis via increasing SDF-1α/CXCR4 signalling (Qiao et al., 2016). CAFs secrete SDF-1α that might cause EMT as shown in breast cancer studies and oral squamous cell carcinoma (Soon et al., 2013, Onoue et al., 2006).

In addition, CAFs may cause metastasis by enhancing angiogenesis within the stroma of tumours. A study by Guo et al, 2008, in a gastric cancer mouse model, showed that after activation of cancer cells, the stromal fibroblasts secrete VEGF-A which enhances angiogenesis (Guo et al., 2008). These studies suggest the important role of CAFs in the mediation of cancer metastasis.

1.5.2.4 CAFs and cancer therapy

In cancer cells, resistance to treatment not only depends on genetic or epigenetic modifications but also on the TME. Although CAFs are more stable genetically than cancer cells, many studies suggested that CAFs make cancer cells more resistant to therapies (Correia and Bissell, 2012, Kerbel, 1997, Li et al., 2015, Kharaishvili et al.,

2014). Some studies showed that the cancer stroma contains cytokines or chemokines that play a role in tumour drug-resistance.

CAFs modulate ECM-cancer cell interaction pathways and chemokines and/or cytokine signaling that cause anti-cancer drug resistance (Paraiso and Smalley, 2013). In BRAF-mutant melanoma cells, a study by Hirata et al., 2015 showed that CAFs play an important role in increasing resistance to BRAF inhibitors (PlLX4720). BRAF is a human gene encoding a protein B-Raf involved in cell growth. Melanoma-associated fibroblasts generate a fibronectin-rich TME, which can sustain cancer cells via fibronectin-activating β1-integrin-FAK-ERK signaling (Hirata et al., 2015). Moreover, culturing melanoma cell lines with CM from tumour fibroblasts caused resistance to treatment. In head and neck cancer, it has been shown that CAFs enhanced anti EGFR drug resistance (Cetuximab) via secretion of MMP-1 (Johansson et al., 2012). In prostatic cancer, Kharaziha and colleagues have shown that culturing of primary CAFs with prostatic cancer cells protected cancer cells from sorafenib cytotoxic effect via over-expression of Bcl-xL (anti-apoptotic protein) (Kharaziha et al., 2012). In ovarian cancer, it has been shown that the level of MCP-1 expression in paclitaxel-resistant ovarian cancer cell lines was increased. Moreover, blocking of MCP-1 caused enhancement of carboplatin and paclitaxel drugs in ovarian cancer (Moisan et al., 2014). MCP-1 was induced by CAFs via activation and phosphorylation of transcription 3 (STAT3). This CAF-derived MCP-1 regulated cancer stem cells via activation of NOTCH signalling and in turn, enhanced cancer progression (Tsuyada et al., 2012). NOTCH signalling regulates apoptosis and cell proliferation and differentiation (Artavanis-Tsakonas et al., 1999).

In the cancer stroma, CAFs are the common source of SDF-1α (CXCL12). It has been shown that SDF-1α and its receptor (CXCR4) signalling activate Akt, ERK, and adhesion kinase signalling pathways that caused resistance to cancer therapies in pancreatic cancer (Weekes et al., 2012, Singh et al., 2010). Moreover, any disruption of SDF-1α/ CXCR4 signalling pathways caused sensitisation of prostate, lung, and colon cancer cells to chemotherapy (Domanska et al., 2012, Burger et al., 2011, Heckmann et al., 2013). In conclusion, as CAFs play a key role in cancer growth, metastasis, invasiveness, and angiogenesis, it is very important to find new anti-cancer therapies that target them.

1.6 Role of mesenchymal stem cells in cancer

Friedenstein and colleagues in 1970 described a group of BM originating cells that have fibroblast features, the ability to differentiate and showed clonal growth (Friedenstein et al., 1970). They called these cells "colony-forming cells". Caplan used the name "mesenchymal stem cells" later for the population of BM-derived cells that were able to differentiate into cartilage and bone (Caplan, 1991). MSCs are multipotent (can produce multiple types of specialized cells) being able to differentiate into muscle, cartilage, bone and connective tissues (Prockop, 1997, Pittenger et al., 2000). MSCs have an important characteristic, namely being able to migrate to sites of tissue injury like heart, kidney and skin, primarily due to inflammatory mediators which are produced locally as a result of tissue damage and remodelling (Wu et al., 2003, Morigi et al., 2004, Li et al., 2006). They express surface markers like CD90, CD105, and CD73 with the absence of hematopoietic markers (Lama et al., 2007).

In wound healing, MSCs recruited to the wound helps in homeostasis, tissue repair, and immune modulation. In cancer, tumour cells that proliferate permanently and invasively produce an inflammatory microenvironment (a wound that never heals) (Karnoub et al., 2007). Once in the TME, MSCs play important roles through their special cellular interactions by either promoting or inhibiting tumour cell growth (Klopp et al., 2011). As inhibitors for tumour growth, MSCs block Akt and Wnt signalling, suppress angiogenesis, and apoptosis through cell cycle arrest (Hass and Otte, 2012, Rhee et al., 2015). Suppression of tumour development has been shown in different cancer types such as colon cancer, lymphoma, and melanoma (Nakamizo et al., 2005, Loebinger et al., 2009, Grisendi et al., 2010). As tumour promoters, MSCs can be recruited to a tumour and activated by TGF-β to form CAFs that play a key role in tumour growth, as described above (Barcellos-de-Souza et al., 2016). Moreover, Karnoub et al and Shinagawa et al showed that MSCs caused immune modulation and stimulation of tumour development (Karnoub et al., 2007, Shinagawa et al., 2010). Local MSCs can secrete bioactive molecules that play an important role in tissue injury by stimulation of angiogenesis and can have immune modulatory effects to maintained homeostasis (Lazennec and Jorgensen, 2008, Uccelli et al., 2008). It has been shown that MSCs enhanced angiogenesis and tumour growth through their abilities to differentiate into pericyte-like, and endothelial cell-like cells (Oswald et al., 2004, Ball et al., 2004, Suzuki et al., 2011). Moreover, co-culture of adipose-derived MSCs with

prostate cancer was shown to cause differentiation of MSC into endothelial-like cells and increased tumour vascularity and enhancement of the tumour growth (Lin et al., 2010, Prantl et al., 2010). Many studies have shown that MSCs enhanced cancer progression via promoting the metastatic ability of cancer cells together with their role in EMT (Karnoub et al., 2007, Kabashima-Niibe et al., 2013). By contrast, other studies have shown that MSCs caused suppressive effects on the tumour growth. Ho and his colleagues have shown that the human glioma tumour volume and vascularity were reduced after administered human BM-derived MSCs with glioma cells (Ho et al., 2013). In Ho et al study, MSCs caused suppression of a tumour through modification of Akt signalling and inhibition of angiogenesis. Furthermore, a study by Otsu et al. showed that MSCs were potentially cytotoxic (anti-angiogenic) by generating ROS that inhibited tumour growth (Otsu et al., 2009).

These opposing results may be because experiments were done under different conditions like differences in the sources of MSCs used; some used BM-derived and other used adipose-derived MSC. In addition, each study used different timing schedules and different cell administration methods. Moreover, culture media for *in vitro* experiments do not have all the active factors that are present in the real stem cell niche (Watt and Hogan, 2000). In murine gastric cancer due to chronic inflammation, it has been reported that 20% of CAFs originated from resident BM-derived MSCs (Quante et al., 2011). These MSC-derived CAFs were recruited to a tumour in response to TGF-β and SDF-1α. These results suggested that epithelial dysplasia due to chronic inflammation leads to relocation of the BM-niche to a tumour and consequent increases in MSC numbers promote cancer progression.

1.6.1 Direct communication of MSC with tumour cells

One of the important ways in which MSCs interact with tumour cells is NOTCH signalling that regulates immune cell functions, cell proliferation, differentiation and tissue repair (Kopan and Ilagan, 2009). It has been shown that the addition of MSCs enhanced breast cancer cells growth both *in vivo* and *in vitro* via NOTCH pathway signalling (Mandel et al., 2013). Moreover, blocking of NOTCH signalling caused decreased CD90 expression by MSCs and inhibited the growth of tumour cells (Mandel et al., 2013, Geling et al., 2002). CD90 (Thy-1) is a cell surface protein expressed by

MSCs, hematopoietic cells, endothelial cells, fibroblasts, and myofibroblasts (Haeryfar and Hoskin, 2004, Craig et al., 1993, Saalbach et al., 1999). CD90 helps to regulate cancer cell proliferation, migration, angiogenesis, apoptosis, metastasis and fibrosis (Rege and Hagood, 2006, Saalbach et al., 1999, Saalbach et al., 1996).

MSCs can also communicate with tumour cells by gap junctions that connect neighbouring cells through gap junctional intercellular communication (GJIC). GJIC maintain tissue homeostasis, and control cell growth and differentiation (Kandouz and Batist, 2010). It has been shown that breast cancer cells can gain CD90 via GJIC signalling when co-cultured with MSC. The expression of CD90 by breast cancer cells was decreased when gap junction inhibitors were used such as carbenoxolone (Mandel et al., 2013).

Another way of communication between MSCs and tumour cells is through nanotubes, which enable neighbouring cells to exchange molecules, organelles, and glycoproteins (Gurke et al., 2008). Breast cancer cells have been reported to use nanotubes to obtain mitochondria from BM-derived MSCs resulting in increased tumour cells proliferation and invasiveness (Caicedo et al., 2015). Formation of fusion between MSC and cancer cells is also a way of communication that is rare and its molecular mechanisms are still poorly understood.

1.6.2 Indirect communication of MSC with tumour cells

This occurs through secretory mediators from tumour cells such as growth factors, chemokines and cytokines that trigger intra-cellular signalling pathways via attaching to receptors of neighbouring MSCs. MSCs activate and secrete different bioactive compounds that regulate adjacent cells such as tumour cells (Melzer et al., 2016). It has been shown that MSCs secrete CC-chemokine ligand 5 (CCL5) or RANTES that interact with receptors (chemotactic cytokine) like CCR5, CCR3, CCR1 (Karnoub et al., 2007). In breast cancer, the G-protein coupled receptor (GPR75) is activated via MSC-derived CCL2 in a paracrine manner, which resulted in enhancement of migration, invasiveness and metastatic ability of breast cancer cells (Karnoub et al., 2007).

Another way of the indirect interplay of MSC with tumour cells is via metabolites. Many metabolites like indoleamine2, 3-dioxygenase or prostaglandins secreted into the tumour stroma motivate other cells in the TME in a paracrine way (Li et al., 2012, Yuan et al., 2013). These metabolites affect tumour growth and lead to an alteration in cancer cell properties such as invasion, self-renewal and survival.

Studies by Bonuccelli et al. (2004) on osteosarcoma cells suggested that human adipose MSC increased production of lactate in response to oxidative stress induced by cancer cells. Subsequently, the lactate efflux receptor expression was increased by osteosarcoma cells that increased their ATP production which enhanced the migratory ability of the tumour cells (Bonuccelli et al., 2014).

MSCs can also interact with tumour cells via microparticles such as microvesicles (50-100 nm in diameter, plasma membrane origin) and exosomes (40-100 nm in diameter, endocytic origin) (Lee et al., 2012). The cellular interaction between cancer cells and MSCs in the TME occurred through microvesicles and exosomes proteins, micro-RNAs, and functional mRNAs (Yang et al., 2015a). It has been shown that MSC-derived exosomes produce MMP-2 and ecto-5'-nucleotidase that modify tumour cell function in TME (Yang et al., 2015a, Friedl and Alexander, 2011). Moreover, in prostatic cancer, differentiation of BM-derived MSC into CAFs was enhanced by cancer cells-derived exosomes and subsequent tumour growth (Chowdhury et al., 2015).

Regarding anti-tumour effects of microvesicles, Wu and his colleagues showed, both *in vitro* and *in vivo* that the growth of bladder cancer cells (T24) was inhibited in response to microvesicles derived from MSCs from human umbilical cords. These cause tumour cell cycle arrest and apoptosis (Wu et al., 2013).

1.7 Tumour microenvironment and radiation

Ionizing radiation is an important modality of curative and palliative cancer treatment. More than half of cancer patients need treatment by radiation therapy (Owen et al., 1992, Delaney et al., 2006). DNA is the main target of radiation leading to cytotoxicity. Radiation effects are either directly through DNA damage, or indirectly by forming free radicals inside cells from interactions with tissue water. These are highly reactive and themselves damaging to DNA. The direct and indirect effects of radiation depend on the linear energy transfer (LET), which is the energy transferred per unit length of track (Eric and Hall, 2006). Moreover, tumour hypoxic cells during RT treatment are about three times more radiation-resistant than normoxic cells (Brown and William, 2004).

Apart from the tumour cells themselves, tumour endothelial cells are relatively radiosensitive, at least partly due to their rapid proliferation (Barker et al., 2015). Tumour blood vessels differ from those in normal tissues, for instance in that, they are tortuous and more permeable (due to structural abnormalities of the basement membrane, a relative paucity of pericytes and stretching of endothelial cells). These features may make them particularly susceptible to radiation damage (Fajardo, 2005, Jain, 2003). The radiation effects on the tumour vasculature depend on many factors such as radiation dose, fractionation, site and size of the tumour, and tumour types (Park et al., 2012, Karam and Bhatia, 2015).

In fact, low dose radiation (<5 Gy) can enhance tumour growth via increased VEGF secretion promoting angiogenesis (Heissig et al., 2005, Vala et al., 2010). Moreover, Lerman and his colleagues showed that low-dose radiation (5 Gy) causes stimulation of HIF-1α in endothelial cells that upregulate SDF-1α and endothelial cell migration (Lerman et al., 2010). In contrast, high dose radiation has been shown to cause severe vascular damage and disruption of the TME (Song et al., 2015, Maeda et al., 2017). Solesvik et al. showed that within a week of irradiation of melanoma xenografts (human) with single doses of 10-15 Gy there was damage to nearly half of all the tumour blood vessels (Solesvik et al., 1984). Another study showed that vascular density of human ovarian cancer xenograft was decreased to a half of control after irradiation with 20 Gy (5 Gy/ fraction) (Dings et al., 2005).

Vascular damage caused by radiation can lead to tumour hypoxia and release of HIF-1α (**Figure 1.3**). As discussed in **section 1.4.4**, HIF-1α enhances secretion of cytokines and chemokines to recruit immune cells like BM-derived cells. These BM-derived cells

can differentiate into endothelial cells by VEGF and PDGF (Ahn and Brown, 2009, Maeda et al., 2017, Conway et al., 2001). Multiple studies have indicated the activation of several genes by HIF-1 during hypoxia, such as erythropoietin, glucose transporters, glycolytic enzymes and VEGF (Sutherland, 1998, Blouin et al., 2004, Williams et al., 2005).

These activated genes make tumour cells more aggressive, radioresistant and able to survive in adverse conditions. RT can cause angiogenesis and radio-resistance (Koukourakis et al., 2001), because radiation makes endothelial cells produce many cytokines that work as proangiogenic factors like PDGF, TGF-β, TNF-α, b FGF, IFN-γ and VEGF (Wachsberger and Burd, 2004, McBride et al., 2004). Moreover, radiation causes sprouting of endothelial cells (the first step in angiogenesis) through stimulation of nitric oxide pathways inside these cells (Sonveaux et al., 2003) **(Figure 1.3)**. Gorski and colleagues reported VEGF up-regulation after radiation in several cancer cell lines (Gorski et al., 1999).

Radiation can also affect CAFs. As mentioned earlier (see **section 1.5**), CAFs represent the most common cells within the TME that play a role in cancer growth, metastasis, and angiogenesis. CAFs have the ability to recruit endothelial cells via secretion of SDF-1; in addition, CAFs enhance secretion of ECM-degrading enzymes (MMPs). It has been found that CAFs and normal fibroblasts are radioresistant (Hawsawi et al., 2008, Papadopoulou and Kletsas, 2011, Tachiiri et al., 2006). However, most of these studies were on fibroblasts rather than CAFs. Irradiation can cause senescence in fibroblasts and release of cytokines, proteolytic enzymes, growth factors and ROS, which all enhance tumour formation (Rodier et al., 2009, Papadopoulou and Kletsas, 2011, Velarde et al., 2013, Liu and Hornsby, 2007). Interestingly, senescence occurs more clearly after single dose radiation of lung CAFs rather than fractionated irradiation with the same dose (Hellevik et al., 2012).

Radiation causes inflammation in the TME and subsequent recruitment of fibroblasts to the site of injury. Fibroblasts differentiate into CAFs via TGF-β that is released in response to inflammation. CAFs secrete ECM proteins like collagen, fibronectin and laminin which cause fibrosis in the long term (Yarnold and Brotons, 2010).

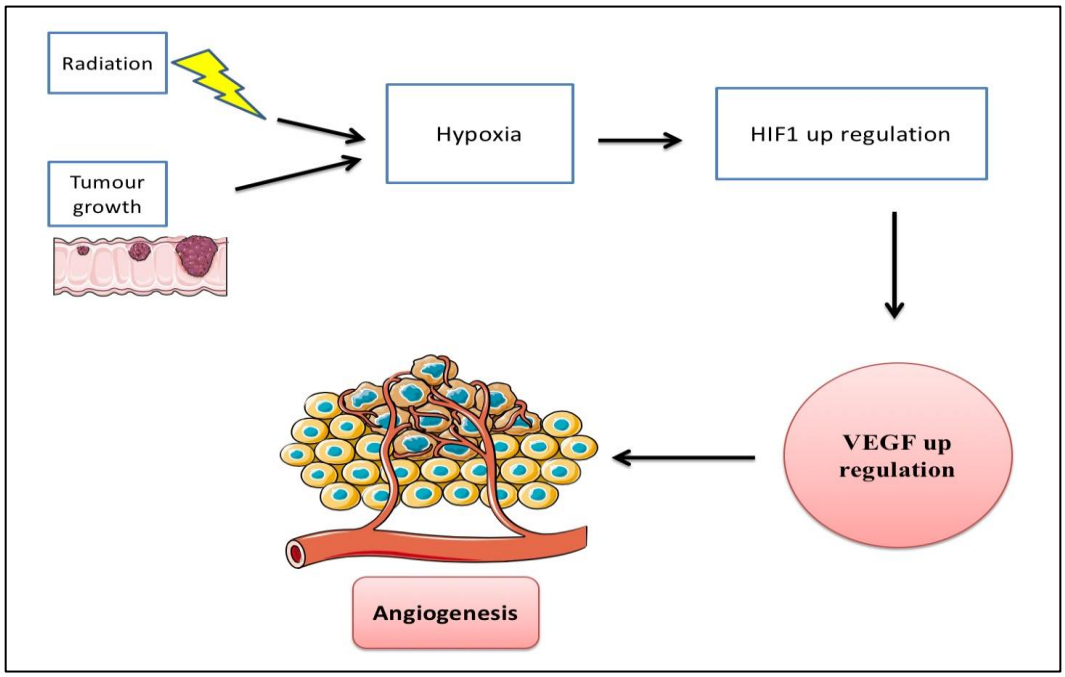


Figure 1.3 The effect of hypoxia on tumour growth: During tumour growth or after radiation, tumour hypoxia increases and this leads to initiation of Hypoxia Inducible Factor 1 (HIF-1), which then cause vascularisation in hypoxic areas through VEGF upregulation, and angiogenesis formation.

1.8 Radiation effect on MSC biology and subsequent fibrosis

Although radiation therapy is an essential component of cancer therapy, side effects are inevitable. Radiation side effects are either acute, like skin erythema and desquamation, mucositis, nausea and diarrhoea or chronic (long term) affecting patient's quality of life such as radiation fibrosis, and vascular damage (Bentzen, 2006). The severity of radiation long-term effects depends on radiation-dose, treatment volume, and fraction size. One of the important and life-threatening radiation late effects is radiation-induced fibrosis (RIF). The development of RIF is a complicated process that involves different growth factors, cytokines, inflammation, fibroblast differentiation, and remodelling of the ECM (Wynn, 2008).

In regards to the response of MSCs to irradiation in human and mouse, it has been shown *in vitro* that MSC survival after high dose irradiation was higher than for other stem cells derived from the BM (Chen et al., 2006, Nicolay et al., 2014). A study by Islam et al. showed that adult human MSCs were more radioresistant than embryonic stem cells (Islam et al., 2015). Fractionated or hyperfractionated radiotherapy of 0.5-2 Gy has been shown to cause radioresistance in MSCs (Tomuleasa et al., 2010, Clavin et al., 2008).

In hypoxic conditions, Sugrue et al. showed that hypoxia increased radioresistance of mouse MSCs *in vitro* by increasing their proliferation, DNA damage repair, and long-term survival after irradiation (Sugrue et al., 2014). Moreover, another study confirmed these results *in vivo* and showed that after irradiation of porcine mandibular bone with single doses of up to 18 Gy, MSCs that survived maintained their proliferation and differentiation abilities (Singh et al., 2012).

1.8.1 Radiation and fibrosis

Fibrosis and tissue repair are strongly related to myofibroblasts, which commonly originate from local fibroblasts. Within normal connective tissue, fibroblasts are considered the most common cell type responsible for the synthesis, remodelling, and degradation of ECM in disease and health (Evans et al., 2003). Fibroblasts differentiate into myofibroblasts (in cancer called CAFs) as a consequence of fibrogenic cytokines secreted by inflammatory cells and other cells (Hinz et al., 2007). This differentiation is called activation and at this stage, α-SMA appears associated with coarse fibres responsible for the contractile properties of myofibroblasts (Hinz et al., 2007). Myofibroblasts are responsible for the production of collagens type I, III, IV and V, fibronectins and other matrix proteins (Sime and O'Reilly, 2001, Wynn, 2008). Myofibroblasts compared to fibroblasts secrete large amounts of collagen and they represent the main source of collagen in tissues (Ramos et al., 2001). Moreover, myofibroblasts represent the main sources of pro-fibrotic cytokines such as TGF-β and MCP-1 in idiopathic pulmonary fibrosis (Goodwin and Jenkins, 2009, Phan, 2002). Presence of myofibroblasts within fibrotic lesions in animal models and in human fibrotic diseases proved them as key cells in the pathogenesis of fibrosis (Zhang et al., 1994). Myofibroblasts play a key role in wound healing through regulation of tissue repair. However, if their extra cellular matrix secretion becomes uncontrolled, it can cause severe impairment of organ function (Hinz et al., 2007, Yarnold and Brotons, 2010). Normally, fibroblasts (that express little or no α-SMA) produce low ECM, while after tissue injury; fibroblasts become activated through cytokines released from epithelial cells in response to the injury. The activated fibroblasts (myofibroblasts) migrate to damaged tissue to synthesise and deposit ECM. To prevent excessive ECM deposition and tissue restoration, apoptosis is activated (Wynn, 2008). It has been shown that myofibroblasts are resistant to apoptosis, which causes excessive ECM production and scar formation (Ramos et al., 2001).

RIF is one of the commonest long-term side effects of radiotherapy treatment that develops by complex molecular processes involving different growth factors, cytokines, chemokines, inflammation, and ECM degradation. The molecular mechanisms of RIF are similar to other fibrotic diseases (Yarnold and Brotons, 2010, Cheresh et al., 2013). The first step in fibrosis is the remodelling of ECM in connective tissue and production of myofibroblasts from different cell-types via fibroblast activation, or EMT (Zvaifler,

2006, Krenning et al., 2010). In healthy tissue, the injury response and wound healing depend on activation of fibroblasts and remodelling of ECM (Bielefeld et al., 2013). In fibrotic diseases, the activity of myofibroblasts remains even after damage repair (Wynn, 2008). Moreover, different types of cells are involved in regulation of tissue such as epithelial and endothelial cells in addition to immune cells (Krenning et al., 2010, Milliat et al., 2006, Xiao et al., 2012). Several growth factors, hormones, and mediators drive or suppress the fibrotic process. TGF-β, connective tissue growth factor (CTGF), and interleukin-6 (IL6) act as radiation fibrosis inducers (Kruse et al., 2009, Haydont et al., 2008, Gaugler et al., 2005), whereas fibrosis suppressors include gamma interferon, hepatocyte growth factor, and thrombomodulin (Hu et al., 2009, Gottlober et al., 2001, Yarnold and Brotons, 2010). A cascade of active cytokines persists after irradiation, which is responsible for long-term radiation tissue damage effects (Devalia and Mansfield, 2008). The initiation of this cascade is still unclear, but may be radiation leads to activation of macrophages and monocytes that secrete cytokines like IL-1, IL-6, and tumour necrosis factor alpha (TNFα), which attract other macrophages, and monocytes. These activated macrophages synthesize cytokines (fibrogenic) such as CTGF, TGF-β, and PDGF which are responsible for the synthesis of ECM proteins, MMPs and their inhibitors, tissue inhibitors of metalloproteinases (TIMPs) (Haase and Rodemann, 2004, Bentzen, 2006).

RIF is a complex process that is driven by multiple intracellular signalling pathways including Smad signalling related to TGF-β, rho/ROCK kinase signalling, integrin signalling, DNA damage response (DDR), and stress response signalling (Milliat et al., 2006, Puthawala et al., 2008, Haydont et al., 2008). Both DDR and stress response are important in radiotherapy treatment of cancer as they determine the normal tissue side effects from irradiation and the effectiveness of the treatment (Moding et al., 2013). Radiation leads to ROS, and different DNA cell-cycle arrest through activation of checkpoints and subsequent apoptosis (Cheresh et al., 2013, Moding et al., 2013). Long-term radiation response leads to delayed onset genomic instability with loss of ability of the tissue to regenerate that caused tissue dystrophy and radiation fibrosis (Koturbash et al., 2006, Sperk et al., 2012, Westbury and Yarnold, 2012). Radiation causes damage to the DNA of the cells within the irradiated areas but cells also secrete factors that cause DNA damage of distant cells through a bystander effect (Koturbash et al., 2006, Dickey et al., 2009). Many studies suggest that long-term radiation effects

rely on loss of stem cells, persistent DNA damage, changes in cellular signalling, and genetic/epigenetic deviations (Koturbash et al., 2006, Dickey et al., 2009, Coppes et al., 2009, Kuhmann et al., 2011, Yarnold and Brotons, 2010). In summary, inflammation plays a key role in the development of RIF.

1.8.2 Origin of myofibroblasts (CAFs) in cancer

There are different types of cells from which CAFs can originate in cancer (**Figure 1.4**). The main source of CAFs is from resident fibroblasts that become activated/differentiated by TGF-β (Postlethwaite et al., 2004). As described previously, in response to the tissue injury, epithelial cells secrete TGF-β that induces differentiation of fibroblasts to CAFs. However, evidence from research have suggested alternative sources that CAFs may originate from. It was shown that epithelial cells, macrophages and pericytes undergo phenotypic differentiation to form CAFs (Flier et al., 2010, Lamouille et al., 2014, Zeisberg et al., 2007b). Epithelial cells can differentiate into CAFs through EMT in the presence of TGF-β. Moreover, it has been suggested that CAFs can derive from endothelial cells through endothelial-mesenchymal transition (En-MT) (Kalluri and Neilson, 2003). In the En-MT process, endothelial cells gain mesenchymal properties and expresses CAFs differentiation markers like α-SMA, collagens and vimentin and decrease endothelial cell markers such as vascular endothelial cadherin (VE-cadherin) (Zeisberg et al., 2007a). Jimenez et al and Kong et al have shown the involvement of En-MT in the development of pulmonary fibrosis (in systemic sclerosis), cardiac fibrosis and intestinal fibrosis in humans (Jimenez, 2016, Kong et al., 2014). It is not yet understood why En-MT plays a role in the pathogenesis of fibrosis in humans. Understanding of molecular mechanisms of En-MT involvement in fibrosis should help in the development of novel anti-fibrotic drugs.

Another source of CAFs is from BM stem cells or tumour MSCs. It has been shown that circulating fibroblasts derived from BM can differentiate into CAFs (Bucala et al., 1994, Ebihara et al., 2006). These mesenchymal stem cell progenitors (fibrocytes) have the phenotype of fibroblast/myofibroblasts and they express collagen I, CD34, and CD35 (Ebihara et al., 2006, Brittan et al., 2002, Forbes et al., 2004). Although it appears that CAFs derive from differentiation of many cellular types, the main source of CAFs remains the resident fibroblasts.

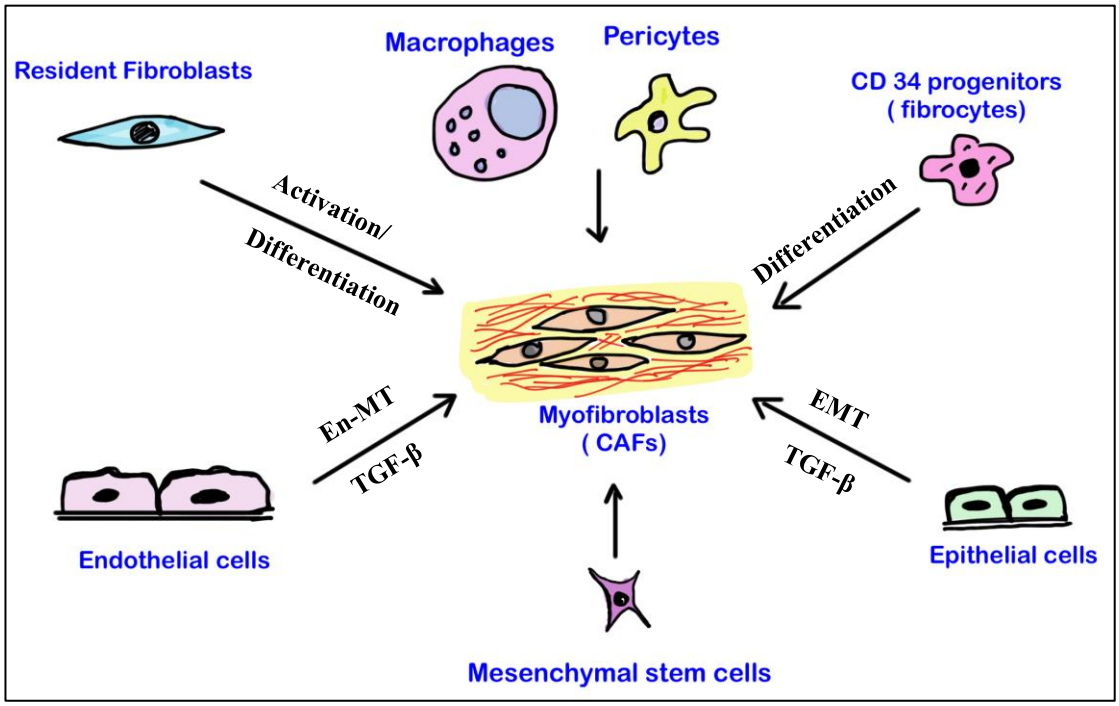


Figure 1.4 Origin of Myofibroblasts (CAFs) in cancer **:** CAFs originate from different sources such as: resident fibroblasts, circulating bone marrow progenitors (fibrocytes), endothelial cells experiencing endothelial-mesenchymal transition (En-MT), pericytes, macrophages, MSCs and epithelial cells experiencing epithelial-mesenchymal transition (EMT).

1.8.3 Role of TGF- β in RIF

1.8.3.1 TGF-β overview and superfamily

TGF-β is 25-kDa multi-functional polypeptide cytokine that plays an important role in fibrosis. It regulates differentiation and proliferation of cells, development and growth of organs, immune response, apoptosis, tumour growth and suppression (O'Sullivan and Levin, 2003, Bentzen, 2006). TGF-β belongs to a superfamily of more than 60 proteins (29 of them encoded by the human genome) that regulate homeostasis, embryonic development, control of cell cycle, and wound healing in multicellular organisms, (Feng and Derynck, 2005, Leask and Abraham, 2004). TGF-β is present as three isoforms (TGF-β1-3) that have similar biological activities (Gorelik and Flavell, 2002). Although in humans all three isoforms are expressed, the TGF-β1 isoform is most prevalent and is associated with tissue fibrosis (Wynn, 2008).

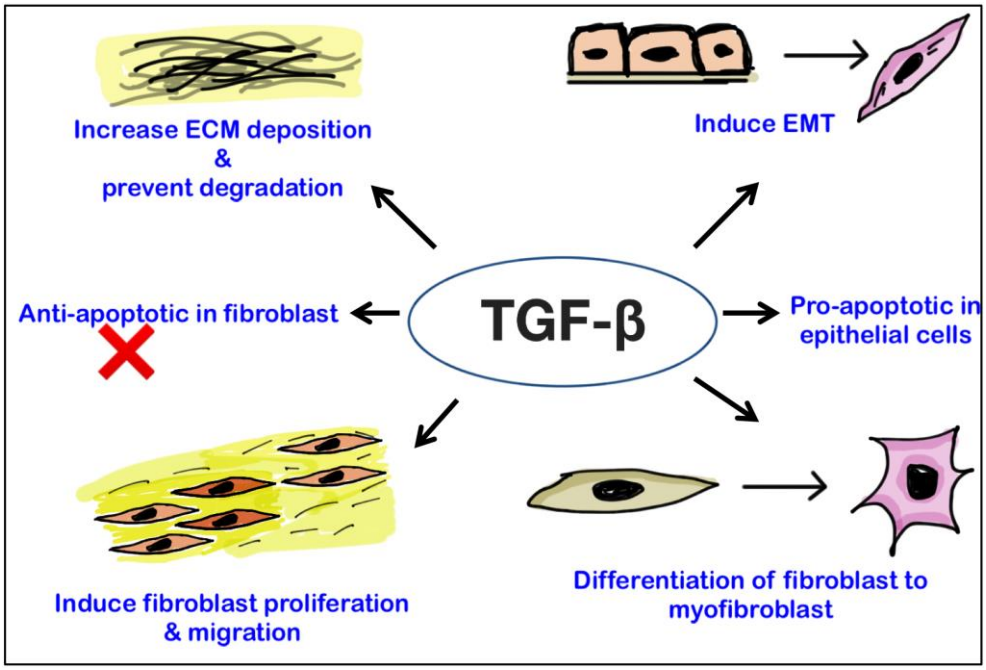


Figure 1.5 Pro-fibrotic effects of TGF-β1

1.8.3.2 TGF-β signalling

The secretion of the TGF-β from most cells (like CAFs and macrophages) occurs in a latent form, which needs to be activated in the extracellular space in order to bind to the receptor. TGF-β is stored inside cells as an inactive homodimer form that is bound to latency-associated peptide (LAP) that keeps TGF-β inactive. Its activation occurs by dissociation of the LAP (Lawrence, 2001). This means that after exposure to triggering factors including ionising radiation, the large amount of latent TGF-β in the extracellular space can be mobilized. It has been shown that within a few minutes or hours after exposure to ionizing radiation with 0.1 Gy, TGF-β becomes activated (Ehrhart et al., 1997, Ewan et al., 2002). When TGF-β becomes activated, it can bind to its receptors TGF-β R1 and TGF-β R2, which then activate a family of transcription factors called Smads. Through Smads, TGF-β signalling transmitted downstream from the receptor to the nucleus. In addition, an alternative non-Smad pathway has been described including p38, MAPK, m-TOR, RAS, P42/p44 MAPK (Zhang, 2009).

Smad pathways regulate translation, transcription, microRNA biogenesis, post-translational modifications and protein synthesis (Hussey et al., 2011, Mu et al., 2012). Within the Smad family, there are three sub-types: inhibitory Smads (I-Smads), receptors that regulate Smads (R-Smads), and common partner Smads (Co-Smads). The phosphorylation of R-Smads, Smad-2 and Smad-3 occur after activation of TGF-β receptor I kinase. Then, a heteromeric complex is formed from Smad-2 and -3 with co-Smad (Smad-4). The resultant complex then imported into the nucleus to regulate gene transcription (Attisano and Wrana, 2002, Verrecchia and Mauviel, 2002). Smad-6 and -7 work as inhibitors for signalling by inhibiting gene induction through preventing phosphorylation of R-Smad (Nakao et al., 1997) (**Figure 1.6**).

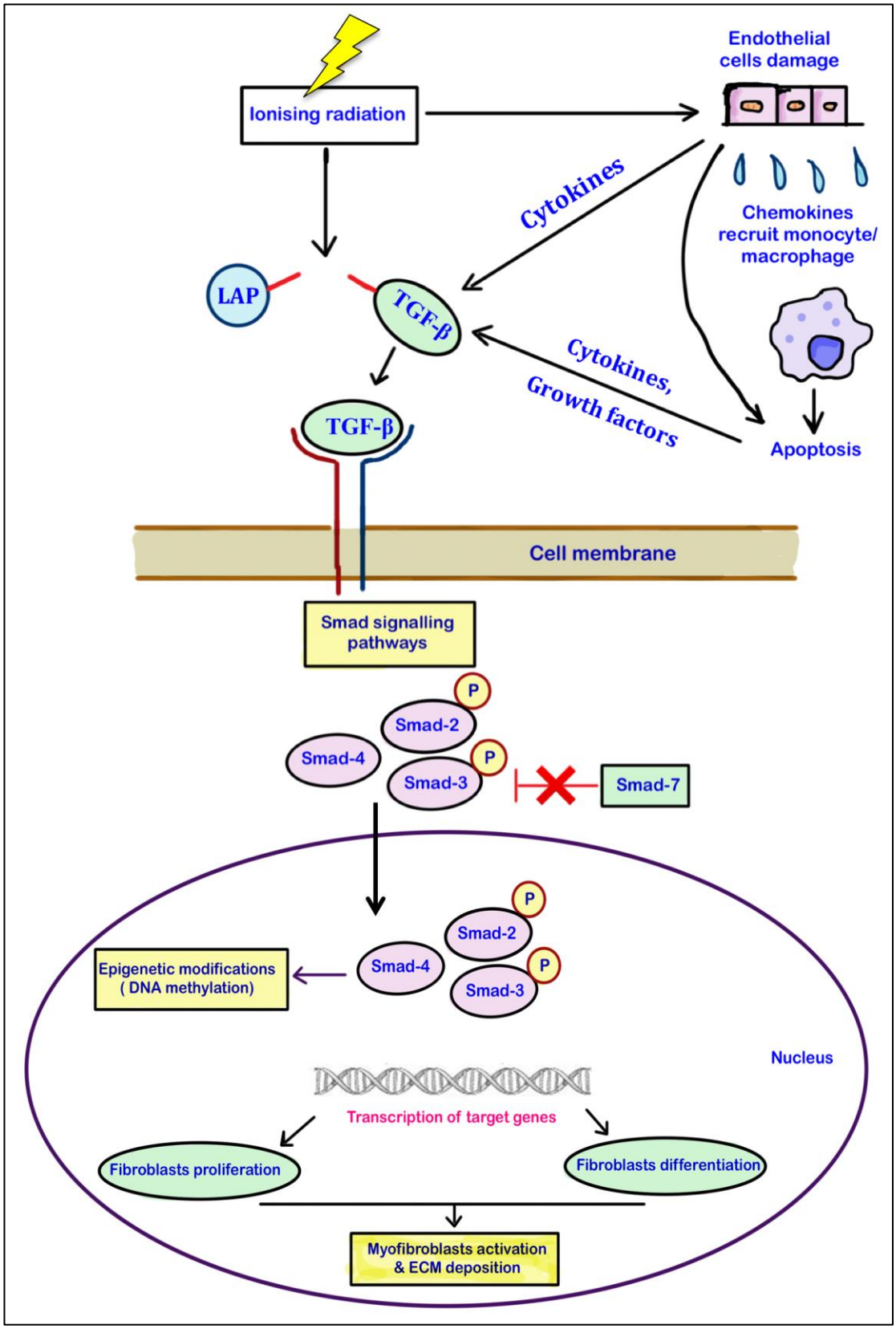

Ionising radiation
Endothelial cells damage
Cytokines
Chemokines recruit monocyte/ macrophage
LAP
TGF-β
Cytokines, Growth factors
Apoptosis
TGF-β
Cell membrane
Smad signalling pathways
P
Smad-2
Smad-4
P
Smad-3
Smad-7
P
Smad-2
Epigenetic modifications (DNA methylation)
Smad-4
P
Smad-3
Nucleus
Transcription of target genes
Fibroblasts proliferation
Fibroblasts differentiation
Myofibroblasts activation & ECM deposition

Figure 1.6 Role of TGF-β in radiation fibrosis:

TGF-β activated by ionizing radiation that leads to dissociation from the latency-associated peptide (LAP). Moreover, radiation causes damage to endothelial cells, which in turn leads to the secretion of chemokines and pro-fibrotic cytokines such as TGF-β. EC-derived chemokines recruit macrophages or monocytes, which cause apoptosis and further release of growth factors and cytokines like TGF-β. These extracellular actions cause TGF-β signaling pathway activation through Smad pathways. The phosphorylation of Smad-2 and Smad-3 occur after activation of TGF-β receptor I kinase. Then, a heteromeric complex formed from Smad-2 and -3 with co-Smad (Smad-4). This resultant complex transmitted into the nucleus to regulate gene transcription. Smad-7 works as an inhibitor for signalling by inhibiting gene induction through preventing phosphorylation of R-Smad and Smad-3. The final result from this process is increased ECM proteins like collagens.

1.8.3.3 TGF-β and fibrosis

As previously mentioned, TGF-β has strong pro-fibrotic effects on epithelial cells and fibroblasts, so it plays an important role in the development of fibrosis. It has been shown, both *in vitro* and *in vivo*, that TGF-β induces fibroblast differentiation into myofibroblasts (Desmouliere et al., 1993, Sime et al., 1997). There are three ways by which TGF-β enhances fibrogenesis. Firstly, TGF-β causes remodelling of the ECM via suppression of the MMPs, enhancing production of tissue inhibitor of metalloproteinases gene (TIMP3), and subsequently inhibits ECM degradation (Martin et al., 2000, Eddington et al., 2007).

Secondly, through Smad signalling, TGF-β leads to the formation of myofibroblasts through EMT. During EMT, the epithelial markers like E-cadherin and cytokeratins are down-regulated while mesenchymal markers like vimentin and α-SMA are up-regulated (Xu et al., 2009). Through EMT, epithelial cells gain invasive and migratory properties of cancer stem cells that can differentiate into different types of cells (Jain et al., 2007, Mani et al., 2008). It has been shown that in adult tissue, both fibrinogenesis and wound healing displaying EMT (Kalluri and Neilson, 2003). Thirdly, TGF-β enhances production of the matrix via Smad-3 dependent or non-Smad mechanism (Lan, 2003). It has been shown that the Smad-3 levels increased in many fibrotic models, moreover, mice with Smad-3 knockout were protected from lung fibrosis induced by bleomycin drug (Liu et al., 2003). A study by (Roberts et al., 2006) showed that fibrinogenesis was reduced in mice after deletion of Smad-3 that blocks EMT.

1.8.4 Connective tissue growth factor in cancer and fibrosis

Connective tissue growth factor (CTGF) or CNN2 is a matricellular protein that plays a role in fibroblast proliferation, and production of matrix and granulation tissue (Leask and Abraham, 2004, Yarnold and Brotons, 2010). It is a 36-38 kDa protein and was discovered in 1991 as a secreted protein in the CM from HUVEC (Human umbilical vascular endothelial cells) (Bradham et al., 1991). Tumour growth and progression is related to CTGF expression. For example, CTGF expression in breast cancer increased in bone metastasis (Kang et al., 2003) and was associated with bad prognosis in oesophageal cancer (Koliopanos et al., 2002), and increased invasiveness in pancreatic cancer (Wenger et al., 1999). On the other hand, some studies showed the reverse. Shakunaga and his colleagues showed, in chondrosarcoma, that the expression of CTGF was negatively related to tumour growth and grade (Shakunaga et al., 2000). Moreover, another study showed that over expression of CTGF was related to decreased oral squamous cell carcinoma tumour growth (Moritani et al., 2003). Lee and his colleagues showed that CTGF was responsible for differentiation of human BM-MSCs into fibroblasts that expressed increased collagen-I, or into myofibroblasts in the presence of TGF-β (Lee et al., 2010).

It has been shown that, in fibroblasts, CTGF enhanced the mRNA expression of α1 collagen-I, fibronectin, and α5 integrin (Frazier et al., 1996). CTGF also mediates endothelial cell migration, proliferation, differentiation and enhanced angiogenesis (Shimo et al., 1999, Babic et al., 1999).

Regarding the role of CTGF in fibrosis, the fibrotic activity of TGF-β is dependent on CTGF activities (Mori et al., 1999, Parada et al., 2013). Moreover, in fibrosis and wound healing the CTGF expression is increased alongside that of TGF-β (Barrientos et al., 2008). Leask and Abraham suggested that some of TGF-β's functions, like secretion of ECM (collagen and fibronectin) and fibroblast proliferation, are mediated by CTGF (Leask and Abraham, 2004). It has been reported that after being secreted from cells, CTGF interacts with different growth factors and cytokines like VEGF, TGF-β, IGF1, BMP-4 and BMP-7 (Yang et al., 2010, Liu et al., 2007). In human skin fibroblasts, previous studies have shown that TGF-β caused a huge increase in the expression of CTGF mRNA (Igarashi et al., 1993). Moreover, TGF-β required CTGF

to activate Smad1-ERK1/2 signalling (no effects on Smad-3 phosphorylation) (Nakerakanti et al., 2011). Through the Smad-3 binding site at the CTGF promotor, TGF-β caused fibroblasts to express CTGF (Verrecchia and Mauviel, 2007).

The up-regulation of CTGF in fibrosis was evident in different studies. A study by Nguyen et al. (2008) in diabetic kidney disease showed that CTGF inhibits BMP-7 signalling that caused altered gene transcription and subsequent reduction in MMP activity (Nguyen et al., 2008). BMP-7 plays a role in neutralizing the pro-fibrotic effects of TGF-β (Wahab and Mason, 2006, Mitu and Hirschberg, 2008). Mori and his colleagues reported that when a single administration of CTGF or TGF-β was injected subcutaneously in new born mice, a transient fibrosis occurred represented by granulation tissue formation (Mori et al., 1999). Furthermore, if CTGF was injected together with TGF-β, a fibrotic response happened and continued for a week. These results suggested that CTGF plays an essential role in the development of fibrosis. Although most studies have shown the positive correlation between TGF-β and CTGF, other studies showed the reverse. A study by Diziadzio et al. (2005) showed that in systemic sclerosis the level of CTGF was increased in the circulation compared to normal levels of TGF-β in the serum from both control healthy and diseased patients (Dziadzio et al., 2005). However, a study by Okada et al. (2005) showed that animals treated by CTGF antisense oligodeoxynucleotide did not affect the levels of TGF-β while it inhibited the expression of CTGF and reduced fibrosis (Okada et al., 2005). These studies are consistent with other studies suggesting that when the activity or synthesis of CTGF was blocked, inhibition of many TGF-β-responding transcriptions such as synthesis of collagen, adhesion, proliferation of fibroblasts and differentiation of myofibroblasts occurred (Frazier et al., 1996, Duncan et al., 1999, Grotendorst et al., 2004, Shi-Wen et al., 2006). Ma et al. suggested that CTGF, TGF-β2, and gremlin (BMPs antagonist) were involved in EMT of lens epithelial cells to form myofibroblasts and synthesis of ECM via Smad pathways (Ma et al., 2014). In summary, CTGF is both a mediator and marker for fibrosis in health and disease. A further understanding of the cellular mechanisms involved in the influence of CTGF on fibrosis is required. CTGF and CTGF-related pathways are also potential targets for the treatment of fibrosis.

1.9 MSCs homing toward cancer

How MSCs migrate to sites of tissue injury or into tumours is not fully understood. However, various different mediators like growth factors, cytokines, and chemokines have been described to be involved in the MSC homing mechanisms to inflammatory sites.

1.9.1 Growth factors involved in 'homing' of MSCs

Growth factors are polypeptide molecules that stimulate cell proliferation /differentiation, migration, and survival through their extracellular signalling (Vanden Berg-Foels, 2014). Growth factors play roles in wound healing and during embryogenesis (Cohn et al., 1995, Cross and Mustoe, 2003). One of the critical factors that play a role in the migration of MSCs is VEGF. It has been shown that MSC migration was enhanced by VEGF secreted by breast cancer cells. Moreover, receptors for VEGF are expressed on MSCs, and blocking of VEGF can cause a reduction in MSC migration (Ritter et al., 2008). PDGF also plays a role in the homing of MSCs. PDGF has been shown to play roles in wound healing and in embryogenesis (Alvarez et al., 2006). It is a polypeptide dimer that has four homo-dimers with their receptors such as PDGF-AA, PDGF-BB, PDGF-CC, and PDGF-DD. Each PDGF dimer has two receptors, α and β. PDGF-AA binds α/α receptors and PDGF-BB binds α/α, α /β, and β/β receptors (Fang et al., 2004). It has been shown *in vitro* that migration and recruitment of human MSCs were enhanced by PDGF-AA, and PDGF-BB (strongest response) (Fiedler et al., 2004). On the other hand, studies by (Ruster et al., 2005) and (Nedeau et al., 2008) have shown that only a minimal number of MSCs migrated in response to PDGF-BB. The differences in the effects of PDGF-BB on the recruitment of MSCs might be due to using different MSCs and culture conditions.

Other growth factors that have a role in MSC homing are shown in **Table 1.1**.

1.9.2 Chemokines

Chemokines are families of chemotactic cytokines with small molecular size (8-10 kDa) which induce chemotaxis of leukocyte cells (Baggiolini, 1998). Based on their cysteine residues number, chemokines are divided into four groups: C, CC, CXC, and

CX3C (Clarklewis et al., 1995). They play key roles in wound healing, tissue homeostasis, and immune response through regulation of cell migration.

1.9.2.1 Stromal cell derived factor 1α

Stromal cell derived factor 1α (SDF-1α) is CXC chemokine 12 (CXCL12) that is expressed at injury sites and is involved in wound healing, embryogenesis, and in response to hypoxia (Gillitzer and Goebeler, 2001, Rezzoug et al., 2011, Abbott et al., 2004). SDF-1α is an 8-12 kDa peptide that enhances the progression of cancer and promotes angiogenesis by recruitment of endothelial cells to the tumour site (Kryczek et al., 2007, Diomedi-Camassei et al., 2008, Meier et al., 2007, Sung et al., 2008). CXCR4 is a protein encoded by the CXCR4 gene and acts as a receptor for SDF-1 α. SDF-1α works on cancer cells through enhancing proliferation via CXCR4 that is expressed on cancer cells.

It has been shown that SDF-1α induces recruitment of human MSCs *in vitro* (Schmidt et al., 2006, Sordi et al., 2005). Moreover, Kitaori et al showed that, *in vivo*, SDF-1α recruited MSCs to a fracture site in mouse and helped the regeneration of the bone. On the other hand, using anti-SDF-1 antibody and anti-CXCR4 caused inhibition of MSC recruitment (Kitaori et al., 2009). Some studies have demonstrated, in mouse models, that in the presence of tissue injury, the migration of MSCs to the area of injury, where SDF-1α was expressed, was increased (Abbott et al., 2004, Ji et al., 2004). It has been shown that tumour cells secreted soluble factors that caused MSCs to secrete SDF-1α that, in turn, activates their migration (Gao et al., 2009).

These studies indicate the importance of SDF-1α chemokine in the recruitment of MSCs.

1.9.2.2 Monocyte chemoattractant protein-1

During tissue injury, myofibroblasts, macrophages, and other active cells that are recruited to the injury site via chemokines work together with pro-fibrotic cytokines. Chemokines allow leukocytes to interact with fibroblasts by recruiting them into tissue across endothelial barriers (Hasegawa and Sato, 2008). One of the important chemokine signalling pathways that play a role in the recruitment of MSCs and fibrosis is the CC- and CXC-chemokine receptor families (Wynn, 2008). (Carulli et al., 2005, Distler et al., 2009, Ong et al., 2003) have demonstrated monocyte chemoattractant protein-1

(MCP-1) or CCL-2 and MCP-3 (CCL-7) as pro-fibrotic mediators. MCP-1 is responsible for recruitment of monocytes to the injury site and its receptor (CCR2) is expressed on the MSCs (Ringe et al., 2007, Ponte et al., 2007). *In vitro*, it has been shown that MCP-1 causes direct and indirect expression of collagen through endogenous signalling of TGF-β or IL-4 (Distler et al., 2006). Moreover, when MCP-1 was neutralized using anti-MCP-1 antibodies, a reduction in fibrosis resulted (Lloyd et al., 1997, Belperio et al., 2001). Similar results were obtained in mice deficient to chemokine receptor 1 and 2 (CCR1, and CCR2) confirming their important roles in fibrosis (Tokuda et al., 2000, Anders et al., 2002, Moore et al., 2001). It has been shown that the migration of human and mice BM-derived MSCs were enhanced by MCP-1 chemokine *in vivo* and *in vitro* (Boomsma and Geenen, 2012, Dwyer et al., 2007, Belema-Bedada et al., 2008). Using anti-CCR2 antibodies caused inhibition of MSC migration via blocking of CCR2 downstream signalling (Belema-Bedada et al., 2008). Conversely, studies by Ringe et al and Takano et al showed that MCP-1 had no significant migratory effect on BM-derived MSCs (Ringe et al., 2007, Takano et al., 2014). A study by Dwyer et al showed that MCP-1 secreted from breast cancer cells enhanced the migration effects of MSCs (Dwyer et al., 2007). Moreover, Klopp et al and Baek et al showed that cytokines and growth factors such as TGF-β, PDGF, and VEGF that were secreted from tumour cells and enhanced MSCs migration were increased post irradiation (Klopp et al., 2007, Baek et al., 2011).

These variations in the MSC migratory response to MCP-1 chemokine need an additional investigation to study the role of MCP-1 in MSCs homing.

Other chemokines are in **Table 1.1**.

Growth factors		
Ligands	**Receptors**	**Reference**
VEGF-A	VEGFR-1, -2	(Sorrentino et al., 2008)
PDGF-AA	PDGF-α	(Ponte et al., 2007)
PDGF-BB	PDGF-β	(Sorrentino et al., 2008)
EGF	EGFR	(Ponte et al., 2007)
TGF	TGFR	(Baek et al., 2011)
BMP-2, -4	BMPR-1a, -1b, -2	(Lavery et al., 2008)
HGF	HGFR	(Neuss et al., 2004)
IGF-1, -2	IGF-1R	(Ponte et al., 2007)
Ang-1	TIE-2	(Sorrentino et al., 2008)
FGF-2	FGFR-1, -2, -3, -4	(Walsh et al., 2000)
Chemokines		
MCP-1 (CCL2)	CCR2	(Ringe et al., 2007)
RANTES (CCL5)	CCR5	(Ponte et al., 2007)
MCP-2, MCP-3, MCP-4	CCR1	(Sordi et al., 2005)
CCL20 (MIP-3α)	CCR6 (CD196)	(Ringe et al., 2007)
CCL25 (TECK)	CCR9 (CDw199)	(Honczarenko et al., 2006)
CCL27 (CTACK), 28 (MEC)	CCR10	(Brooke et al., 2008)
CXCL12 (SDF-1)	CXCR4 (CD184)	(Ponte et al., 2007)
CXCL13 (BLC)	CXCR5 (CD185)	(Ringe et al., 2007)
Others		
HMGB-1	RAGE; TLR2,	(Meng et al., 2008)
LPA	LPA-1 (Edg2)	(Song et al., 2010)
TNF-α	TNFR1	(Croitoru-Lamoury et al., 2007)
Lipoproteins, peptidoglycans	TLR1 (CD281)	(Tomchuck et al., 2008)

Table 1.1 Growth factors, chemokines and other chemoattractant ligands and receptors expressed by human bone marrow MSCs.

Adapted from (Vanden Berg-Foels, 2014). VEGF, vascular endothelial growth factor; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; TGF, transforming growth factor; BMP, bone morphogenetic protein; HGF, hepatocyte growth factor; IGF, insulin-like growth factor; FGF, fibroblast growth factor; MCP, monocyte chemoattractant protein; RANTES, regulated on activation normal T-cell expressed and secreted; MIP, macrophage inflammatory protein; MDC, macrophage-derived chemokine; SLC, secondary lymphoid tissue chemokine; IL, interleukin; SDF, stromal cell derived factor; TNF, tumour necrosis factor; RAGE, receptor for advanced glycation end products; HMGB, high mobility group box; LPA, lysophosphatic acid; S1P, sphingosine 1-phosphate; TLR, Toll-like receptor.

1.9.3 Radiation and MSCs homing toward cancer

It has been shown that radiation induces the expression of many cytokines like EGF, pro-inflammatory cytokines and fibroblast growth factor (Wang et al., 1998). Moreover, in tissues exposed to high or low doses of ionizing radiation, there is early and persistent activation of TGF-β1 (Anscher et al., 1990, Wang et al., 1998). Klopp et al. studied the effects of radiation on the migratory ability of MSC. They showed, both *in vitro* and *in vivo*, that radiation enhanced MSC migration through inflammation cytokines and chemokines that released after tissue injury in response to radiation (Klopp et al., 2007). Furthermore, they were able to identify the chemokines and cytokines responsible for the recruitment of MSC toward irradiated tumours. They showed that after irradiation of the tumour cells, their ability to produce cytokines that recruit MSCs (like VEGF, TGF-β1, and PDGF) were increased (Klopp et al., 2007). Also, they found that the level of chemokine receptor CCR2 (MCP-1 receptor) was up-regulated in irradiated MSCs. Ren et al. demonstrated that blocking of CCR2 *in vitro* caused decreased MSC migration (Ren et al., 2012). These studies indicate that the post inflammation mediators were increased in response to irradiation, which enhanced MSCs migration toward tumour microenvironment.

However, there is still more to be studied about the molecular mechanisms behind the recruitment of MSCs to the tumour site post-irradiation, and whether MSCs play a key role in the fibrotic changes that occur post-irradiation via their differentiation into CAF-like cells.

1.10 Aims of the study

As described earlier, radiotherapy is one of the standard treatments for patients with intermediate and high-grade soft tissue sarcoma. Some studies have shown that radiation recruits MSCs to tumours (Klopp et al., 2007, Fenton and Paoni, 2007), but the mechanism behind this event is still unclear. MSCs can differentiate into CAFs, which is responsible for ECM secretion and deposits of collagen and other matrix proteins with subsequent fibrosis (Azorin-Vega et al., 2015). CAFs play a key role in cancer initiation, invasion and metastasis (Luo et al., 2015) and the exact origin of CAFs in irradiated tumours has not been established. In normal tissues, growth factors such as PDGF and TGF-β play a key role in the differentiation of myofibroblasts, so similar factors may be involved in response to radiation in the tumour.

The general hypothesis to test in this project is that radiation alters MSC protein expression and behaviour, either directly or indirectly via radiation effects on other cell types present in solid tumours. These alterations have the potential to play a significant role in radiation-induced changes within the TME linked to CAF-induced tumour progression.

The aims of this project were, therefore:

- Using *in vitro* models, determine whether radiation induces migration of MSCs and differentiation of MSCs into CAF-like cells.

- Determine the influence of irradiated tumour cells and/or endothelial cells on these processes, utilizing mouse fibrosarcoma cells (FS120 and FS188) previously shown to have differential effects on the composition of the TME, when propagated *in vivo* (Tozer et al., 2008)*.

- Investigate the role of specific cytokines/chemokines previously shown to be induced by irradiation of tumour cells on the above processes and determine the effect of irradiation on expression/secretion of these factors in FS120 and FS188 cells.

- Identify the differences between FS120 and FS188 tumour cells and solid tumours (i.e. *in vitro* and *in vivo*) that could potentially influence the recruitment /differentiation

of MSCs.

* FS188 tumour sections showed more positive cellular staining for α-SMA than FS120 tumour sections. A proportion of these α-SMA-positive cells were closely associated with blood vessels, suggestive of pericytes.

CHAPTER TWO:

Materials and methods

2.1 Materials

Reagents for cell culture	Supplier
Cells	
Balb/c Mouse Mesenchymal Stem Cells	Purchased from Cyagen Biosciences Cat. No. MUCMX-01001
C3H/10T1/2, clone 8 Mouse Mesenchymal Stem Cells	Purchased from ATCC® (ATCC® CCL-226™)
HDMEC (Human Dermal Microvascular Endothelial Cells)	PromoCell®
H5V mouse cardiac endothelial cell	A kind gift from Dr Annuciatta Vecchi (Garlanda et al, 1994, PNAS, 91)
Mouse fibrosarcoma cells	These cells were developed in our laboratory (Tozer et al., 2008).
Antibodies	
PDGF Receptor β Rabbit mAb (Cat. 3169)	Cell Signalling Technology®
Monoclonal Anti- β Tubulin mAb (Cat. T4026)	SIGMA-ALDRICH®
Anti-Actin, α-Smooth Muscle mAb (A 2547)	SIGMA-ALDRICH®
GAPDH (14C10) Rabbit mAb (Cat. 2118)	Cell Signalling Technology®
Anti-Mouse Collagen Type I pAb (Cat.AB765P)	Millipore®
Anti-TGF beta antibody pAb (ab 66043)	Abcam®
Anti-Fibronectin antibody pAb (ab 2413)	Abcam®
MCP-1 Antibody pAb (Cat. 2029)	Cell Signalling Technology®
Mouse TGF-β2 Antibody mAb (Cat. MAB73461)	R & D systems®
Purified Rat Anti-Mouse CD31 (Cat. 553370)	BD Pharmingen ™
Anti-TGF beta 1 antibody pAb (ab 155264)	Abcam®
Anti-alpha smooth muscle actin antibody mAb (ab 124964)	Abcam®
Anti-CCR2 antibody mAb (ab 203128)	Abcam®
Anti-CTGF antibody pAb (ab 6992)	Abcam®
SDF1 Antibody pAb (Cat. 3740)	Cell Signalling Technology®
Recombinant Murine JE/MCP-1 (CCL2) (Cat. 250-10)	Peprotech®

Recombinant Murine SDF-1α (CXCL12) (Cat. 250-20A)	Peprotech®
Recombinant Human TGF-β2 (Cat. 100-35B)	Peprotech®
Recombinant Human TGF-β1	PeproTech®
DuoSet® ELISA kits	
Mouse CCL2/JE/MCP-1 Antibody (Cat. AB-479-NA)	R & D systems®
Normal Goat IgG Control (Cat. AB-108-C)	R & D systems®
Goat Anti-Mouse JE Capture Antibody (Cat. DY479-05)	R & D systems®
Biotinylated Goat Anti-Mouse JE Detection Antibody (Cat. DY479-05)	R & D systems®
Recombinant Mouse JE Standard (Cat. DY479-05)	R & D systems®
Wash buffer (Cat. WA126)	R & D systems®
Reagent Diluent (Cat. DY008)	R & D systems®
Streptavidin-HRP (Cat. DY479-05)	R & D systems®
Colour Reagent A & B (Cat. DY999)	R & D systems®
Coating buffer (Cat. DY479-05)	R & D systems®
Stop solution (Cat. DY994)	R & D systems®
Masson's trichrome reagents	
Weigert`s Iron Hematoxylin Set	Sigma-Aldrich®
Ponceau Xylidine	Sigma-Aldrich®
Light Green SF Yellowish	Sigma-Aldrich®
Acid Fuchsin	Sigma-Aldrich®
Phosphotungstic acid solution	Sigma-Aldrich®
Phosphomolybdic acid hydrate	Sigma-Aldrich®
Western blotting, IHC and IF materials	
Cell Extraction Buffer (Cat.FNN0011)	Invitrogen®
Ultra Pure ProtoGel®	National Diagnostics
ProtoGel® Resolving Buffer	National Diagnostics
ProtoGel® Stacking Buffer	National Diagnostics
Ammonium persulfate	Sigma-Aldrich®
M.O.M ™ Immunodetection kit	Vector®
Nitrocellulose membrane	Hybond® ECL™

PVDF membrane	Immobilon®-P Polyvinylidene difluoride membranes
Thick blotting paper	Invitrogen™
Laemmli Sample Buffer (4X)	Bio-Rad
NuPAGE™ Sample Reducing Agent (10X)	Fisher Scientific
Centrifugal filter tube amicon Ultra-4 ultracel 3KD	Millipore®
Bovine serum albumin	Sigma-Aldrich®
Immun-Blot® PVDF Membranes for Protein Blotting	Bio-Rad
Nitrocellulose Membranes, 0.2 µm	Bio-Rad
Dimethyl sulfoxide	Sigma-Aldrich®
Texas Red® Streptavidin (Cat. SA-5006)	Vector®
Goat Anti-Rat IgG H&L (FITC) (ab 97056)	Abcam®
Precision Plus Protein™ Standards	Bio-Rad®
TEMED	Sigma-Aldrich®
Tween 20	Bio-Rad
Isopropanol	Fisher Scientific
Ethanol 96% vol	Fisher Scientific
Methanol	Fisher Scientific
Triton X-100	Fisher Scientific
Bovine serum albumin	Sigma-Aldrich®
Glycerol	Fisher Scientific
DAB	Vector Laboratories
Avidin–biotinylated enzyme complex (ABC)	Vector Laboratories
Cell culture reagent	
Dulbecco's Phosphate Buffered Saline	BioWhittaker®
Cell culture insert 8.0 µm	Falcon®
Multiwell 24 well	Falcon®
Cell Scraper	Fisherbrand®
DMEM	BioWhittaker®
HDMEC growth media (Endothelial cell medium MV Catalogue No. C-39220)	PromoCell®
Minimum essential medium eagle	Sigma-Aldrich®

2.2 Methods

2.2.1 Cell culture

2.2.1.1 Cell lines

All cells were cultured and subcultured using pre-warmed (37°C) growth media, PBS and trypsin. A biosafety cabinet was used to perform all cell culture work.

A) C3H/10T1/2 Clone 8 (ATCC® CCL-226™) cell line

C3H/10T1/2 cells subsequently referred to as C3H10 cells, are a multipotent mesenchymal stem cell line derived from C3H mouse embryos (Reznikof.Ca et al., 1973), often used in research as a model of differentiation *in vitro*. The morphology of these cell lines is fibroblast-like; they are flat and polygonal with long cytoplasmic processes (**Figure 2.1**). These cells grown in the following medium: Eagle's Minimum Essential Medium (EMEM) that is modified to contain Earle's Balanced Salt Solution, non-essential amino acids and 1500 mg/L sodium bicarbonate (Sigma M5650). 500 ml of Sigma M5650 MEM was supplemented with 2mM L-glutamine (5 ml glutamine aliquot), 5 ml Penicillin/Streptomycin, 1mM sodium pyruvate (5 ml of 100 mM solution Sigma S8636), and 50 ml FCS (final concentration of 10%). It is important to trypsinise and subculture C3H10 cells before they become confluent otherwise they can start differentiation.

B) OriCell ™ Strain Balb/c Mouse Mesenchymal Stem Cells

Subsequently referred to as Balb/c MSCs, these cells are multipotent mesenchymal stem cells that derived from Balb/c mouse BM. They have the ability to differentiate into different types of cells such as chondrocytes, osteocytes, and adipocytes (Prockop, 1997) (**Figure 2.2**). Moreover, these cells have abilities to migrate to the injury-site of tissue like skin, heart and kidney (Li et al., 2006, Wu et al., 2003, Morigi et al., 2004) For regular use, Balb/c MSCs were cultured in Eagle's Minimum Essential Medium (Sigma, M8042), 20% foetal bovine serum supplemented with the penicillin-streptomycin mixture, and L-glutamine (Klopp et al., 2007).

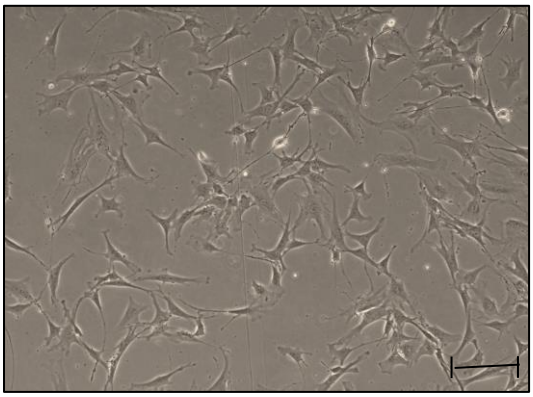


Figure 2.1 Early passage C3H/10T1/2 Clone 8 in their media.

They are flat and polygonal with long cytoplasmic processes. Also they have fibroblasts-like morphology.

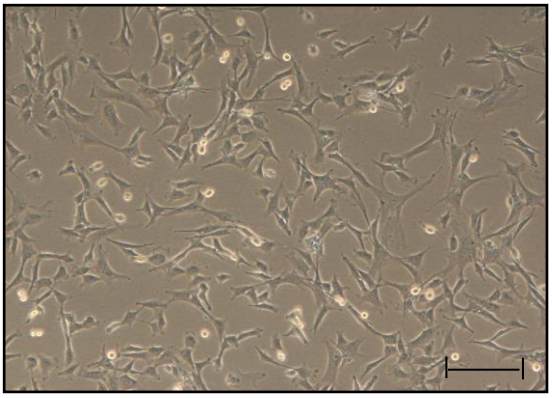


Figure 2.2 Balb/c Mouse Mesenchymal Stem cells, BM- derived cells passage 9

C) Mouse fibrosarcoma cells

Mouse fibrosarcoma (FS) cells that express single isoforms of VEGF, either VEGF 188 or VEGF 120 were used. These cells were developed in our laboratory through separation of primary mouse embryo fibroblasts that express a single isoform of VEGF (VEGF 120, or VEGF 188) from mouse embryos. These fibroblasts were genotyped, immortalised, and transformed to cancer cells using retroviral transduction with Simian Virus 40 HRAS. The tumour cell lines (fibrosarcoma) which resulted were sustained in a medium made of DMEM (high glucose), L-glutamine, FCS, and the antibiotics puromycin, and G-418 (Tozer et al., 2008).

In vivo, fibrosarcomas that express VEGF 188 have been shown to recruit more pericytes and CAFs than fibrosarcoma cells which express VEGF 120 (Tozer et al., 2008). Moreover, Kanthou et al have shown that tumour cell expression of VEGF 188 is linked to increasing apoptosis levels and slower proliferation rate compared with tumour cells expressing VEGF 120, proliferate rapidly and have increased survival (Kanthou et al., 2014).

Kanthou and colleagues also showed that FS188 cells display typical mesenchymal features (spindle-shaped cells with extended processes and ruffles) compared to FS120 cells, which display less mesenchymal features (a mixture of rounded-elongated cells with less extended processes) (Kanthou et al., 2014). Recently, English et al. (2017) showed (in mouse) that tumour cells expressing VEGF 120 (FS120) metastasised to the lungs and produced lung colonies more than tumour cells expressing VEGF 188 (FS188). Likewise, these FS120 tumour cells that metastasised to the mice lungs showed more sensitivity to anti-VEGFA therapy than that FS188 (English et al., 2017). These features made these cells good models to study the indirect response of MSCs to radiation, as each line potentially provides a different array of growth factors affecting the differentiation of MSCs to CAF-like cells.

The culture media for these cells was DMEM (high glucose), supplemented with Foetal calf serum (FCS) final concentration 10%, 5 ml Glutamine (stock 200 mM; final concentration 2mM), 3ml G-418 (stock 100 mg/ml; final concentration 600 μg/ml), and 1 ml puromycin (stock 1mg/ml; final concentration 2 μg/ml).

D) H5V (mouse cardiac endothelial cells)

H5V are immortalized transformed endothelial cells which were derived from hearts of C57BL/6 mouse and were a kind gift from Dr Annuciatta Vecchi (Garlanda et al., 1994). They were grown in DMEM (Dulbecco's Modified Eagle's Medium) with L-glutamine (2mM), 10 % FCS, Streptomycin (100µg/ml), and penicillin (100U/ml) (**Figure 2.3**).

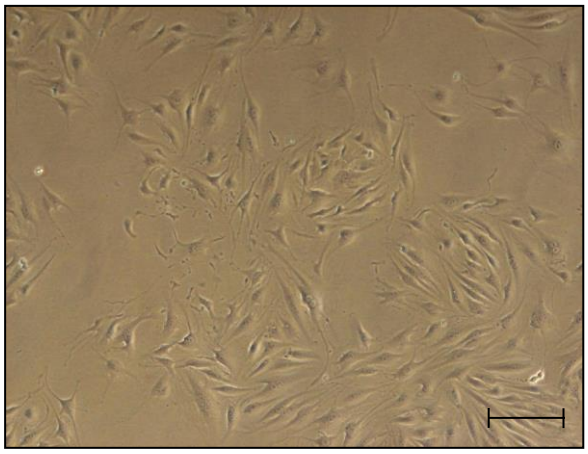


Figure 2.3 Mouse cardiac endothelial cells (H5V)

E) Human Dermal Microvascular Endothelial Cells (HDMEC)

HDMEC are primary microvascular endothelial cells of the blood and lymphatic origin derived from human adult skin (PromoCell Catalogue number C-12210). They express vascular endothelial growth factor (VEGF), which plays an important role in angiogenesis, cell migration and vasculogenesis (Nor et al., 1999, Bernatchez et al., 1999) (**Figure 2.4**).

HDMEC were used up to passage 10 and were grown in their media (Endothelial cell medium MV Catalogue No. C-39220). The MV growth medium supplemented with 10 %FCS-DMEM: (Dulbecco's Modified Eagle's Medium) supplemented with 10 % FCS, 2mM L-glutamine, 100 U/ml penicillin and 100 µg/ml Streptomycin.

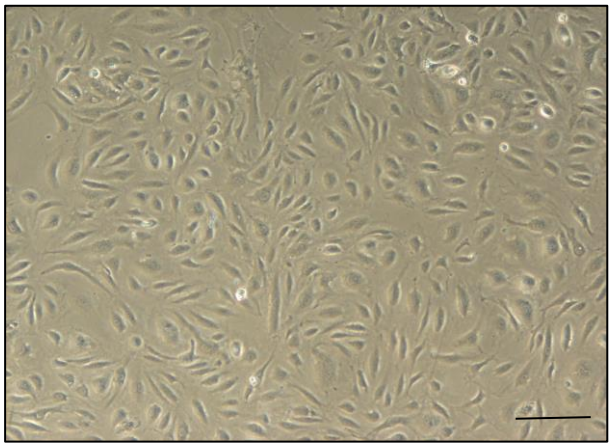


Figure 2.4 Human Dermal Microvascular Endothelial Cells (HDMEC) passage 5

2.2.1.2 Freezing cells in liquid nitrogen

For additional experiments, early passage and healthy cells were frozen using liquid nitrogen. Before freezing cells, their media were changed the day before, in order to ensure that the cells would be healthy.

First, the freezing solution (10% DMSO) was prepared by adding 1ml of DMSO to 9 ml of the full fresh medium. Then, the freezing solution was mixed and filtered by 0.22-μm filters, before placing on ice. The early passaged cells (75-80% confluent) were washed with PBS, trypsinsed and centrifuged. The pellet was suspended in a suitable volume of ice-cold freezing medium (10^6 cells/ml). Thereafter, cells were aliquoted in 1ml aliquots and placed in pre-labelled cryovials. The vials were labelled with cell type, a number of passage, and date. After that, the cryovials were placed immediately into a freezing container (Mr Frosty™, Thermo Fisher Scientific™) which was transferred to -80°C freezer overnight. The cryovials were then stored in liquid nitrogen.

2.2.1.3 Thawing cells

Cryovials were removed from liquid nitrogen and thawed quickly in a 37°C water bath. After complete thawing of the cells, the outside of the cryovial was disinfected using 70% ethanol. The cells were then transferred to a T-75 flask that contains a cell-growing medium. The flask was incubated at 37°C in a 5% CO2 humidified incubator. To remove the effects of DMSO, the medium was changed with fresh, pre-warmed medium the next day. After the confluence of the cells reached 80-90%, they were trypsinised, aliquoted (1 x 10^6 cells/mL) and stored in liquid nitrogen (1x 10^6 cells/ml) for long-term use.

2.2.1.4 Cell counting

Cell counting is a critical step to determine the number of cells within a given volume of a sample. A haemocytometer was used as an accurate tool to calculate cell number. Trypan blue was used to colour the dead cells since live cells have intact cell membranes and exclude the dye. 1:1 ratio was used (one volume cell suspension to one volume Trypan Blue) to be loaded into the haemocytometer chamber. Subsequently, the cells were counted inside four outer squares plus the central striped square using a Nikon phase contrast microscope (**Figure 2.5**).

In order to calculate the total number of cells/ml:

$$\text{Total number of viable cells counted/square} \times \frac{\text{Dilution Factor (2)}}{\text{Number of squares}} \times 10^4$$

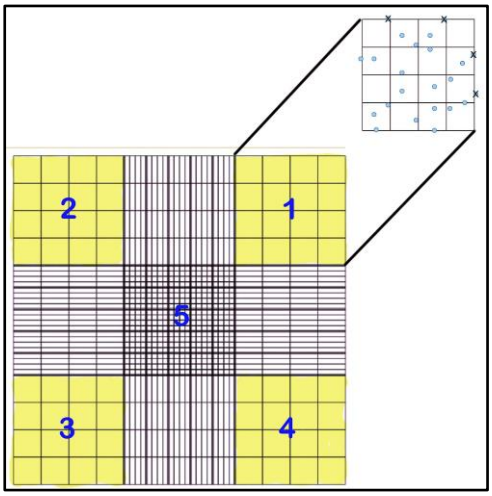


Figure 2.5 The Counting chamber of haeocytometer (grid layout). were counted in 1, 2, 3, 4 and 5 squares. Cells touching the outer lines on two sides of each square were excluded from counting. (Adapted from The Hemocytometer, 2010).

2.2.1.5 Sub-culturing of the cells

In order to prevent overgrowth of cells, the cells were subcultured every 3 days. Cell flasks were maintained in the incubator with routine daily observation to evaluate the density of the cells. When the cells reached ~80% confluence, they were subcultured. For routine sub-culturing of the cells, their media were changed by discarding the old medium and washing the monolayer with PBS twice. In order to dislodge cells from the flask surface, a pre-warmed trypsin solution was added to the cells before incubating the cells for 3-4 minutes. Next, when the cells were dislodged completely from the flask, they were collected in a 5 ml tube that contained a pre-warmed full medium. The cells were syringed to make them a single-cell suspension before they were counted by either haemocytometer (see above), or by a ViCell cell counter machine (Bio-Rad TC20™ Automated Cell Counter). Then, cells were subcultured to a new T25 flask by adding 25x 10^4 cells to 5 ml pre-warmed fresh medium and placed in the incubator.

2.2.1.6 *In vitro* irradiation of the cells

All cells were irradiated using an AGO 250 kV X-ray machine (Model No. CP160/1, Gulmay LTD) (**Figure 2.6**).

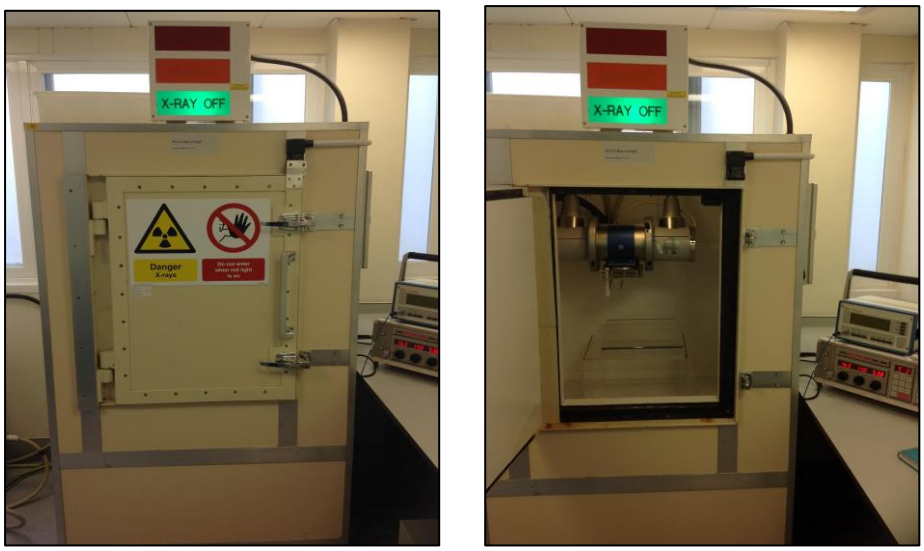


Figure 2.6 The AGO X-Ray machine and its chamber

Each sample of cells in the T25 flask was positioned at 40 cm distance from the radiation source. Cells were irradiated at a dose rate of 537.6 mGy per minute (200kV and 12.8 mA). Different radiation doses were used that ranged from 0.5 to 4 Gy (**Table 2.1**).

Table 2.1 The radiation dose used by AGO X-ray machine

Kv	mA	Dose (Gy)	Time (minutes)
200	**12.8**	**0.5**	**0.7**
200	**12.8**	**1**	**1.5**
200	**12.8**	**4**	**6**

2.2.1.7 Collection and concentration of conditioned media (CM)

Tumour cells (FS120 cells and FS188 cells) were plated in T75 flasks (5 x 10^5 /ml) and when they reached 80% confluence the cells were washed carefully with PBS twice (2X) and with pre-warmed serum-free medium three times (3X).

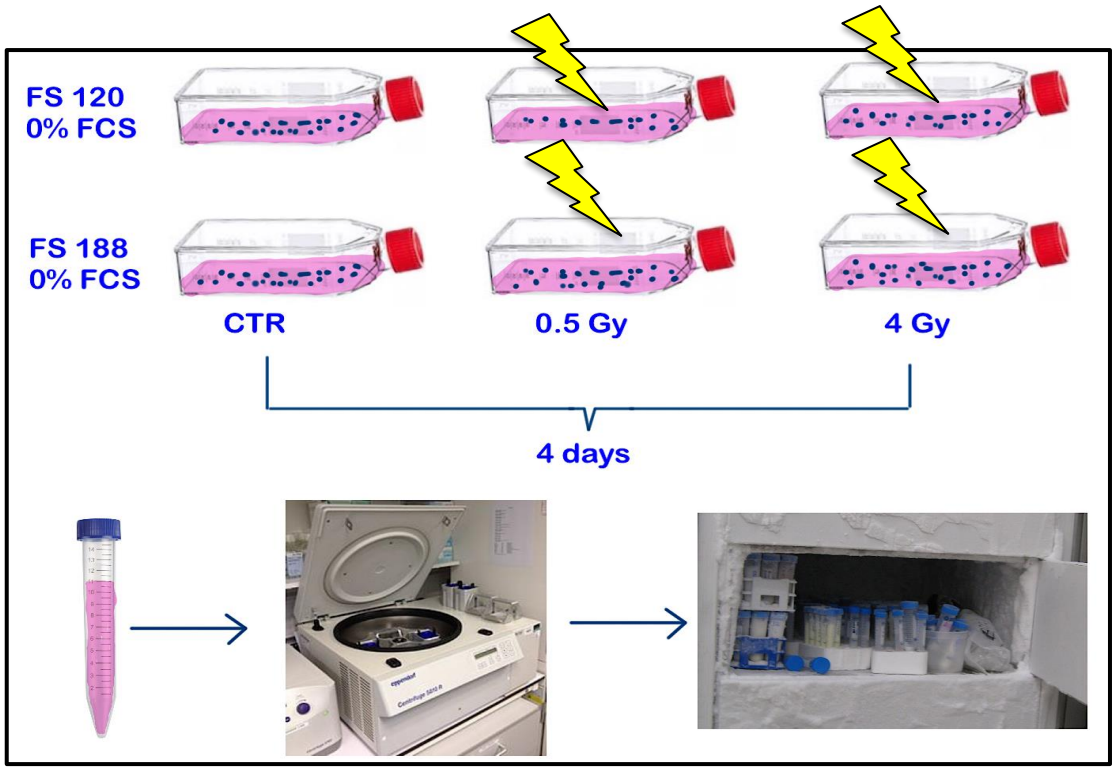
Then the cells were incubated with serum-free medium for 2-3 hours in the incubator before irradiation. After irradiation of the cells with 0, 0.5 Gy, or 4 Gy, they were incubated as above. 3-4 days' post-irradiation, the conditioned media were collected and cells were counted (as described above). In order to eliminate any debris or dead cells, the CM was centrifuged at 2000 r.p.m. for 5 minutes and the supernatant stored at -80 ^{o}C. The CM was normalised according to the counted cells as follow:

$$\frac{\text{lowest cell number value}}{\text{cell number value}} \text{ X CM amount in } \mu l$$

Therefore, if we decided to use 200 µl as a total volume, the amount of CM needed will be 130 µl, see an example below:

Total amount	Laemmli buffer (4X)	Reducing agent (10X)	CM amount
200 µl	50 µl	20 µl	130 µl

To concentrate the CM, 4 ml of CM sample were added to an Amicon Ultra-4 ultracel centrifugal filter tube (3 KD, Millipore®). The device was spun at 4000 r.p.m. for 30 minutes. The concentrated solute (1 ml) collected in the filter was extracted and stored at -80 ^{o}C (**Figure 2.7**).



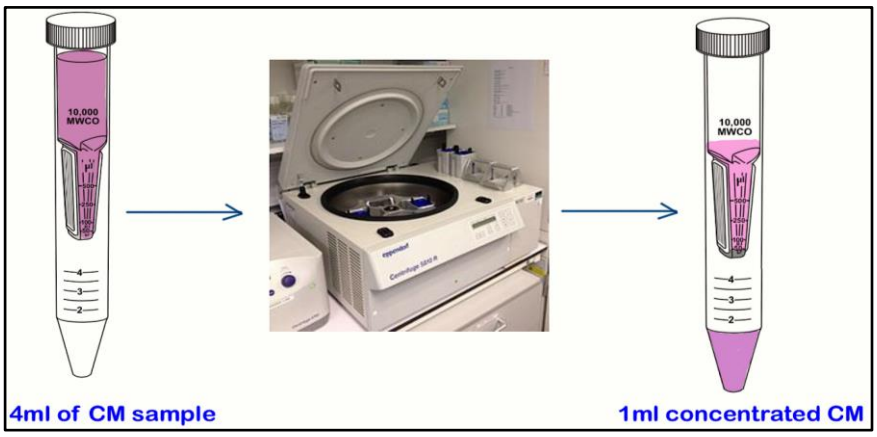


Figure 2.7 Schematic representatives of concentration of CM. First, tumour cells were plated in T75 flask. When the cells reach 80 % confluence, they were washed with PBS (2X), and pre-warmed serum-free medium (3X). 3 hours later, the cells were irradiated and incubated for 4 days in serum-free medium. Subsequently, CM was collected and centrifuged at 2000 r.p.m. for 5 minutes and stored at -80 ^{o}C. Later, 4 ml of CM sample were added to Amicon Ultra-4 ultracel centrifugal filter tube (3 KD). The device was spun at 4000 r.p.m. for 30 minutes. The concentrated solute (1 ml) collected from the filter was extracted and stored at -80 ^{o}C.

2.2.2 Clonogenic assay

The aim of doing this *in vitro* biological technique is to study the radiation effects on the proliferation and survival of tumour cells (fibrosarcoma), and MSCs (Balb/c & C3H10 cells). First, cells were plated in several T-25 flasks at a density of 250,000 cells per flask. When the cells became almost confluent (80%), their media were removed and changed with a pre-warmed medium to 37°C in a water bath. Then, the cells were incubated for one hour before irradiation with 0, 0.2, 0.5,1, 2 and 4 Gy. Afterwards, the cells were returned to the cell culture incubator until ready to start the clonogenic assay (about one hour). Cells were trypsinised using 1ml trypsin and re-suspended in 4 ml of full serum-containing medium. The cell suspension was then syringed using 21 gauge needles to achieve a single-cell suspension. Viable cells per ml were counted using a haemocytometer (as described earlier).

Because there were differences in the smallest number of cells required to form colonies and to survive after irradiation between each cell line, different cell numbers were used for each radiation dose (**Table 2.2**). The cells were plated in 6-well plates, then the plates were left in the incubator at 37°C without moving them for 10-14 days. One 6-well plate was used for each radiation dose per experiment (triplicate for each group).

Table 2.2 Cells number per radiation dose used for clonogenic assay

	0 Gy	0.2 Gy	0.5 Gy	1 Gy	2 Gy	4 Gy
FS120 cells & FS188 cells	50/100		50/100	100/150	150/200	200/400
C3H10 MSCs	50/100	100/150	100/150	150/200	150/200	300/400
Balb/c MSCs	50/100	100/150	100/150	150/200	150/200	300/400

It is important not to do any movement of the plates during these 10-14 days, as any movement may disturb colony formation through dislodging them.

After 10-14 days, and when the colonies were formed, the plates were taken out from the incubator and their media were removed. The colonies were fixed in 100% iced cold methanol in a freezer for 15 minutes. Subsequently, the methanol was removed and the cells were stained with 0.25% crystal violet in 25% methanol. The stain was left for 10 minutes before it was washed off in tap water, and the plates were left to dry at room temperature to be ready for counting the next day manually (**Figure 2.8**).

Lastly, all colonies (a colony is a group of approximately 50 cells or more) in all plates were counted manually. Plating efficiency (PE) with survival fraction (SF) was calculated using the following equations:

$$\mathrm{PE} = \frac{\text{Number of colonies formed}}{\text{Number of cells seeded}} \text{ X } 100$$

$$\mathrm{SF} = \frac{\text{PE after irradiation}}{\text{PE of control (0Gy)}}$$

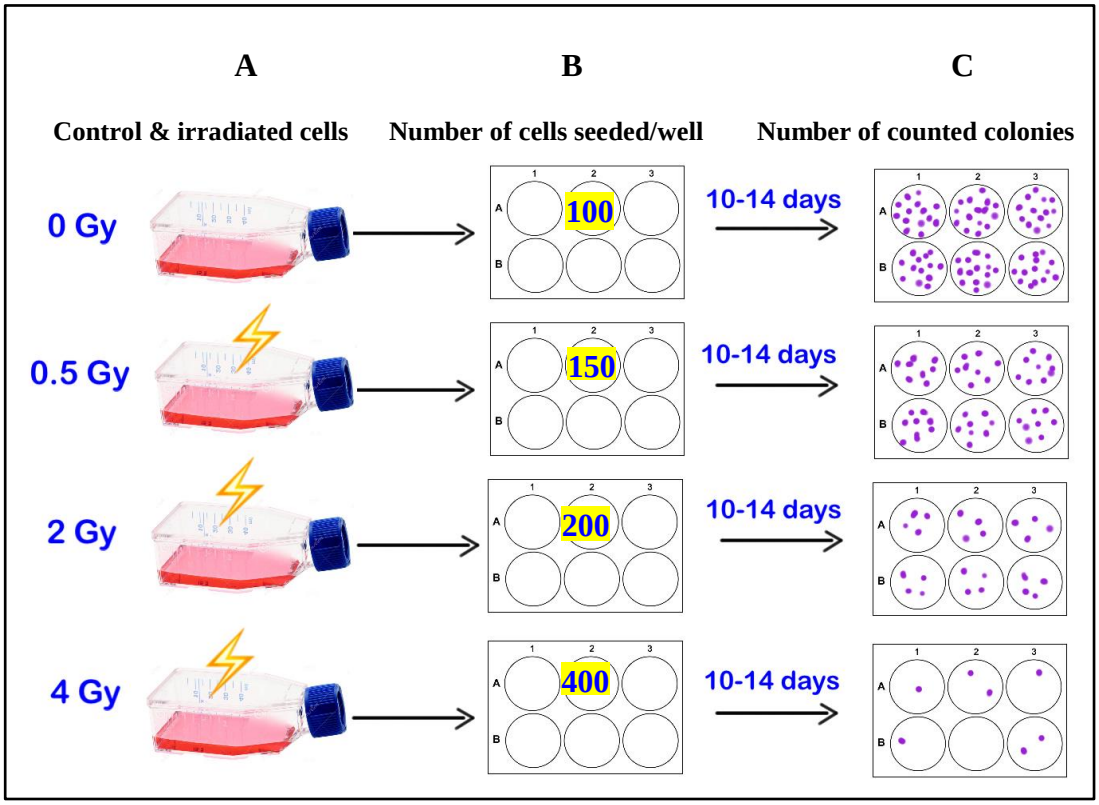


Figure 2.8 Diagram of clonogenic assay steps: The confluent cells (~80%) inside T-25 flasks were irradiated (except for 0 Gy as a control) with different radiation doses. **B**- After irradiation, the cells were trypsinised, and different cells number were seeded in each well of the 6-well plates. The plates were incubated for 10-14 days (without movement or changing media). **C**- After 10-14 days, and when the colonies were formed, the colonies were fixed in 100% iced cold methanol in a freezer for 15 minutes. Subsequently, methanol was removed and the cells were stained with 0.25% crystal violet in 25% methanol. The stain was left for 10 minutes then washed off in tap water, and the plates were left to dry at room temperature. A colony is described as 50 cells or more.

2.2.3 Growth Curve assay

The viability and proliferation of the cells can be determined through counting of the cells. In order to measure the growth rates of cells, Balb/c MSCs and C3H10 MSCs were plated individually at a density of 3x 10^4 cells/well in triplicate at 6-well plate (4 plates were used). Three wells of each plate were treated with TGF-β1 (human TGF-β 1, Cat. No.100-21). The viability of the cells was evaluated on a daily basis. Each day, one plate was rinsed twice with PBS after removal of the medium. Then, the cells were trypsinised, and collected in a tube, centrifuged for 5 minutes at 1000 rpm. Next, media from the pellet were removed and the full medium was added to the pellet, mixed well and cells were counted.

2.2.4 Radiation effects on MSC differentiation

2.2.4.1 Irradiation of cells

Cells were plated in two wells of 6 well-plates. The cells were left in the incubator for 3 days to become ~80% confluent. One hour before radiation, the medium was changed to a pre-warmed medium. After that, the plates were irradiated (as described above, **2.2.1.5**) with different radiation doses (0-4 Gy). The plates were placed in the incubator 3-4 days' post-irradiation.

2.2.4.2 Collection of cell extracts and sample preparation for western blotting

Protease inhibitors and working on the ice were used to minimise protein degradation. To extract proteins from cells, the monolayer of the cells was washed twice with ice-cold PBS. The cold PBS was pipetted to the side of the well without detaching the cells. After that, tilting the plate and aspirating any traces of PBS from the corner. The cells in each well were lysed with an appropriate volume of ice-cold cell extraction buffer (Cat. No. FNN0011, Invitrogen®) that contained 10 mM Tris, 100 mM NaCl, 1 mM EDTA, 1 mM NaF, 20 mM NaPO, 2 mM NaVO, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate. I supplemented this cell extraction buffer with protease inhibitor and 1 mM PMSF before use.

The volume of lysis buffer used to lyse the cells depending on the size of the plate used and how dense the cells were. 250 µl was used for each well of a six-well plate. To prepare the lysis buffer, a complete protease inhibitor tablet (Roche) was dissolved in 200 µl of PBS. Then, 100 µl of the dissolved complete tablet was added to 5 ml of lysing buffer that was inside a tube in the ice. The rest of the dissolved tablet stored at -20 ^{o}C to be used later.

PMSF (Phenyl Methyl Sulfonyl Fluoride) stock was a 0.3 M solution in DMSO and is an irreversible inhibitor of serine proteinases. For every 5 ml of the total, 17 µl was added to lysing buffer tube (final concentration ~1 mM). This ice-cold cell extraction buffer was kept on the ice and used within 30 minutes of preparation to lyse the cells (**Figure 2.9**). The cell monolayer was scraped using a cell scraper and the lysates were collected in pre-cooled Eppendorf tubes. Tubes were then centrifuged for 10 minutes at 10,000 rpm in a micro-centrifuge after it was cooled down to 4^{o}C. Supernatants were aliquoted for each sample into three Eppendorf tubes and immediately stored in -80^{o}C freezer.

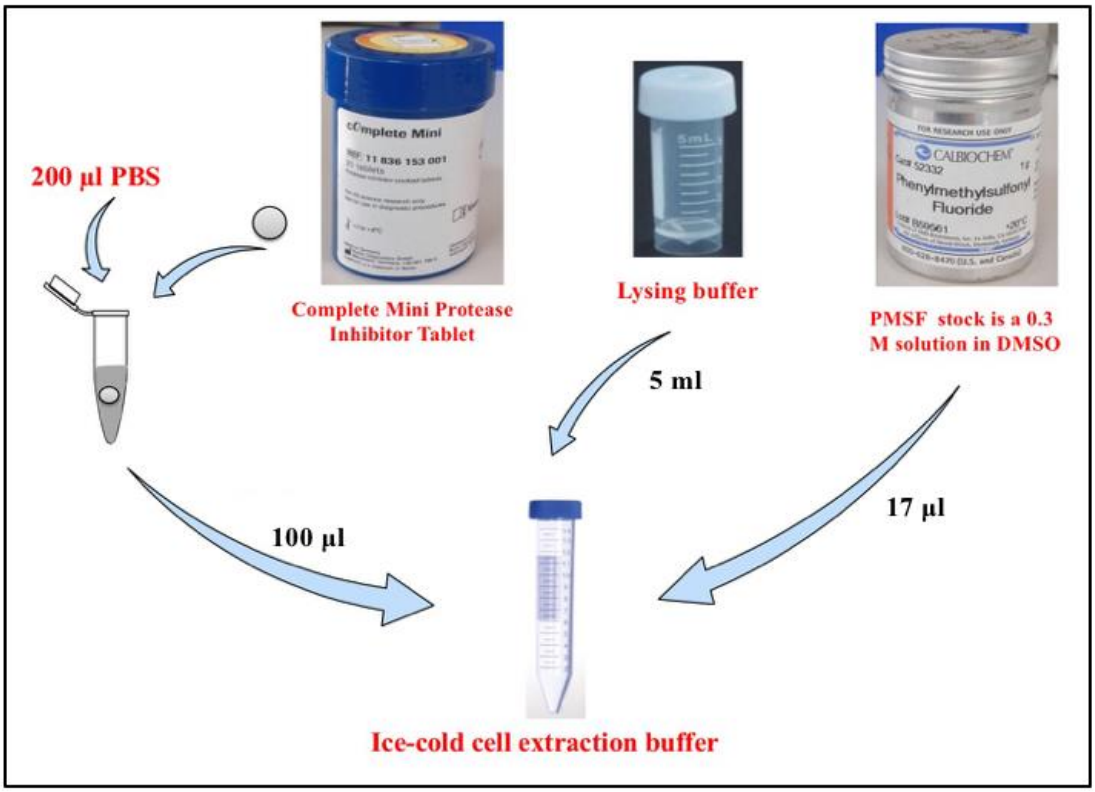


Figure 2.9 Schematic demonstrative of cell extraction buffer preparation.

5 ml lysis buffer was added to the tube on the ice (total volume of the buffer depends on the size of plate was used and how dense the cells were). After that, a Complete Mini Protease Inhibitor Tablet was dissolved in 200 µl PBS (each tablet is enough for 10 ml = 200 µl→ 10 ml), and 100 µl was taken and added to the tube. Finally, 17 µl from PMSF stock was added to the mixture and put on ice.

2.2.4.3 Pierce Micro BCA™ Protein Assay

The Thermo Scientific™ Micro BCA Protein Assay Kit (Product No. 23225) is a detergent-compatible bicinchoninic acid (BCA) formulation for the colourimetric detection and quantitation of total protein. The principle of this assay is the detection of cuprous ion (Cu^{+1}) through BCA reagent to form a purple-coloured solution. Cu^{+1} is formed by reduction of cupric ion (Cu^{+2}) by protein in an alkaline environment (Smith et al., 1985). This water-soluble complex exhibits a strong absorbance at 562 nm that is linear with increasing protein concentrations. From the provided stock solution of standard BSA that is 2 mg/ml, a 1:10 dilution in dH_2O was done to obtain a stock of 0.2 mg/ml (or 200 µg /ml) (100 µl stock plus 900 µl dH_2O).

Further standards of 20, 15, 10, 7.5, 5, 2.5 and 0 µg/ml were set up (**Table 2.3**).

The unknown samples were prepared by diluting into 1 ml of dH2O

- 10 µl sample + 990 dH_2O (1:100)
- 5 µl sample + 995 dH_2O (1:200)

Further dilutions were prepared if the readings were not on the standard curve. 150 µl of standards and diluted samples were put into wells of a 96 well plate. Triplicates of each were used. 150 µl of working reagent, prepared following the manufacturer's instructions, was added to each well and mixed. The 96 well-plates was covered with the lid and incubated at 37°C for 2 hours. The absorbance was measured at 562 nm using a spectrophotometer. Linear regression analysis was done using GraphPad Prism software to interpolate concentrations of protein samples from BSA standards.

Table 2.3 BCA standard preparations

Standard (µg/ml)	µl of 200 µg /ml stock	µl dH2O
0	0	1000
2.5	12.5	987.5
5.0	25	975
7.5	37.5	962.5
10	50	950
15	75	925
20	100	900

2.2.4.4 SDS-PAGE and western blotting

Western blotting, also known as immunoblotting or protein blotting, is an important technique in cell and molecular biology. It is used to detect the presence of a specific protein in a complex mixture extracted from cells.

In order to analyse equal amounts of protein by western blotting, the samples that were already quantified by BCA protein assay were kept on ice and prepared in sample buffer as described below. So the aim is to make each sample contain the same amount of total protein in a similar volume and a final concentration of 1X sample buffer. The stock Laemmli sample buffer was 4X (Laemmli Sample buffer, Biorad, USA, catalogue #161-0747). The reducing agent (NuPAGE® Sample reducing agent 10X, Cat. No. NP0005) was added and the remaining volume was made up with dH_20.

Single-use cassettes (Invitrogen™) and Handcasting Systems (Bio-Rad) were used for casting gels. Each cassette takes approximately 10 ml of gel volume. For large molecular weight proteins (>100 kDa), a low percentage acrylamide gel (8%) with large pores was used. While for smaller proteins (< 20 kDa), a higher percentage gel (12%) was used. The reagents used are shown in (**Table 2.4**).

Table 2.4 Reagents used for casting gels in western blotting	
ProtoGel 30% (acrylamide with bis-acrylamide)	National Diagnostics, Geneflow, UK
10% glycerol solution	Made by measuring out 10 ml of glycerol using a graduated cylinder and adding 90 ml of dH_20
Resolving buffer	4X concentrate, National Diagnostics, Geneflow
Stacking gel buffer	4X concentrate, National Diagnostics, Geneflow
10% Ammonium persulphate APS	SIGMA-ALDRICH®
TEMED (Tetramethylethylenediamine)	SIGMA-ALDRICH®
Isopropanol	SIGMA-ALDRICH®, UK

A gel cassette was placed upright on a rack and the gel solution was prepared by adding the ingredients in the order given in **Table 2.5**. The APS was added first and the TEMED was the last and immediately the solution was mixed gently then poured carefully into the cassette leaving enough space for the stack.

The stacking gel was then prepared according to the recipe in **Table 2.5** and poured. Immediately the comb was put in the slot to form wells and left for 30 minutes at room temperature to polymerize.

Table 2.5 Different gel and ingredients concentrations used in experiments for a total 10 ml gel (single gel)				
	8%	**10%**	**15%**	**Stack**
Protogel	2.7 ml	3.3 ml	5 ml	1.3 ml
dH2O	4.7 ml	4.1 ml	2.4 µl	6.1 ml
10% glycerol	100 µl	100 µl	100 µl	50 µl
Resolving buffer	2.5 ml	2.5 ml	2.5 ml	
Stacking buffer				2.5 ml
10% APS	50 µl	50 µl	50 µl	50 µl
TEMED	5 µl	5 µl	5 µl	10 µl
Total volume	**10 ml**	**10 ml**	**10 ml**	**10 ml**

The reagents used for loading the gels were as in **Table 2.6**.

Table 2.6 Reagents used for loading the gels in western blotting	
Tris/Glycine/SDS PAGE Buffer	10 X, National Diagnostics, Geneflow, UK
Sample buffer	4X Laemmli Sample buffer, Biorad, USA, catalog #161-0747
Reducing agent	NuPAGE® Sample Reducing Agent (10X), Cat. No. NP0005
Protein molecular weight markers	Precision Plus Protein™ Prestained Standards BioRad, USA, Cat. #161-0374

The gel cassette was assembled in the tank and 600 ml 1X Tris-Glycine-SDS running buffer was added to the outer chamber (prepared from a 10X stock by diluting 1:10, ie 100 ml buffer plus 900 ml dH_2O). 20 μg of protein samples were loaded into each well and a similar volume (30 μl) of 1X Laemmli reducing sample buffer was also loaded in the unused wells. 6 μl of Precision plus protein™ standards (Bio-Rad) was mixed with 24 μl of 1X Laemmli LDS reducing sample buffer and this was loaded in the last well.

The gel was run at 150 V (constant voltage). The running time was around 60 minutes until the bromophenol blue dye has reached the bottom of the gel. After running, proteins on the gel were transferred to a PVDF or nitrocellulose membrane using Bio-Rad transfer machine (Trans-Blot® Turbo™ Transfer System). The transfer was done according to the manufacturer`s instructions.

One time transfer buffer was prepared by adding 100 ml Tris Glycine 10X transfer buffer to 700 ml dH_20 and 200 ml methanol to make up to 1 Litre. The flat gel placed in a dish containing 10-20 ml of cold 1X transfer buffer. PVDF membrane needs to be soaked in methanol first for 3-5 minutes.

Electro-blotting was for 30 minutes at 150 volts. After the transfer of proteins was finished, the membrane placed (protein side up) in TBS containing 0.1% Tween-20 (TBS-T) buffer.

The membrane incubated with PBS-T or TBS-T buffer containing blocking agent, which was 5% dried milk and was prepared by dissolved 2.5 g dry-powdered milk in 50 ml of TBS-T. This was used as a blocking solution and an antibody-binding buffer (5% milk solution).

The membrane was blocked for one hour at room temperature on a shaker. The blocking agent removed and the antibody was added. The antibodies concentrations were used as in **Table 2.7**.

Table 2.7 Primary antibodies used in western blotting					
Primary antibody	**Molecular Weight**	**Dilution**	**Catalogue No.**	**Secondary antibody**	**Gel concentration**
Anti-Fibronectin	285 kDa	(1:2000) in 5% non-fat milk in TBS-Tween	(ab2413)	Anti-rabbit	8%
Anti-Mouse Collagen Type I	140-210 kDa	(1:1000) in 5% non-fat milk in TBS-Tween	(Cat.ABP)	Anti-rabbit	8%
α-SMA	42 kDa	(1:3000) in 5% non-fat milk in TBS-Tween	(ab12496)	Anti-rabbit	10%
Anti-TGF beta antibody	13 kDa	(1:1000) in 5% non-fat milk in TBS-Tween	(ab6604)	Anti-rabbit	15%
PDGFR-β	190 kDa	(1:1000) in 5% non-fat milk in TBS-Tween	(Cat.316)	Anti-rabbit	8 %
Monoclonal Anti-β Tubulin	55 kDa	(1:2000) in 5% non-fat milk in TBS-Tween	(Cat.T42)	Anti-mouse	10 %
MCP-1 Antibody	13 kDa	(1:1000) in 5% non-fat milk in TBS-Tween	(Cat.2029)	Anti-rabbit	15%
Mouse TGF-β2 Antibody	25 kDa	(1:1000) in 5% non-fat milk in TBS-Tween	(Cat.MAB73461)	Anti-rat	15%
Anti-CCR2 antibody	43 kDa	(1:1000) in 5% non-fat milk in TBS-Tween	(ab20312)	Anti-Rabbit	15%
Anti-CTGF antibody	36 kDa	(1:1000) in 5% non-fat milk in TBS-Tween	(ab 6992)	Anti-Rabbit	15%
SDF1 Antibody	9 kDa	(1:1000) in 5% non-fat milk in TBS-Tween	(Cat.3740)	Anti-Rabbit	15%

Antibody incubations were performed on a shaker at 4^{o}C overnight. After that, the antibody was removed and the membrane rinsed with TBS-T buffer 4 x 5 minutes' washes. The secondary HRP-coupled antibody was prepared in buffer containing the same blocking agent used for blocking primary antibody. DAKO HRP coupled antibodies was used at 1:2000 dilution and incubated for one hour at room temperature on the shaker.

Then, the membrane rinsed 4 x 5 minutes. The membrane was left in the buffer to be used for ECL detection. The work was done in the darkroom to detect the proteins through chemiluminescence (ECL). The ECL reagent was left on the membrane for three minutes and exposed to ECL film for 1-5 minutes. The membrane was developed and fixed by Kodak X-ray developer and fixer reagents. The developed film then rinsed in water and dried. To analyse western blotting results through densitometric quantification, the x-ray films were scanned using a scanner (HP Scanjet 4850).

Then, the scanned picture where uploaded to Fiji image j software where the density of peak area of the band was quantified. The percentage value of the target protein was divided by the value of the loading control to get a relative intensity. In order to re-probe the membrane with another antibody, the membrane was stripped for 15 minutes on the shaker at RT with 20 ml of Pierce stripping buffer (Thermo Scientific, Cat.no. 21059). Subsequently, the membrane was washed with TBS-T and then incubates with the antibody as above.

2.2.5 Radiation effects on the migration of MSCs

2.2.5.1 Trans-well migration assay

The aim of this assay is to see whether the secreted factor(s) from irradiated tumour cells mediate MSCs migration. This assay is sometimes named the Boyden chamber assay, as Boyden originally introduced it (Boyden, 1962). In this assay, media in two chambers (upper and lower) are separated by a filter membrane through which cells can migrate. For the migration assay, C3H10 MSCs, and Balb/c MSCs were cultured separately on trans-well plates (Falcon® 8.0 μm pore size), while the chemo-attractant was in the lower compartment.

Thus, vertical cell migration was expected toward the attractant. Different laboratory

techniques and radiation doses were used (**Figure 2.10**). After the incubation period (8-16 hours), the MSCs located in the upper compartment (Falcon® 8.0 μm pore size filter) were fixed by ice-cold methanol for 10 minutes. Then, the filters were washed with PBS and stained with haematoxylin staining for 20 minutes. Next, the filters were washed with PBS and left to dry at room temperature. The cells from the topside of the inserts were wiped off using a damp cotton bud. The filters were removed from the filter cup using a sharp razor blade and mounted on a glass slide using DPX. The cells, which were migrated to the bottom of the filter, were counted using the microscope (40X objectives). All migration experiments were done in duplicates, and cells were counted in 10 random views per filter at 40 X objective lens.

- Direct irradiation: MSCs were cultured on trans-well plates and irradiated directly using an Ago x-ray machine (see above) with 0, 0.5 Gy, and 4 Gy. The migration of MSCs was calculated after fixation and staining of the migrated cells.

- Incubation with conditioned media (neat or concentrated) from irradiated tumour cells: MSCs were cultured on trans-well plates. A CM obtained from irradiated tumour cells (FS120 cells or FS188 cells) was moved to the lower compartment. Migration of cells was assessed as above.

- Co-culture of MSCs with irradiated tumour cells: Tumour cells (FS120 cells or FS188 cells) were plated in the lower chamber. When they became ~80% confluent, they were irradiated with different radiation doses. 24 hours later, MSCs were seeded into the insert and their migration was assessed.

- Co-culture of MSCs with irradiated mouse endothelial cells (H5V): Mouse endothelial cells were seeded in the lower chamber and irradiated after they became confluent. After 24 hours, MSCs were seeded into the insert and their migration was evaluated as above.

- Co-culture of MSCs with irradiated human endothelial cells (HDMEC): The method was the same as for H5V, except the incubation time was different.

- Co-culture of MSCs with irradiated HDMEC+ FS: Both tumour cells (FS120 cells or FS188 cells) and human endothelial cells (HDMEC) were plated together (with HDMEC medium) at the lower chamber. As the tumour cells were growing faster than HDMEC, a 1:4 ratios were used to seed them together. Two days later, the media were changed to MSCs media and irradiated. 24 hours post radiation the MSCs were seeded into the insert and their migration was assessed as described above.

- Effects of TGF-β1, MCP-1, and SDF1 on the migration assay: MSCs were stimulated with TGF-β1, MCP-1 and SDF-1α.

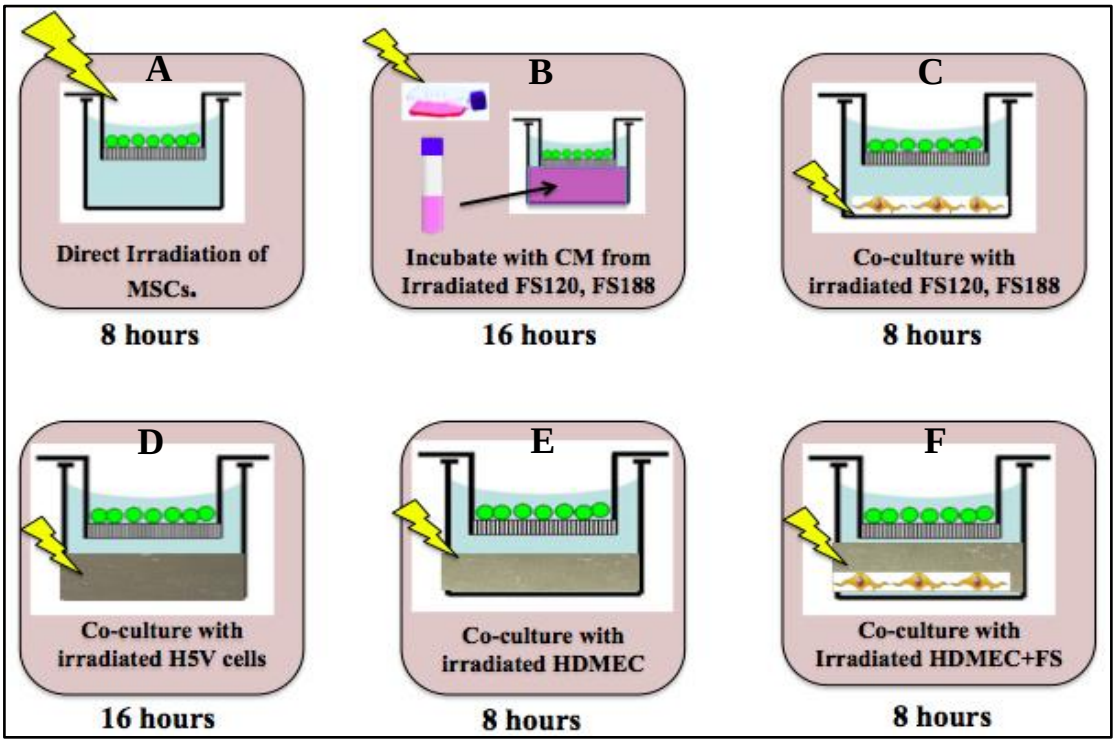


Figure 2.10 Schematic representatives of different C3H10 MSCs and Balb/c MSCs migration assay procedures.

A- Direct irradiation of MSCs **B-** Incubation of MSCs with conditioned media (neat and concentrated) from irradiated FS 120 cells, and FS188. **C-** Co-culture of MSCs with irradiated FS120 cells, and FS188 cells. **D-** Co-culture of MSCs with irradiated moues endothelial cells (H5V). **E-** Co-culture of MSCs with irradiated HDMEC. **F-** Co-culture of MSCs with irradiated FS120 cells, FS188 cells and HDMEC. The radiation doses used was 0, 0.5 Gy, and 4 Gy. The time under each scheme, represent incubation period after seeding of MSCs.

2.2.5.2 Trans-well migration assay using neutralizing antibodies to MCP-1

Neutralizing antibody was used to block the activity of MCP-1 in certain experiments. A fresh CM extracted from irradiated tumour cells (FS120 cells or FS188 cells) was concentrated first (as in **section 2.2.1.7**). Then, the concentrated CM was tested using western blotting to check that the MCP-1 protein is present. Before starting the assay, the concentrated CM was incubated with the non-immune IgG or antibodies in polypropylene tubes for 2 hours on a roller at room temperature. Using a 24-well plate, the migration assay was performed. Therefore, 3x 10^4 Balb/c MSCs in 200-µl medium were cultured on trans-well plates (Falcon® 8.0 µm pore size) and then incubated for 16 hours in the incubator. Fixation, staining, and counting of the migrated cells were done as previously explained.

2.2.6 Measuring MCP-1 release from irradiated tumour cells via enzyme-linked immunosorbent assay (ELISA)

Sandwich ELISA was done to detect and quantify mouse MCP-1 chemokine in the CM. The DuoSet® development system was used to detect mouse MCP-1 in the CM from irradiated fibrosarcoma tumours. Firstly, a 96-well plate was coated with the capture antibody (goat anti-mouse) that was diluted to the working concentration (**Table 2.8**). 100 µl per well of the diluted capture antibody was used, and then the plate was sealed and incubated at room temperature overnight.

Table 2.8 Working concentration and preparation of reagents for ELISA

Reagent	Amount per vial	Working concentration	Preparation
Capture antibody	50 µg	200 ng/ml	Reconstitute in 0.5 ml PBS
Detection antibody	3 µg	50 ng/ml	Reconstitute in 1 ml Reagent diluent
Standard	45 ng	3.91-250 pg/ml	Reconstitute in 0.5 ml Reagent diluent
Streptavidin-HRP	N/A	40-fold	Dilute in Reagent diluent

Next day, the wells were washed 3 times with 400 µl washing buffer after removal of the capture antibody and put to dry on a paper towel. Then, the wells were blocked for one hour with 300-µl reagent diluent at room temperature before washing with 400 µl washing buffer as earlier explained.

Standards were prepared as described in (**Figure 2.11**), while CM samples were diluted 1:10, 1:50, 1:100, 1:150 and 1:200 with the reagent diluent. Each sample 100 µl was tested in duplicate and incubated for 2 hours at room temperature followed by three washings with 400 µl washing buffer. 100 µl of the detection antibody (biotinylated goat anti-mouse JE) diluted in reagent diluent was added to each well and left for 2 hours at room temperature prior to washing 3 times with washing buffer (as above).

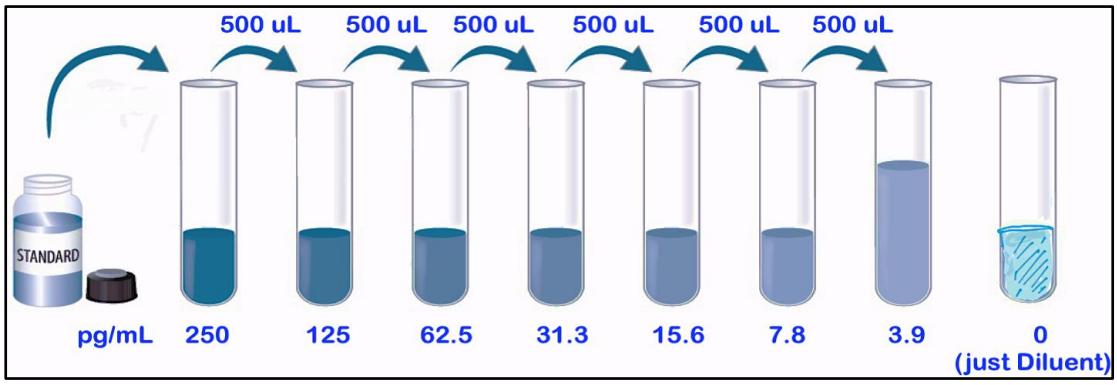


Figure 2.11 Preparation of ELISA standards by serial dilution. Initially, the recombinant mouse JE Standard was reconstituted with 0.5 mL of reagent diluent. According to manual instructions, a standard curve as prepared starting from 250 pg/mL to 3.9 pg/mL. 2.77 µl of standard was added to 1 mL of diluent reagent (first tube), and was mixed well. Next, 500 µl was taken from the first tube and added to the second tube (which already contained 500 µl of reagent diluent) followed by the same for the next tubes. One tube was without standard, and was used as a negative control for the curve (only reagent diluent).

Subsequently, 100 µl of Streptavidin-HRP diluted to 40-fold with reagent diluent was added to the wells and incubated for 20 minutes at room temperature away from light. Washing with washing buffer was repeated as above and 100 µl of substrate solution (1:1 mixture of colour reagent A and colour reagent B) was added to each well and incubated for 20 minutes at room temperature (in the dark). When enough yellow colour was detected in each well, the reaction was stopped by adding 50 µl of stop solution to each well and the plate was tapped smoothly to ensure full mixing.

Using a plate reader, the optical density of each well was determined immediately at 450 nm with background correction at 570 nm. To remove the background, the absorbance at 450 nm was subtracted from the absorbance at 570 nm. GraphPad Prism (Linear regression test) was used to determine the concentrations of the unknown protein samples from the standard curve.

2.2.7 Staining procedures for tumour sections from an *in vivo* experiment

All tumour sections (paraffin and frozen) that were used to analyse fibrosis and CAFs *in vivo* were provided by Mr Matthew Fisher, Senior Technician, Department of Oncology and Metabolism. These sections were taken from an experiment done by Dr Debayan Mukherjee who injected the fibrosarcoma cells (FS120 cells or FS188 cells) subcutaneously in CD1 nude mice.

After tumours had grown to ~100 mm^3 in volume, they were irradiated *in situ* with 20 Gy over 4 days (8 x 2.5 Gy fractions) starting from day 1. When they reached ~1000-1200 mm^3, they were excised, embedded in paraffin and sectioned by a microtome. Mrs Maggie Glover, Histology Technician, Department of Oncology and Metabolism did tumours sectioning and embedding in paraffin. While, Mr Matthew Fisher, Senior Technician prepared tumours frozen sections.

2.2.7.1 Immunohistochemical staining of formalin-fixed, paraffin-embedded fibrosarcoma tumour sections

IHC is a method for demonstrating the presence and location of proteins in tissue sections. It analyses and identifies cell types based on the binding of antibodies to specific components of the cell. Before starting with the staining protocol, the slides were deparaffinised and rehydrated, as incomplete removal of paraffin can lead to poor staining of the section.

The IHC protocol was given to me by Mr Matthew Fisher, which I then optimised. The sections were dewaxed by placing the slides in a rack, and performed the following washes:

- Xylene 2 X 10 minutes.
- 100% ethanol 2 X 5 minutes.
- 95% ethanol for 5 minutes.
- 70% ethanol for 5 minutes.
- Distilled water for one minute.
- Rinse slides in PBS for 2 minutes.

The next step was antigen retrieval, which is important due to the formation of methylene bridges during fixation, which cross-links proteins and therefore masks antigenic sites. Heat-mediated retrieval by pressure cooker was used with target retrieval solution (DAKO® Target Retrieval Solution, 10X concentrate, Code No. S 1699) which was diluted at 1:10 in dH2O.

700 ml of dH2O were added inside the pressure cooker while the slides were put in the target retrieval solution. Heating with pressure cocker was for 2 hours, then, the slides were removed and rinsed once in PBS. Sections were circled by wax pen and left for 10 minutes to dry at room temperature before rinsing with PBS.

In order to block endogenous peroxidase activity, the slides were blocked with 3% H_2O_2/PBS for 20 minutes (dilution was 1:10 dilution from 30% H_2O_2 stock solution). Subsequently, the slides were rinsed with PBS and blocked with the mouse on mouse (M.O.M. ™) IgG for 60 minutes at room temperature (Vector®M.O.M ™ Immunodetection kit, Cat. No. BMK-2202) to prevent non-specific antibody binding

to tissue. The primary antibody (α-SMA 2 mg/ml anti-mouse, made in mouse) was diluted 1: 10,000 in PBS plus M.O.M. ™ diluent. The blocking solution was removed and the primary antibody was added on the slides and incubated at 4 °C overnight.
Next day, the primary antibody was removed and slides were rinsed three times in PBS. The secondary antibody (biotinylated anti-mouse IgG reagent 1:250) was put over the sections and incubated for 10 minutes. The slides were rinsed twice in PBS and then incubated with avidin-biotin complex (ABC) for 60 minutes and washed three times with PBS. Incubation with DAB (3, 3'-diaminobenzidine) from 1-10 minutes (visualised under a microscope) was the next step.
The slides then washed with tap water and counterstained with Mayer`s haematoxylin stain for 30 seconds to visualize the nuclei, and then washed in tap water for 5 minutes. The slides finally dehydrated with 70% ethanol for 5 minutes, 95% ethanol for 5 minutes, 100% ethanol 2 X 5 minutes, and Xylene 2 X 5 minutes. The slides were then mounted with DPX (distyrene, plasticizer, and xylene) and cover slipped then left to dry at room temperature.

2.2.7.2 Collagen staining (Masson's trichrome staining) of paraffin-embedded fibrosarcoma tumour sections

Masson's trichrome consists of three-colour staining; dark brown-black for cell nuclei, orange-red for cytoplasm and green-blue for collagen. Samuel F Harding, Research Technician at the Diabetes, Endocrinology & Metabolism Unit, Department of Oncology and Metabolism, provided me with a basic protocol which I then optimised.

Fibrosarcoma tumour sections were dewaxed in xylene for 10 minutes (X2), followed by ethanol 100%, 95%, 75%, and water for 2 minutes each. Then, the slides were incubated for 10 minutes in Weigert's iron haematoxylin staining, followed by one-minute washing in tap water, and one minute blue in Scott's tap water. The slides were then placed in Ponceau stain (Fucshin 2:1 0.5% Ponceau 2R in 1% Acetic acid, 0.5% Acid fucshin in 1% Acetic acid) for 10 minutes, followed by washing in tap water. Phosphomolybdic acid-phosphotungstic acid (2.5%) was used for 2 minutes and the slides were transferred quickly to tap water to remove the acid. After that, the sections were stained with 2% light green in 2% acetic acid for 4 minutes, rinsed in tap water (one dip only).

Dehydration of the samples was through ethanol 75%, 95%, and 100% (very quickly) followed by xylene for 5 minutes (X2). Finally, the slides were mounted using DPX.

To prepare solutions:

- Ponceau:Fucshin 2:1 (0.5% Ponceau 2R in 1% Acetic acid, 0.5% Acid fucshin in1% Acetic acid)

- ❖ 1% Acetic acid: 10 ml of glacial acetic acid was added to 1000 ml of distilled water.
- ❖ 2.5 g of Fuschin was weighted and added to 500 ml of 1% acetic acid.
- ❖ 2.5 g of Ponceau 2R was weighted and added to 500 ml of 1% acetic acid.
- ❖ Fuscin and Ponceau red solutions were mixed at a ratio of 2:1 (200 ml Fuschin and 100 ml Ponceau).

- 5% Phosphomolybdic acid: (5% Phosphotungstenic acid in 2% Acetic acid)

- ❖ 2% Acetic acid solution was prepared by adding 20 ml of glacial acetic acid to 1000 ml of dH_2O.
- ❖ 100 ml of the 10% Phosphomolybdic acid was mixed with 100 ml of the 10% Phosphotungstenic acid and then 200 ml of 2% acetic acid was added.

- 2% light green in 2% acetic acid

- ❖ 10 g of light green powder was weighed and dissolved in 500 ml of 2% acetic acid.

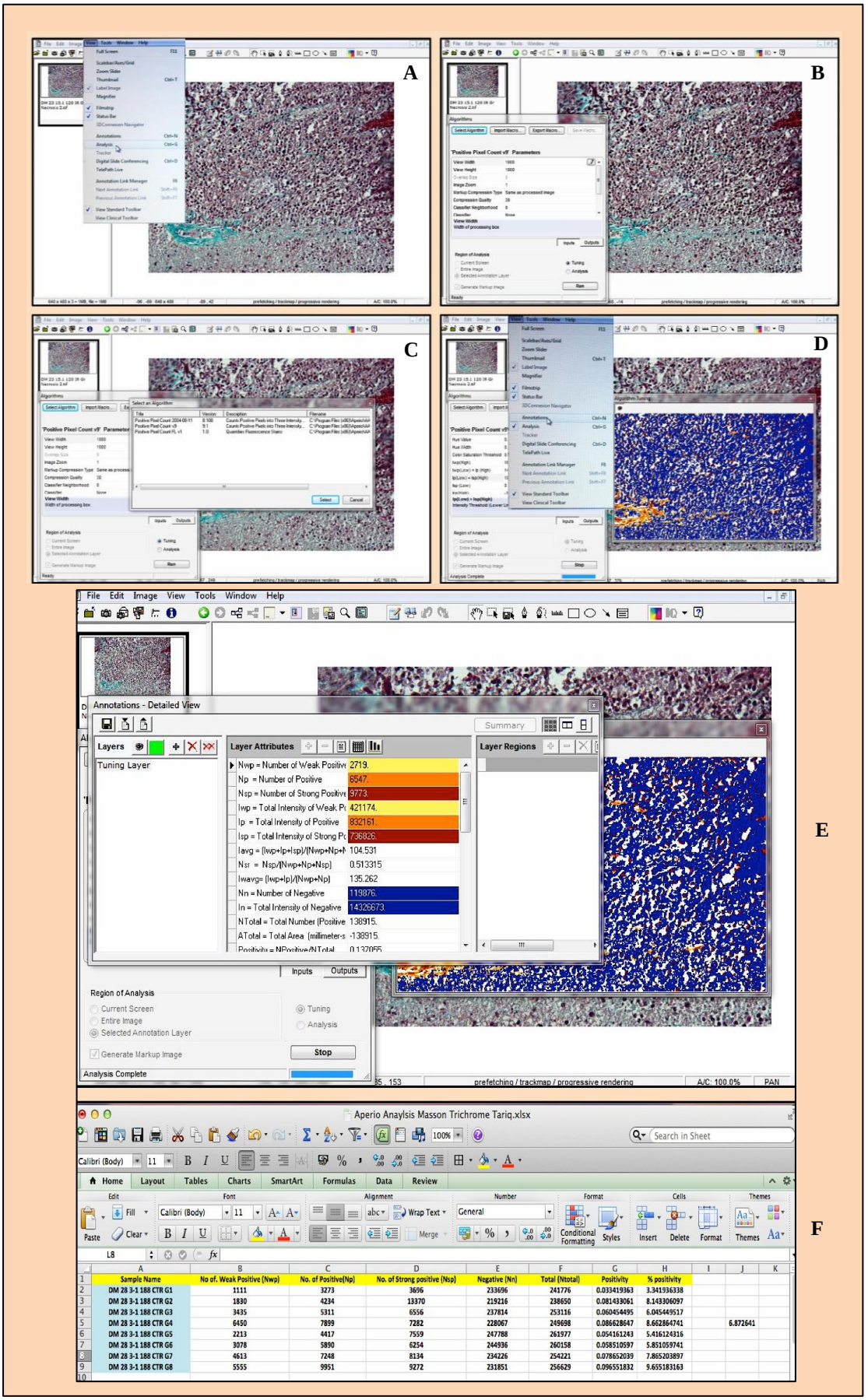
To analyse images from Masson's trichrome staining:

Two ways were used, the first method was via Aperio ImageScope software (version 12.2), in which eight images were taken from each stained tumour section (16 tumour sections were used, 4 for FS120 CTR, 4 FS120 irradiated, 4 FS188 CTR and 4 FS180 irradiated) using a 10X objective and a Nikon microscope (Nikon Optiphot-2). First, the image to be analysed was uploaded to the Aperio ImageScope software by clicking the "File" menu and selecting "Open Image". In order to analyse the image on a spectrum, an algorithm was created. The parameters were adjusted (Hue value, Hue width, colour saturation threshold, Iwp, and Isp) of the algorithm and saved the setting to apply for all subsequent images. To do that, from the "View" menu "Analyse" was

chosen and “Select Algorithm”, another window opened with three choices. “Positive Pixel count V9 Parameters” was selected followed by “Run”.

The parameters were adjusted according to what wanted to analyse (analyse the green-blue collagen area) by clicking it in the list and selecting the parameter. These parameters were set as the default parameters and were used for all slides images. After achieving the analysis, from the ImageScope “View” menu, “Annotations” was selected. Next to each parameter, the number of positive pixels for the chosen colour for that analysed section result appeared. Results were exported to an Excel spreadsheet for further analysis. A schematic representation of images analysis via Aperio ImageScope is shown in **Figure 2.12**. The results from the analysis were divided into either positive areas (presence of collagen), or negative areas (no collagen). In the positive areas, there were three areas, weakly positive, positive or strongly positive. The positivity was calculated by summation of all the positive areas (weak positive + positive + strong positive) then divided by the total area (all positive areas + negative areas). The percentage of positivity was estimated by multiplying positivity by 100 (**Figure 2.12**).

The positivity was calculated by summation of all the positive areas (weak positive + positive + strong positive) then divided by the total area (all positive areas + negative areas). The percentage of positivity was estimated by multiplying positivity by 100 (**Figure 2.12**).



	A	B	C	D	E	F	G	H	I	J	K
1	Sample Name	No of. Weak Positive (Nwp)	No. of Positive(Np)	No. of Strong positive (Nsp)	Negative (Nn)	Total (Ntotal)	Positivity	% positivity			
2	DM 28 3-1 188 CTR G1	1111	3273	3696	233696	241776	0.033419363	3.341936338			
3	DM 28 3-1 188 CTR G2	1830	4234	13370	219216	238650	0.081433061	8.143306097			
4	DM 28 3-1 188 CTR G3	3435	5311	6556	237814	253116	0.060454495	6.045449517			
5	DM 28 3-1 188 CTR G4	6450	7899	7282	228067	249698	0.086628647	8.662864741		6.872641	
6	DM 28 3-1 188 CTR G5	2213	4417	7559	247788	261977	0.054161243	5.416124316			
7	DM 28 3-1 188 CTR G6	3078	5890	6254	244936	260158	0.058510597	5.851059741			
8	DM 28 3-1 188 CTR G7	4613	7248	8134	234226	254221	0.078652039	7.865203897			
9	DM 28 3-1 188 CTR G8	5555	9951	9272	231851	256629	0.096551832	9.655183163			
10											

Figure 2.12 Schematic representative of analysing Masson's trichrome staining analysis of paraffin embedded fibrosarcoma tumour sections using Aperio ImageScope software version 12.2.

Aperio ImageScope version 12.2 software was used for the analysis. After the image has been uploaded, from "View" menu "Analyse" and "Select Algorithm" were chosen (A & B). "Positive Pixel count V9 Parameters" was selected followed by Run (C). The parameters were adjusted according to what we wanted to analyse (we wanted to analyse the green-blue collagen area) by clicking it in the list and select the parameter. These parameters were used as default parameters for all other images later. After finishing the analysis, from ImageScope "View" menu, "Annotations" was selected (D). Next to each parameter, the colour for that analysed section result will appear (E). To save the results, "Export Grid to Excel Spreadsheet" was chosen. The results from the analysis were divided into either positive area (presence of collagen), or negative area (no collagen). Within the positive areas, there were three areas, weak positive, or positive, or strong positive. The positivity was calculated by summation of whole positive areas (weak positive + positive + strong positive) then divided by the total number (all positive areas + negative areas). The percentage of the positivity was estimated by multiplying positivity by 100 (F).

The second method used to analyse Masson's trichrome staining in paraffin sections was by scanning sections with the TissueGnostics Confocal Slide scanner (TISSUE FAX 200, Tissue Gnostics Vienna, Austria) (**Figure 2.13**) and using HistoQuest software analysis system V4.0. Using this more sensitive method of analysis allows us to analyse more tumour sections in less time. The system is a multi-colour high-resolution slide scanner with 5 µm confocal sectioning, and up to 50 µm optical slice in sample thickness. Dr Maya Boudiffa, a post-doctoral research associate, Bone Biology Unit, Department of Oncology and Metabolism, scanned the sections using the Slide Scanner and provided training in data analysis using the HistoQuest software.

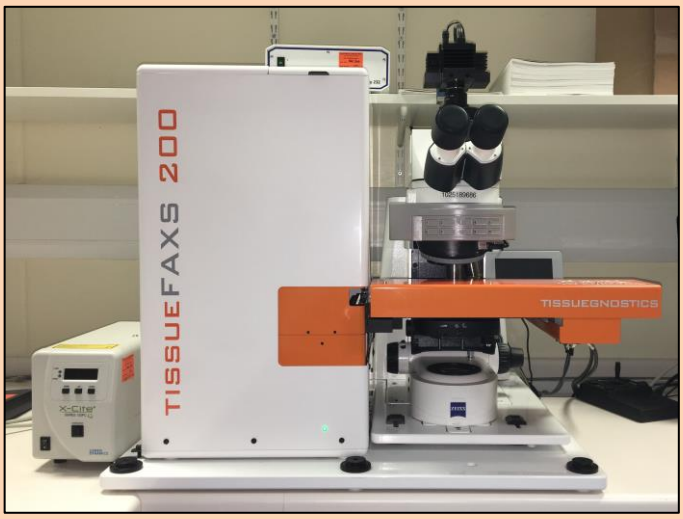


Figure 2.13 TissueGnostics Confocal Slide Scanner with the TissueFAXS 200 autoloader.

The high-resolution images from the scanned slides were sent to the computer-assisted analysis HistoQuest software system (TissueGnostics GmbH). All images were analysed using specific protocol in the software.

As shown in **Figure** (**2.14 C**), for each tissue sections the total area was first measured followed by necrotic and non-necrotic (viable) area measurements. Each area was measured as mm^2. The green area (collagen) was measured within the total tissue area, necrotic area, and non-necrotic (viable) areas. The percentage of the green areas (collagen) in each tissue section was calculated as follows:

$$\frac{\text{Green area (mm2)}}{\text{Total tissue area (mm2)}} \text{ X } 100$$

A

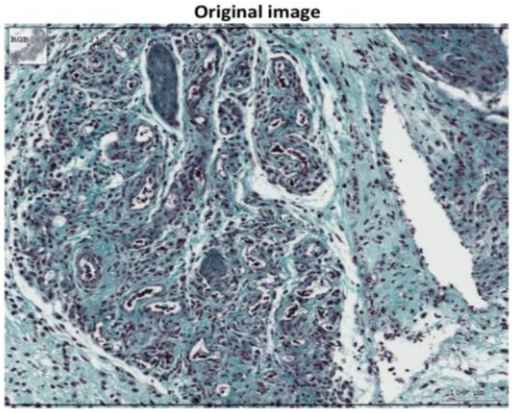
Analysis collagen content in tissue sections (green/blue)

Method:

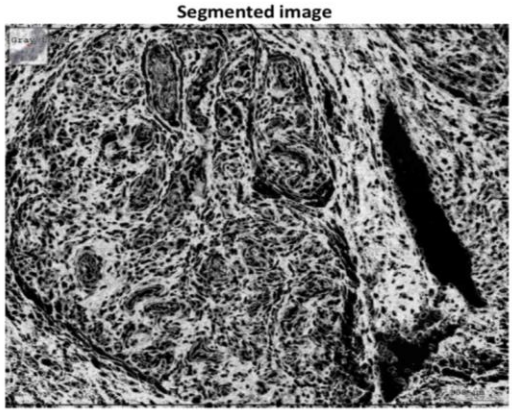
Using histoquest, a multiple shades segmentation was applied to the images and area measurements were performed.

Example of segmentation:

Original image



Segmented image



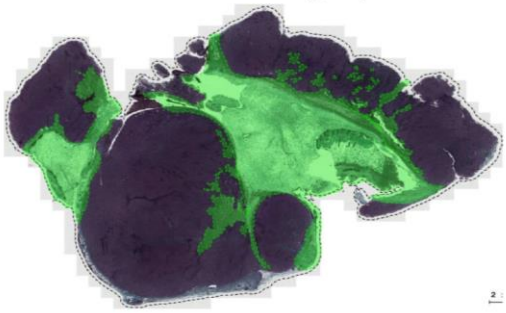
B

Analysis collagen content in tissue sections (green/blue)

Sample 10916001 showing all necrosis area identified:

Necrotic area outlined in green

Necrotic area filled (green)



C

Analysis collagen content in tissue sections (green/blue)

Results analysis:

Sample	Region analysed	Total tissue area (mm²)	Green Area (mm²)	% Green Area
10916001	Total tissue	153.53	20.29789361	13.22076805
	Necrotic area	51.95948107	14.77636169	28.43823953
	Non Necrotic area	101.57	5.52	5.436136461
10916002	Total tissue	147.65	20.38758426	13.80805384
	Necrotic area	58.18634786	13.49710668	23.19634618
	Non Necrotic area	89.46	6.89	7.701990081

Figure 2.14 Analysis collagen content of the Masson's trichrome stained paraffin sections using HistoQuest software.

The stained slides were scanned via TissueGnostics Confocal Slide Scanner (TISSUEFAXS 200). Then the high-resolution images were analysed by HistoQuest software V 4.0. Multiple shades segmentation was applied to the images and area measurements were performed (A). The viable or necrotic areas within the section were outlined (B). The results of the analysis measured the total tissue area, and the green area (collagen) in mm^2 (C). For each sample the whole tissue area, the necrotic areas and viable areas were analysed. The green area (collagen) was measured within the total tissue area, necrotic area, and non-necrotic (viable) areas. The % of the green areas (collagen) in each tissue section was calculated: $\frac{\textbf{Green area (mm2)}}{\textbf{Total tissue area (mm2)}}$ X 100.

2.2.7.3 Immunofluorescence staining of frozen fibrosarcoma tumour sections

Tumour sections (frozen) were left to dry at room temperature for 15 minutes. Then, they were circled with a wax pen and allowed to dry for 10 minutes at room temperature. The sections were fixed in ice-cold acetone for 10 minutes before they were washed twice with PBS.

Blocking was with MOM IgG (Vector®M.O.M™ Immunodetection kit, Cat. No. BMK-2202) for one hour at room temperature. Thereafter, incubation with the antibody (α-SMA 2 mg/ml anti-mouse, made in mouse) was done overnight at 4°C and was diluted 1: 10,000 in PBS plus M.O.M.™ diluent. Next day, the slides were washed with PBS X3 and incubated with the secondary antibody (1:250 biotinylated anti-mouse IgG reagent) for 10 minutes at room temperature. The slides then were incubated with Texas Red® Streptavidin (diluted 1:200 in 1% BSA/PBS) for one hour at room temperature, after washing the slides with PBS X3.

Blocking the slides with 10% goat serum in 1% BSA/PBS for one hour was done at room temperature followed by incubation with the CD31 antibody (diluted 1:200 in 1% BSA/PBS) overnight at 4°C.

Washing the slides with PBS X3 was done next day, and then the slides were incubated for one hour with goat anti-rat IgG H&L (FITC) diluted 1:200 in 1% BSA/PBS. After washing the slides with PBS X3, they were mounted with Vector shield hard set with DAPI (4', 6-diamidino-2-phenylindole).

2.3 Statistical analyses

Statistical analyses were achieved using GraphPad Prism 7.0 Software. To compare between two groups, unpaired two-tailed t-test was used. While one-way analysis of variance (ANOVA) was used to see whether there are any significant differences (statistically) between the means of two or more independent groups.

For clonogenic assay, the linear-quadratic model was used to analyse data. This was done in GraphPad Prism via changing in the Format Axis dialog.

CHAPTER THREE:

The effects of radiation on the differentiation of MSCs into cells with CAF-like characteristics and its consequences on MSC migration/recruitment

3.1 Introduction & aims

Radiotherapy is one of the standard effective treatments for intermediate and high-soft tissue sarcoma. Around 60% of patients with cancer will receive radiotherapy as a part of their treatment (Robbins et al., 2012). More importantly, as discussed formerly, radiotherapy can cause fibrosis, which is characterised by deposits of collagen and other matrix proteins and presence of activated fibroblasts known as CAFs, which are major producers of collagen in tumours (Azorin-Vega et al., 2015). CAF play a key role in cancer initiation, invasion and metastasis (Luo et al., 2015). The underlying cellular mechanisms of radiation fibrosis in tumours are not fully understood. MSCs can differentiate into CAF-like cells (Mishra et al., 2008), but the exact origin of CAFs in irradiated tumours has not been established. In normal tissues, growth factors such as PDGF and TGF-β1 play a key role in the differentiation of MSCs to CAFs, therefore, similar factors may be involved in response to radiation in a tumour. Moreover, Klopp et al and Fenton and Paoni showed that irradiation induced recruitment of MSCs to tumours (Klopp et al., 2007, Fenton and Paoni, 2007). The mechanisms that rule migration of MSCs into the sites of injury have not been fully explained (Karp and Teol, 2009). Some studies have shown that chemotaxis is a mode of recruitment for MSCs in response to chemoattractants like chemokines or growth factors (Vanden Berg-Foels, 2014). Presence of the MSCs in the tumour microenvironment will promote tumour angiogenesis, suppress the immune response, enhance the stemness of tumour cells, induce chemotherapy resistance and endorse EMT (Sun et al., 2014).

The aims of this chapter are:

- To determine whether radiation induces the differentiation of MSCs into cells with CAF-like characteristics using sarcoma cells *in vitro* to model the tumour microenvironment.
- Use *in vitro* models, to investigate the role of post-radiation injury cytokines and chemokines in radiation-enhanced MSC recruitment to tumours.

3.2 Results

3.2.1 Effects of radiation on tumour cells and MSC colony formation

It has long been established that radiation causes DNA damage and cell death (Nikjoo et al., 2001, Warters and Hofer, 1977). In order to choose appropriate radiation doses for later experiments, the radio-sensitivities of tumour cells and MSCs were tested. The clonogenic assay was used as a cellular response technique to test the effectiveness of radiation on the proliferation and survival of cells. A colony means a group of at least 50 cells.

Cells were irradiated with different radiation doses (0-4 Gy), plus a non-irradiated group, which was used as a control. Next, the cells were trypsinised and counted by haemocytometer, before being sub-cultured into 6-well plates and incubated for 12 days. **Table 2.2** shows cell numbers plated for each radiation dose condition. Fixation and staining of the cells with crystal violet was done followed by manual counting of the colonies. The plating efficiency (PE) with survival fraction (SF) was calculated as described in chapter two (**section 2.2.2**).

Cell survival curves were fitted with Linear Quadratic modelling and plotted as a logarithm of the surviving fraction versus radiation dose in Gray (Gy) (**Figure 3.1**). The survival fraction for all cells was decreased in a dose-dependent manner. At 4 Gy, both Balb/c and C3H10 MSCs were more radio-resistant than fibrosarcoma cells (P = 0.014 for Balb/c, and 0.020 for C3H10 cells, using Linear regression test). In addition, at 4 Gy FS120 cells were more radioresistant than FS188 cells (P = 0.0123, Linear regression test). The results were averages of 5 independent experiments each plated in triplicate (**Figure 3.1**). The survival fraction at 4 Gy were 40.0%, 25.6%, 3.1%, and 0.98% for Balb/c MSCs, C3H10 MSCs, FS120 cells, and FS188 cells respectively.

Although 4 Gy was lethal-sublethal for tumour cells and there was moderate cell killing for MSCs, it was chosen for subsequent experiments as the upper limit dose to mimic the radiation dose used in the clinic. The data presented in **Figure 3.1**.

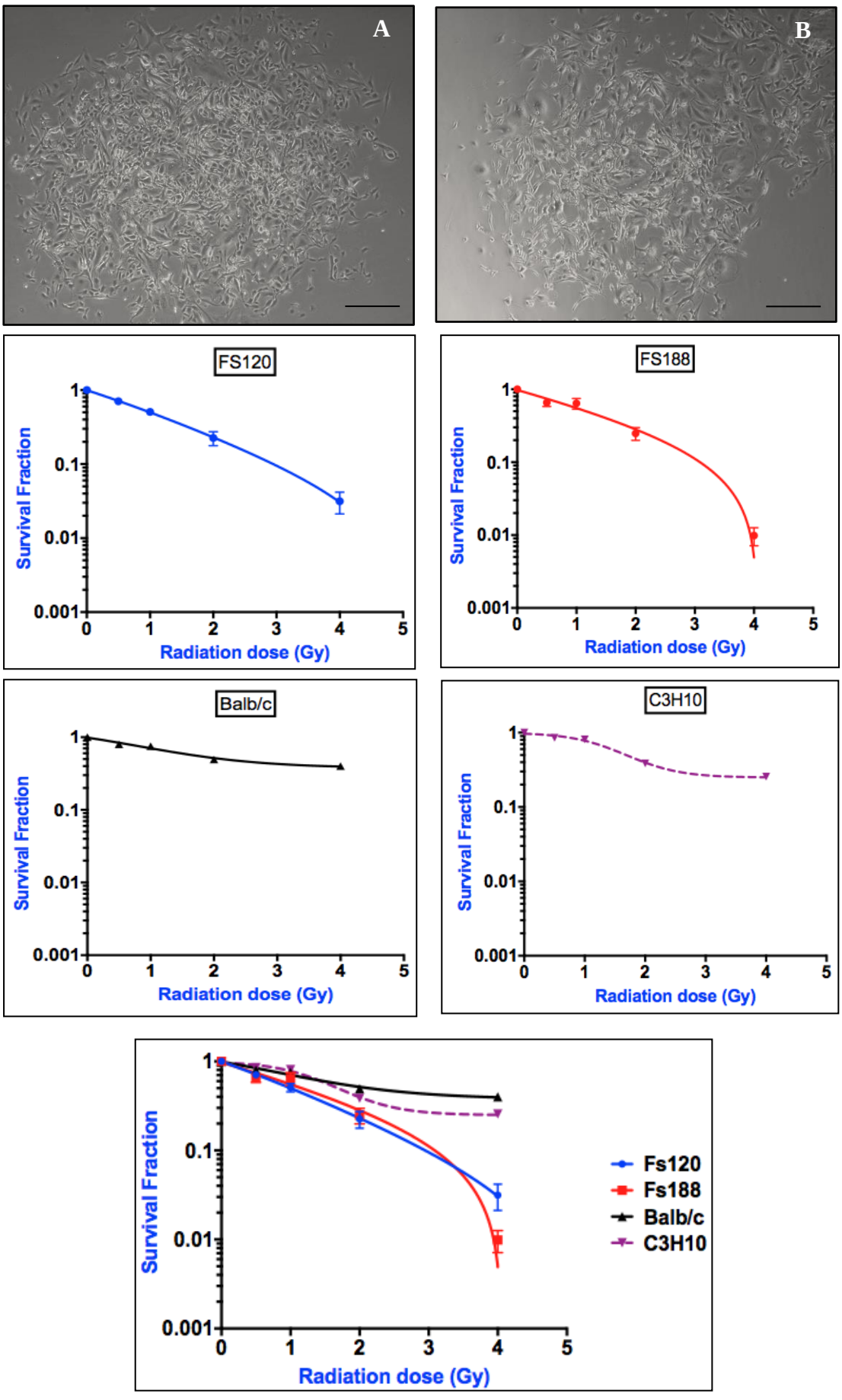

A
B
FS120
FS188
Balb/c
C3H10
Survival Fraction
Radiation dose (Gy)
1
0.1
0.01
0.001
0
1
2
3
4
5
Fs120
Fs188
Balb/c
C3H10

Figure 3.1 Radiation effects on survival of FS 120 cells, FS 188 cells, C3H10 MSCs, and Balb/c MSCs cells.

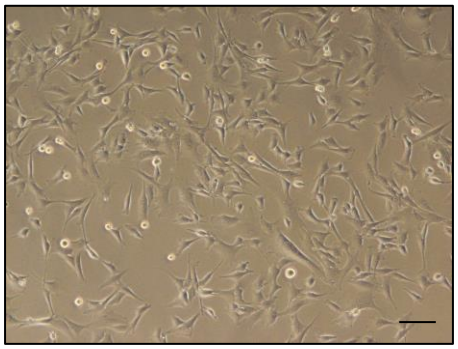
Representative images of colonies from FS 120 cells (A), and FS 188 cells (B). Cells were seeded after irradiation with 0, 0.5, 1, 2 and 4 Gy (0 represent control un-irradiated cells). Colonies were fixed, stained and counted 12 days after radiation. 50 cells or more were representing a colony. The survival fraction (SF) was calculated and plotted for each cell line as a logarithm of the survival fraction versus radiation dose in Gray using Linear Quadratic model. At 4 Gy, both Balb/c (P = 0.014) and C3H10 MSCs (P = 0.020) were more radio-resistant than fibrosarcoma cells. In addition, at 4 Gy FS120 cells were more radioresistant than FS188 cells (P = 0.0123). The data points are the averages of 5 independent experiments each plated in triplicate. The dose-response curves were fitted by linear regression analysis, the mean ± SEM is presented *P < 0.05. The survival fraction for all cells was decreased in a dose-dependent manner. The survival fractions at 4 Gy were 40.0%, 25.6%, 3.1%, and 0.98% for Balb/c MSCs, C3H10 MSCs, FS120 cells, and FS188 cells respectively.

3.2.2 Effects of TGF-β1 on the growth, morphology and differentiation of MSCs

TGF-β1, a pro-fibrotic cytokine, plays a key role in post-radiation injury (Martin et al., 2000) and in the growth of MSCs (Ng et al., 2008). It has been shown that TGF-β1 induced morphological changes in MSCs into cells with a CAF-like morphology and leads to an increase in collagen and fibronectin production (Desai et al., 2014). First, to establish whether TGF-β1 is playing a role in the growth of C3H10 and Balb/c MSCs, *in vitro* growth curve assays were done. MSCs were cultured in triplicate in the presence of TGF-β1 (10 ng/ml) and left for 24 hours in the incubator to allow cells to adhere to the plate. Untreated cells acted as controls.

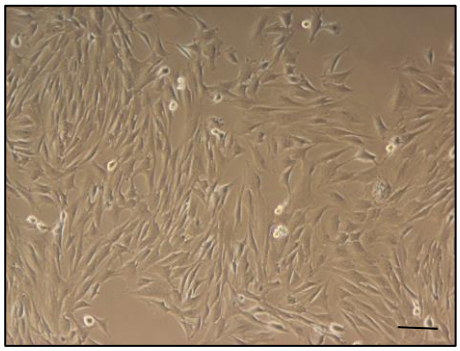
Counting cells was done on a daily basis (days 1, 2, 3 and 4) as explained in **section 2.2.3**. The results show that there was no significant effect of TGF-β on the growth of either C3H10 or Balb/c MSCs (**Figure 3.2)**. However, altered cell morphology displaying elongated spindle-shaped and compacted cells in contrast to control cells that were flat, polygonal, and less spindle-shaped was observed. These results suggested that TGF-β induced differentiation of both MSCs into CAF-like cells (**Figure 3.2 A-D**). These morphological changes were associated with changes in the expression of α-SMA (see later in this chapter), which support that MSCs differentiated into CAF-like cells in the presence of TGF-β1. Presence of CAF cells in the TME is strongly associated with excessive ECM deposition and later fibrosis (De Wever et al., 2008).

A)



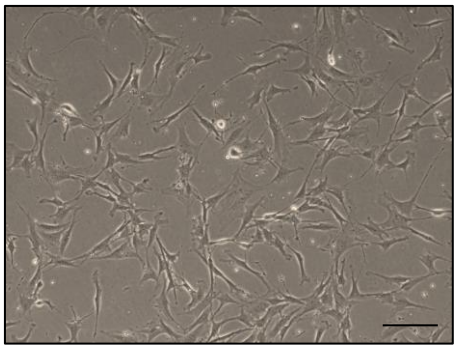
Balb/c MSCs control without TGF-β

B)



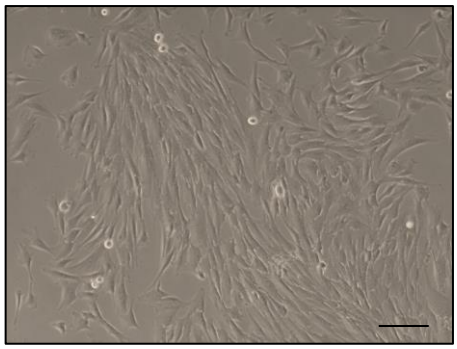
Balb/c MSCs treated with 10ng/ml TGF-β

C)



C3H10 MSCs control without TGF-β

D)



C3H10 MSCs treated with 10ng/ml TGF-β

E)

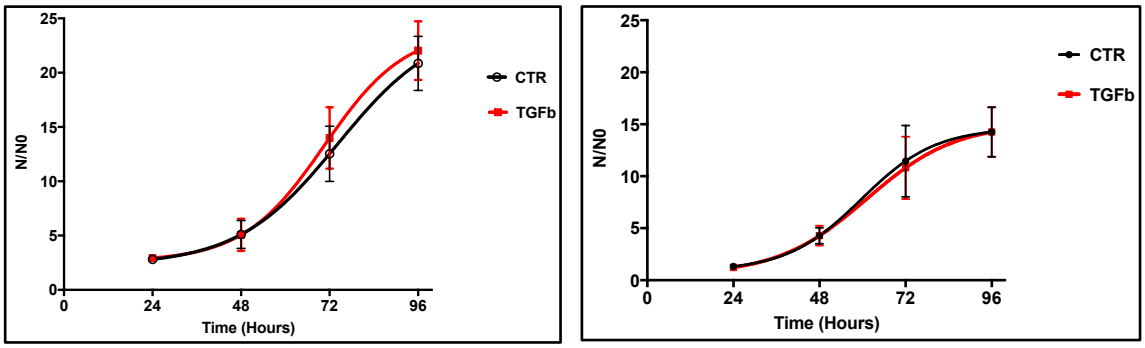


Figure 3.2 Effects of TGF-β1 on the growth and morphology of Balb/c and C3H10 MSCs.

MSCs were plated in 6-well-plates, and either treated with TGF-β (10 ng/ml), or control cells (no TGF-β). MSCs grown under normal conditions without TGF-β displayed a flattened fibroblastic morphology (**A & C**), while MSCs grown in the presence of TGF-β displayed distinct spindle morphology (**B & D**). (**E**) Starting at 24 hours after plating, and then at daily intervals (for total of 4 days) cells were trypsinised and counted. The result revealed that TGF-β had no effects on the growth of Balb/c or C3H10 MSCs. Results are expressed as mean cell counts ± SEM of three individual experiments (each done in triplicate). N0= the number of the cells that were seeded (3 x 10^4 cells/well). N= number of the cells counted on a daily basis whether controls or after treatment with TGF-β.

3.2.3 Radiation effects on C3H10 and Balb/c MSC morphology and expression of CAF-like cells differentiation markers

MSCs can differentiate into activated fibroblasts or CAFs (as discussed earlier) (Sugimoto et al., 2006, Mishra et al., 2008). CAFs are large spindle-shaped cells, which express α-SMA and, PDGFR-β (Sugimoto et al., 2006, Anderberg et al., 2009). Moreover, CAFs secrete collagen, fibronectin and other ECM proteins which contribute to tissue fibrosis (Miles and Sikes, 2014). With respect to radiation effects on the MSCs' differentiation ability, western blotting was done on the cell lysates that were extracted from both C3H10, and Balb/c MSCs. As shown in **section 3.2.2**, TGF-β1 altered the morphology of both MSCs into CAF-like cells. These changes suggested that TGF-β1 induced differentiation of both MSCs into CAF-like cells. So, TGF-β1 was used as a positive control of MSCs differentiation in subsequent experiments.

3.2.3.1 Effects of direct irradiation on the expression of CAF markers by mouse MSCs

Both C3H10 MSCs and Balb/c MSCs were seeded in 6-well plates individually until they became ~80% confluent. Then, they were X-ray irradiated directly with 0-4 Gy (**section 2.2.1.6**). One well was treated with 10 ng/ml TGF-β1 alone without radiation used as a positive control for differentiation. Another well was left un-irradiated as a control. A further well was irradiated with 4 Gy and also treated with TGF-β1 (10 ng/ml). Plates were incubated for 3 days before extraction of proteins using cell extraction buffer (**section 2.2.4.2**). 20 µg (30 µl/lane) of proteins was loaded in the western blotting. Normalisation was done through calculating the ratio of target protein to internal control (i.e., dividing or "normalizing" by the loading control).

As seen in **Figure 3.3**, only TGF-β1 had a significant effect in the expression of collagen-I, fibronectin, PDGFR-β and α-SMA in C3H10 MSCs. However, in Balb/c cells 0.5 Gy also increased the expression of collagen-I (one-way ANOVA, Dunnett's multiple comparisons test, $P = 0.037$) (**Figure 3.4**). Some of the markers showed a tendency to increase after low doses of radiation and decrease after high dose radiation (2-4 Gy), although these effects were not significant.

Direct irradiation of C3H10 MSCs

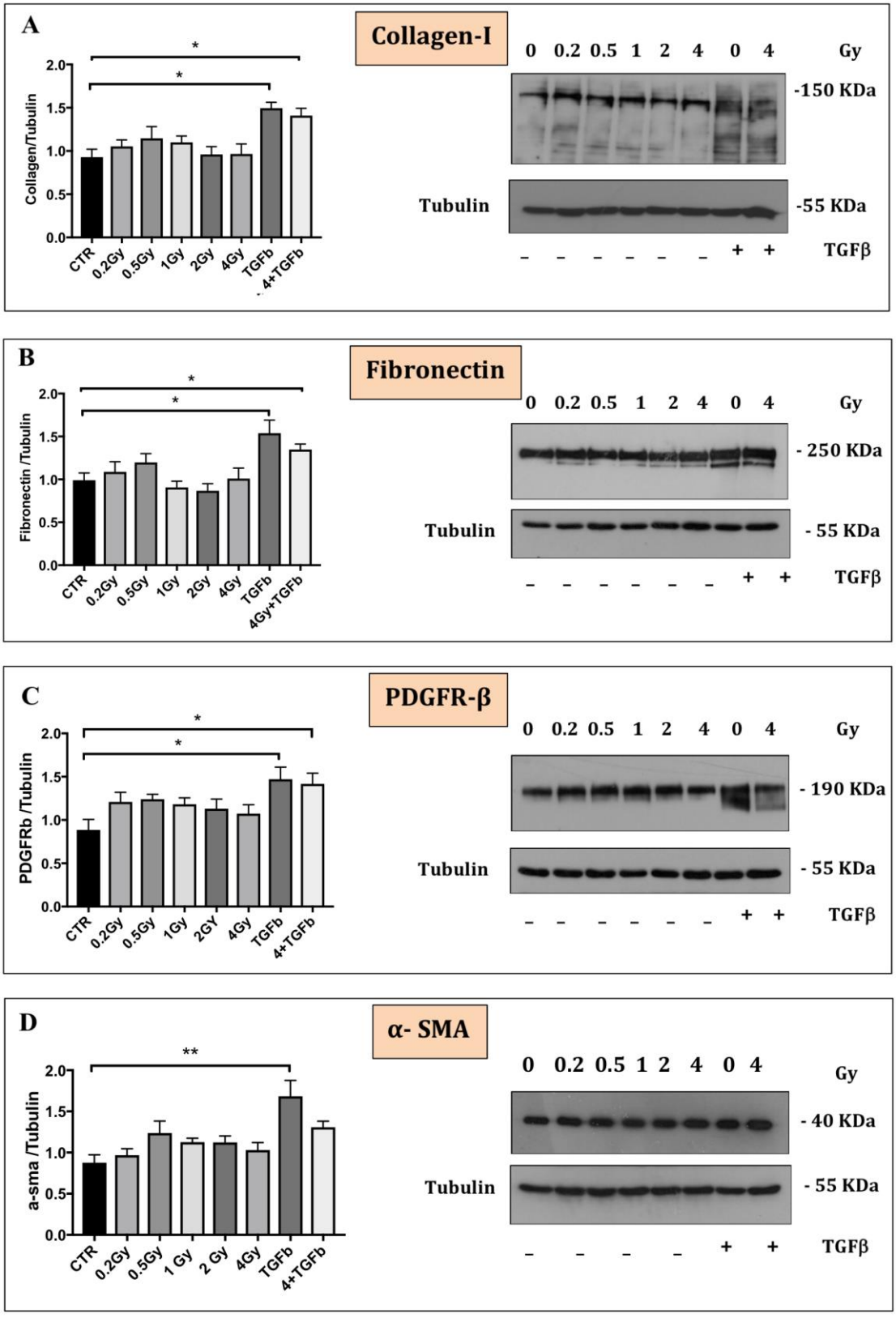


Figure 3.3 Effects of direct radiation on the expression of CAF markers by C3H10 MSCs.

Cells were irradiated and/or treated with TGF-β (10 ng/ml). Protein extracts were prepared three days' post-irradiation and analysed for expression of various markers expressed by CAFs (collagen-I, fibronectin, PDGFR-β and α-SMA). 20 μg (30 μl/lane) of proteins were loaded. Radiation had no effect on the expression of all markers tested although collagen-I, fibronectin and PDGFR-β tended to increase after low radiation doses (0.2, and 0.5 Gy) (A, B, C, and D). There was also a tendency of the expression markers to decrease after higher radiation doses (1, 2, and 4 Gy). TGF-β1 caused a significant increase in the expression of collagen-I, fibronectin, PDGFR-β and α-SMA in un-irradiated cells, with similar effects when administered in combination with radiation (A, B, C, and D). One-way ANOVA test followed by a Dunnett's multiple comparisons test was used to analyse data. Results expressed as means ± SEM and were considered statistically significant when $P \leq 0.05$ (* $P<0.05$, ** $P<0.01$). Each graph represents 4 independent experiments (N=4).

Direct irradiation of Balb/c MSCs

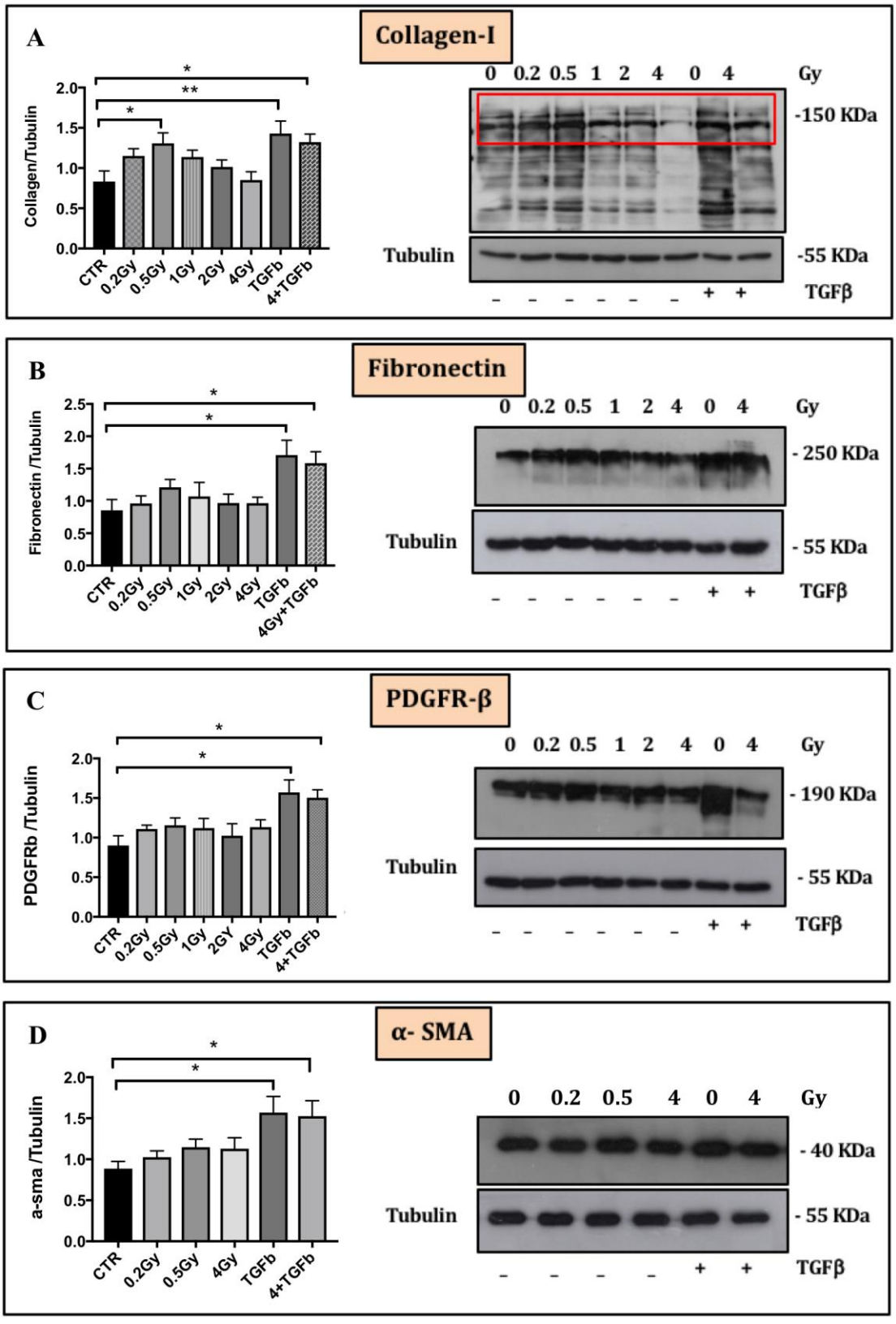


Figure 3.4 Effects of direct radiation on the expression CAF markers by Balb/c MSCs.

Cells were irradiated and/or treated with TGF-β (10 ng/ml). Protein extracts were prepared three days post-irradiation and analysed for expression of various markers expressed by CAFs (collagen-I, fibronectin, PDGFR-β and α-SMA). 20 µg (30 µl/lane) of proteins were loaded. Low radiation dose (0.5 Gy) caused a significant increase in the collagen-I expression significantly 3-4 days after radiation (A). Collagen-I, fibronectin, PDGFR-β and α-SMA were also increased significantly after treatment of the MSCs with TGF-β (A, B, C, and D). 4 Gy tended to decreased the expression of differentiation markers, while TGF-β1 caused a significant increase in the expression of collagen-I, fibronectin, PDGFR-β and α-SMA in un-irradiated cells, with similar effects when administered in combination with radiation (A, B, C, and D). One-way ANOVA test followed by a Dunnett's multiple comparisons test was used to analyse data. Results expressed as means ± SEM and were considered statistically significant when $P \leq 0.05$ (* $P<0.05$, ** $P<0.01$). Each graph represents four independent experiments (N=4).

3.2.3.2 Effects of irradiated tumour cells on the expression of CAF markers by mouse MSC

In the previous experiments, the direct irradiation of the MSCs caused only very modest effect on the expression of CAF markers. For a further understanding of the development of the radiation fibrosis within a tumour, it is essential to understand the interaction of individual components of the TME with the MSCs, as the development and progression of the tumours are determined by the interaction between the cells within the TME (Bissell and Hines, 2011). The aim of this experiment was to see whether irradiated tumour cells (FS120 cells & FS188 cells) secreted growth factors or cytokines that could affect both C3H10 and Balb/c MSCs differentiation marker expression. Therefore, FS188 cells, as well as FS120 cells, were cultured in 24-well plates and when they became 80% confluent, their media were changed and then irradiated with 0, 0.5 and 4 Gy.

24 hours later, C3H10 MSCs and Balb/c MSCs were seeded separately (3×10^4 in 200 µl medium) in filter inserts (Falcon® 1.0 µm pore size) and were co-cultured with the tumour cells for 3-4 days before cell lysates were prepared. 20 µg of sample protein (30 µl/lane) was loaded for each lane of the gel in the western blotting. Normalisation done by calculating the ratio of target protein to internal control.

As can be seen from **Figure 3.5**, the irradiated tumour cells had no significant effects on the expression of the various differentiation markers in C3H10 cells (**Figure 3.5**). However, when Balb/c MSCs were co-cultured with irradiated FS188 cells (0.5 Gy), there were significant increase in the expression of collagen-I, PDGFR-β and α-SMA (but not fibronectin) (**Figure 3.6**) (One-way ANOVA, Tukey's multiple comparisons test, $P = 0.021$, 0.036, and 0.022 respectively). Additionally, irradiation of FS188 by 4 Gy caused an increase in the expression of collagen-I ($P = 0.016$) and PDGFR-β ($P = 0.026$) in co-cultured Balb/c MSCs. On the other hand, co-culture with FS120 cells did not cause a significant increase in Balb/c MSC differentiation markers, although there was a tendency towards such an increase (**Figure 3.6**).

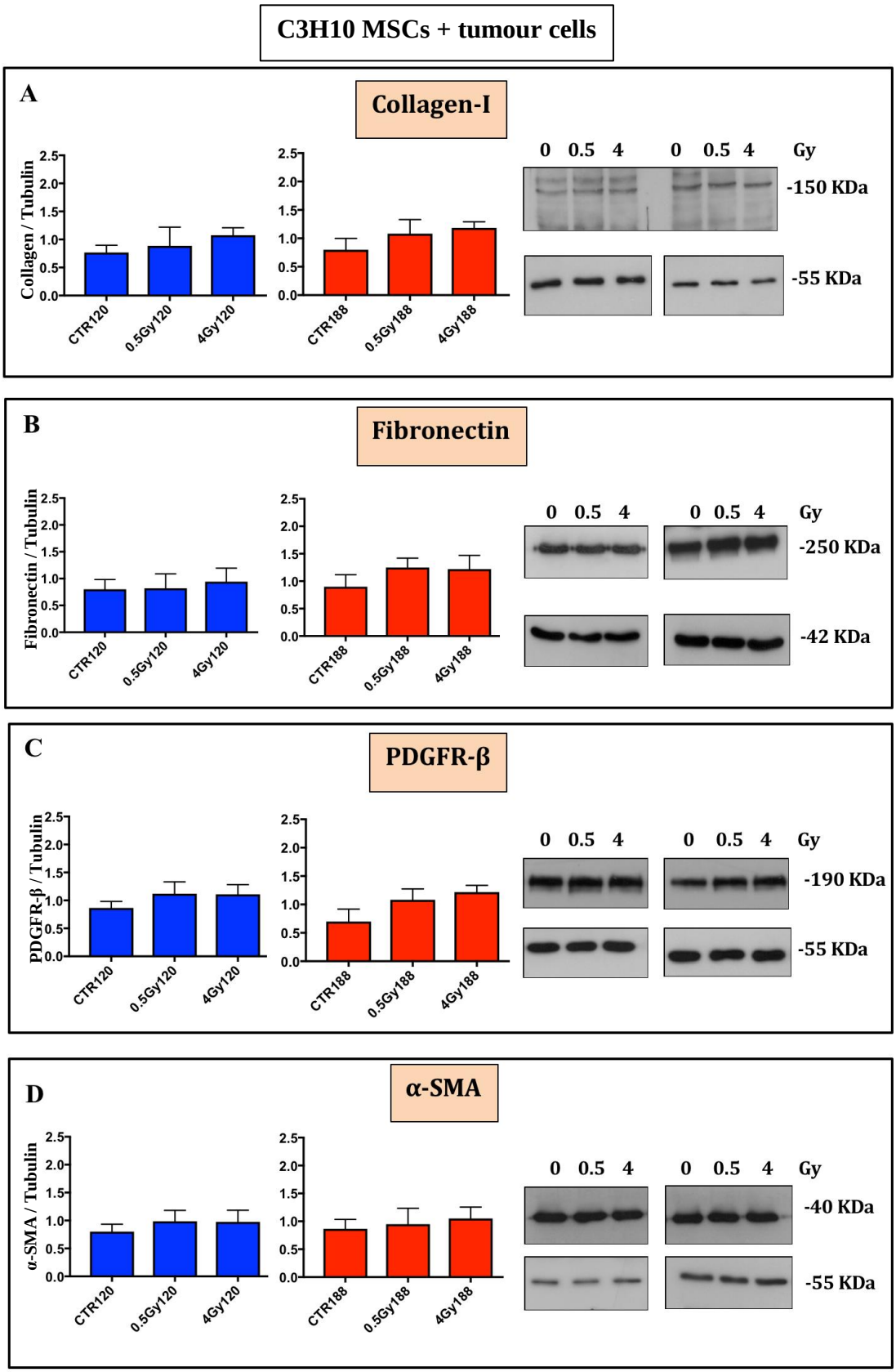

C3H10 MSCs + tumour cells
A
Collagen-I
Collagen / Tubulin
2.5
2.0
1.5
1.0
0.5
0.0
CTR120
0.5Gy120
4Gy120
CTR188
0.5Gy188
4Gy188
0 0.5 4 0 0.5 4 Gy
-150 KDa
-55 KDa
B
Fibronectin
Fibronectin / Tubulin
0 0.5 4 0 0.5 4 Gy
-250 KDa
-42 KDa
C
PDGFR-β
PDGFR-β / Tubulin
0 0.5 4 0 0.5 4 Gy
-190 KDa
-55 KDa
D
α-SMA
α-SMA / Tubulin
0 0.5 4 0 0.5 4 Gy
-40 KDa
-55 KDa

Figure 3.5 Effects of irradiated tumour cells (FS120 cells, and FS 188 cells) on the expression of CAF markers by C3H10 MSCs.

Tumour cells were cultured in 24-well plates and when they became 80% confluent, their media were changed and then irradiated with 0, 0.5, and 4 Gy. 24 hours later, the C3H10 MSCs were seeded (3×10^4 in 200 µl medium) in filter inserts (Falcon® 1.0 µm pore size) and were co-cultured with the tumour cells for 4 days before extraction of their cell lysates. 20 µg of sample protein (30 µl/lane) was loaded for each lane of the gel. Normalisation was done by calculating the ratio of target protein to internal control. Presence of irradiated tumour cells (FS120 cells, and FS188 cells) with the C3H10 MSCs did not cause any changes in the MSCs differentiation markers. One-way ANOVA test followed by a Tukey's multiple comparisons test was used to analyse data. Results expressed as means ± SEM. Each graph represents three independent experiments (N=3).

Balb/c MSCs + tumour cells

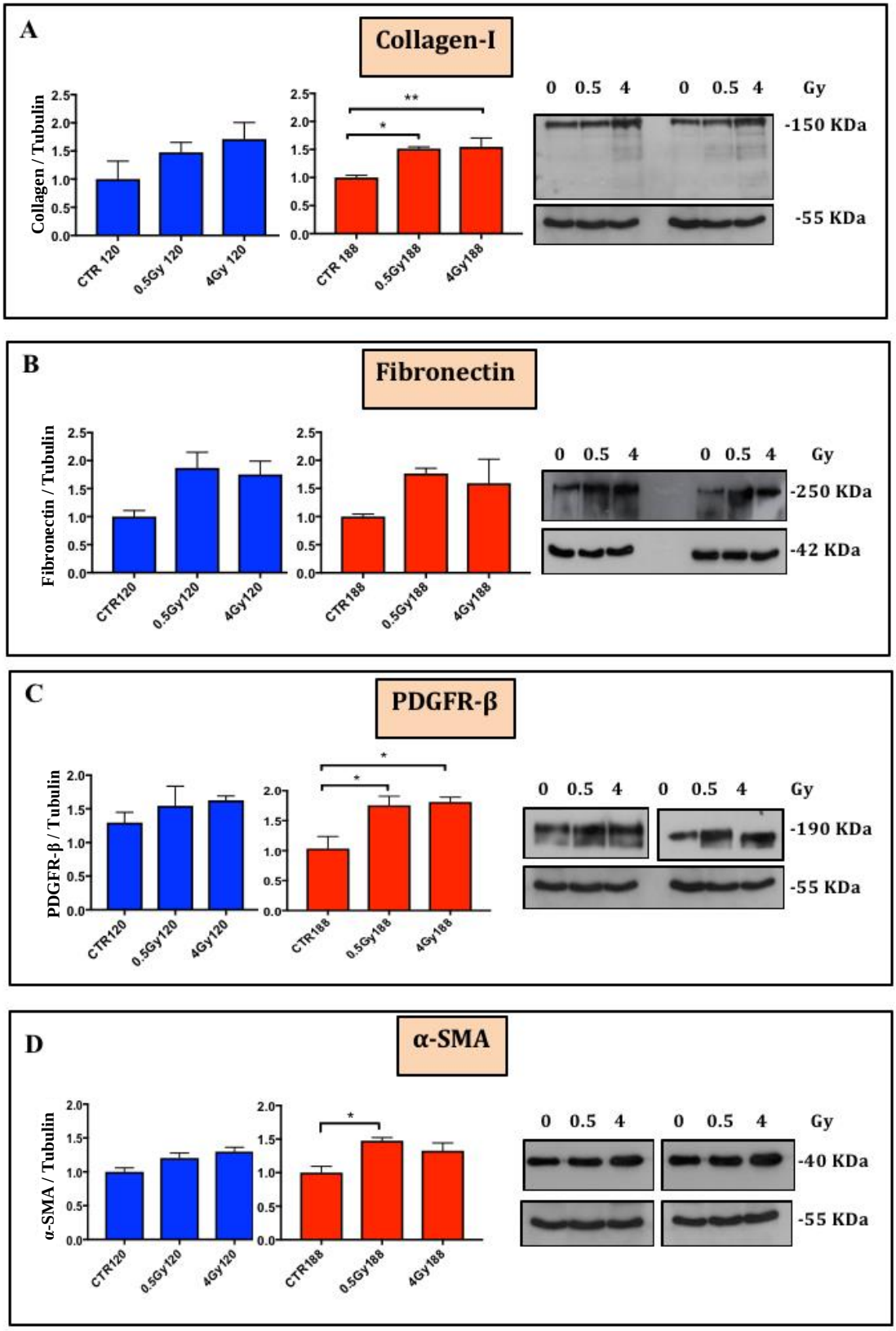


Figure 3.6 Effects of irradiated tumour cells (FS120 cells, and FS 188 cells) on the expression of CAF markers by Balb/c MSCs.

Tumour cells were cultured in 24-well plates and when they became 80% confluent, their media were changed and then irradiated with 0, 0.5, and 4 Gy. 24 hours later, the MSCs were seeded (3×10^4 in 200-µl medium) in filter inserts (Falcon® 1.0 µm pore size) and were co-cultured with the tumour cells for 4 days before extraction of their cell lysates. 20 µg of sample protein (30 µl/lane) was loaded for each lane of the gel. Normalisation was done through calculating the ratio of target protein to internal control (tubulin). From the results, the presence of irradiated FS188 cells significantly increased the expression of collagen-I, and PDGFR-β after 0.5 Gy, and 4 Gy (A and C), and α-SMA after 0.5 Gy (D), while it has no effects on fibronectin expression (B). There were no significant effects of FS120 cells on MSCs differentiation markers. One-way ANOVA test followed by a Tukey's multiple comparisons test was used to analyse data. Results expressed as means ± SEM, and were considered statistically significant when $P \leq 0.05$ (* $P<0.05$, ** $P<0.01$). All experiments were accomplished in triplicate and repeated three times and analysed using GraphPad Prism 7.0 software.

3.2.3.3 Effects of secreted factor(s) produced by irradiated tumour cells on the expression of CAF markers by MSCs

In the previous experiment (**section 3.2.3.2**), the presence of irradiated FS cells (especially FS188 cells) was shown to cause an increase in the expression of Balb/c MSCs differentiation markers, which implies that secreted factors produced by irradiated tumour cells might be responsible. It was important to confirm that, by testing conditioned media (both neat and concentrated) from irradiated tumour cells. In the light of that, serum-free conditioned media were collected from control and irradiated FS cells and concentrated using Amicon Ultra-4 centrifugal filters as described in **section 2.2.1.7**. In the lower chamber of the 24-well plate, either neat or concentrated CM was placed, and in the upper chamber filters C3H10 MSCs or Balb/c MSCs (3 x 10^4 / 200 µl medium) were seeded in their corresponding media. Incubation was for 3 days before extraction of the cell lysate. 20 µg of sample protein (30 µl/lane) was loaded for each lane of the gel in the western blot. Normalisation was done through calculating the ratio of target protein to internal control (tubulin).

As the neat medium had no effect of the expressions of CAF markers by MSCs, I focused on the concentrated CM data. Data from three experiments is shown in **Figure 3.7** and **3.8**. There was no effect of the CM (neat or concentrated) extracted from irradiated tumour cells on the C3H10 expression markers (**Figure 3.7**). Concentrated CM from irradiated FS120 and FS188 cells had no significant effect on α-SMA expression in Balb/c MSCs, However, concentrated CM from irradiated (0.5 and 4 Gy) FS188 cells, caused a significant increase in the expression of collagen-I (P = 0.0014, and 0.0067) and fibronectin (P = 0.038, and 0.031). A similar increase in the expression of collagen-I (P = 0.047) and PDGFR-β (P =0.033) was observed using concentrated CM from irradiated FS120 cell with 4 Gy (**Figure 3.8 A** and **C**). In this experiment, I missed to use a non-irradiated medium alone to see whether presence of tumour cells was the cause of the increase in the CAFs expression markers expressed by MSCs. This work will be done in the future.

One-way ANOVA followed by Tukey's multiple comparisons test was used.

C3H10 MSCs + concentrated CM from irradiated tumour cells

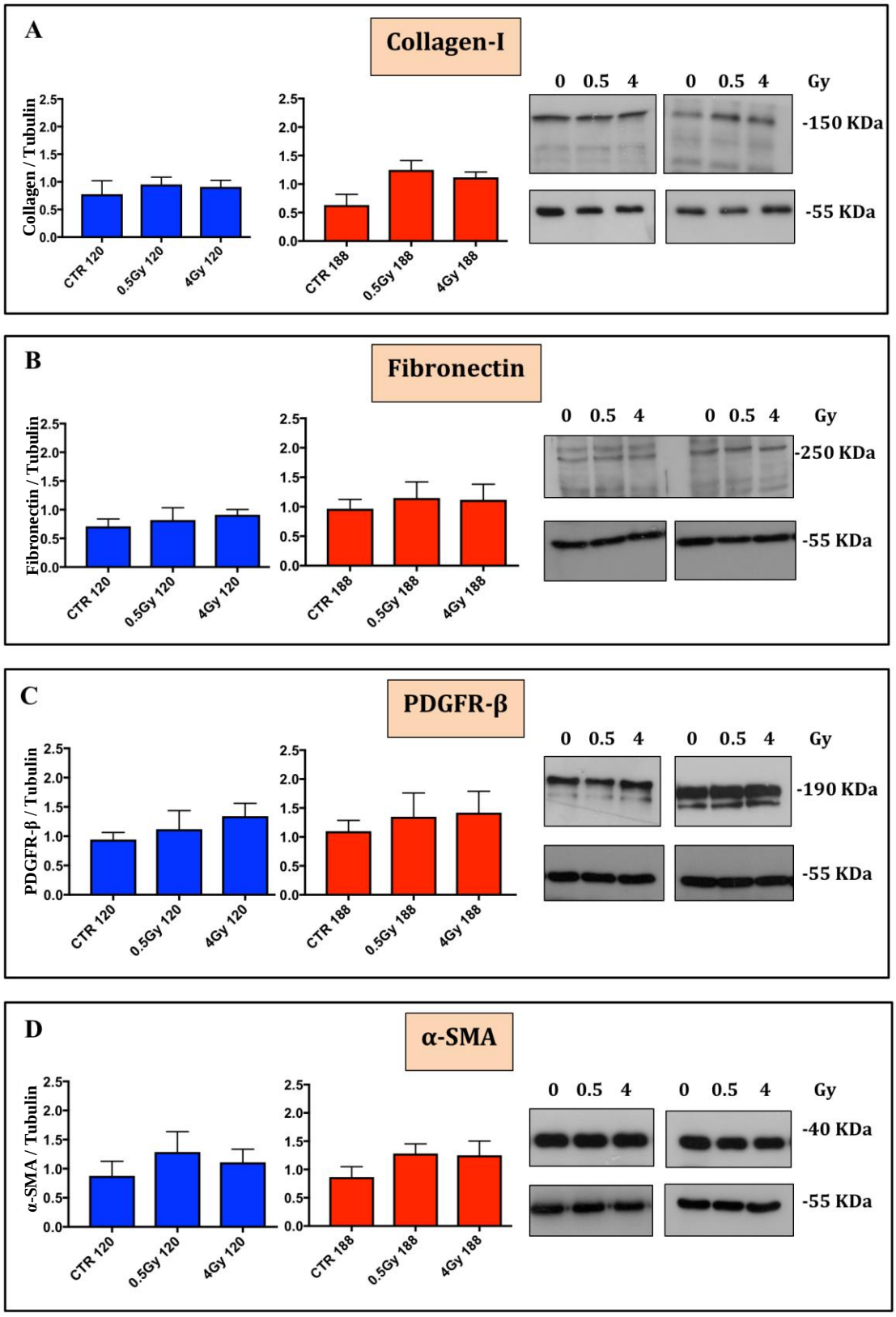


Figure 3.7 Effects of factors (s) produced from irradiated tumour cells on the expression of CAF markers by C3H10 MSCs.

C3H10 MSCs were exposed to concentrated CM from irradiated FS120 cells and FS188 cells for 3 days. Normalisation was done through calculating the ratio of target protein to internal control (i.e., dividing or "normalizing" by the loading control). No effects of the concentrated CM extracted from irradiated tumour cells were seen on the expression of collagen-I, PDGFR-β, fibronectin or α-SMA. One-way ANOVA test followed by a Tukey's multiple comparisons test was used to analyse data. Results expressed as means ± SEM of 3 experiments, each done in triplicate and were considered statistically significant when $P \leq 0.05$.

Balb/c MSCs + concentrated CM from irradiated tumour cells

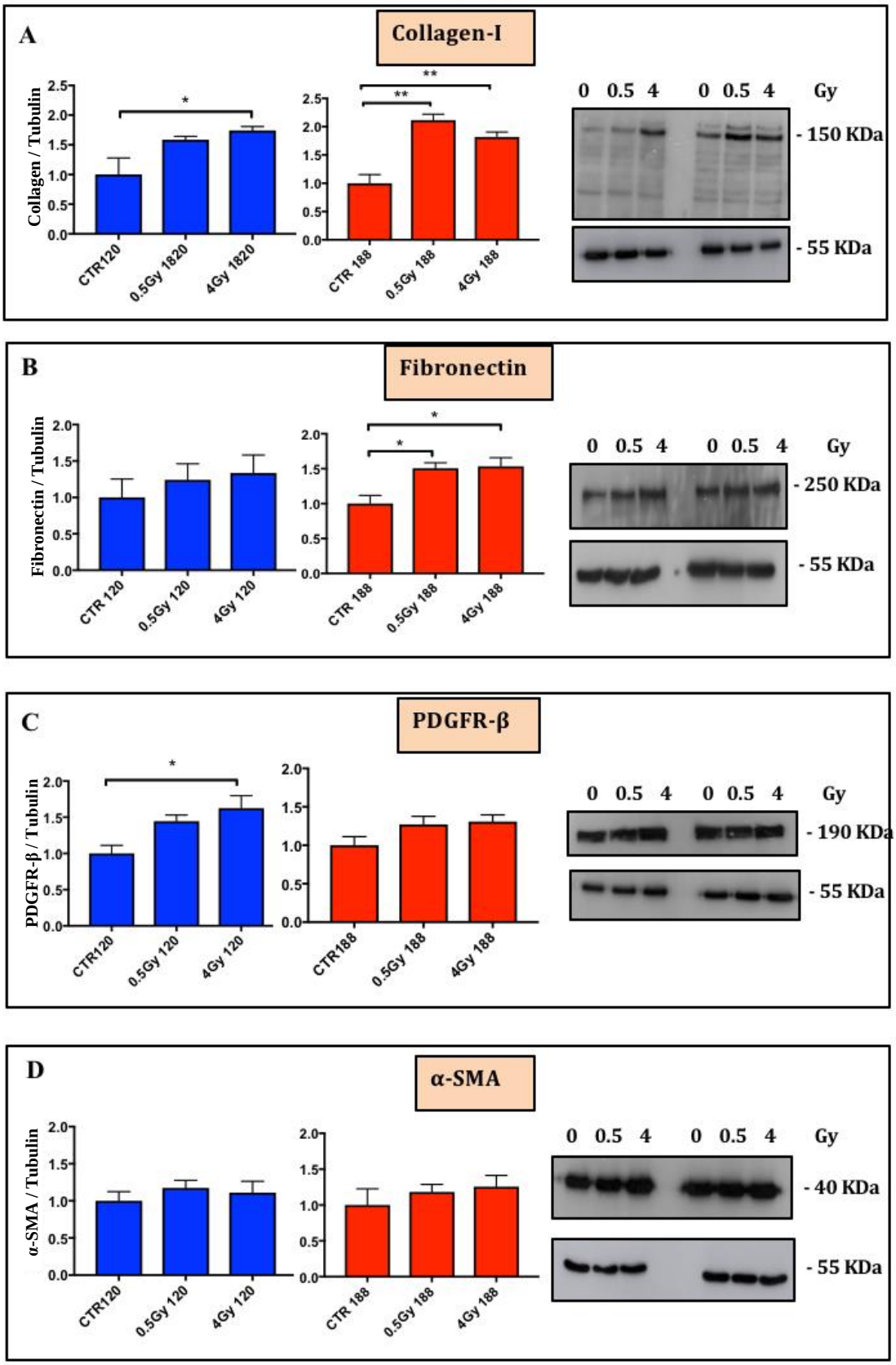


Figure 3.8 Effects of factors (s) produced from irradiated tumour cells on expression of CAF markers by Balb/c MSCs.

Balb/c MSCs were incubated with concentrated CM extracted from irradiated FS120 cells, and FS188 cells for 3 days. The results showed an increase in the expression of collagen-I, and fibronectin in cells treated with concentrated CM from 0.5 Gy and 4 Gy- treated FS188 cells. Likewise, a significant increase in collagen-I and PDGFR-β for MSCs treated with concentrated CM from irradiated FS120 cells with 4 Gy. One-way ANOVA test followed by a Tukey's multiple comparisons test was used to analyse data. Results expressed as means ± SEM of 3 experiments, each done in triplicate and were considered statistically significant when $P \leq 0.05$ (* $P<0.05$, ** $P<0.01$).

3.2.3.4 Effects of irradiated HDMEC on the expression of differentiation markers by Balb/c MSCs

Endothelial cells are important tumour-stromal cells (Hanahan and Weinberg, 2011). It has been shown that pro-inflammatory mediators (chemokines and cytokines) were increased in the supernatant of irradiated HDMEC (Haubner et al., 2013b). Moreover, the presence of endothelial cells co-cultured with MSCs significantly affected their differentiation and proliferation (Saleh et al., 2011). Firstly, HDMEC were cultured in their standard medium in 24 well plates. Then, when they became 80% confluent, their medium was changed to Balb/c medium and incubated for 2 hours and then irradiated. 24 hours after radiation, Balb/c MSCs were seeded at 3 x 10^4 / 200 µl medium in the 1.0 µm pore size filter inserts (upper chamber). Inserts containing the Balb/c MSCs were incubated with HDMEC for 3 days and then the cell lysates were extracted. For the western blotting, the amount of sample protein loaded in each lane was 20 µg (30 µl/lane). To normalise the samples, the ratio of target protein was divided by internal control (i.e., dividing or "normalizing" by the loading control).

Fibronectin and PDGFR-β were significantly increased after radiation (0.5 Gy and 4 Gy) whilst, neither collagen-I nor α-SMA showed any significant changes after 0.5 Gy and 4 Gy (although they showed the tendency to increase) (**Figure 3.9**).

Because the results from C3H10 MSCs were not robust and not promising (as shown in previous experiments), I stopped working with them and continue with Balb/c MSCs as a model of stem cells.

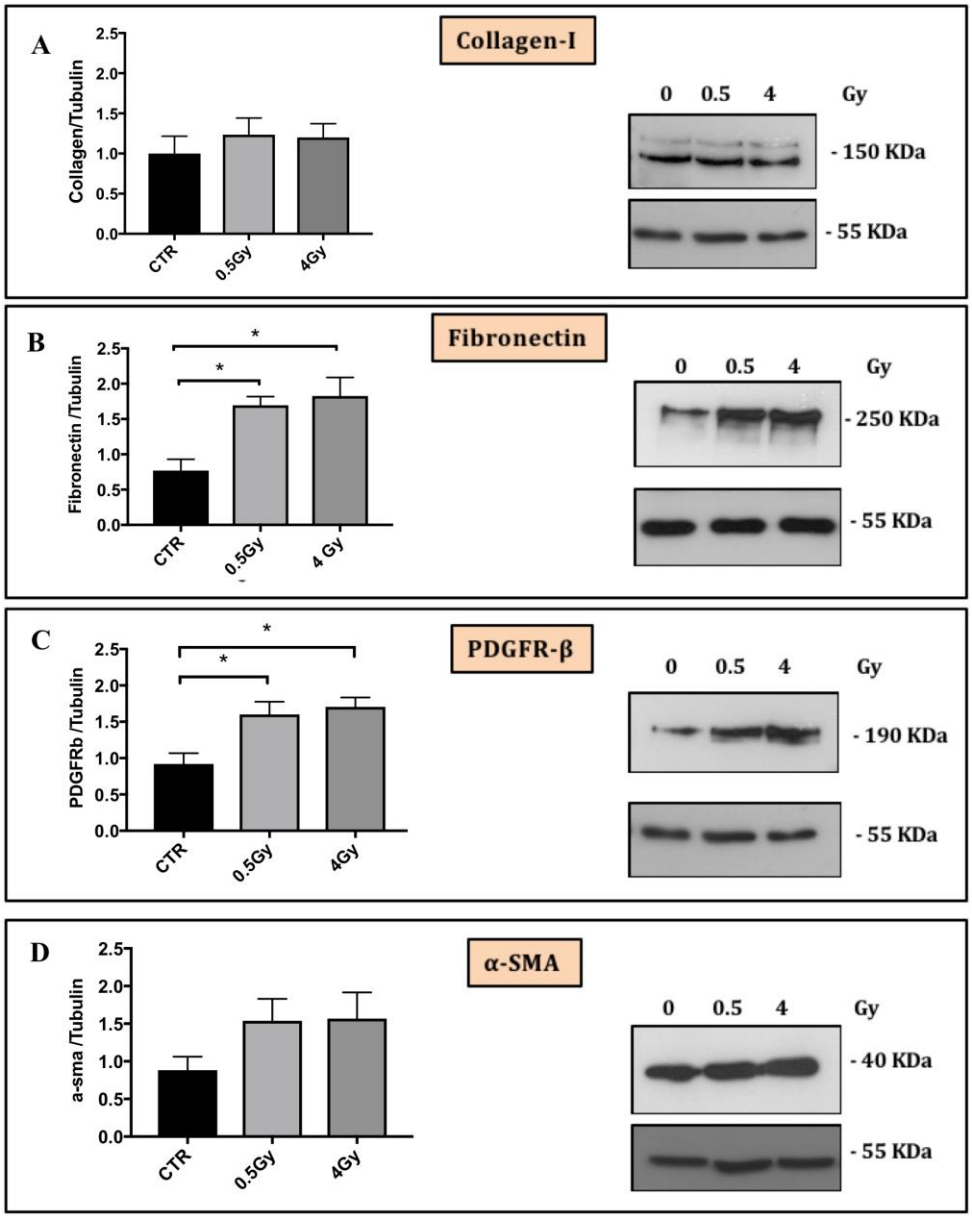


Figure 3.9 Effects of irradiated HDMEC on the expression of CAF markers by Balb/c MSCs.

Balb/c MSCs co-cultured with irradiated HDMEC (0.5 Gy & 4 Gy) for 3 days showed increased expression of fibronectin and PDGFR-β (B & C). There were no significant changes in the expression of collagen-I & α-SMA (A & D). One-way ANOVA test (Tukey's multiple comparisons test) was used to analyse data. Quantitative data represent mean value ± SEM of 3 experiments, each done in triplicate and were considered statistically significant when $P \leq 0.05$.

3.2.3.5 Effects of irradiated HDMEC co-cultured with the irradiated tumour cells on the MSCs differentiation markers

To model the TME further, FS120 & FS188 cells were co-cultured with HDMECs. As described previously, FS tumour cells were established from transgenic mouse embryos by immortalisation with SV40 and H-ras transformation. The cells are routinely cultured in medium containing G418, and pyromycin to maintain transgene selection. When the cells became 80% confluent, they were trypsinised, centrifuged and re-suspended with HDMEC medium (without G418 and pyromycin in which HDMEC do not survive). At the same time, HDMEC were trypsinised and re-suspended with their media. Since endothelial cells were growing slower than FS cells, they were mixed together at a ratio of 1:4 (12×10^3 FS + 48×10^3 HDMEC) (**Figure 3.10**).

The cell mixture was placed in the lower chamber of 24 well plates and incubated for 2 days. Then, the media were changed to Balb/c media and incubated for 2 hours before they were irradiated with (0.5 Gy or 4 Gy). The cells were incubated for 24 hours, then Balb/c MSCs were seeded at 3×10^4 / 200 µl medium into 1.0 µm pore size filter and co-cultured with the fibrosarcoma/endothelial cultures for 3 days. For the western blotting, the amount of sample protein loaded in each lane was 20 µg (30 µl/lane). Normalisation was done by dividing the ratio of target protein to internal control (i.e., dividing or "normalizing" by the loading control). Western blot analyses showed that collagen-I, fibronectin, and PDGFR-β but not α-SMA were significantly increased after incubation of MSCs with HDMEC + FS188 cells irradiated with 0.5 Gy and 4 Gy (**Figure 3.11**). In contrast, only collagen-I and PDGFR-β were significantly upregulated in Balb/c MSCs that were incubated with HDMEC + irradiated FS120 cells (**Figure 3.11 A and C**).

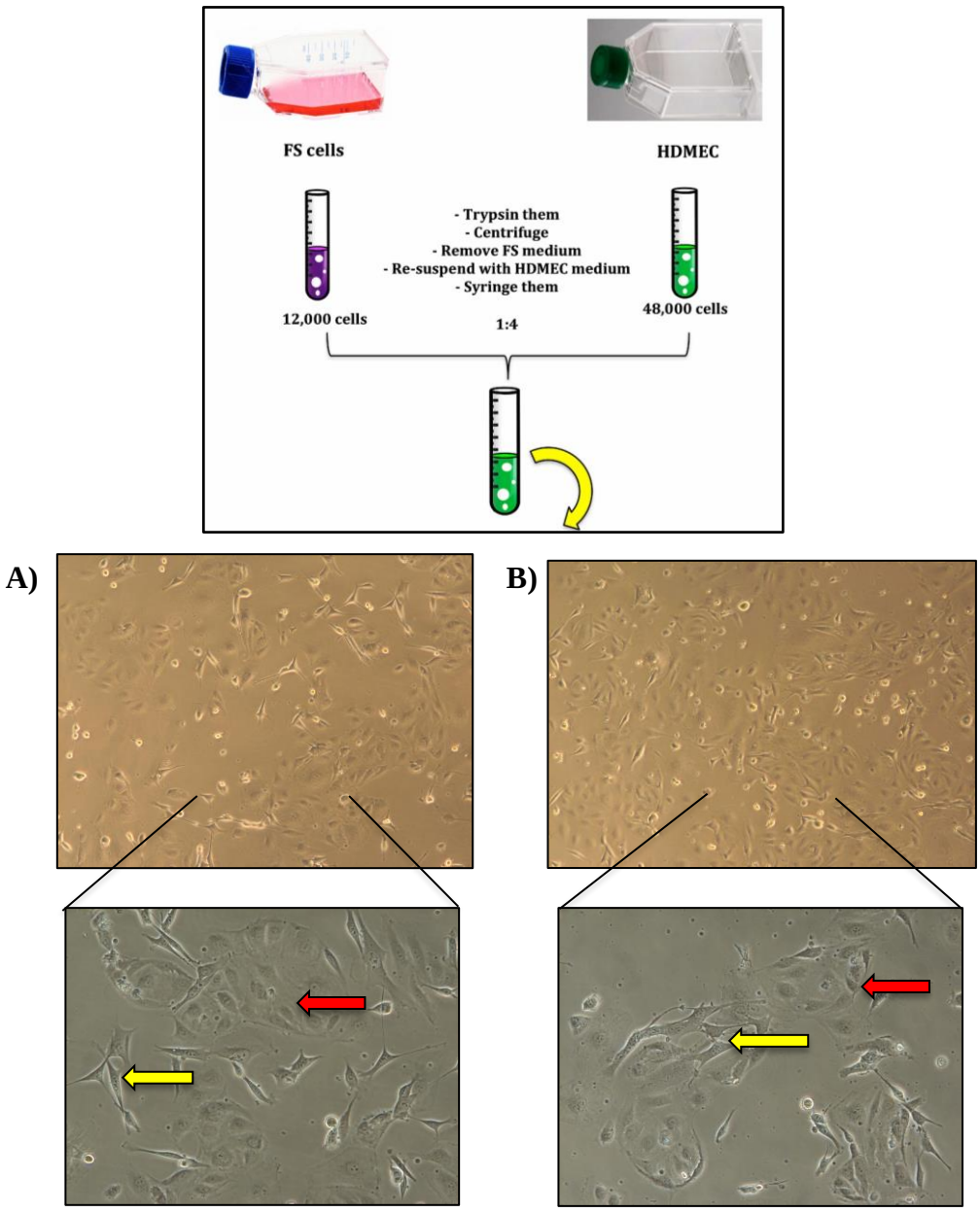


Figure 3.10 Co-culture of HDMEC with tumour cells.

Both tumour cells (FS120 cells & FS188 cells) and early passage HDMEC were co-cultured together at ratio of 1:4 respectively using HDMEC medium. A) FS 120 cells co-cultured with HDMEC. B) FS 188 cells co-cultured with HDMEC.

⇦ Represent FS cells and ⇦ represent HDMEC

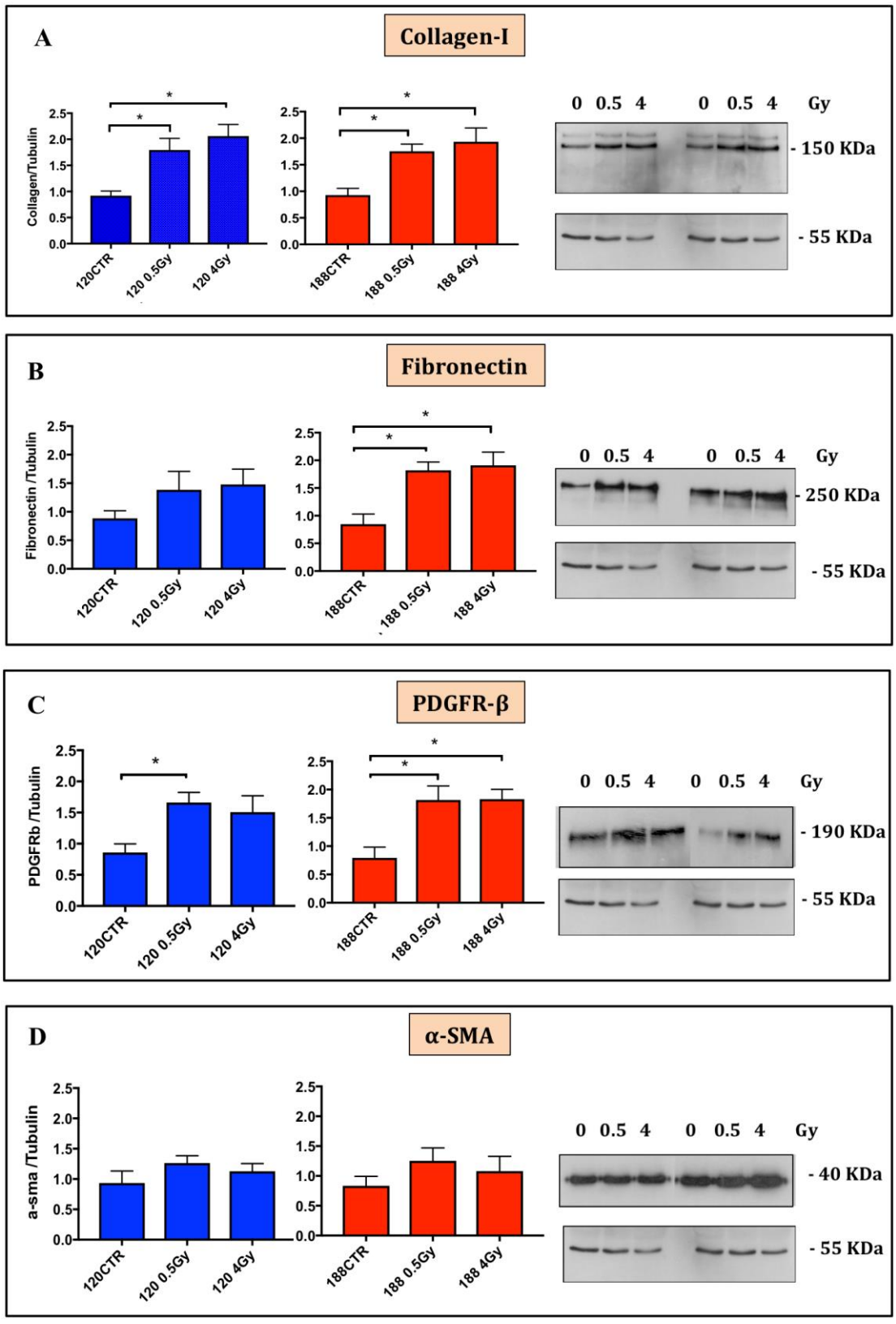

A
Collagen-I
Collagen/Tubulin
120CTR
120 0.5Gy
120 4Gy
188CTR
188 0.5Gy
188 4Gy
0 0.5 4 0 0.5 4 Gy
- 150 KDa
- 55 KDa
B
Fibronectin
Fibronectin /Tubulin
- 250 KDa
- 55 KDa
C
PDGFR-β
PDGFRb /Tubulin
- 190 KDa
- 55 KDa
D
α-SMA
a-sma /Tubulin
- 40 KDa
- 55 KDa

Figure 3.11 Co-culture of Balb/c MSCs with both irradiated HDMEC plus tumour cells.

Balb/c MSCs that were incubated with irradiated HDMEC + FS188 cells showed a significant increase in the expression of collagen-I, fibronectin, and PDGFR-β (except α-SMA) compared to cells co-cultured with non-irradiated HDMEC and FS188 cells (A, B, and C). In contrast, there were no changes in fibronectin and α-SMA expression in Balb/c MSCs that were incubated with irradiated HDMEC + FS120 cells, only significantly increased in collagen-I after 0.5 Gy and 4 Gy and for PDGFR-β after 0.5 Gy (A and C). One-way ANOVA test (Tukey's multiple comparisons test) was used to analyse data. Quantitative data represent mean value ± SEM and were considered statistically significant when $P \leq 0.05$.

3.2.3.6 Effects of chemokines and cytokines on the differentiation markers expressed by Balb/c MSCs

It was reported by Klopp et al, that irradiation of tumour cells caused an increase in the secretion of cytokines like VEGF, TGF-β1, and PDGF. Moreover, exposure of MSCs to irradiated tumour cells caused up-regulation of CCR2, a receptor for MCP-1) (Klopp et al., 2007). Some studies have demonstrated that tumour cells secrete stromal cell-derived factor 1α (SDF-1α) that enhance MSCs migration (Kitaori et al., 2009, Abbott et al., 2004). Moreover, I showed that both irradiated tumour cells and their concentrated CM increased the expression of differentiation markers by MSCs (see **section 3.2.3.2**, and **3.2.3.3**). Therefore, the effects of MCP-1 and SDF-1 on the differentiation markers expressed by Balb/c MSCs were important to determine.

For this experiment, 1 x 10^5 /ml Balb/c MSCs were seeded in 6-well plates, and when the cells reached 80% confluently, cells were treated with either TGF-β1 (10 ng/ml), MCP-1 (10 ng/ml), or SDF-1 (100 ng/ml) or left untreated as controls. Cells were incubated for 3 days prior to protein extraction. 20 μg of cell lysates were analysed by western blotting as described previously. The value of the target protein was divided by the loading control to get a relative intensity (target/loading).

From the western blot analysis, TGF-β1 was the only cytokine that significantly up-regulated all differentiation markers (**Figure 3.12**). The morphological changes which were seen in both MSCs (C3H10, and Balb/c) (towards CAFs-like cells) after treatment with TGF-β1 (see **section 3.2.2**) in addition to increasing in the expression of α-SMA, together indicated that MSCs were acquiring characteristics of CAF cells but only after TGF-β1 treatment.

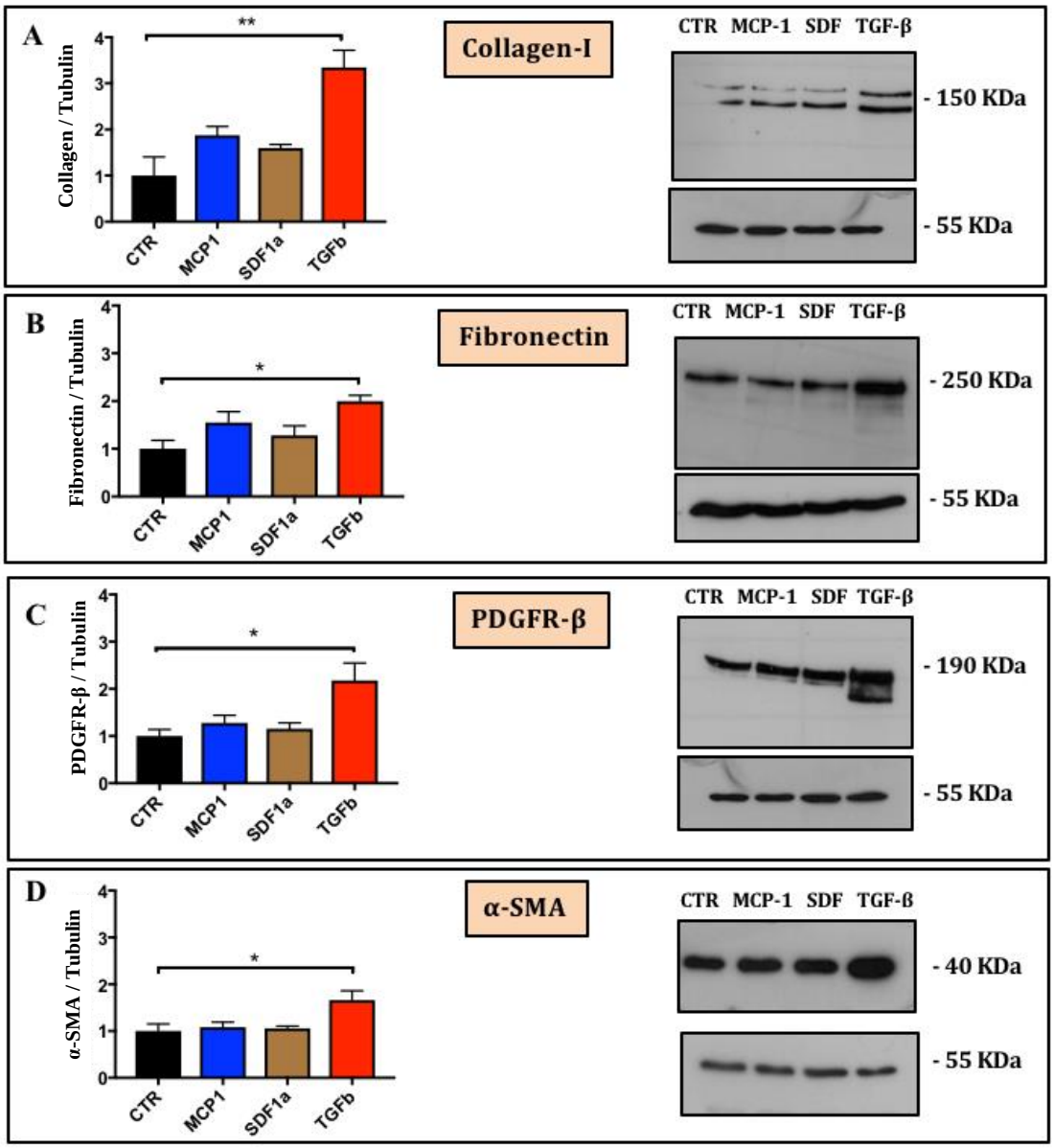


Figure 3.12 Effects of TGF-β, MCP-1, and SDF-1 on the differentiation markers expressed by Balb/c MSCs.

Balb/c MSCs were seeded in 6-well plates, each well was treated with either TGF-β (10 ng/ml), or MCP-1 (10 ng/ml), or SDF-1 (100 ng/ml) and incubated for 3 days. Cell lysates were extracted and run for western blot. Analyses using one-way ANOVA test followed by a Tukey's multiple comparisons test showed that TGF-β was the only cytokine that significantly up-regulated all differentiation markers (**A**, **B**, **C**, and **D**). Results expressed as means ± SEM of 3 experiments, each done in triplicate and were considered statistically significant when $P \leq 0.05$ (* $P<0.05$, ** $P<0.01$).

3.2.4 *In vitro* radiation effects on Balb/c MSCs migration (transwell migration assay)

Migration in biology usually describes the movement of the cells directly through substrates like ECM fibers or basal membranes (Kramer et al., 2013). Recently, there has been increased relevance into the migration and homing ability of MSCs into tumours (De Becker and Van Riet, 2016). The interaction between MSC and the TME play an important role in the development and progression of tumours. It has been shown that recruitment of the MSC to the injury sites is important to support tissue repair, angiogenesis, immune modulation and stem cell homeostasis (Karnoub et al., 2007, Mandel et al., 2013). Moreover, migration of MSC to the site of injury or inflammation causes either direct or indirect interaction between MSC and tumour cells (Melzer et al., 2016). The direct interaction occurs via membrane receptors and gap junctions while the indirect interaction is through secretion of cytokines, chemokines and growth factors (Mandel et al., 2013, Yang et al., 2015b) (see chapter one).

MSCs can stimulate neighbouring cells with pro-tumorigenic and/or anti-tumorigenic behaviours by releasing these cytokines, chemokines and growth factors. Furthermore, tumour cells in the TME have the abilities to stimulate MSCs to develop a tumour-associated phenotype (Hass and Otte, 2012). It has been shown that MSCs have the ability to migrate to sites of tissue injuries like kidney, skin, and heart as a result of inflammatory mediators that are produced locally due to tissue damage (Morigi et al., 2004, Li et al., 2006). Moreover, Mouiseddine et al showed that the migration of MSCs to the radiation-injured tissues was enhanced by local irradiation of the mouse abdomen (Mouiseddine et al., 2007). One of the potential methods to induce inflammation and tissue injury is local irradiation. Therefore, I tested the effect of irradiation (directly and indirectly) on the recruitment of the MSCs using an *in vitro* transwell migration assay model. In addition, I tested the role of the inflammatory cytokines and chemokines in radiation-induced MSC migration.

3.2.4.1 Effects of direct irradiation on the migration of MSCs *in vitro*

To investigate the ability of direct radiation to induce migration of MSCs, the *in vitro* transwell migration model was used. C3H10 and Balb/c MSCs were seeded individually as 3 x 10^4 cells / 200-µl medium in transwell dishes (Falcon® 8.0 µm pore filters). Next, the cells were irradiated with 0.5 Gy and 4 Gy, with the un-irradiated group as control. Migration was observed 8 hours' post-irradiation as explained in **section 2.2.5.1**. As shown in **Figure 3.13**, direct radiation of the C3H10 MSCs and Balb/c MSCs did not show a significant increase in the migration of these cells.

3.2.4.2 Irradiated tumour cells increase the migration of Balb/c MSCs *in vitro*

Our aim was to detect paracrine factor(s) secreted by tumour cells that influence the migration of C3H10 and Balb/c MSCs *in vitro*. So, the *in vitro* co-culture assay was carried out using tumour cells (FS120 cells, and FS188 cells) seeded at the lower chamber of the 24-well plate with 2 wells left with medium alone (no tumour cells) serving as a negative control. When the cells became 80% confluent, they were irradiated with 0.5 and 4 Gy, with the non-irradiated group as a positive control. After 24 hours, the upper chamber (8.0 µm filter) was seeded with either C3H10 MSCs or Balb/c MSCs (3 x 10^4 cells / 200µl medium) and incubated for 8 hours. Migrated cells were then fixed, stained and counted. Results of this experiment are shown in **Figure 3.14**. Regarding the migration of the C3H10 MSCs, there were no effects of irradiated tumour cells on the recruitment of the C3H10 MSCs. Also, the presence of un-irradiated tumour cells in the lower transwell section did not show any effects on the migration of the C3H10 MSCs (**Figure 3.14 A**). On the other hand, the presence of un-irradiated FS120 cells or FS188 cells in the lower transwell compartment increased the migration of Balb/c MSCs compared to medium alone. In addition, irradiation of a tumour cells with low-dose (0.5 Gy), and high dose (4 Gy) enhanced the migratory capacity of Balb/c MSCs (**Figure 3.14 B**). These results suggest that tumour cells secreted growth factors in their media that positively enhanced Balb/c MSCs migration. This means that the growth factors produced by tumour cells were potentially upregulated and secreted as a consequence of irradiation. Therefore, studying the effects of these growth factors within the conditioned medium from irradiated FS cells on the migration of MSCs was the next step.

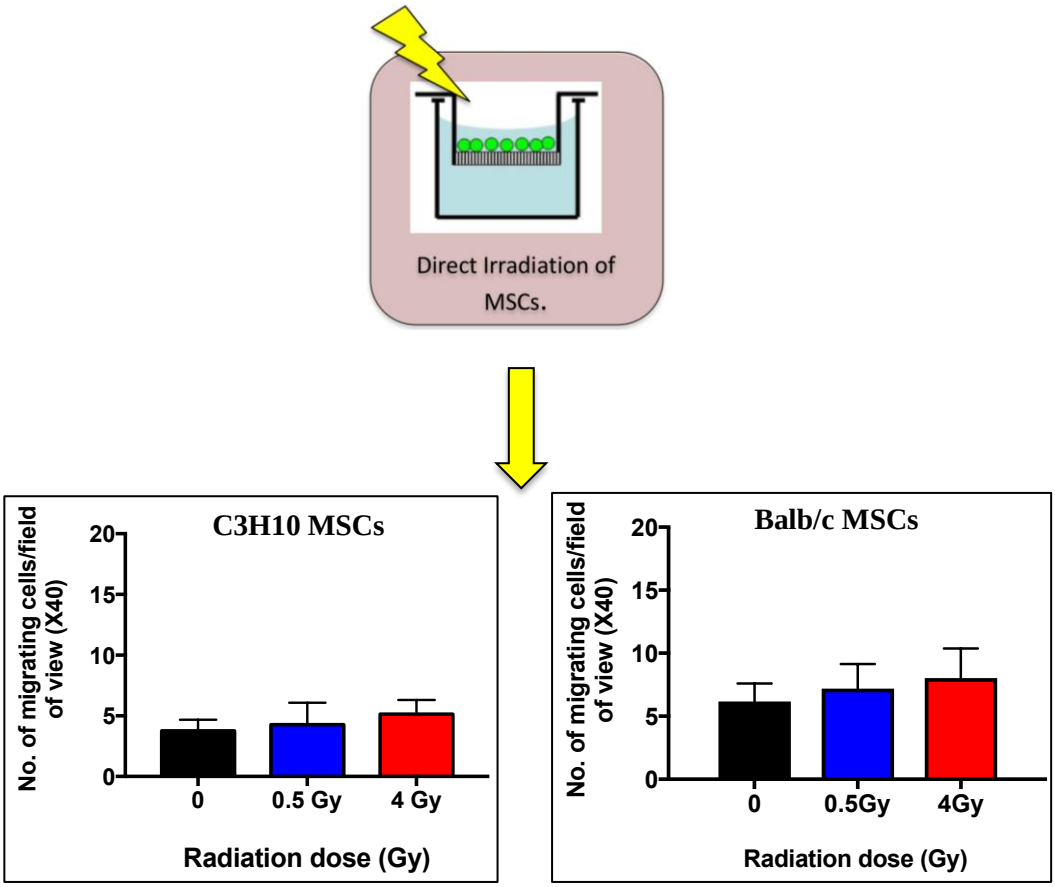


Figure 3.13 Direct irradiation does not increase MSCs migration in vitro.

C3H10 MSCs and Balb/c MSCs were seeded separately in the 8.0-µm pore filters, before they were irradiated with 0, 0.5, and 4 Gy. Migrated cells were fixed, stained and counted 8 hours after irradiation. The average number of migrated cells per 10 fields of view (40 X objective) were counted. Analyses using one-way ANOVA test followed by a Tukey's multiple comparisons test showed that there was no significant increase in the migrated cells after direct radiation. Results expressed as means ± SEM of 3 experiments.

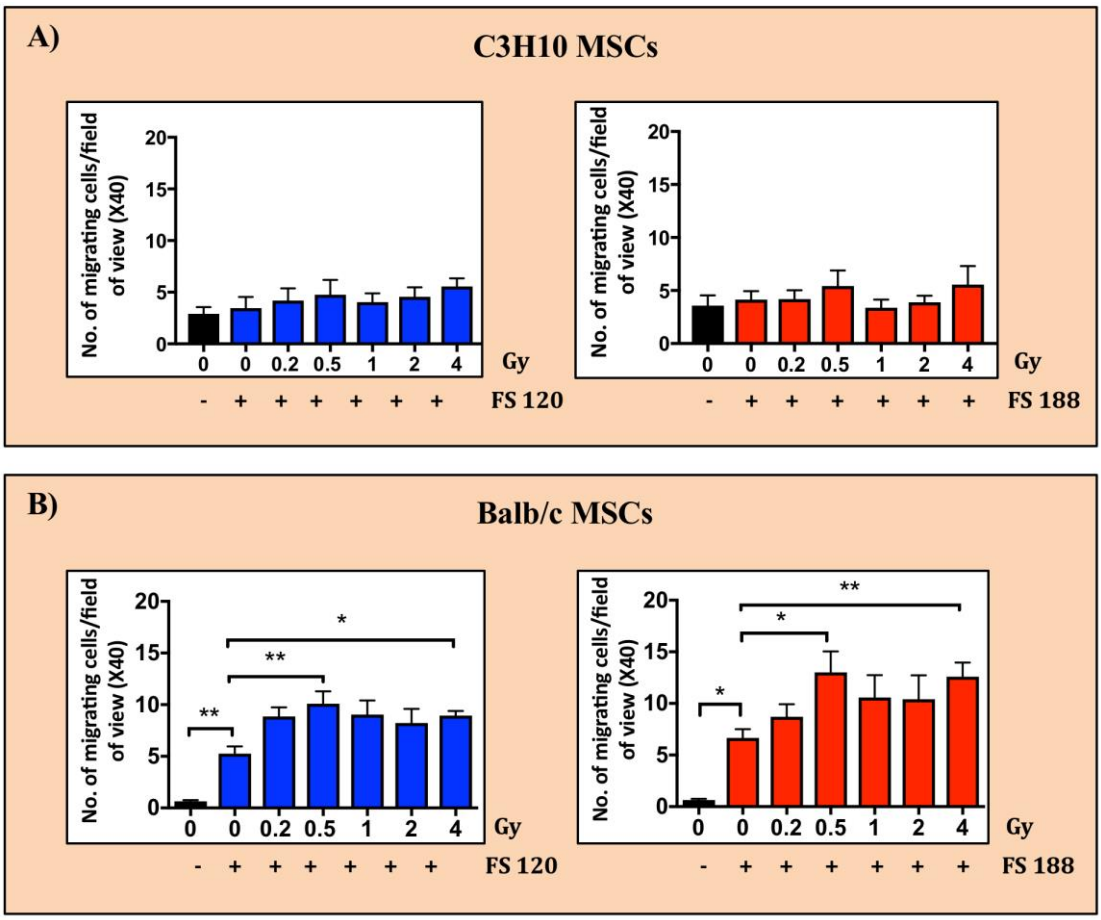


Figure 3.14 Migration capacity of C3H10 and Balb/c MSCs exposed to irradiated tumour cells.

In vitro migration assay for C3H10, and Balb/c MSCs was measured through transwells. Co-culture of C3H10 MSCs with FS120 cells and FS188 cells for 8 hours had no effect on the migration of these cells (compared to cells that migrated in the absence of tumour cells) (A). Balb/c MSCs co-cultured with tumour cells for 8 hours increased their migration compared to medium alone. Irradiation of FS 120, and FS 188 cells with 0.5 Gy, and 4 Gy significantly increased the migration of Balb/c cells further. Statistical analysis was done using one-way ANOVA test followed by Tukey`s multiple comparisons test. Results expressed as means ± SEM, of 6 experiments, each done in triplicate and were considered statistically significant when $P \leq 0.05$ (* $P<0.05$, ** $P<0.01$).

3.2.4.3 Fibrosarcoma cell-conditioned media enhance Balb/c MSCs migration

Presence of irradiated tumour cells caused an increase in migration of Balb/c MSCs as shown in **section 3.2.4.2**. Therefore, CM (neat and concentrated) from irradiated tumour cells were used to see whether growth factor(s) secreted from irradiated fibrosarcoma cells accelerated MSCs chemotaxis. Conditioned media from irradiated FS120 cells and FS188 cells were extracted and concentrated as described in **section 2.2.1.7**. For this experiment, 3 x 10^4 C3H10 MSCs or Balb/c MSCs were plated onto transwell filters (8.0 μm filter) with CM (neat or concentrated) at the lower chamber and incubated for 16 hours. In the previous experiments, the incubation time after irradiation was 8 hours and so, in this experiment, the MSCs were initially incubated with the CM for 8 hours. However, at this time, not enough cells had migrated and so it was decided to double the time to 16 hours.

Neither neat nor concentrated CM had significant effects on the migration of C3H10 MSCs (**Figure 3.15 A**). While both neat and concentrated CM from irradiated tumour cells caused significant migration of the Balb/c MSCs (**Figure 3.15 B**). There were significant differences between the groups for neat CM (comparing FS120 cells and FS 188 cells), i.e. more MSCs were migrated when they were treated with CM from FS188 cells (control and irradiated) than treated with CM from FS120 cells. Moreover, there was a significant increase in migrated MSCs treated with CM from FS188 cells compared to MSCs treated with their medium alone or with concentrated DMEM alone (**Figure 3.15 A**). Likewise, concentrated CM from irradiated tumour cells enhanced the migration of Balb/c MSCs, although effects were similar for FS120 and FS188 cells. The presence of concentrated CM caused a significant increase in the migrations of MSCs compared to using media alone. All experiments were done using duplicate filters and were repeated three times.

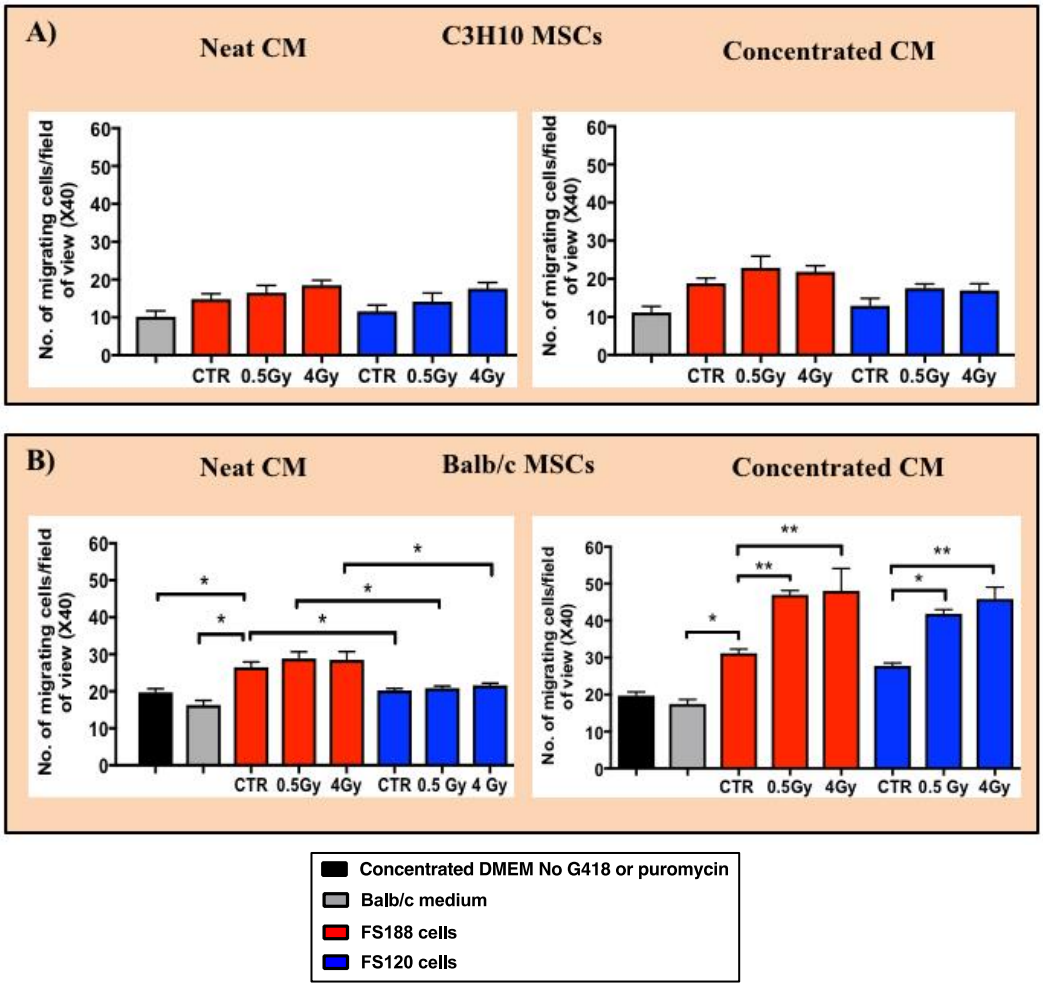


Figure 3.15 Effects of CM (neat and concentrated) on the migration of C3H10 MSCs (A), and Balb/c MSCs (B).

In vitro migration of both C3H10 MSCs and Balb/c MSCs was measured through transwells. Both MSCs were treated individually with CM (neat & concentrated) from irradiated FS120 cells and FS188 cells for 16 hours. CM (neat or concentrated) had no effect on the migration of C3H10 MSCs (**A**). However, the presence of CM (neat or concentrated) increased the migration of Balb/c MSCs compared to medium or DMEM alone (**B**). Moreover, concentrated CM from control and irradiated with 0.5 Gy, and 4 Gy tumour cells significantly increased migration of Balb/c MSCs compared to non-concentrated CM. One-way ANOVA test followed by Tukey`s multiple comparisons test was done using GraphPad Prism 7 software. Results expressed as means ± SEM, of 3 experiments, each done in duplicates and were considered statistically significant when $P \leq 0.05$ (* $P<0.05$, ** $P<0.01$).

3.2.4.4 Effects of irradiated endothelial cells on the migration of Balb/c MSCs

It has been shown that endothelial cells increased the migration of MSCs cells (Yuan et al., 2015, Kamprom et al., 2016). Additionally, co-culture of MSCs with endothelial cells affect their proliferation and differentiation (Saleh et al., 2011). The effect of two types of irradiated endothelial cells (H5V, and HDMEC) on the migration of Balb/c MSCs was determined by transwell assays. H5V cells and HDMEC were seeded separately in 24 well plates with their media and incubated to become confluent. Then, 2 hours before irradiation, their media were changed to Balb/c medium and irradiated with 0.5 and 4 Gy. Two wells were left without irradiation as a positive control, and another two wells left with only Balb/c medium (no cells) as a negative control. 24 hours after irradiation, Balb/c MSCs were cultured into the insert and incubated for 16 hours as before.

The result revealed that there were no effects of the irradiated H5V endothelial cell on the migration of Balb/c MSCs (**Figure 3.16 A**). However, exposure of Balb/c MSCs to HDMEC for 8 hours caused significantly increased in the migration of MSCs compared to Balb/c medium alone (**Figure 3.16 B**). Additionally, irradiation of the endothelial cells with 0.5 Gy and 4 Gy caused more MSCs migration compared to control non-irradiated cells. These results indicated that the presence of the HDMEC enhanced the migration of the Balb/c MSCs, while irradiation increased this migration ability of these cells.

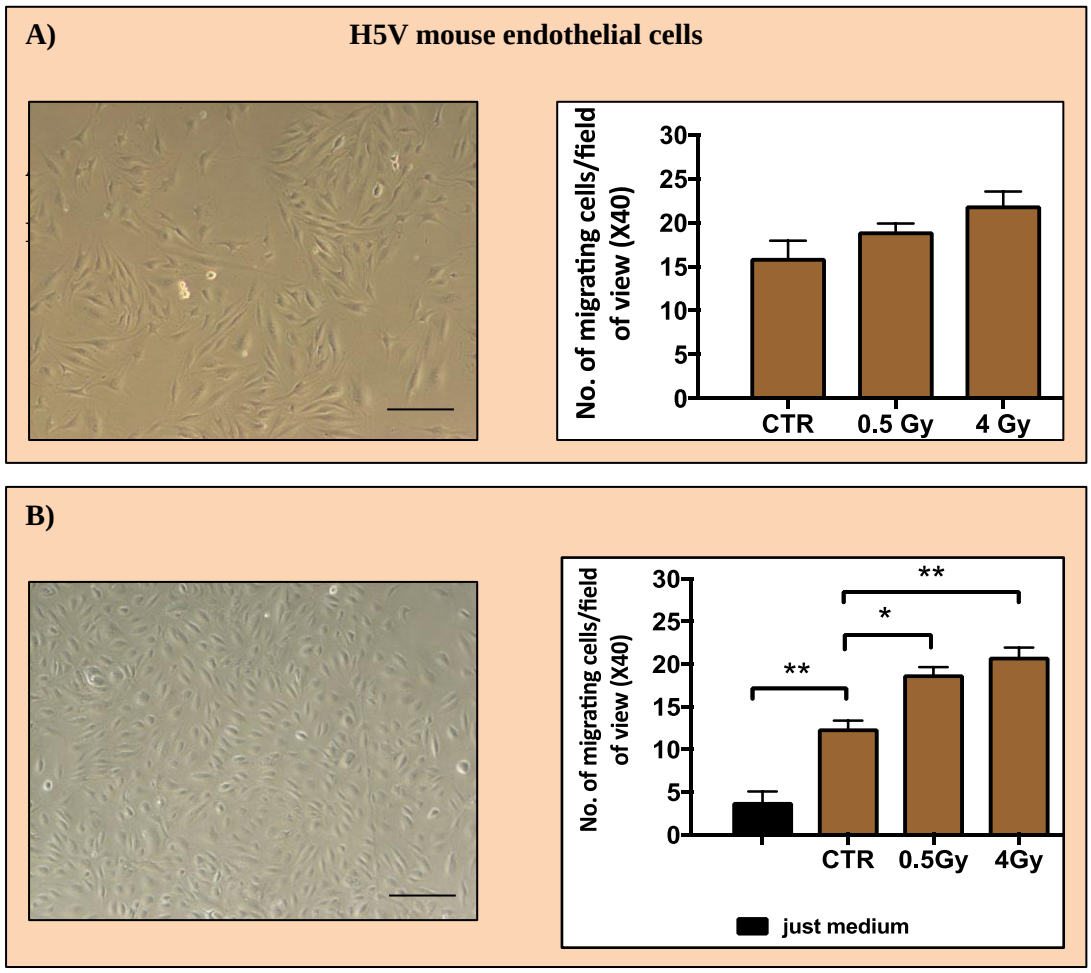


Figure 3.16 Migration ability of Balb/c MSCs increased when co-cultured with irradiated A) H5V cells, and B) HDMEC.

Transwell migration assay was done for Balb/c MSCs co-cultured with control (no irradiation), and irradiated H5V (for 16 hours) or HDMEC (for 8 hours). There were no effects of the irradiated H5V on the balb/c MSCs migration (**A**). While, enhancement of MSCs migration by HDMEC was shown, compared to MSCs incubated with medium alone (no HDMEC). Besides, low and high dose radiation (0.5, and 4 Gy) significantly increased the migration of MSCs (**B**). The results were statistically analysed using one-way ANOVA test (Tukey`s multiple comparisons test) with GraphPad Prism 7. All data are presented as mean ± SEM, of 3 experiments, each done in duplicates and were considered statistically significant when $P \leq 0.05$ (* $P<0.05$, ** $P<0.01$).

3.2.4.5 Co-cultured tumour cells and endothelial cells stimulate migration of Balb/c MSCs *in vitro*

In the tumour microenvironment, cross-talk between tumour cells and endothelial cells is essential for angiogenesis (Folkman, 1995, Zeng et al., 2005). Angiogenesis is mediated by soluble factors secreted from tumour cells which enhance migration and proliferation of endothelial cells (Lutsenko et al., 2003). As the interactions between endothelial cells and tumour cells are complicated, and to better understand the intercellular interactions between tumour cells and endothelial cells, and whether irradiation modifies it, I modelled an *in vitro* co-culture assay consisting of tumour cells (FS120 cells and FS188 cells) and HDMEC. By this assay, I studied if tumour cells cultured with HDMEC produce factor(s) that increase recruitment of the Balb/c MSCs and whether irradiation modified this factor(s).

To further evaluate the synergistic effect of both tumour cells and HDMEC on the migration of the MSCs (as they have shown significant effects on the migration of the MSCs as discussed in **section 3.2.4.2**, and **3.2.4.4**), both cells were co-cultured together (as described in **section 3.2.3.5**). The mixture of cells (FS120 cells + HDMEC) or (FS188 cells + HDMEC) were seeded at the lower chamber of 24 well plates and incubated till the cells became confluent. Next, their media were changed to MSCs medium and incubated for 2 hours before irradiated with 0.5, and 4 Gy with non-irradiated cells used as controls. The cells were incubated for 24 hours after irradiation, and then Balb/c MSCs were seeded at 3 x 10^4/ 200 µl medium on the filter (8.0 µm pores) and incubated for 8 hours. A group of MSCs were seeded with their medium only (no tumour cells or HDMEC) to see whether the migration occurs due to the presence of cells of via factors present in the medium itself. After 8 hours' incubation, the migrated cells were fixed, stained and counted.

As shown in **Figure 3.17**, presence of both endothelial cells and tumour cells together, caused significantly more Balb/c MSC to migrate through the transwell filter compared to cells were exposed to medium alone (**Figure 3.17**). Furthermore, irradiation of the co-cultures resulted in further significant increases in migration (**Figure 3.17**). The table below (**Table 3.1**) compares the results from MSCs exposed to irradiated tumour cells alone (**Figure 3.15**), to irradiated HDMEC alone (**Figure 3.16**) with the results

shown in **Figure 3.17**. It can be seen that the number of migrated Balb/c MSCs were further increased after co-culture tumour cells with HDMEC than alone. This observation suggests that the presence of both tumour cells + endothelial cells enhanced the migratory ability of the Balb/c MSCs.

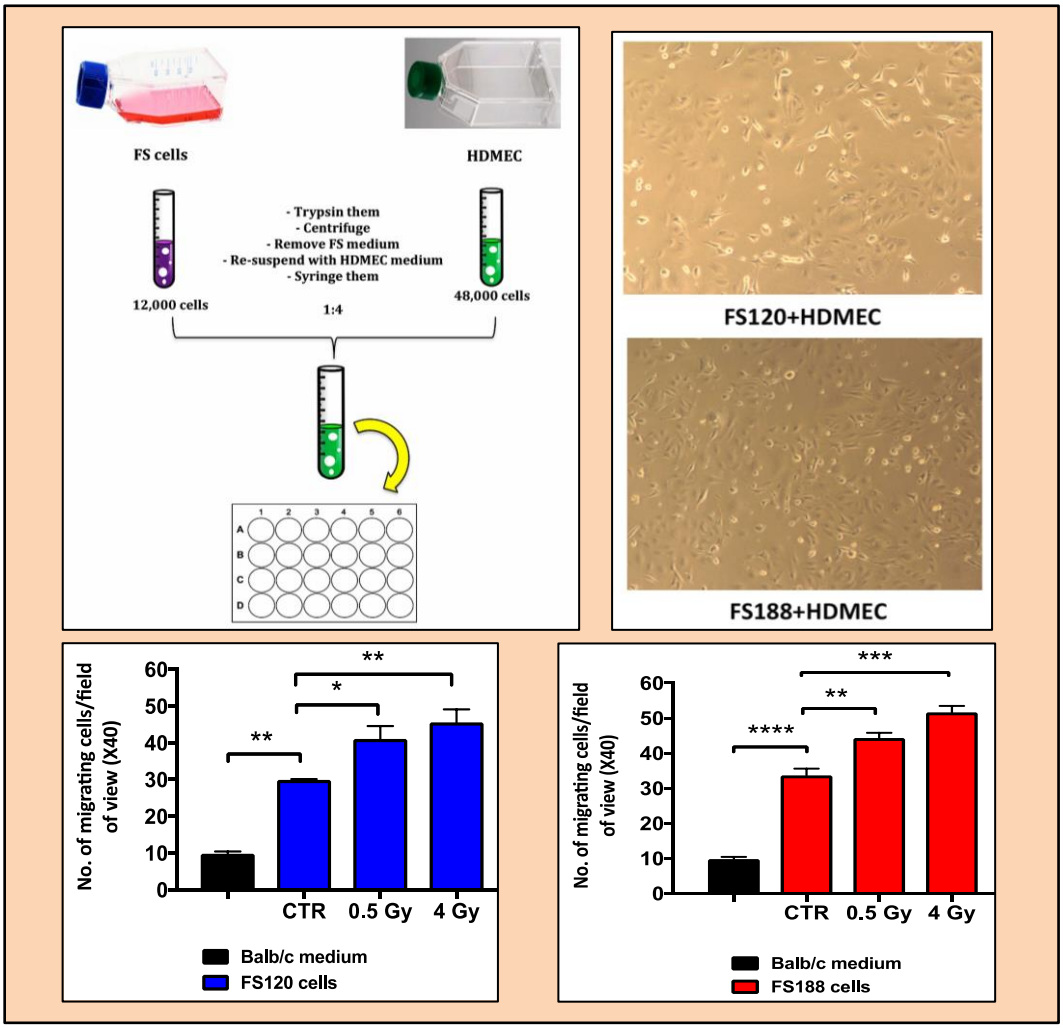


Figure 3.17 Effects of tumour cells co-cultured with endothelial cells on the migration ability of Balb/c MSCs.

Tumour cells (FS120 cells, and FS188 cells) were co-cultured with HDMEC at a ratio of 1:4. Then, the co-culture mixture was irradiated by 0, 0.5, and 4 Gy. After 24 hours, Balb/c MSCs were seeded on the filter and incubated for 8 hours with the control and irradiated co-cultures. Enhancement of MSCs migration by both endothelial cells and tumour cells was shown, compared to MSCs incubated with medium alone. Low and high dose radiation (0.5 and 4 Gy) also significantly increased the migration of MSCs. The results were statistically analysed using one-way ANOVA test (Tukey`s multiple comparisons test) with GraphPad Prism 7. All data are presented as mean ± SEM, of 3 experiments, each done in duplicates and were considered statistically significant when $P \leq 0.05$ (* $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$).

Table 3.1 Average numbers of migrated Balb/c MSCs after exposed to different cells types with their statistical significance.

	FS120 cells	FS188 cells	HDMEC	HDMEC + FS120 cells	HDMEC + FS188 cells
Control	5.25	6.6	12.48	30	33.7
0.5 Gy	10	13	18.8	41	44.3
4 Gy	9	12.58	20.8	45.5	51.6
one-way ANOVA test (Tukey`s multiple comparisons test)					
FS120 vs. HDMEC	FS188 vs. HDMEC	FS120 vs. HDMEC+FS120	FS188 vs. HDMEC+FS188	HDMEC vs. HDMEC + FS120	HDMEC vs. HDMEC + FS188
P=0.0373 *	P=0.0486 *	P<0.0001 ****	P<0.0001 ****	P<0.0002 ***	P<0.0001 ****

3.2.4.6 Role of cytokines and chemokines enhanced MSCs migration

In the previous experiments, it was successfully shown that MSCs exposed to irradiated endothelial cells co-cultured with irradiated tumour cells resulted in increased numbers of migrated MSCs. These results imply that factors secreted from tumour cells and/or endothelial cells facilitate MSCs migration. It was reported by Abbott et al and Kitaori et al that tumour cells secreted SDF-1α, which enhance MSCs migration, and the migration of MSCs was decreased when SDF-1α was blocked by the relevant antibody (Abbott et al., 2004, Kitaori et al., 2009). Also, Dwyer et al revealed that primary breast cancer cells secreted MCP-1 that caused migration of the MSCs (Dwyer et al., 2007). Furthermore, Baek et al and Klopp et al determined that the tumour cells secreted cytokines that enhanced the migration of MSCs, like TGF-β1, PDGF, and VEGF, which were increased after irradiation (Baek et al., 2011, Klopp et al., 2007).

In this study, an *in vitro* migration assay was used (as described previously) using transwell filters. In the lower chamber of the transwells, there was Balb/c medium

treated with TGF-β1 (20 ng/ml), MCP-1 (20 ng/ml), or SDF-1α (100 ng/ml, or 200 ng/ml). The non-treated cells were used as controls. MSCs were incubated with growth factor for 16 hours (the time was optimized) before the cells were fixed, stained, and counted. The results (**Figure 3.18**) showed that all these factors enhanced stem cell migration significantly. The next step was to test for these growth factors in the CM from irradiated tumour cells and establish their involvement in the recruitment of MSCs towards them (see **chapter 4**).

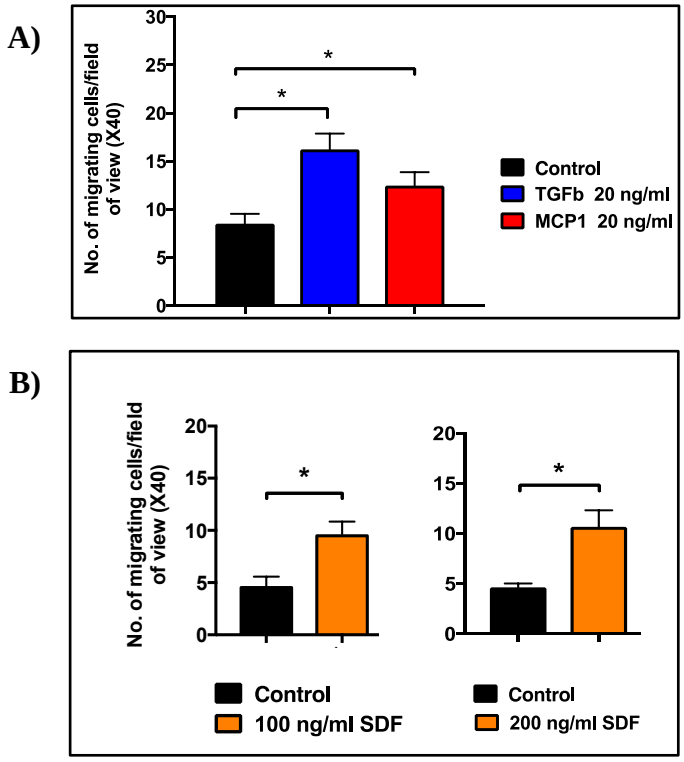


Figure 3.18 Cytokines and chemokines in enhanced MSCs migration.

Balb/c MSCs were treated for 16 hours with 20 ng/ml TGF-β1, 20 ng/ml MCP-1 (A), or 100 ng/ml, or 200 ng/ml SDF-1α (B). Enhancement of MSCs migration by these cytokines or chemokines was shown compared to MSCs with medium alone (no treatment). One-way ANOVA test (Tukey`s multiple comparisons test) for (A), while unpaired t-test (two-tailed) for (B), were used with GraphPad Prism 7.0. All data are presented as mean ± SEM, of 3 experiments, each done in duplicates and were considered statistically significant when $P \leq 0.05$.

3.3 Summary of results

In this chapter, the radiation sensitivity of tumour cells and MSCs were tested using a clonogenic assay. Then, the effect of TGF-β1 on the growth and differentiation of tumour cells and MSCs was done using a growth curve assay. The western blotting technique was used for both C3H10 and Balb/c MSCs to study the expression of the MSC differentiation markers collagen-I, fibronectin, PDGFR-β and α-SMA. Different techniques were used to determine direct and indirect effects of irradiation on MSCs: i) direct irradiation, ii) incubation with CM (neat or concentrated) from irradiated tumour cells, iii) co-culture with irradiated tumour cells, iv) co-culture with irradiated endothelial cells or v) co-culture with both irradiated tumour cells + irradiated endothelial cells. While both C3H10 and Balb/c MSCs expressed high basal levels of CAFs-like cell expression markers, still the results from C3H10 MSCs were not robust. Therefore, Balb/c MSCs used instead for subsequent experiments. The direct irradiation of both C3H10 MSCs and Balb/c MSCs caused minimal changes in the expression of various proteins associated with differentiated CAFs. The response of Balb/c MSCs to irradiated tumour cells and/or their CM was clearer. The results showed that direct irradiation had no significant effects on the differentiation markers of MSCs, except for collagen I (significant increase after 0.5 Gy). However, irradiation of tumour cells and/or endothelial cells did increase the expression of some differentiation markers in MSCs, under certain conditions. This encouraged further study into the roles of specific cytokines and chemokines on the differentiation and migration of MSCs.

TGF-β1, MCP-1, and SDF-1α were studied. The results showed that only TGF-β1 significantly increased all differentiation markers (collagen-I, fibronectin, PDGFR-β, and α-SMA). However, TGF-β1, MCP-1, and SDF-1α all significantly enhanced the migration of MSCs through trans-well plates *in vitro*. Although there were no effects of the direct irradiation on the MSC migration, indirect radiation showed significant effects on the migration ability of the MSCs *in vitro*. Presence of tumour cells or endothelial cells (non-irradiated or irradiated) increased the migration of the MSCs compared to incubation of MSCs with medium alone.

3.4 Discussion

The main objectives of this chapter were to study the *in vitro* effects of radiation (directly or indirectly) on the expression of CAF differentiation markers by MSCs, and, to investigate radiation effects on the homing and migration of MSCs.

First, models of mesenchymal stem cells and tumour cells were developed. Fibrosarcoma cells that expressed a single isoform of VEGF were used as models of soft tissue sarcoma. Pathologist has checked these mouse tumours and she agreed that they are similar to human fibrosarcoma but she did not classified them into grades like in humans. *In vivo*, FS188 cells express only VEGF 188 and produce tumours with more CAF-like cells and perivascular pericytes than those produced by FS120 cells (Tozer et al., 2008). Collagen I levels are also higher in FS188 tumours versus FS120 tumours and this correlates with the higher abundance of CAFs in the FS188 tumours (English et al, 2017). The differences between these tumour cells make them a good model to study the indirect response of MSCs to radiation, by potentially providing different growth factors to drive the differentiation of MSCs to CAFs. As discussed in chapter two, FS188 cells are more apoptotic and proliferate slower than FS120 cells that proliferate rapidly and apoptose less (Kanthou et al., 2014). Furthermore, FS188 cells showed spindle-shape features with extended processes and ruffles (typical mesenchymal features) compared to FS120 cells which area rounded to elongated cells with less extended processes (Kanthou et al., 2014). A recent study by English et al has shown that FS120 cells have the ability to metastasise in mice to lung and these cells form micrometastases in lung more readily than the FS188 cells These metastasised FS120 tumours colonies were more sensitive to anti-VEGFA therapy than colonies of FS188 (English et al., 2017).

At the beginning of this research project, C3H10 MSCs were used, which are a cell line established in 1973 and were used by many researchers as a stem cell model of MSCs that can differentiate into CAF-like cells (Reznikof.Ca et al., 1973). Although both C3H10 and Balb/c MSCs expressed high basal levels of CAFs-like cell expression markers, still the results from C3H10 MSCs were not as expected. Therefore, Balb/c MSCs were used instead. Balb/c MSCs were used by Klopp et al. and have shown a good response to radiation as seen by an increase in expression of CCR2, a receptor for

MCP-1, and enhancement of Balb/c MSCs migration by cytokines secreted by irradiated tumour cells (Klopp et al., 2007). Although direct irradiation of both C3H10 MSCs, and Balb/c MSCs caused minimal changes in the expression of various proteins associated with differentiated CAFs (see **section 3.2.3.1**), the response of Balb/c MSCs to irradiated tumour cells and/or their CM was more robust. Neither irradiated tumour cells, nor their CM had any effects on the differentiation markers of the C3H10 MSCs (**Figure 3.5** & **3.7**). On the other hand, Balb/c MSCs showed promising results after exposure to irradiated tumour cells (especially irradiated FS188) or their CM such as an increase in collagen-I, fibronectin, PDGFR-β, and α-SMA expression as shown in **Figures 3.6** & **3.8**. Compared with the above study, a study by Barcellose-de-Souza showed that treatment of MSC with CM from human prostatic cancer cells caused enhancement the expression of α-SMA (CAF marker) (Barcellos-de-Souza et al., 2016). Furthermore, C3H10 MSCs, unlike Balb/c MSCs, did not respond to either direct irradiation, or to treatment with irradiated tumour cells and/or their CM in terms of migration (**Figure 3.13**, **3.14**, and **3.15**). Since C3H10 cells were rather unresponsive, subsequent experiments were performed using only the Balb/c MSC model.

An *in vitro* clonogenic cell survival assay was chosen to assess viability and to enable a selection of appropriate radiation doses to expose the cells to. In this study, the radiation sensitivity of mouse fibrosarcoma cells (FS120 cells, and FS188 cells), C3H10 MSCs, and Balb/c MSCs were tested. The results showed no differences in radiation sensitivity between FS120 cells and FS188 cells, except at 4 Gy, where FS188 cells were more radiosensitive than FS120 cells (**Figure 3.1**). Many studies showed that normal fibroblasts and CAFs could tolerate high radiation doses (radioresistant cells) (Hawsawi et al., 2008, Papadopoulou and Kletsas, 2011, Tachiiri et al., 2006). Similarly, both C3H10 MSCs and Balb/c MSCs were more radio-resistant than the tumour cells (**Figure 3.1**). These results support a study by Islam et al in which adult human MSCs were more radioresistant than embryonic stem cells (Islam et al., 2015). Furthermore, it has been shown that fractionated or hyperfractionated radiotherapy of 0.5-2 Gy causes radioresistance in MSCs (Tomuleasa et al., 2010, Clavin et al., 2008).

Data from clonogenic assays might suggest that most cells die at higher radiation doses. However, irradiated endothelial cells and fibrosarcoma cells still produce growth factors and cytokines that enhanced MSCs migration and differentiation. The

explanation of this could be because irradiation caused senescence of the tumour cells and endothelial cells. Senescent cells exhibit increased inflammatory cytokines and ROS, decreased nitric oxide production and increased ECM protein production through stimulation of TGF-β1 (Wang et al., 2016, Davalos et al., 2010). Several studies showed that senescent cells play an important role in radiation fibrosis. Schafer et al showed, in lung tissue, that fibrogenesis was enhanced via senescent fibroblasts (Schafer et al., 2017). Moreover, Beach et al showed that radiotherapy of the lungs caused cellular senescence via DNA damage and an inflammatory response which later caused pulmonary fibrosis (Beach et al., 2017). Radiation can also cause senescence of the endothelial cells (Korpela and Liu, 2014). On the other hand, a study by Krizhanovsky et al showed the reverse, in which senescent hepatic stellate cells limit fibrotic processes in acute liver damage (Krizhanovsky et al., 2008). It is well known that senescent cells secrete growth factors and chemokines such as TGF-β1 and MCP-1 as a part of senescence-associated secretory phenotype (SASP) (Acosta et al., 2013). Radiation also induces senescence of MSCs (Wang et al., 2010, Wang et al., 2011).

In the current study, doses between 0.2 and 4 Gy were chosen for either irradiating MSCs directly or irradiating tumour and endothelial cells to stimulate MSCs. Although 4 Gy was a lethal/sublethal dose on tumour cells (SF was 3.1%, and 0.98% for FS120 cells, and FS188 cells respectively) and a moderate dose for MSCs (SF was 40.0%, and 25.6% for Balb/c MSCs, and C3H10 MSCs respectively) it was selected to mimic radiation doses used in clinical treatment.

In vitro clonogenic assays may not reflect the actual *in vivo* radiosensitivity of the cells within the TME, for instance because the TME is more hypoxic. It has been shown that hypoxia increased radioresistance of MSCs, both *in vitro* and *in vivo*, by increasing their proliferation, DNA damage repair, and maintained their proliferation and differentiation abilities after irradiation (Sugrue et al., 2014, Singh et al., 2012).

In this chapter, TGF-β1 growth factor was tested for both growth and differentiation of MSCs. TGF-β1 has been shown to be an important pro-fibrotic cytokine (Yarnold and Brotons, 2010) involved in organ growth and development, differentiation and proliferation of the cells, and immune modulation (Lewis et al., 2004). While TGF-β1 growth factor has been shown to have a significant effect on MSCs growth (Ng et al., 2008) in this study, an *in vitro* growth curve showed no significant effect of TGF-β1 on

the proliferation of C3H10 and Balb/c MSCs. A possible explanation of this may be because in the previous study, human MSCs were used while in this study mouse MSCs were used.

Also, in both previous studies and this study 10 ng/ml of recombinant TGF-β1 were used, however, the technique, MSCs seeding densities, passage number, and the time limit were different (Ng et al., 2008). Moreover, while TGF-β1 is an important cytokine for the growth of MSCs it can also act as a negative growth regulator in some cell types (Datto et al., 1995, Hannon and Beach, 1994). Kulterer et al and Ng et al showed that TGF-β1 is important growth factors in the differentiation of the MSCs (Kulterer et al., 2007, Ng et al., 2008). In this study, it was demonstrated that TGF-β1 induced morphological differentiation of both C3H10 and Balb/c MSCs so that the cells became more spindle shaped, which is a characteristic of CAFs. This observation was further supported by a western blot study through up-regulation of extracellular matrix proteins such as collagen-I, fibronectin, and α-SMA (**Figure 3.12**), and was also supported by a trans-well migration assay, where TGF-β enhanced the migration of the MSCs significantly (**Figure 3.18**). Similarly, a recent study by Barcellose-de-Souza has shown that MSCs were recruited to the tumour site and differentiated into CAF-like cells through TGF-β1 (Barcellos-de-Souza et al., 2016). In this study, higher expression of collagen-I, fibronectin, PDGFR-β and α-SMA was evident after treatment of MSCs with 10 ng/ml TGF-β for 4 days.

Despite a substantial amount of research in this area, still the role MSCs in cancer growth and metastasis is not fully understood. It has been shown that MSCs enhance cancer growth and metastasis, while other studies showed the reverse (Karnoub et al., 2007, Secchiero et al., 2010, Clarke et al., 2015, Shinagawa et al., 2010). Moreover, MSCs also play a key role in the TME via their special cellular interactions to cause either promotion or inhibition of tumour growth (Klopp et al., 2011). As tumour inhibitors, MSCs block AKT and Wnt signalling, suppress angiogenesis, and apoptosis through cell cycle arrest (Hass and Otte, 2012, Rhee et al., 2015). As tumour promoters, MSCs can be recruited to a tumour and activated by TGF-β1 to form CAFs that play a key role in tumour growth, as described above (Barcellos-de-Souza et al., 2016). It has been shown that MSCs enhanced angiogenesis and tumour growth through their abilities to differentiate into pericyte-like and endothelial-like cells (Oswald et al., 2004,

Ball et al., 2004, Suzuki et al., 2011). Moreover, co-culture of adipose-derived MSCs with prostate cancer was shown to cause differentiation of MSC into endothelial-like cells and increased tumour vascularity and enhancement of the tumour growth (Lin et al., 2010, Prantl et al., 2010).

So, in order to further understand the relation between individual cells of the TME with the MSCs, and because the development of tumours determined by the interaction and cross talk between the cells within TME, MSCs were co-cultured with irradiated tumour cells and/or endothelial cells. Our study demonstrated that co-culture of Balb/c MSCs with tumour cells (especially FS188 cells) irradiated by 0.5 Gy, and 4 Gy caused a significant increase in the ECM protein (collagen-I, PDGFR-β and α-SMA), which suggested that the tumour cells are induced to secrete factors in response to irradiation that work in a paracrine manner to cause differentiation of MSCs into CAF-like cells.

Our results were supported by a transwell migration assay with either, tumour cells, or with their concentrated CM, where more MSCs were migrated in the presence of the tumour cells (controls or irradiated) compared to medium alone suggesting that irradiated tumour cells produce factors that stimulate MSCs to home towards the tumour. Paracrine factors were also established to cause migration of MSCs into parenchyma of irradiated tumours in previous studies (Klopp et al., 2007). Moreover, I have found that FS188 cells caused MSCs to express higher levels of differentiation markers and they recruited more MSCs than FS120 cells. These results might explain the ability of FS188 cells to produce more α-SMA positive cells (CAFs) *in vivo* as shown by Tozer et al, 2008 (Tozer et al., 2008), by potentially producing and secreted more cytokines or chemokines in response to radiation.

In this chapter, the effect of irradiated endothelial cells on the MSC expression markers was also studied. Endothelial cells display a high sensitivity to irradiation but also cross-talk with MSCs therefore studying the cellular events due to endothelial radiation injury is crucial (Barker et al., 2015). It has been shown that co-culture of endothelial cells (HUVEC) with MSCs significantly affected their differentiation and proliferation, suggesting presence of cross-talk between endothelial cells and MSCs, which regulate MSCs in the TME (Saleh et al., 2011). Moreover, Saleh et al showed that HUVECs secreted paracrine factors in their CM that induced enhancement of the MSCs proliferation when MSCs treated by this CM. Furthermore, an *in vitro* study showed

that low dose radiation (<5 Gy) increases VEGF secretion and enhances tumour growth (Heissig et al., 2005, Vala et al., 2010). In this study, Balb/c MSCs co-cultured with irradiated HDMEC (0.5, and 4 Gy) displayed an upregulated expression of fibronectin and PDGFR-β.

The effects of radiation on the C3H10 MSCs and Balb/c MSCs migration were assessed using an *in vitro* transwell migration assay. C3H10 MSCs did not show significant migration after direct radiation, or co-cultured with irradiated tumour cells or with their CM, whereas using irradiated tumour cells or their CM strongly enhanced MSCs migration at 0.5 and 4 Gy after 8-16 hours. These results are in agreement with Klopp et al (2007). In our results, Balb/c MSCs migrated more if they were treated with CM extracted from irradiated FS188 cells (see **Figure 3.15 B**).

Two models of endothelial cells were used to study recruitment of MSCs, H5V mouse endothelial cells, and HDMEC. Both control and irradiated HDMEC recruited Balb/c MSCs, while H5V did not. This is might be because H5V cells are endothelial cells that were immortalised using Polyoma virus (Garlanda et al., 1994), while HDMEC were primary cells taken from the human adult skin. This study was able to show that co-culture of both; irradiated HDMEC with irradiated tumour cells caused a significant increase in the migration of MSCs in a dose-dependent manner as shown in **Table 3.1**. It has been shown that MSC can be recruited to inflammatory sites by crossing the endothelium via a multistep process (Henschler et al., 2008, Aldridge et al., 2012). Moreover, Luu et al showed that the cross-talk between endothelial cells and MSCs caused a decrease in the cytokine-induced leukocyte recruitment (Luu et al., 2013).

The effects of cytokines and chemokines (TGF-β1, MCP-1, and SDF-1α) on the migration of MSCs were reported by (Baek et al., 2011, Wang et al., 2002, Kitaori et al., 2009) respectively. Several studies demonstrated MCP-1 as pro-fibrotic mediator (Carulli et al., 2005, Distler et al., 2009, Ong et al., 2003). Moreover, studies have shown that the MCP-1 chemokine enhance the migration of MSCs, both *in vitro* and *in vivo* (Boomsma and Geenen, 2012, Dwyer et al., 2007, Belema-Bedada et al., 2008). Klopp et al showed that TGF-β1 that secreted from tumour cells and enhanced MSCs migration was increased post irradiation (Klopp et al., 2007, Baek et al., 2011). In addition, studies demonstrated that MSCs were recruited by SDF-1α *in vitro* (Schmidt et al., 2006, Sordi et al., 2005). Furthermore, Gao et al showed that tumour cells

secreted soluble factors that cause MSCs to secrete SDF-1α that, in turn, activates their migration (Gao et al., 2009). Similar results were observed in this study, where TGF-β1, MCP-1, and SDF-1α enhanced the migration of MSCs after 16 hours.

In summary, the results presented here suggest that radiation caused a significant increase in the expression of MSC differentiation markers and enhanced the migration of MSCs through secreted factors from tumour cells and endothelial cells.

In the next chapter, factors produced by irradiated tumour and endothelial cells were investigated further for their potential contribution towards MSC recruitment.

CHAPTER FOUR:

Analysis of factor(s) responsible for MSC differentiation/migration within the tumour microenvironment

4.1 Introduction and Aims

In the previous chapter, it was shown that radiation significantly increased the expression of several differentiation markers in Balb/c MSCs. Specifically, co-culture of Balb/c MSCs with irradiated fibrosarcoma cells or incubation with fibrosarcoma CM significantly increased the expression of proteins such as collagen-I, fibronectin, PDGFR-β, and α-SMA suggesting that MSCs were induced to acquire CAF-like characteristics. In addition, co-culture of Balb/c MSCs with irradiated endothelial cells or a mixed culture of irradiated tumour cells and endothelial cells, significantly increased the expression of collagen-I, fibronectin and PDGFR-β proteins. In chapter three, Balb/c MSC migration was shown to be enhanced by co-culture with fibrosarcoma cells or by incubation with fibrosarcoma cell CM. Migration was further enhanced by prior fibrosarcoma irradiation. Moreover, incubation of Balb/c MSCs either with endothelial cells alone, or with both endothelial cells and tumour cells increased their migration. Recombinant growth factors like MCP-1, TGF-β1, and SDF-1α that might be secreted from tumour cells were tested and found to significantly increase migration of Balb/c MSCs *in vitro* (**Figure 3.18**).

In this chapter, the expression of cytokines including MCP-1, TGF-β1 and SDF-1α by fibrosarcoma cells and their potential involvement in MSC recruitment and differentiation were studied in more detail. Multiple studies have shown that the MCP-1 receptor CCR2 is expressed on MSC (Ringe et al., 2007, Ponte et al., 2007, Shen et al., 2016, Klopp et al., 2007). Moreover, it has been shown that radiation can up-regulate the expression of CCR2 in MSCs (Klopp et al., 2007, Connolly et al., 2016). Therefore, the CCR2 expression by MSC was studied in order to investigate whether its expression is altered after radiation.

Because of potential differences in the ability of FS120 and FS188 cell to enhance the migration of MSCs, data obtained from a previous study involving RNA sequencing of FS 120 and FS188 solid tumours was analysed further. RNA sequencing using NGS is a technique used to analyse and quantify millions of RNA transcripts in cells and tissues. The RNA sequencing data from un-irradiated FS120 and FS188 tumours was checked for differences in the expressions of specific genes that might be involved in MSC

recruitment and differentiation, which might clarify the development of more CAF-like cells in FS188 compared with FS120 tumours (Tozer et al, 2008).

Subsequently, quantification of MCP-1 chemokine was done using an ELISA assay in order to quantify the amount of MCP-1 secreted from tumour cells in response to irradiation. Finally, to confirm that MCP-1 chemokine is an important factor that mediates MSCs migration to TME, blocking of MCP-1 activity by neutralizing anti-MCP-1 antibody was performed.

Thus, the aims of this chapter were:

- Establish whether radiation modifies the expression of chemokines & cytokines involved in chemotaxis of MSCs toward irradiated tumour cells.
- Study the effect of radiation (direct and indirect) on the expression of chemokine receptor CCR2 by Balb/c MSCs.
- Identify any differences between FS120 and FS 188 tumours that could cause differential recruitment of the MSCs
- Study whether MCP-1 chemokine that is secreted by tumour cells (control, and irradiated) plays a key role in the recruitment of MSCs to TME.

4.2 Results

4.2.1 Radiation enhanced the expression/secretion of cytokines and chemokines by irradiated tumour cells.

In the previous chapter, cytokines including TGF-β1, MCP-1 and SDF-1α were shown to enhance the migration of MSCs *in vitro* (see **section 3.2.4.6**). The aim of this experiment was to study the role of cytokines and chemokines involved in radiation-enhanced MSC migration and whether they were up-regulated in the conditioned media from irradiated tumour cells. To do that, two methods were used, western blot technique and an ELISA assay. For analysis of secreted TGF-β1, MCP-1, and SDF-1α by western blotting, I tested both neat and concentrated CM. The CM samples (whether neat or concentrated) were mixed with reducing Laemmli buffer and heated at 70°C for 10 minutes. The amount of CM was adjusted according to cell numbers (see **2.2.1.7**), so that 30 µl of each sample was analysed per lane (30 µl /0.5 x 10^6 cells). As shown in **Figure 4.1**, FS120 and FS188 cells produced MCP-1, TGF-β1, and SDF-1α, and furthermore, radiation significantly increased their expression/secretion.

This increase was radiation dose-dependent. There was a tendency for higher levels of growth factors within concentrated CM from FS188 cells (**Figure 4.1**). These results agreed with previous array work that showed MCP-1 from FS188 cells was more than from FS120 cells (unpublished data from our group) and were confirmed by us using an ELISA assay (see later). Within the neat CM, these factors could not be detected, so only results from concentrated CM are shown. One-way ANOVA test followed by Tukey's multiple comparisons test was used to analyse data, and were considered statistically significant when $P \leq 0.05$ (* $P<0.05$, ** $P<0.01$).

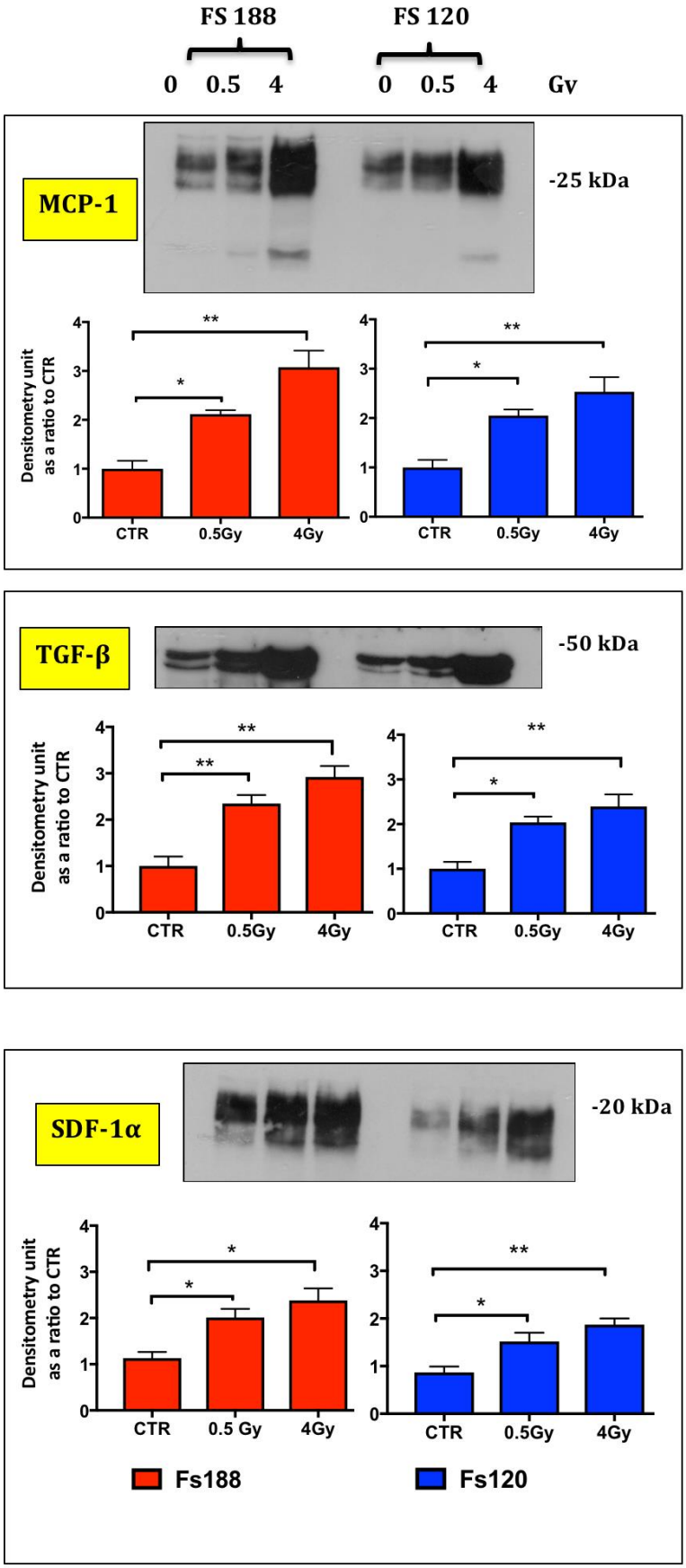

FS 188
FS 120
0 0.5 4
0 0.5 4 Gy
MCP-1
-25 kDa
Densitometry unit as a ratio to CTR
CTR
0.5Gy
4Gy
TGF-β
-50 kDa
SDF-1α
-20 kDa
0.5 Gy
Fs188
Fs120

Figure 4.1 Radiation up-regulated the expression of cytokines and chemokines in the serum-free concentrated CM from fibrosarcoma cells.

Serum-free CM collected from control and irradiated tumour cells (4 days after irradiation) was concentrated and mixed with reducing Laemmli sample buffer. 30 µl (corresponding to /0.5 x 10^6 cells) was analysed by western blotting to detect MCP-1, TGF-β, and SDF-1α. A significant increase in the expression of the cytokines and chemokines was revealed for both CM extracted from irradiated FS120 cells or from irradiated FS188 cells. One-way ANOVA test followed by a Tukey's multiple comparisons test was used to analyse data. Results expressed as means ± SEM of 3 experiments, each done in triplicate and were considered statistically significant when $P \leq 0.05$ (* $P<0.05$, ** $P<0.01$).

4.2.2 Radiation increased levels of MCP-1 secreted from tumour cells in a dose-dependent manner

As established in the previous work (see **chapter 3**), MCP-1 plays a key role in the migration and differentiation of the Balb/c MSCs. In order to quantify the amount of MCP-1 chemokine (chemotactic cytokine) that was secreted from irradiated tumour cells, an ELISA was performed as described in chapter two (**section 2.2.6**). Serum-free CM from irradiated FS120 and FS188 tumour cells 4 days' post-irradiation was analysed to detect mouse MCP-1 protein. At the beginning, both concentrated and neat serum-free CM were tested, but as the results from concentrated CM were too high (above the range of the standards), so only neat CM were analysed. Different dilutions were tested for CM samples with the reagent diluent (1:10, 1:50, 1:100, 1:150, and 1:200) as described in **section 2.2.6**. Dilutions of the neat CM were performed until the values were within the linear range of the standard curve. Results from two dilutions (1:150 and 1:200) were found to be within the range of the assay and were used to calculate the amount of MCP-1 (pg/ml) in CM using GraphPad Prism software (Linear regression test). The values obtained were multiplied by the dilution factor (150 or 200) and divided by the number of the cells from which the CM was extracted (in millions). As shown in **Figure 4.2**, FS188 tumour cells secreted significantly more MCP-1 than FS120 tumour cells as established by analysing the values obtained from both dilutions. MCP-1 secreted from irradiated FS188 cells significantly increased after 4 days of irradiation compared to control un-irradiated FS188 cells in a dose-dependent manner. There was no significant induction of MCP-1 production by irradiation in FS120 cells although there was a trend for an increase (**Figure 4.2**).

One-way ANOVA test followed by Tukey's multiple comparisons test was used to analyse data, and were considered statistically significant when $P \leq 0.05$ (* $P<0.05$, ** $P<0.01$). To compare between two controls groups, unpaired t-test (two-tailed) was used.

A) **B)**

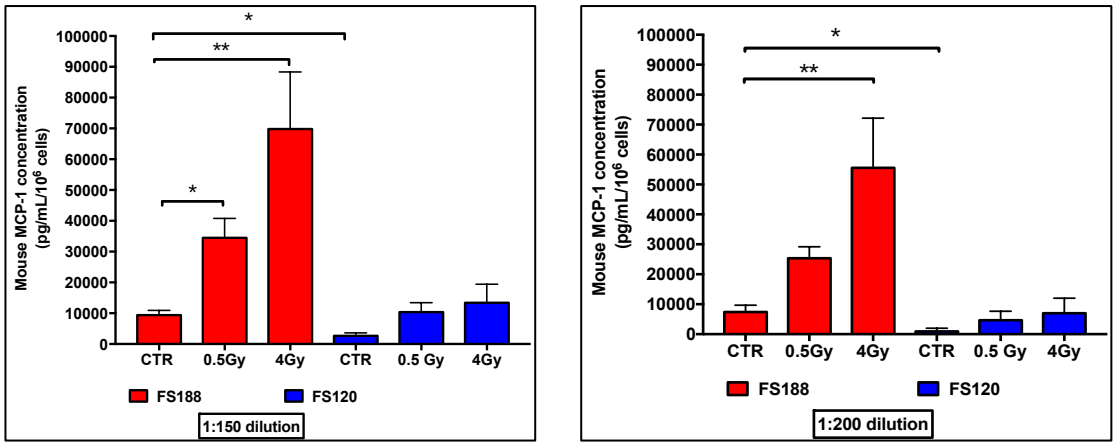


Figure 4.2 MCP-1 proteins production from tumour cells under normal (un-irradiated) and irradiated conditions.

The R&D ELISA assay kit for MCP-1 was used to analyse CM from FS120 and FS188 cells. The results were obtained from two dilution sets, 1:150 (**A**) and 1:200 (**B**). MCP-1 produced from irradiated FS188 tumour cells increased in a radiation dose-dependent manner 4 days after irradiation. Furthermore, FS188 tumour cells secreted significantly more MCP-1 than FS120 tumour cells as established using both dilutions. One-way ANOVA test followed by Tukey's multiple comparisons test was used to analyse data and unpaired t-test (two-tailed) was used to compare between control FS188 cells and control FS120 cells. All data are presented as mean ± SEM of 3 experiments, each done in duplicate and were considered statistically significant when $P \leq 0.05$ (* $P< 0.05$, ** $P< 0.01$).

4.2.3 MCP-1 secreted from tumour cells plays a role in enhancing MSC migration toward TME

I hypothesized that the chemokine MCP-1 secreted from tumour cells is a potential mediator of MSC migration. In Chapter 3, it was shown that the fibrosis and differentiation markers (collagen-I, PDGFR-β, and α-SMA) expressed by MSCs were increased after co-culture of MSCs with irradiated tumour cells (especially FS188 cells) (**Figure 3.5**). Additionally, presence of tumour cells (control & irradiated) or their CM significantly increased MSC migration (**Figure 3.14**, & **3.15**). Also, I showed that migration was significantly enhanced by 20 ng/ml recombinant MCP-1 protein (**section 3.2.4.6**). In this chapter, ELISA revealed that radiation significantly increased the amount of MCP-1 secreted from irradiated FS188 tumour cells. In the light of that, and to determine whether MCP-1 is a potential mediator of Balb/c MSCs migration, MCP-1 activity was blocked using a neutralizing antibody.

Serum-free concentrated CM from irradiated tumour cells (FS188 cells & FS 120 cells) was used. Before starting the assay, the concentrated CM was tested using western blotting to make sure that MCP-1 is present. As the results from ELISA assay showed that FS188 tumour cells produce more MCP-1 protein than FS120 tumour cells, concentrated CM extracted from FS188 cells were tested only. Using the 24-well plate, the following samples were used:

- Concentrated CM from control Fs188 cells (un-irradiated).
- Concentrated CM from irradiated Fs188 cells (4 Gy).
- Concentrated CM from control FS188 cells + non-immune IgG (10 µg/ml).
- Concentrated CM from irradiated FS188 cells (4 Gy) + non-immune IgG (10 µg/ml).
- Concentrated CM from control FS188 cells + anti-MCP-1 antibody (10 µg/ml).
- Concentrated CM from irradiated FS188 cells (4 Gy) + anti-MCP-1 antibody (10 µg/ml).
- Recombinant MCP-1 in serum-free medium (20 ng/ml).
- Recombinant MCP-1 in serum-free medium (20 ng/ml) + non-immune IgG (10 µg/ml).
- Recombinant MCP-1 in serum-free medium (20 ng/ml) + anti-MCP-1 antibody

(10 µg/ml).

- Control (just serum-free medium).

Non-immune IgG was used to ensure that the results are specific and not caused by non-specific effects on the cells by any immunoglobulin. Neutralizing antibody to MCP-1 was used to show that this protein is involved in driving the migration of the cells after incubation with CM. The trans-well migration assay was done as previously explained (see **section 2.2.5.2**).

As shown before (**Figure 3.15**) there was a significant increase in the Balb/c MSCs migration after treatment with concentrated CM from FS188 cells irradiated with 4 Gy whether alone or with non-immune IgG compared to CM from un-irradiated tumour cells (**Figure 4.3**). However, blocking the activity of MCP-1 using anti-MCP-1 antibody (ab203128) prevented the radiation-induced increase in the migration of the Balb/c MSCs (**Figure 4.3**). Likewise, recombinant MCP-1 protein either alone or combined with non-immune IgG significantly enhanced the migration of Balb/c MSCs. As a control for recombinant MCP-1, its activity was blocked with an anti-MCP-1 antibody, which resulted in suppression in the migration of MSCs.

MSCs exposed to concentrated CM from un-irradiated tumour cells significantly increased their migration compared with MSCs treated with serum-free medium alone. These results agreed with our previous results in which presence of tumour cells or their concentrated CM enhanced the migratory ability of Balb/c MSCs (see **3.2.4.2**, and **3.2.4.3**). Blocking the activity of MCP-1 had no effect on MSC migration, where cells were exposed to CM from un-irradiated tumour cells. These results strongly suggest that MCP-1 chemokine secreted from FS188 tumour cells is an important mediator of radiation-induced Balb/c MSCs migration toward TME. For statistical analysis, One-way ANOVA test followed by Tukey's multiple comparisons test was used to analyse data and were considered statistically significant when $P \leq 0.05$ (* $P<0.05$, ** $P<0.01$, *** $P< 0.001$).

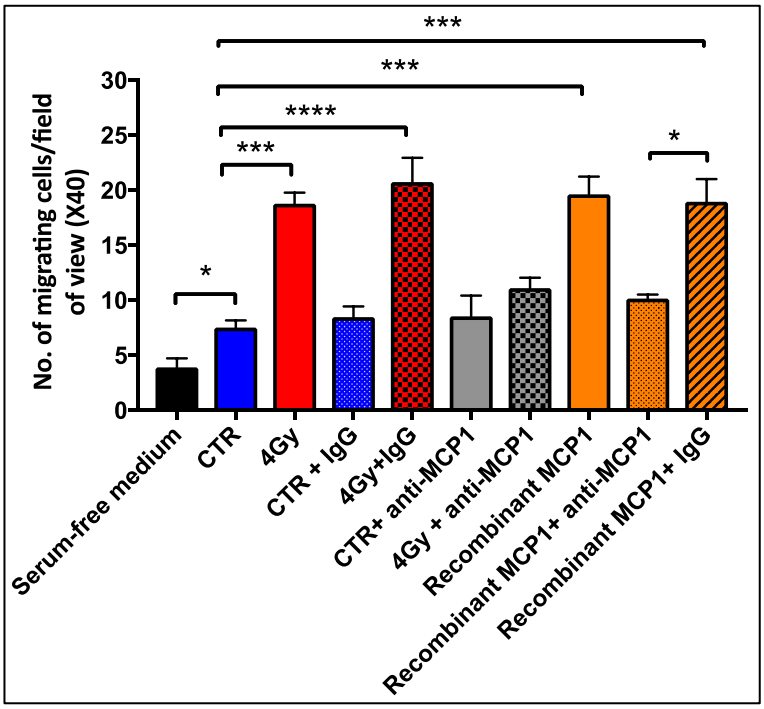


Figure 4.3 MCP-1 produced by FS188 cells enhanced Balb/c MSCs migration.

A significant increase in the MSCs migration was seen after treatment with concentrated CM from FS188 cells irradiated with 4 Gy whether alone or with non-immune IgG compared to CM from unirradiated tumour cells. However, blocking the activity of MCP-1 by anti-MCP-1 antibody produced no increase in the migration of the MSCs. Likewise, recombinant MCP-1 protein either alone or combined with non-immune IgG significantly enhanced the migration of MSCs. As a control for recombinant MCP-1, its activity was blocked with anti-MCP-1 antibody, which resulted in suppression in the migration of MSCs. Interestingly, MSCs exposed to concentrated CM (no radiation) significantly showed migration ability more than those treated with free-serum alone. These results strongly suggest that MCP-1 chemokine secreted from FS188 tumour cells is an important mediator of Balb/c MSCs migration toward TME and it is significantly increased after irradiation. The statistical test was done using One-way ANOVA test followed by Tukey's multiple comparisons test. Unpaired t-test (two-tailed) was used to compare the results from concentrated CM from control FS188 cells with serum-free medium only. Quantitative data represent mean value ± SEM of 3 experiments, each done in duplicates and were considered statistically significant when $P \leq 0.05$ (* $P< 0.05$, ** $P< 0.01$, *** $P< 0.001$, **** $P< 0.0001$).

4.2.4 Radiation effects on the expression of the chemokine receptor CCR2 by Balb/c MSCs.

There are many chemokines associated with cancer progression and fibrosis. Among them, MCP-1 and its receptor (CCR2) signalling has been recognised as a key player in stimulating tumour formation and metastasis (Zhang et al., 2010, Borsig et al., 2014). So far, this project has shown that MCP-1 plays an essential role in the increased migration of the Balb/c MSCs when exposed to CM from irradiated tumour cells. Here, I investigate the effects of radiation (direct & indirect) on the expression of the chemokine receptor CCR2 by Balb/c MSCs. Cell lysates from Balb/c MSCs were collected from different experimental conditions, 3-4 days after:

- ❖ Direct irradiation of the Balb/c MSCs.
- ❖ Co-culture of Balb/c MSCs with irradiated tumour cells.
- ❖ Incubation of Balb/c MSCs with concentrated CM from irradiated tumour cells.

Anti-CCR2 antibody (1:1000 concentration) was used. MSCs treated with Balb/c medium alone or with no radiation, were regarded as control cells.

Normalisation was done by dividing the ratio of target protein to internal control (i.e., dividing or "normalizing" by the loading control). Western blotting analysis revealed a main band present in all samples at ~ 45 kDa, which is the predicted molecular size of CCR2. There was no significant increase in the expression of CCR2 in Balb/c MSCs exposed to direct irradiation (**Figure 4.4, A**). In contrast, MSCs co-cultured with FS188 tumour cells irradiated with 0.5 Gy or their concentrated CM showed a significant increase in the expression of CCR2 (**Figure 4.4, B & C**). On the contrary, there was no effect of the irradiated FS120 tumour cells or their concentrated CM on the expression of CCR2 by MSCs. One-way ANOVA test followed by Tukey's multiple comparisons test was used to analyse data and were considered statistically significant when $P \leq 0.05$.

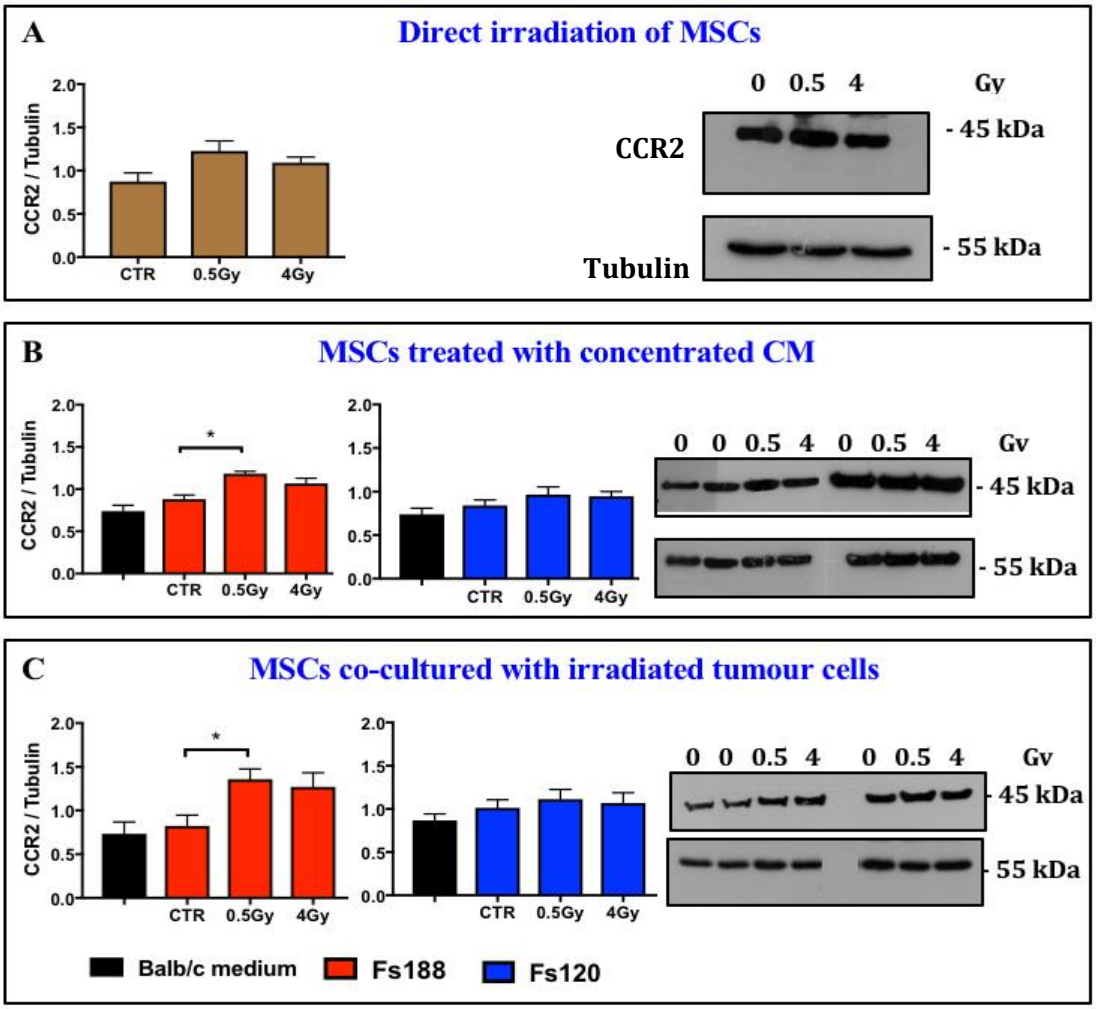


Figure 4.4 Effects of (A) direct radiation, (B) co-culture with concentrated CM from irradiated tumour cells, and (C) co-culture of MSCs with irradiated tumour cells on the chemokine receptor CCR2 expressed by Balb/c MSCs.

Cell lysates from different experiments (**A, B** and **C**) were tested for CCR2 using western blot technique. Direct irradiation of the Balb/c MSCs did not show an increase in the expression of the chemokine receptor CCR2 (**A**). In contrast, Balb/c MSCs co-cultured with FS188 tumour cells irradiated with 0.5 Gy or their concentrated CM showed a significant increase in the expression of CCR2 (**B & C**). There was no effect of the FS120 tumour cells or their concentrated CM on the expression of CCR2 by Balb/c MSCs. Statistical analysis was done using one-way ANOVA test followed by Tukey's multiple comparisons test. Results expressed as means ± SEM of 3 experiments, each done in triplicate and were considered statistically significant when $P \leq 0.05$ (* $P<0.05$).

4.2.5 Searching for genes that might explain the variations between FS120 and FS188 tumours using NGS information.

Tumour samples from an experiment carried out by a post-doctoral scientist, Dr Debayan Mukherjee, were used. He injected FS120 and FS188 cells tumour cells subcutaneously into separate groups of CD1 nude mice. When the tumours reached ~1000-1200 mm^3, they were excised and frozen. Dr Will English extracted the RNA from the un-irradiated tumour sections for sequencing by the NGS service at the Sheffield Children's Hospital. Dr James Bradford, carried out the bioinformatics analysis.

Through literature searching, a list of all factors (chemokine receptors & ligands) that might be involved in recruitment or differentiation of MSCs to tumours was prepared (see **chapter one**, **Table 1.1**). Using this list, the NGS database was explored in order to find whether gene expression levels of any of these factors was significantly different between FS120 and FS188 tumours. The highlighted genes that showed significant differences between the two groups are shown in **Figure 4.4**. The results considered statistically significant when $P \leq 0.05$.

Although this project is interested in growth factors MCP-1, TGF-β1, and SDF-1α and have shown that they were significantly increased the migration of the MSCs *in vitro*, the NGS results did not show significant differences between FS120 and FS188 tumours in regard to those factors.

From the data shown in **Figure 4.4**, CTGF and TGF-β2 were chosen for further study, as both of them play an important role in radiation fibrosis (Hill et al., 2001, Westbury et al., 2014, Bonniaud et al., 2004). Thus, CTGF and TGF-β2 were tested in both concentrated CM and cell lysates from irradiated tumour cells. The CM was concentrated as described in **section 2.2.1.7**. For western blotting, a 15% SDS-PAGE gel was prepared as explained in **section 2.2.4.4**. Recombinant Human TGF-beta2 protein was used as a positive control and run at 2 μg/lane and resolved with the gel under reducing conditions. Each membrane was blocked by either anti-CTGF antibody (ab 6992) or anti-TGF-β2 antibody. Western blotting results from cell lysates were normalised by calculating the ratio of target protein to internal control (i.e., dividing or "normalizing" by the loading control).

The western blot analysis for CTGF showed a significant increase in the expression of CTGF for cell lysate samples from FS120 cells versus FS188 cells (unpaired t-test, P = 0.041) (**Figure 4.5**, **A**). This means that FS120 cells produce more CTGF than FS188 cells. Moreover, irradiation of FS120 cells with 0.5 Gy caused an increase in the expression of CTGF in both cell lysates and concentrated CM (One-way ANOVA, Tukey's multiple comparisons test, P = 0.027) (**Figure 4.5**, **A** & **B**). In contrast, there was no increase in the expression of CTGF for both concentrated CM and cell lysates extracted from irradiated FS188 tumour cells.

Regarding TGF-β2 expression, as noticed in **Figure 4.5 C**, TGF-β2 protein is expressed at its expected molecular weight size (~50 kDa) for the cell lysate samples. There was also a large band running at ~37 kDa, and a very faint lower molecular weight bands at ~25 kDa. No TGF-β2 was detected in the CM (neat & concentrated) extracted from irradiated tumour cells (FS120 and FS188 cells) as shown in **Figure 4.5.D** whereas the positive control (Recombinant Human TGF-beta 2) protein was expressed at its molecular size, 13 kDa for the monomer, and 25 kDa for the dimer (**Figure 4.5 D**).

Western blot analysis for TGF-β2 was done for the band at ~50 kDa, which is the expected molecular size of the target protein. There was no difference in the expression of TGF-β2 between FS120 and FS188 cells. In addition, there was no expression of TGF-β2 in the concentrated CM extracted from irradiated tumour cells (positive control expressed only) (**Figure 4.5**, **D**).

A)

ligands and Receptors	188 CTR				120CTR				log2FC
CCR1	2.95	3.69	3.83	3.06	5.13	4.05	3.33	3.23	-0.55
CCL3	1.42	1.5	2.41	1.26	2.76	1.53	1.66	1.21	-0.41
CCR2	1.66	2.57	2.25	1.35	3.94	3.13	2.23	2.42	-0.94
CCL2 (MCP1)	5.77	5.71	6.42	5.61	8.04	5.73	6.15	6.12	-0.71
CCR3	0.16	0.23	0.21	0.22	0.26	0.23	0.14	0.2	0.02
CCL5 (RANTES)	4.43	3.43	4.05	4.01	7.8	2.71	3.9	4.25	-1.21
CCR4	0.16	0.17	0.14	0.14	0.19	0.19	0.14	0.14	-0.18
CCR5	3.22	3.73	3.48	2.82	5.35	4.44	3.79	3.71	-0.95
CCL3	1.42	1.5	2.41	1.26	2.76	1.53	1.66	1.21	-0.41
CCR6	0.2	0.14	0.14	0.14	0.26	0.32	0.14	0.14	-0.30
CCL20	0.14	0.14	0.14	0.14	0.14	0.51	0.14	0.14	-0.08
CCR7	0.76	0.33	0.64	0.39	1.58	0.91	0.49	0.8	-0.80
CCL19	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.00
CCR8	0.14	0.14	0.14	0.14	0.26	0.14	0.14	0.14	-0.17
CCL1	0.14	0.14	0.57	0.14	0.14	0.35	0.52	0.14	-0.39
CCR9	0.14	0.22	0.23	0.25	0.4	0.19	0.2	0.22	-0.26
CCL25	1.64	1.11	2.5	1.54	1.34	1.44	1.86	1.44	0.71
CCR10	0.14	0.25	0.27	0.19	0.22	0.36	0.39	0.25	-0.48
CXCR1	0.14	0.14	0.14	0.14	0.27	0.14	0.14	0.14	-0.04
CXCR2	0.18	0.59	0.41	0.33	2.07	1.07	0.28	0.38	-1.11
CXCL1	2.52	3.56	3.08	3.07	3.8	2.41	2.59	3.12	0.10
CXCR4	2.49	2.48	2.56	2.32	3.92	3.69	3.13	3.38	-1.18
CXCL12 (SDF-1)	1.9	2.98	2.37	2.22	4.67	3.67	2.79	2.97	-0.90
CXCR5	0.19	0.18	0.14	0.14	0.23	0.2	0.14	0.14	-0.18
CXCL13	0.39	0.23	0.14	0.14	1.2	0.6	0.14	0.14	-0.58
CXCR6	0.28	0.24	0.26	0.23	1.49	1.1	0.74	0.95	-2.30
CXCL16	3.06	2.85	4.07	2.9	5.09	4.58	4.06	4.16	-1.09
CX3CR1	0.91	0.81	1.52	0.92	0.91	2.53	1.67	2.32	-1.02
CX3CL1	2.18	1.79	1.93	2.89	3.81	3.6	3.65	4.53	-1.67
XCR1	0.38	0.47	0.28	0.29	0.43	0.34	0.23	0.14	0.51
XCL1	0.14	0.14	0.66	1.02	1.16	0.96	0.14	1.23	-0.70
PDGFRb	2.54	2.44	2.04	2.38	3.87	3.73	4.4	4.59	-1.90
PDGFb	1.36	1.05	1.48	1.2	2.78	2.77	2.83	2.6	-1.87
IGF-1	1.38	2.04	1.5	1.37	2.2	1.35	0.55	1.45	0.33
TGFb2	1.34	1.47	1.38	1.8	0.69	0.55	1.48	0.88	2.47
CTGF	2.33	1.76	2.48	2.02	4.43	2.77	4.48	3.56	-1.72
TGFb1	4.41	4.57	3.68	4.32	5.19	4.96	5.04	4.86	-0.61
BMP-2	4.3	4.11	3.67	4.41	3.39	2.44	2.97	3.06	1.29
BMP-4	0.57	0.8	0.74	0.91	2.46	1.65	2.94	2.44	-2.35
BMP-7	0.63	0.73	0.38	0.67	0.66	0.79	0.43	0.19	0.22
EGF	0.38	0.65	0.81	0.22	0.39	0.61	0.36	0.66	0.34
EGFR	0.81	1.14	1.15	1.54	1.83	1.32	1.17	0.67	0.15
FGF-2	0.63	0.46	0.68	0.78	1.59	1.17	1.69	1.67	-1.69
HGF	1.35	2.03	1.34	2.15	1.62	1.25	1.61	0.8	0.62
VEGF-A	6	5.66	6.55	5.79	5.05	4.79	5.54	5.37	0.65
CCR7	0.76	0.33	0.64	0.39	1.58	0.91	0.49	0.8	-0.80
TLR2	1.88	2.14	2.18	1.72	4	2.81	2.87	2.84	-1.30
LPAR-1	5.18	5.32	4.53	5.26	3.71	3.44	3.76	3.71	1.57
S1PR1	2.79	2.94	2.3	2.87	4.6	4.53	4.38	4.48	-1.80
TLR1	1.22	1.73	1.66	1.23	2.44	1.94	1.23	1.62	-0.32
TLR2	1.88	2.14	2.18	1.72	4	2.81	2.87	2.84	-1.30

B)

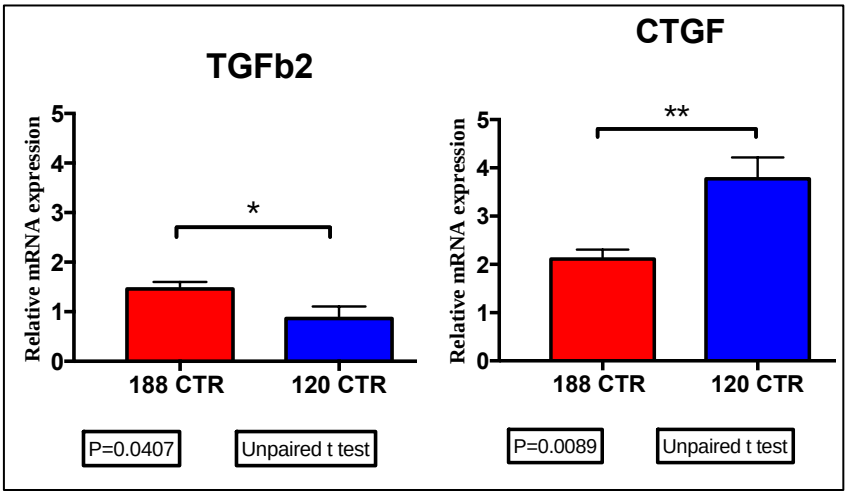
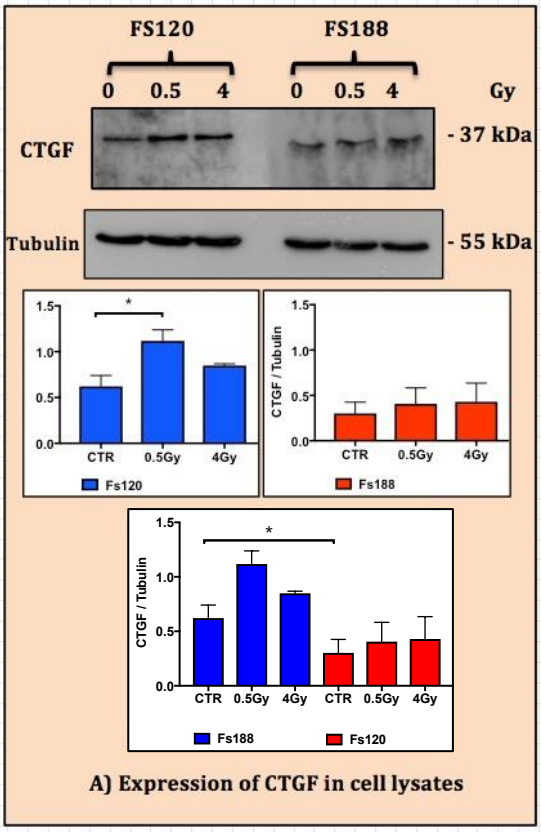
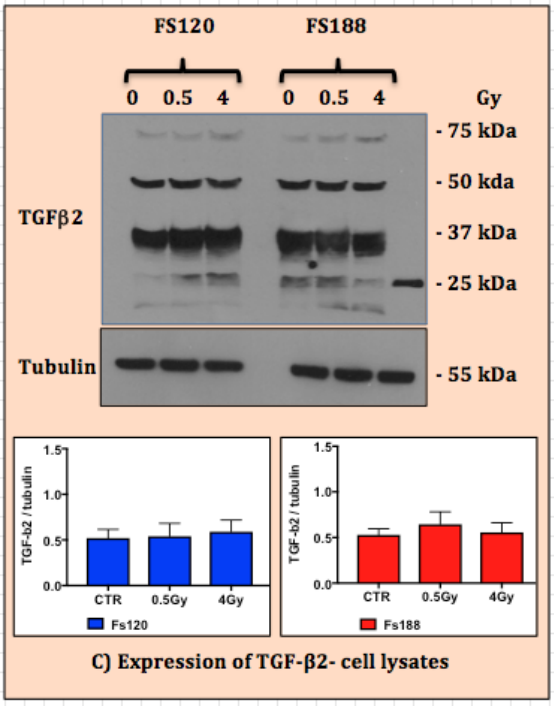
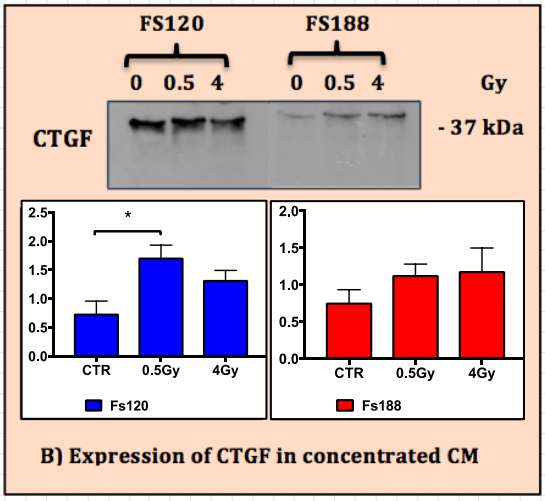


Figure 4.5 NGS database results for chemokine receptors and ligands expressed by control (non-irradiated) mouse fibrosarcoma tumours (FS120 tumours & FS188 tumours).

A) Quadruplicate data from NGS analysis of murine fibrosarcoma control sections. The genes that showed significant differences between the two tumour types are highlighted. The results considered statistically significant when $P \leq 0.05$. **B)** CTGF and TGF-β2 were examples of two genes that were significantly different between FS188 and FS120 tumours values from the RNAseq analysis are plotted for comparison (**B**).


FS120
FS188
0 0.5 4
0 0.5 4
Gy
CTGF
- 37 kDa
Tubulin
- 55 kDa
CTR
0.5Gy
4Gy
Fs120
Fs188
CTGF / Tubulin
A) Expression of CTGF in cell lysates


FS120
FS188
0 0.5 4
0 0.5 4
Gy
TGFβ2
- 75 kDa
- 50 kda
- 37 kDa
- 25 kDa
Tubulin
- 55 kDa
TGF-b2 / tubulin
CTR
0.5Gy
4Gy
Fs120
Fs188
C) Expression of TGF-β2- cell lysates


FS120
FS188
0 0.5 4
0 0.5 4
Gy
CTGF
- 37 kDa
CTR
0.5Gy
4Gy
Fs120
Fs188
B) Expression of CTGF in concentrated CM

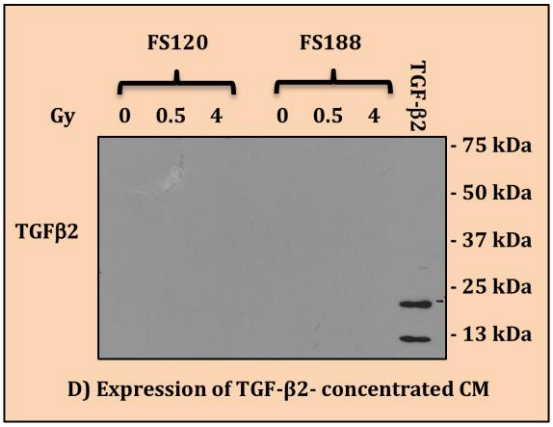

FS120
FS188
TGF-β2
Gy
0 0.5 4
0 0.5 4
TGFβ2
- 75 kDa
- 50 kDa
- 37 kDa
- 25 kDa
- 13 kDa
D) Expression of TGF-β2- concentrated CM

Figure 4.6 Expressions of CTGF, and TGF-β2 proteins in the concentrated CM and cell lysates obtained from irradiated tumour cells.

The expression of the CTGF protein is significantly increased in the cell lysates from FS120 cells versus from FS188 cells (un-paired t-test, P = 0.041) (**A**). Moreover, the expression of the CTGF protein (cell lysates & concentrated CM) is significantly increased after irradiation of the FS120 cells with 0.5 Gy (One-way ANOVA, Tukey's multiple comparisons test, P = 0.027) (**A** & **B**). On the other hand, TGF-β2 was expressed in the cell lysates extracted from FS188 cells and FS120 cells (no differences between the two groups) (**C**). No expression of the TGF-β2 in the concentrated CM extracted from irradiated tumour cells (positive control expressed only) **(D)**. TGF-β2 antibody detected in the cell lysate samples showed a big band at ~37 kDa, in cell lysates as well as very faint lower molecular weight bands at ~25 kDa. Western blot analysis was done for TGF-β2 band at ~50 kDa (real molecular weight). One-way ANOVA test followed by Tukey's multiple comparisons test was done to analyse data from each group individually. While unpaired, two-tailed t-test was used to analyse two control groups (control FS12, and control FS188). Results expressed as means ± SEM of 3 experiments, each done in triplicate and were considered statistically significant when $P \leq 0.05$.

4.3 Summary of results

Radiation up-regulated the expression and secretion of cytokines and chemokines (TGF-β1, MCP-1, and SDF-1α) by fibrosarcoma cells. This up-regulation of MCP-1 and its ability to enhance the migration of MSCs (as previously shown) was followed by a study of the expression of its receptor (CCR2) by MSCs. CCR2 expression by Balb/c MSCs was increased significantly after exposure to FS188 tumour cells irradiated by 0.5 Gy or their concentrated CM. While exposure of MSCs to FS120 tumour cells or their concentrated CM did not show any significant changes in the CCR2 expression.

Because of potential differences in the ability of FS120 and FS188 cell to enhance the migration of MSCs, data obtained from a previous study involving RNA sequencing of FS 120 and FS188 solid tumours was analysed further. NGS analysis was done using control unirradiated tumours from *in vivo* experiment. The NGS analysis showed significant differences in the expressions of certain genes (chemokine receptors & ligands) between FS120 and FS188 tumour sections. The expression of CTGF and TGF-β2 were studied in cell lysates and concentrated CM from irradiated tumour cells. CTGF protein levels were higher in the extracts and CM from FS120 cells versus FS188 cells. These results are in the agreement with the NGS database results for gene expression in un-irradiated solid tumours. On the other hand, the TGF-β2 protein was expressed in cell lysates of both FS188 and FS120 tumour cells at similar levels, with no expression in their CM, as assessed by western blotting. In order to quantify the amount of the MCP-1 protein secreted from tumour cells in their CM, an ELISA assay was used. The results showed that FS188 tumour cells secreted significantly more MCP-1 protein than FS120 cells. Likewise, irradiation of the FS188 tumour cells increased the secretion of MCP-1 in a dose-dependent manner. Finally, the migration assay using blocking antibodies suggested that MCP-1 chemokine secreted from tumour cells (especially FS188 cells) is an important factor that facilitates radiation-induced MSC migration toward TME.

4.4 Discussion

In the previous chapter, it was successfully shown on the basis of western blotting analysis, that irradiation modifies the secreted factor(s) produced from tumour cells that caused an increase in the expression of CAF-like differentiation factors expressed by Balb/c MSCs. It was also seen, *in* vitro, that Balb/c MSCs display a particular tendency to migrate after exposure to irradiated tumour cells or their concentrated CM (**Figure 3.14**, and **3.15**). Moreover, this Balb/c MSCs migration mediated by paracrine factors secreted by irradiated tumour cells.

It has long been thought that radiation causes tissue injury and subsequent local inflammation. As a consequence of radiation-responding inflammation, a number of paracrine mediators including inflammatory cytokines secreted from fibroblasts, macrophages, and epithelial cells are up-regulated (Chen et al., 2002). It has been shown that the level of serum cytokines and their receptors (TNF-α, IL-6, IL-8, VEGF, M-CSF and bFGF) were elevated in STS patients compared to healthy controls (Rutkowski et al., 2002). The next step was to study whether irradiation of fibrosarcoma cells could modify the expression/secretion of paracrine factors such as MCP-1, TGF-β1, and SDF-1α in their CM. To investigate that, these cytokines were first analysed in the CM from control and irradiated tumour cells using a western blotting technique. The results showed that the expression of MCP-1, TGF-β1, and SDF-1α was increased significantly in the concentrated CM of irradiated tumour cells in a radiation dose-dependent manner (**Figure 4.1**). However, the amount of these factors was undetected in the neat CM possibly due to their low levels.

In the previous chapter (see **section 3.2.4.6**), it was demonstrated that after 16 hours treatment of Balb/c MSCs either with MCP-1, or TGF-β1 or SDF-1α their migration abilities significantly increased compared to control (**Figure 3.18**). Because many studies have already been performed on TGF-β1 and its role in recruitment/ differentiation of MSC, MCP-1 cytokine was chosen to study further here. MCP-1 protein is expressed and upregulated in CAFs (Li et al., 2014). Furthermore, studies showed that MCP-1 expressed by human ovarian cancer cells (Furukawa et al., 2013) and by human endometrial cancer cells and affect their treatment with chemotherapy (Wang et al., 2006). Recently, it has been shown that MCP-1 is upregulated in triple-

negative breast cancer cells with enhanced its metastasis and invasiveness (Dutta et al., 2018). Moreover, the role of MCP-1 in fibrosis and recruitment of MSC to the site of injury is still controversy; some studies support its role in recruitment of MSCs while others not (Tokuda et al., 2000, Anders et al., 2002, Moore et al., 2001, Ringe et al., 2007, Takano et al., 2014).

Many methods are available to detect and quantify the amount of the MCP-1 chemokine secreted from tumour cells in response to radiation such as ELISA (sandwich), cytokine bead arrays and microarrays, antibody array and flow-cytometry. ELISA (sandwich) was chosen because it is a reliable, less expensive, colorimetric method that requires less time to set up and optimize than some other methods. ELISA results showed that the secretion of MCP-1 chemokine by tumour cells increased significantly after 4 days of irradiation. Moreover, ELISA assays revealed that the increase in the secretion of MCP-1 chemokine was in a radiation dose-dependent manner (**Figure 4.2**). This dose-dependent increase in the amount of MCP-1 chemokine was only with the CM from irradiated FS188 tumour cells and not FS120 tumour cells (although there was a tendency to increase in the amount of MCP-1).

These results are different from the data obtained from the western blotting analysis in which both tumour cell types were shown to produce MCP-1 cytokine to similar levels and increased in a radiation dose-dependent manner (**Figure 4.1**). Proteins in the western blot methods are denatured using SDS-PAGE, while in ELISA, the proteins in its native form. This big difference in the amount of MCP-1 measured by the above two methods could be due to the sensitivity of ELISA assays in quantification of proteins compared to the western blot. Also, for ELISA, a neat non-concentrated CM was used while in the western blot, a concentrated CM was used, so it is possible that proteins were lost or degraded during the concentration of the CM. Our previous results showed an enhancement of MSC migration in a radiation-dependent manner after treatment with cell lysates or CM from control and irradiated tumour cells (**Figure 3.14**, and **3.15**). This dose-dependent increase in MCP-1 levels in the CM from FS188 cells might explain the ability of these cells to recruite more α-SMA positive cells (CAFs) than FS120 cells (Tozer et al., 2008). Moreover, the differences between the two-fibrosarcoma isoforms, FS188 and FS120, might explain this, independent of radiation.

CAFs are responsible for the synthesis of ECM components like collagen (Bentzen, 2006) and MCP-1 was shown to be expressed and upregulated in CAFs (Li et al., 2014). ELISA confirmed that tumour cells secrete MCP-1 in response to irradiation. As shown in chapter three, MCP-1 caused migration of MSCs *in vitro* (see **section 3.2.4.6**). Moreover, it has been shown that inhibition of MCP-1 caused a decrease in the migration of MSCs (Ren et al., 2012, De Becker and Van Riet, 2016). To address this issue, migration assays were done using a blocking MCP-1 antibody in order to establish whether it is the potential mediator of Balb/c MSCs migration or not. The same time scale that was used before (see **section 3.2.4.6**) were used for incubation (16 hours). As ELISA assays showed that FS188 produce more MCP-1 than FS120 tumour cells, concentrated CM from FS188 was tested. The results showed that the anti-MCP-1 antibody significantly suppressed Balb/c MSCs migration and suggest that MCP-1 is potentially a major chemokine that caused the migration of MSCs in the co-culture systems that were used in this study. These results (**Figure 4.3**) supported the results obtained from the migration assay that was done before (see **section 3.2.4.2,** and **3.2.4.3**) in which presence of the tumour cells or their CM cause a significant increase in the migration of MSCs compared with using serum-free medium alone. To establish the differences between FS188 and FS120 tumour cells, blocking the activity of MCP-1 using concentrated CM from FS120 needs to be done in the future. Moreover, it has been shown, in chapter three, that TGF-β1 and SDF-1α also enhanced the migration of MSCs significantly. Therefore, the next step was to test both TGF-β1 and SDF-1α using the migration assay with blocking antibody and if they show significant effects, ELISA assays would be the next step. This work will be done in the future to establish the differences between the two fibrosarcoma tumour cells *in vitro* and whether MCP-1, TGF-β1 or SDF-1α play a role in the migration/recruitment of MSCs.

Because chemotaxis involves the release of signals or chemokines recognised by receptors expressed on the migrating cells, and as it was established in this chapter that tumour cells up-regulated MCP-1 chemokine signalling in their concentrated CM in response to radiation, it was important to establish whether MCP-1 receptor (CCR2) was also expressed by the MSCs cells. It has been shown that CCL2-CCR2 signalling plays an important role in cancer progression, fibrosis and metastasis (Zhang et al., 2010, Borsig et al., 2014). In this chapter, it established that treatment of Balb/c MSCs with either irradiated FS188 tumour cells (0.5 Gy) or their concentrated CM cause a

significant increase in the expression of the CCR2 by Balb/c MSCs. These results are in agreement with Klopp et al and Connolly et al, who showed that chemokine receptor CCR2 was expressed by MSCs and its levels of expression increased after irradiation (Klopp et al., 2007, Connolly et al., 2016). These finding could indicate that after irradiation, the tumour cells secrete MCP-1 cytokine that leads to up-regulation of its receptor (CCR2) on MSC, and eventually cause recruitment of the MSC to the MCP-1 ligand-bearing tumour. To prove that, migration assay using anti-CCR2 antibody needs to be done to see whether that blocking of CCR2 will suppress MSCs migration after irradiation *in vitro*. Inflammatory cytokines like TNF-α, and other growth factors secreted by tumour cells can also up-regulate CCR2 (Ringe et al., 2007, Ponte et al., 2007).

NGS analysis was done using control unirradiated tumours from *in vivo* experiment done by Dr Mukherjee (as discussed in chapter 2). The NGS analysis showed significant differences in the expressions of certain genes (chemokine receptors & ligands) between FS120 and FS188 tumour sections. Multiple studies have shown that CTGF and TGF-β2 play an important role in radiation fibrosis (Hill et al., 2001, Westbury et al., 2014, Bonniaud et al., 2004, Ihn, 2002). Moreover, the fibrotic activity of TGF-β depends on the CTGF activities (Mori et al., 1999, Liu et al., 2013, Parada et al., 2013). Recently, it has been shown that blockage of the CTGF activities can reverse the fibrotic process in the lungs (Bickelhaupt et al., 2017). In the light of that, I decided to study the expression of CTGF and TGF-β2 in cell lysates and concentrated CM from irradiated tumour cells. Although the results were against what expected (FS188 cells produce more CTGF), it was in agreement with the NGS data analysis, in which the expression of CTGF was greater in FS120 cells than FS188 cells.

Comparing our results with previous results, Tozer et al showed that FS188 tumours produce more α-SMA positive cells (more fibrotic) than FS120 tumours *in vivo* (Tozer et al., 2008). However, *in vitro* results in this work were controversial. This project showed, using ELISA, that FS188 produce more MCP-1 than FS120 tumour cells. However, in contrast, it is shown in this work that FS120 secreted more CTGF than FS188 cells. Furthermore, NGS data showed complex differences between FS188 and FS120 tumours, and the ability of fibrosarcoma tumours to recruit CAF-like cells and pericytes depends not only on one factor, but it is a combination of many factors such

as TGF-β1 and SDF-1α. Many studies showed that tumour cells growth, migration and metastasis depends mainly on cross-talk between tumour cells and local TME which is more complex *in vivo* than in *in vitro* models (Chambers et al., 2002, Fidler, 2003, Steeg, 2006, Wirtz et al., 2011).

On the other hand, TGF-β2 was expressed in the cell lysates from both tumour cells, with no radiation modification of its expression (**Figure 4.5**). There was no expression of TGF-β2 in the CM (neat or concentrated), although the positive control (recombinant human TGF-β2 protein) was detected at its correct molecular size, 13 kDa for the monomer, and 25 kDa for dimer (**Figure 4.5 D**). This might be either because the TGF-β2 isoform is secreted as a latent precursor large molecule (Roberts, 1998) or it was cleaved by ionising radiation.

In summary, this work found that MCP-1 chemokine and its receptor CCR2 play a key role in the recruitment of Balb/c MSCs toward the irradiated tumour environment, with a dose-dependent manner, as the MCP-1 amount was increased. Moreover, this work also demonstrated that CTGF secretion from FS120 tumour cells was more than FS188 tumour cells *in vitro*. In the next chapter, staining and analysing of the fibrosarcoma tumour sections from an *in vivo* experiment for fibrosis markers was done and analysed.

CHAPTER FIVE:

In vivo fibrosarcoma model

5.1 Introduction & aims

In the previous work, it was shown that radiation significantly increased the expression of differentiation markers in Balb/c MSCs *in vitro* such as collagen-I, fibronectin, PDGFR-β, and α-SMA suggesting that the cells were induced to acquire CAF-like characteristics. ELISA assay showed that FS188 tumour cells secreted more MCP-1 protein than FS120 cells. Likewise, irradiation of the FS188 tumour cells increased the secretion of MCP-1 in a dose-dependent manner. It was also shown that MCP-1 induced migration of MSCs toward tumour cells *in vitro*. As detailed in chapter one, CAFs are the primary source of ECM proteins such as collagen and fibronectin in TME. So, it was important to investigate levels of CAFs within FS188 and FS120 tumours *in vivo* and the response to irradiation.

Paraffin-embedded and frozen tumour sections (control and irradiated) from an experiment done by a post-doctoral scientist, Dr Debayan Mukherjee were used. Immunohistochemical staining for α-SMA, as a marker for CAF-like cells, was carried out in paraffin-embedded tumour sections. Masson's trichrome staining, originally used in smooth muscle tissue (Puchtler and Isler, 1958), was used to detect collagen fibres within tumour sections. Masson's trichrome staining is still used to detect collagen fibres in cardiac, renal, and liver fibrosis (de Jong et al., 2012, Zhou et al., 2013, Lo and Kim, 2017).

Analysis of Massson's staining in images of tumour sections was done using Imagescope and HistoQuest software after scanning of the slides with a TissueGnostics confocal slide scanner. Many studies have used Imagescope or HistoQuest to quantify tissue injury and inflammation (Daunoravicius et al., 2014, Hernandez-Morera et al., 2016, Vranceanu et al., 2014, Chen et al., 2016). In this chapter, immunofluorescent staining with CD31 and α-SMA was used to study radiation effects on the TME. Moreover, vascular staining with CD31 enabled us to study vascular differences between two tumour types expressing single isoforms of VEGF (FS188 and FS120 tumours) and to identify whether radiation increased ECM produced from CAF-like cells (α-SMA staining).

Accordingly, the aims of this chapter were to:

- Analyse α-SMA expression within control and irradiated paraffin-embedded mouse fibrosarcoma tumour sections using immunohistochemistry to see whether radiation enhances the CAF-like cells and if there are any differences in the α-SMA expression between FS188 and FS120 tumours.
- Establish whether radiation modifies the expression of collagen in both FS120 and FS188 tumours using Masson's trichrome staining on paraffin tumour sections.
- Identify any differences in vasculature and ECM contents post-irradiation between FS120 and FS188 tumours *in vivo* that could explain their abilities to recruit MSCs using immunofluorescent staining with CD31 and α-SMA for frozen tumour sections.

5.2 Results

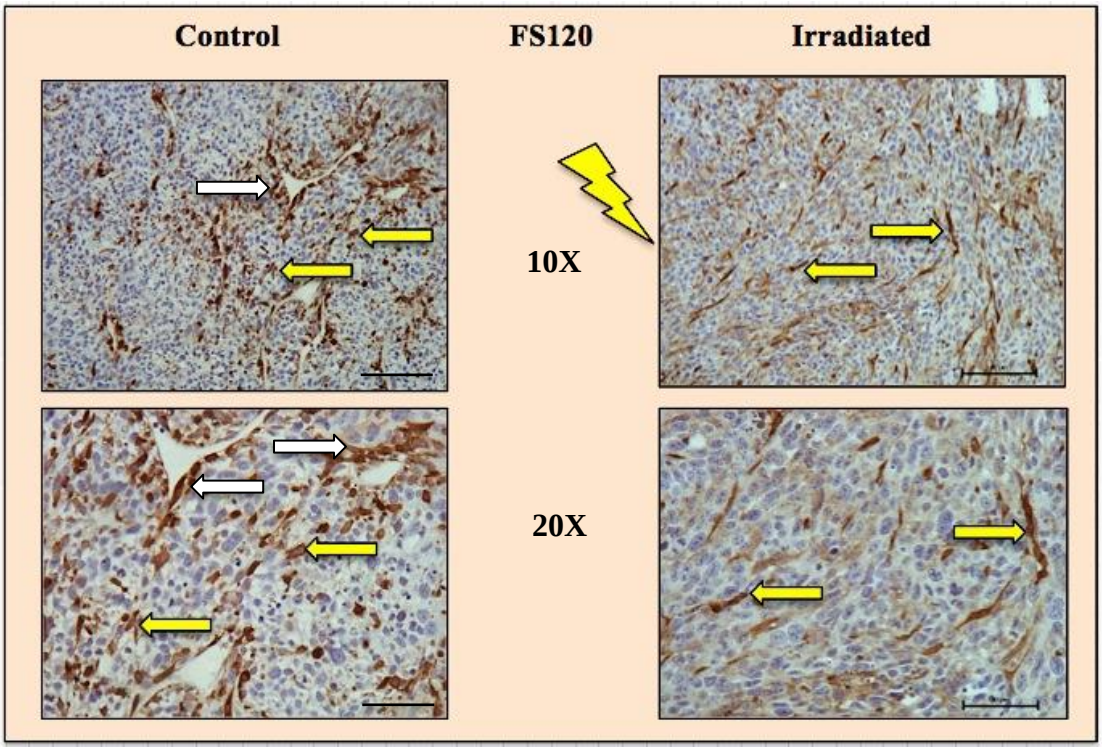
5.2.1 Immunohistochemical staining of fibrosarcoma tumour sections

In the previous chapters, the results from *in vitro* experiments verified that irradiation of the tumour cells caused them to secrete factor(s) that enhanced the migration of the Balb/c MSCs. Moreover, it was shown that irradiation (indirectly) increased the CAF-like differentiation markers expressed by Balb/c MSCs. The aim of this experiment was to study the radiation effects on the recruitment of CAF-like cells in fibrosarcomas, using expression of α-SMA. As discussed in chapter two **section 2.2.7**, Dr Debayan Mukherjee performed the *in vivo* experiments and Mrs Maggie Glover provided formalin-fixed, paraffin-embedded tumour sections, while Mr Matthew Fisher prepared frozen tumour sections.

Mr Matthew Fisher provided the original IHC protocol. Formalin-fixed and paraffin-embedded fibrosarcoma sections were deparaffinised and rehydrated as discussed in chapter two, **section 2.2.7.1**. Randomly assigned fields were chosen with different magnifications (10X and 20X objectives) and captured using a Nikon microscope (Nikon Optiphot-2). A positive reaction for α-SMA was observed, but unfortunately, there was a lot of background, which made identification of truly α-SMA positive cells difficult. In order to decrease the background (Menon and Fisher, 2015), the followings were done:

- ✓ Increased the incubation period with IgG blocking from 60 minutes to 90 minutes at room temperature.
- ✓ Decreased the concentration of primary antibody (α-SMA) from 1:10,000 to 1: 20,000.
- ✓ Decreased the concentration of secondary antibody (biotinylated anti-mouse IgG) from 1:250 to 1:300 and 1:350.
- ✓ Increased washing time with PBS and decreased incubation with DAB staining.

However, the background staining did not disappear.


Control
FS120
Irradiated
10X
20X

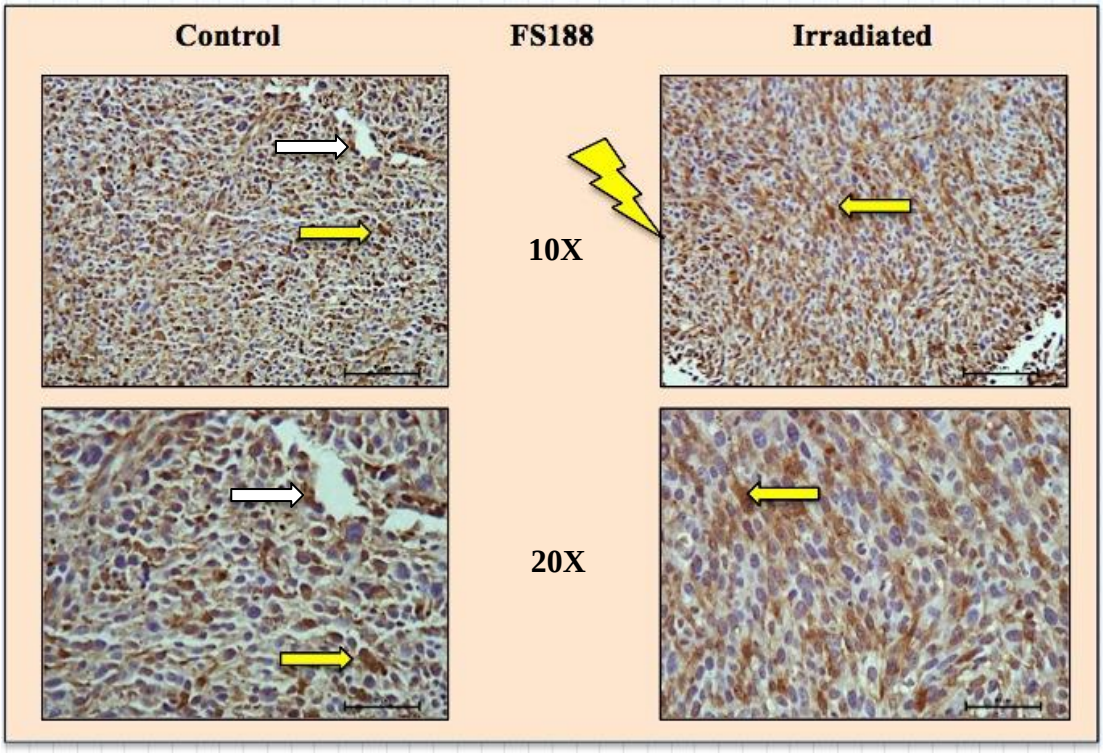

Control
FS188
Irradiated
10X
20X

Figure 5.1 α-SMA distributions within irradiated and un-irradiated fibrosarcoma tumour sections.

FS120 and FS188 tumour cells were implanted subcutaneously into CD1 nude mice. Once tumours had grown to ~100 mm^3 in diameter, they were irradiated *in situ* with 20 Gy over 4 days (8 X 2.5 Gy fractions). Tumours were excised when they reached ~1000-1200 mm^3, and were embedded in paraffin, sectioned and stained for α-SMA. Random fields were chosen with different magnifications (10X and 20X objectives) using a Nikon microscope. A positive reaction for α-SMA was observed. Stromal spindle cells, which were positive for α-SMA, were regarded as CAF-like cells (yellow arrows). Perivascular cells positively reacted α-SMA were regarded as pericytes (white arrow). Unfortunately, there was a lot of background for most of the slides, which made identification of α-SMA positive cells difficult.

5.2.2 Collagen staining (Masson's trichrome staining) of paraffin-embedded fibrosarcoma tumour sections

This technique detects collagen fibres tissues and it consists of three-colour staining, dark brown-black for cell nuclei, orange-red for cytoplasm, and green-blue for collagen (Puchtler and Isler, 1958). Through this triple staining, Masson's can provide a clear morphology of stroma (normal and reactive). To measure collagen deposition/fibrosis within the tissues, FS120 and FS188 paraffin tumour sections were de-waxed, fixed, and stained as explained in **Chapter two, section 2.2.7.2**. The stain worked well after optimising the protocol (**Figure 5.2**).

The ways of analysis of images from Masson's trichrome staining was described in chapter two (**section 2.2.7.2**) using both, Aperio Imagescope and HistoQuest software analysis system V 4.0.

The difference in percentage collagen staining areas between viable and necrotic areas within the control and irradiated tumour sections is shown in **Figure 5.3**. The results revealed that there was a significant increase in the average collagen positivity areas within the viable regions after irradiation of the tumours *in vivo* (FS188 & FS120 tumours) (**Figure 5.3 A**). Moreover, within the necrotic areas, the percentage area stained positive for collagen was significantly higher in the irradiated FS188 tumours compared to control (un-irradiated) tumours (**Figure 5.3 B**). There were no significant differences in collagen staining between FS188 and FS120 tumours, although there was a tendency for it to be higher in FS188 tumours for both unirradiated and irradiated tumours.

To analyse data, one-way ANOVA test followed by Tukey's multiple comparisons test was used. Results expressed as means ± SEM and were considered statistically significant when $P \leq 0.05$.

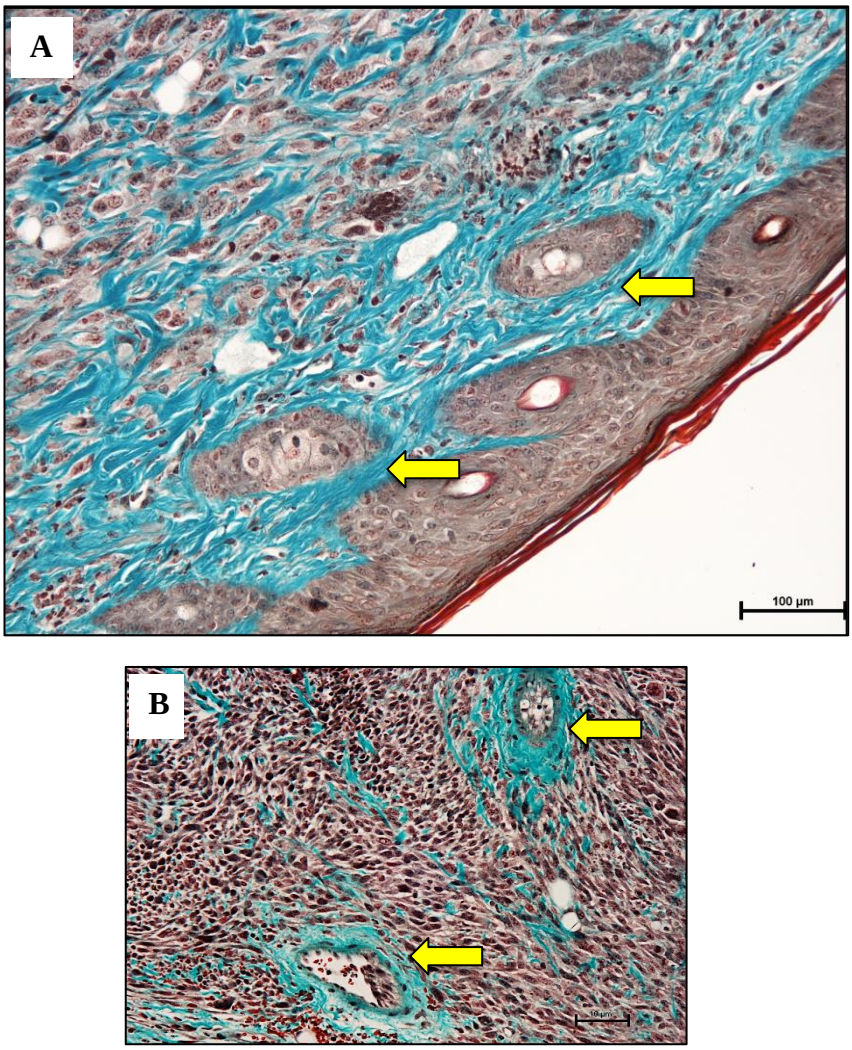
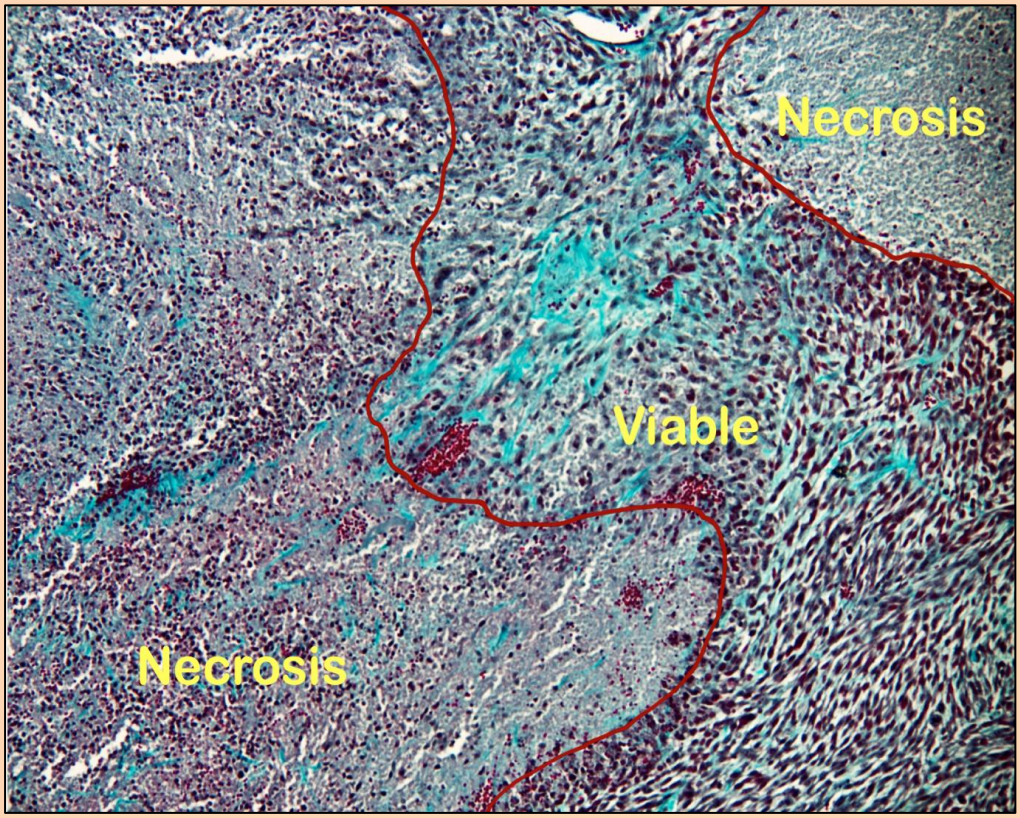


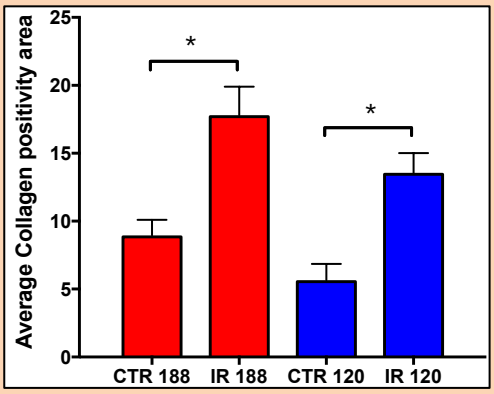
Figure 5.2 Masson's trichrome staining for paraffin-embedded fibrosarcoma tumour sections (irradiated FS188 tumour) showing collagen deposition (magnification A- 10X and B- 20X).

Note that green-blue fine and coarse fibres represent collagen, which is clearly visible. Abundant amount of collagen surrounding blood vessels (arrow).

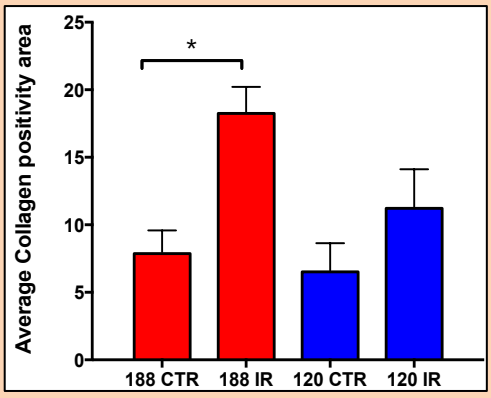


Imagescope Results

B) Percentage of viable tumour regions stained for collagen



A) Percentage of necrotic tumour regions stained for collagen



FS188 FS120

Figure 5.3 Differences in collagen staining (Masson's trichrome) within viable and necrotic regions of control and irradiated fibrosarcoma FS188 and FS120, the section established using Aperio ImageScope software.

Eight images were taken from each stained tumour section (16 tumour sections were used) using a 10X objective and a Nikon microscope (Nikon Optiphot-2). Collagen stained areas were significantly increased within the viable regions of the tumours (FS188 & FS120 tumours) after irradiation compared to control areas (non-irradiated) (**A**). Likewise, the collagen stained areas were significantly increased within the necrotic areas in the irradiated FS188 tumour sections compared to control (un-irradiated) areas of the tumour (**B**). The positivity was calculated by summation of all the positive areas (weak positive + positive + strong positive) then divided by the total area (all positive areas + negative areas). The percentage of positivity was estimated by multiplying positivity by 100. One-way ANOVA test followed by Tukey's multiple comparisons test was used for data analysis. Results expressed as means ± SEM, and were considered statistically significant when $P \leq 0.05$.

The second method used to analyse Masson's trichrome staining in paraffin sections was by scanning sections with the TissueGnostics Confocal Slide scanner (TISSUE FAX 200, Tissue Gnostics Vienna, Austria) (see **Chapter two, Figure 2.13 and 2.14**) and using HistoQuest software analysis system V4.0.

By HistoQuest software, 38 stained tumour-sections were analyzed (10 sections for control FS188, 10 sections for control FS120, 9 sections for irradiated FS188, and 9 sections for irradiated FS120 tumours). Radiation significantly increased the collagen content of the FS188 tumours and FS120 tumours (**Figure 5.4**). Furthermore, there was more collagen within the control (non-irradiated) FS188 tumours than within control FS120 tumours (**A**, **B** & **C**). Irradiated FS188 tumours showed more collagen percentage than irradiated FS120 tumours in both, viable and necrotic areas (**Figure 5.4**, **A**, **B** and **C**). One-way ANOVA test followed by Tukey's multiple comparisons test was used to analyse the data.

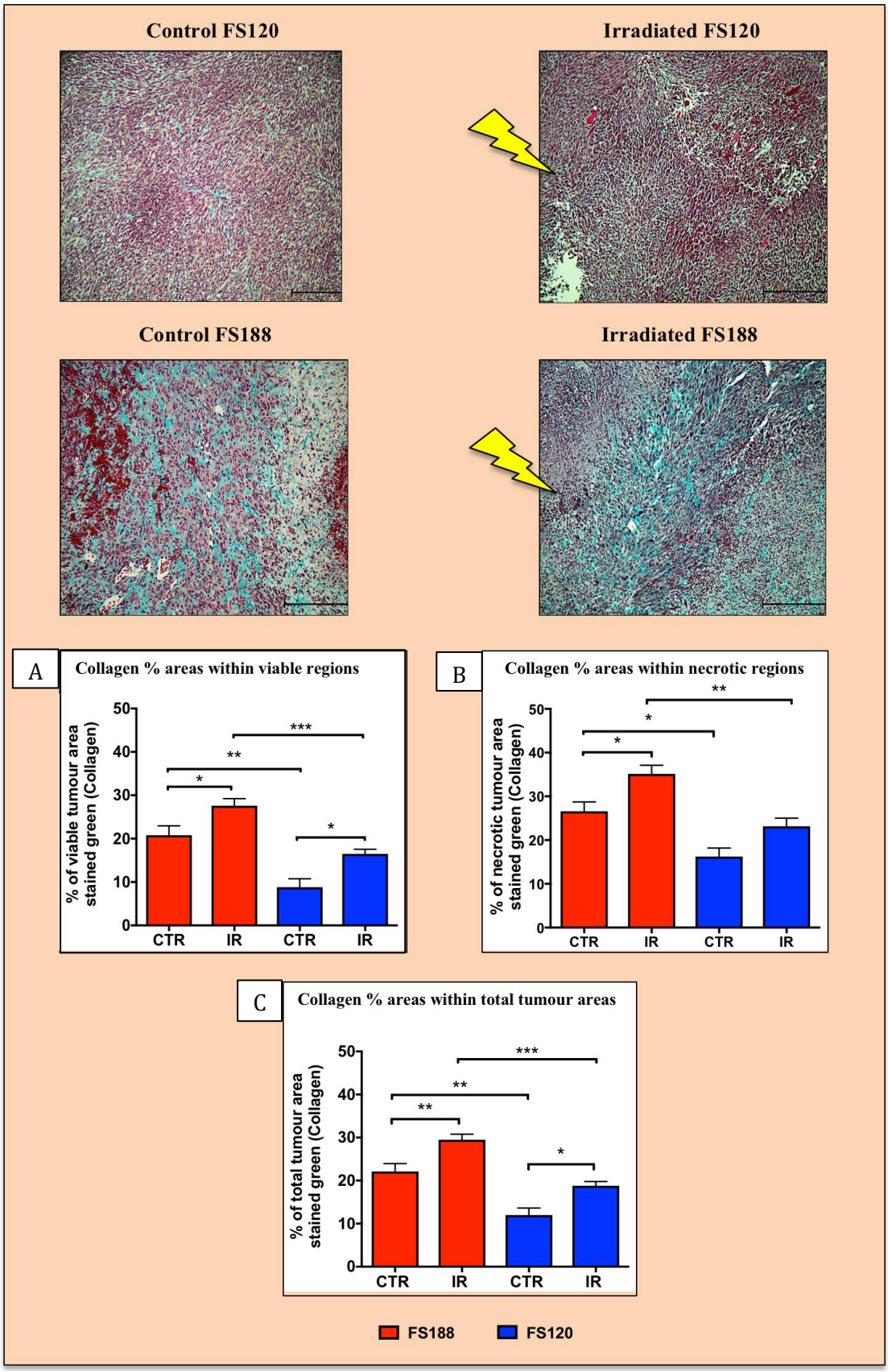

Control FS120
Irradiated FS120
Control FS188
Irradiated FS188
A
Collagen % areas within viable regions
% of viable tumour area stained green (Collagen)
B
Collagen % areas within necrotic regions
% of necrotic tumour area stained green (Collagen)
C
Collagen % areas within total tumour areas
% of total tumour area stained green (Collagen)
CTR
IR
CTR
IR
FS188
FS120

Figure 5.4 Percentage of collagen staining (Masson's trichrome) within the viable and necrotic regions of the paraffin embedded fibrosarcoma tumour sections using HistoQuest software analysis system.

Thirty-eight stained tumours sections (10 sections for control FS188, 10 sections for control FS120, 9 sections for irradiated FS188, and 9 sections for irradiated FS120 tumours) were analyzed using HistoQuest software. The graphs showed the percentage of green stained (collagen) areas within viable regions of the tumour (A), within necrotic regions (B) and as a fraction of the total tumour area (C). Radiation caused a significant increase in collagen for both FS188 tumours and FS120 tumours. Furthermore, the percentage of collagen content within the control (non-irradiated) FS188 tumours was more than within control FS120 tumours (A, B & C). There was a significant difference between irradiated FS188 and irradiated FS120 (more collagen content within irradiated FS188). One-way ANOVA test followed by Tukey's multiple comparisons test was used to analyse data. Results expressed as means ± SEM and were significant when $P \leq 0.05$ (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$).

5.2.3 Immunofluorescence staining of frozen fibrosarcoma tumour sections using CD31 & α-SMA

Frozen tumour sections (see **section 2.2.7**) were provided by Mr Matthew Fisher. The tumour sections were dried at room temperature, fixed and stained for immunofluorescence as discussed previously (see **section 2.2.7.3**). Slides were visualised using an Olympus BX61 microscope that has an Olympus laser-based autofocus unit (**Figure 5.5**). First, areas with high cell density were identified under bright field. Then, sections were observed using microscope filters of different excitation and emission wavelengths to detect DAPI stained nuclei (excitation 350/50 and emission 460/50 nm), FITC for CD31 (excitation 470/40 and emission 620/60 nm), and TX-RED filter for α-SMA (excitation 545/30 and emission 525/50 nm). Fourteen tumour sections were stained (3 FS188 control, 2 FS120 control, 4 FS188 irradiated, and 5 FS120 irradiated), and eight images were taken per each tumour section. CD31-positive cells represent endothelial cells. The red colour α-SMA-positive cells either represent CAF-like cells if they were within the stroma or pericytes if surrounding the blood vessels. Images from each tumour are shown in **Figure 5.6**. Because of time issues and problems with scanning the slides, quantification of staining was not possible. Instead, subjective visual assessment of α-SMA and CD31 positivity was performed.

Images were processed and merged using Adobe Photoshop CS6 software.

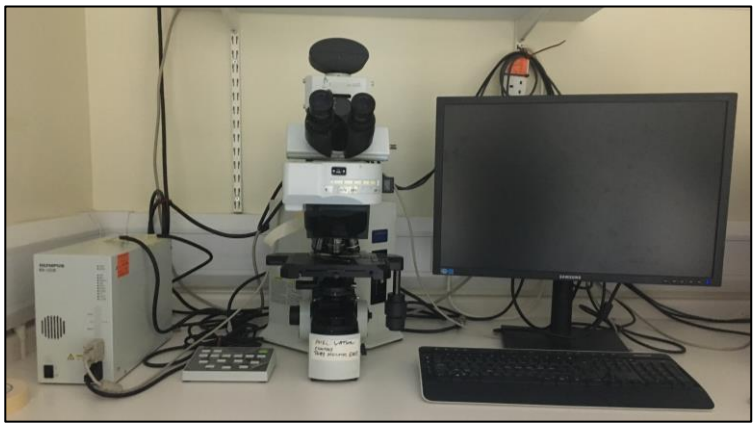


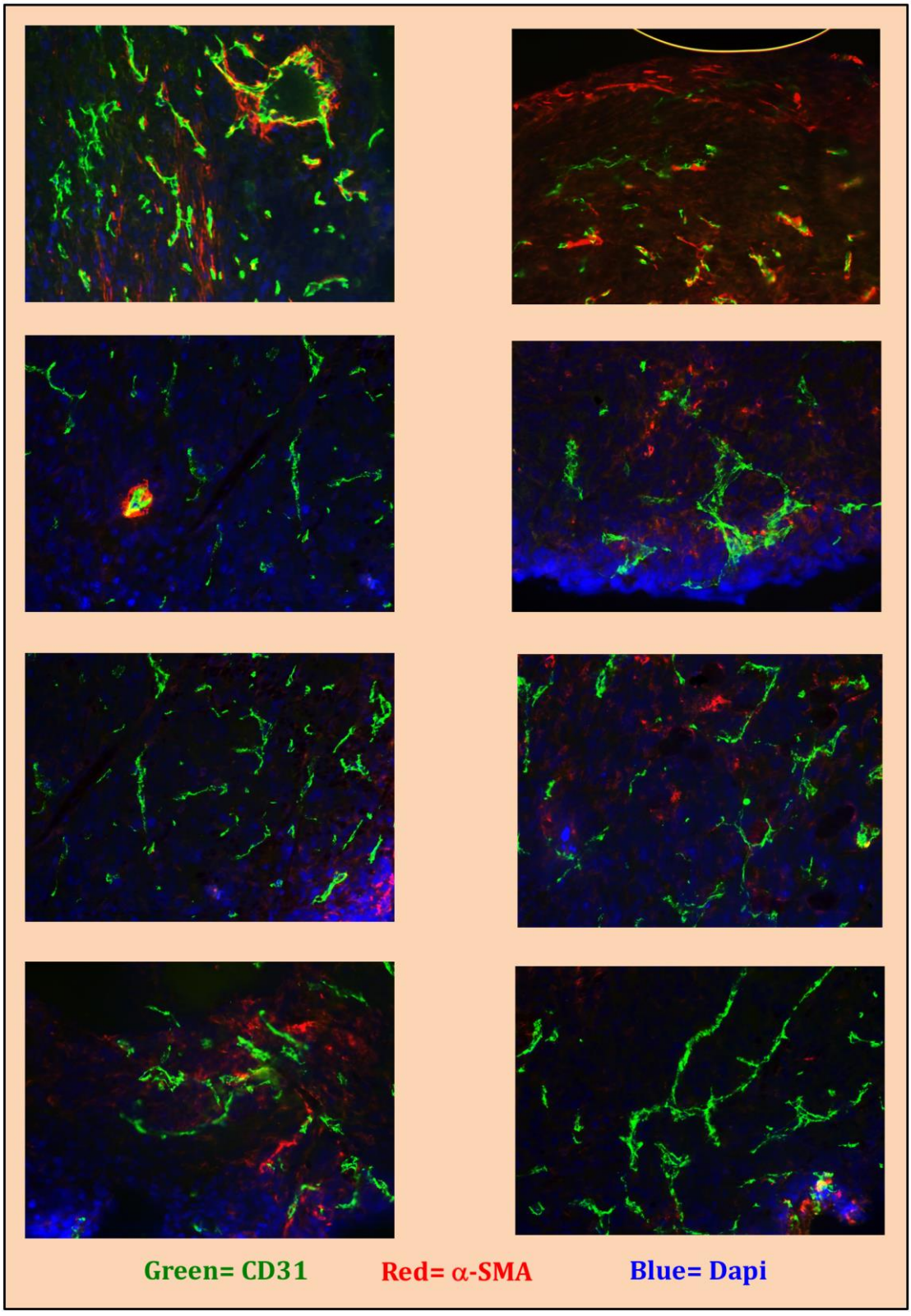
Figure 5.5 Olympus BX61 microscope used to visualise IF slides

On observation, it appeared that irradiated FS188 and FS120 tumours had an abundance of pericytes and CAFs. In some tumour sections, numerous CAFs were located around the tumour edges (tumours 4, 6, and 10). CD31 staining in all tumour sections shows that all tumours were well vascularised (especially FS188 tumours). Because of the small number of tumours that were included in this analysis it is not possible to say whether irradiation caused any changes in α-SMA-stained cells or blood vessels, although in irradiated tumours perivascular α-SMA stained cells (pericytes) appeared particularly abundant (see tumour 4, 5, 6, 10, and 13).

More tumour sections need to be stained and analysed with specific quantification of blood vessels and α-SMA stained cells to establish if radiation recruited CAFs and pericytes to these tumours.

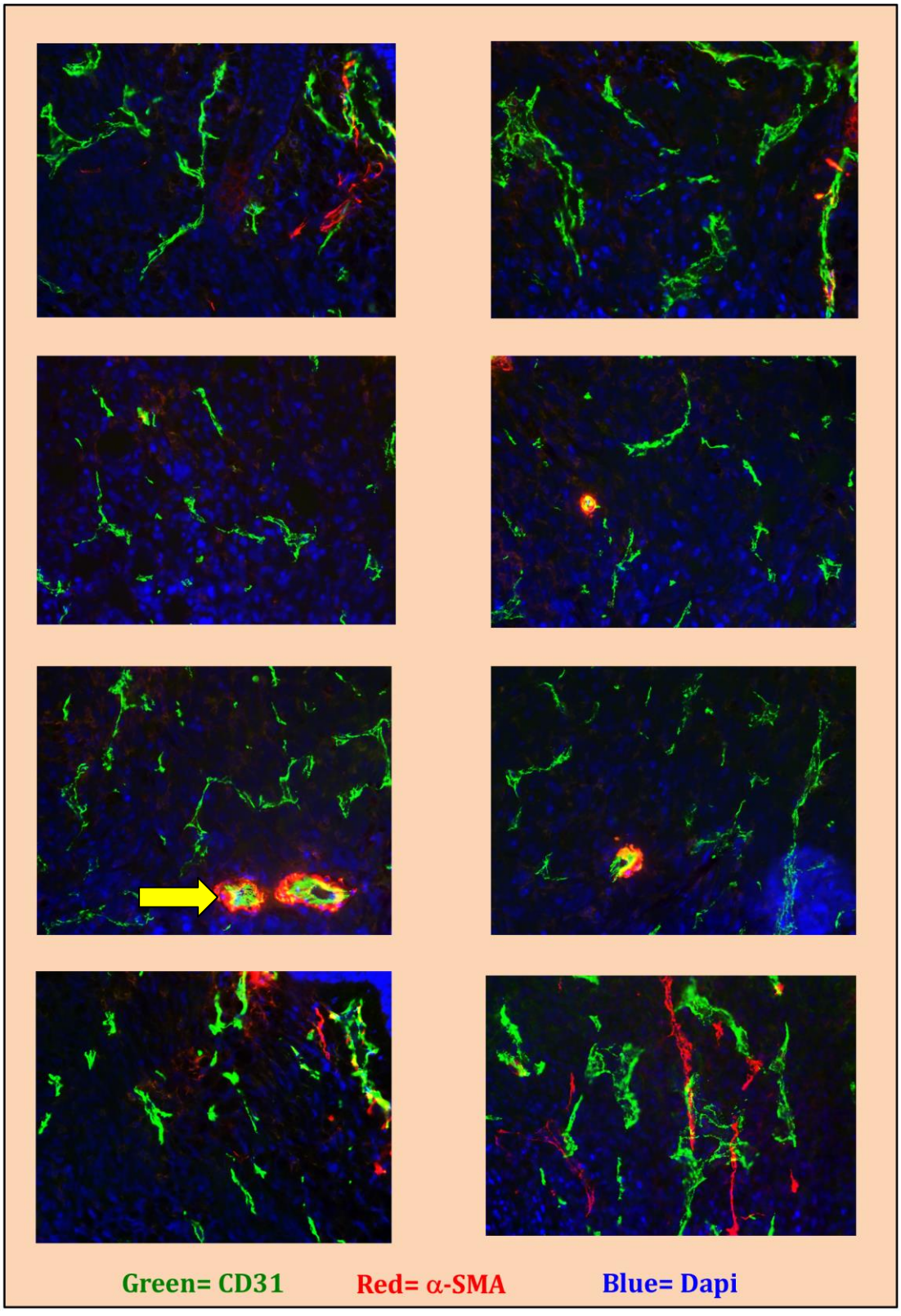
Tumour 1

FS 188 control



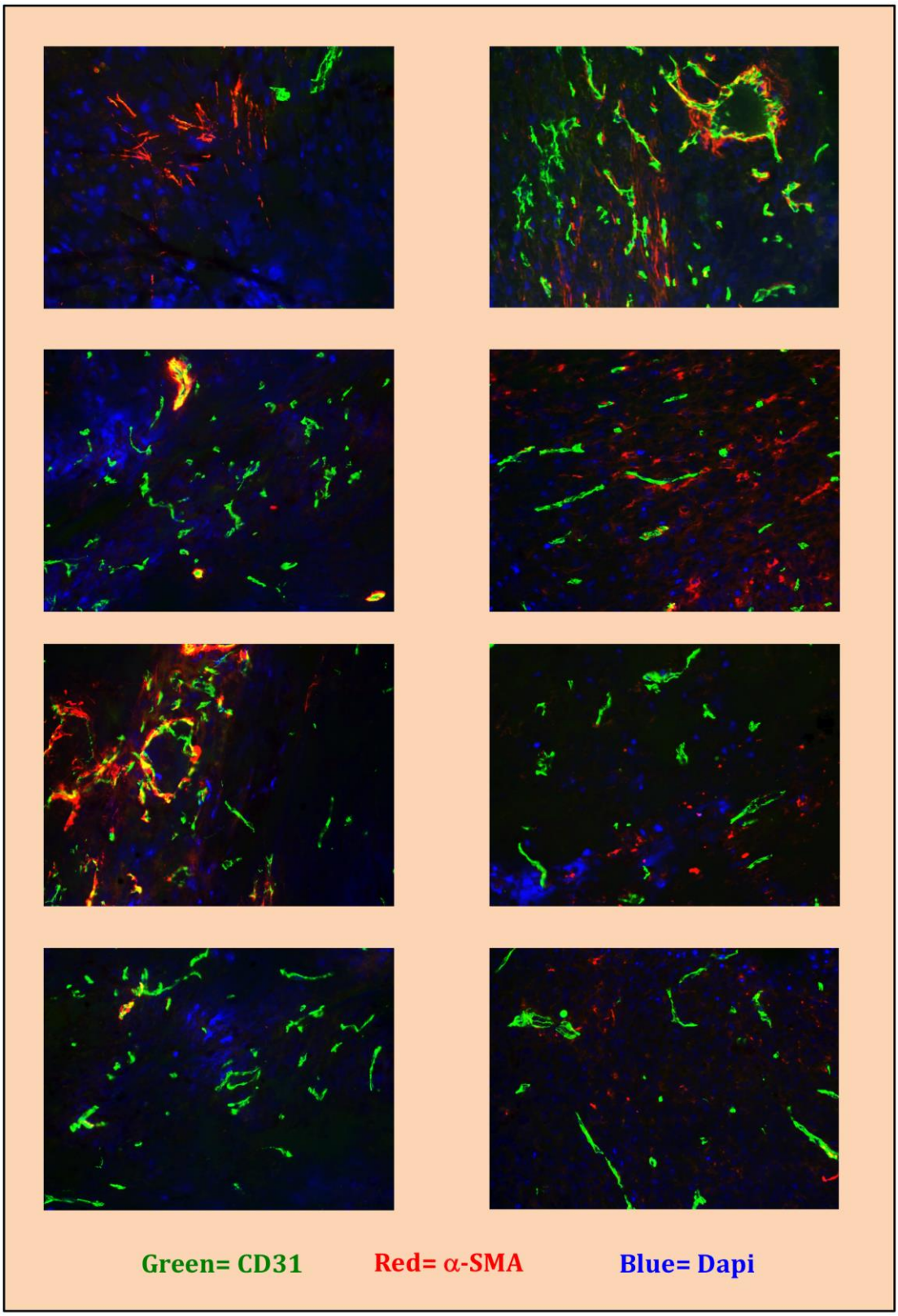
Tumour 2

FS 188 control



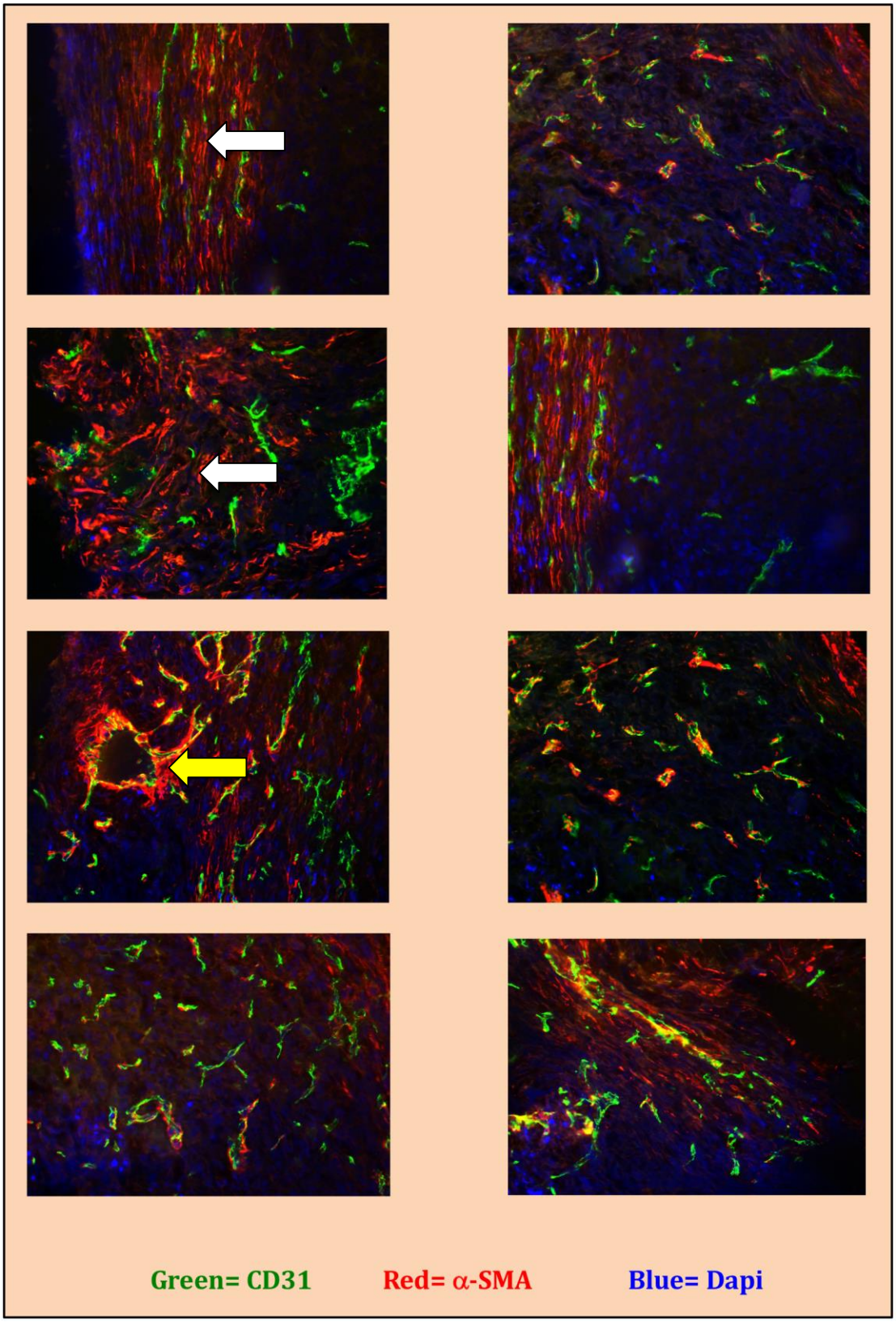
Tumour 3

FS 188 control



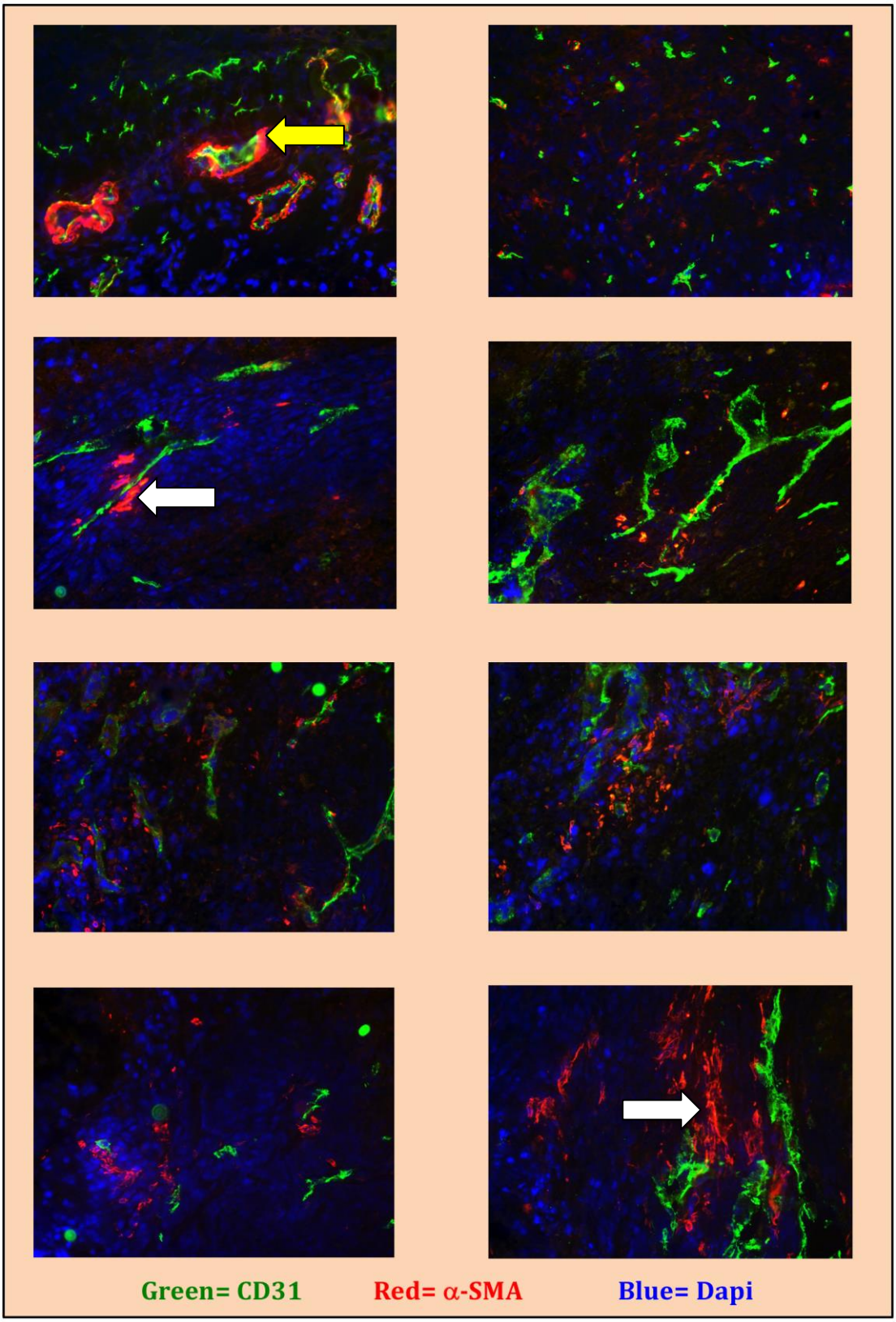
Tumour 4

FS 188 irradiated



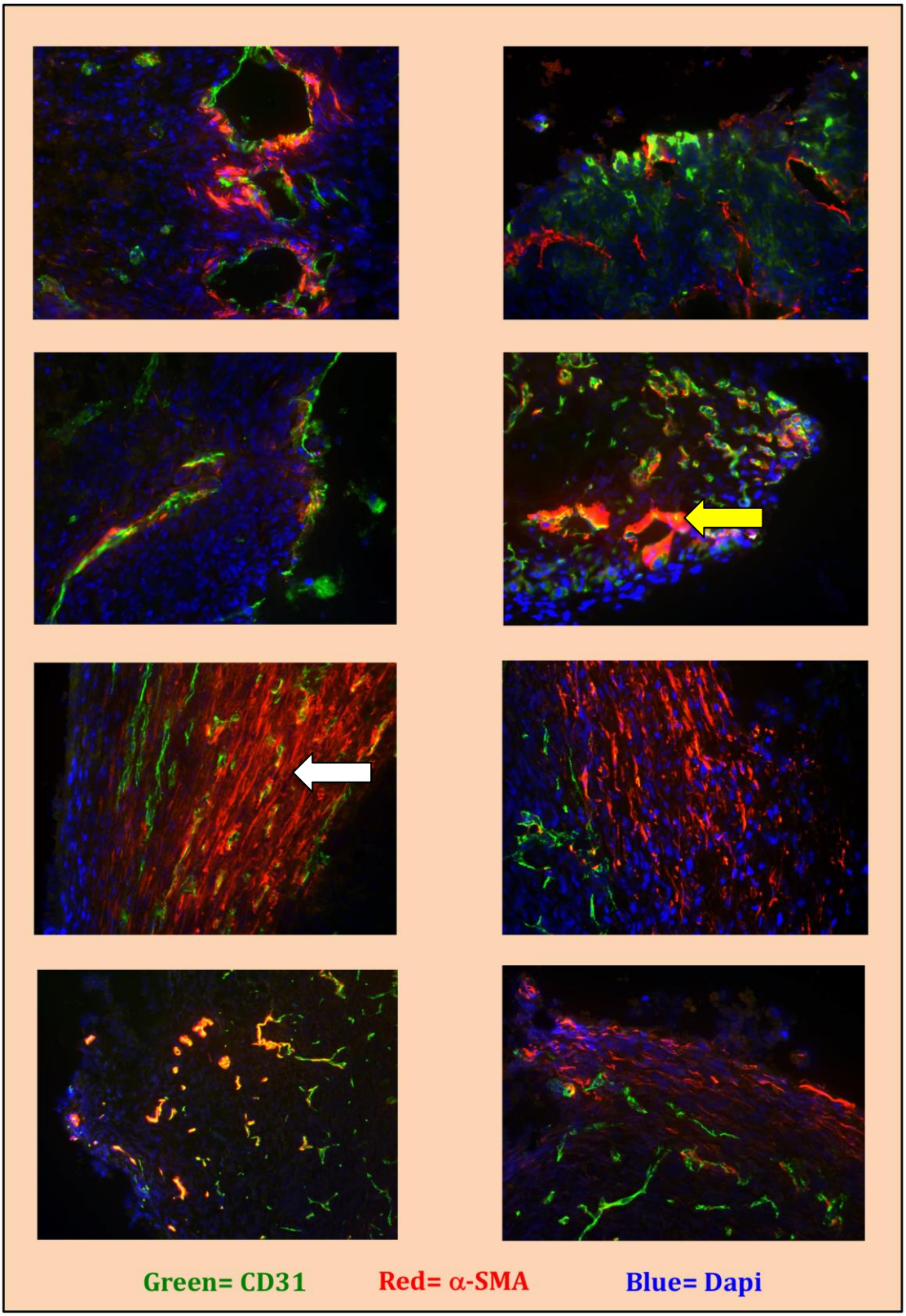
Tumour 5

FS 188 irradiated



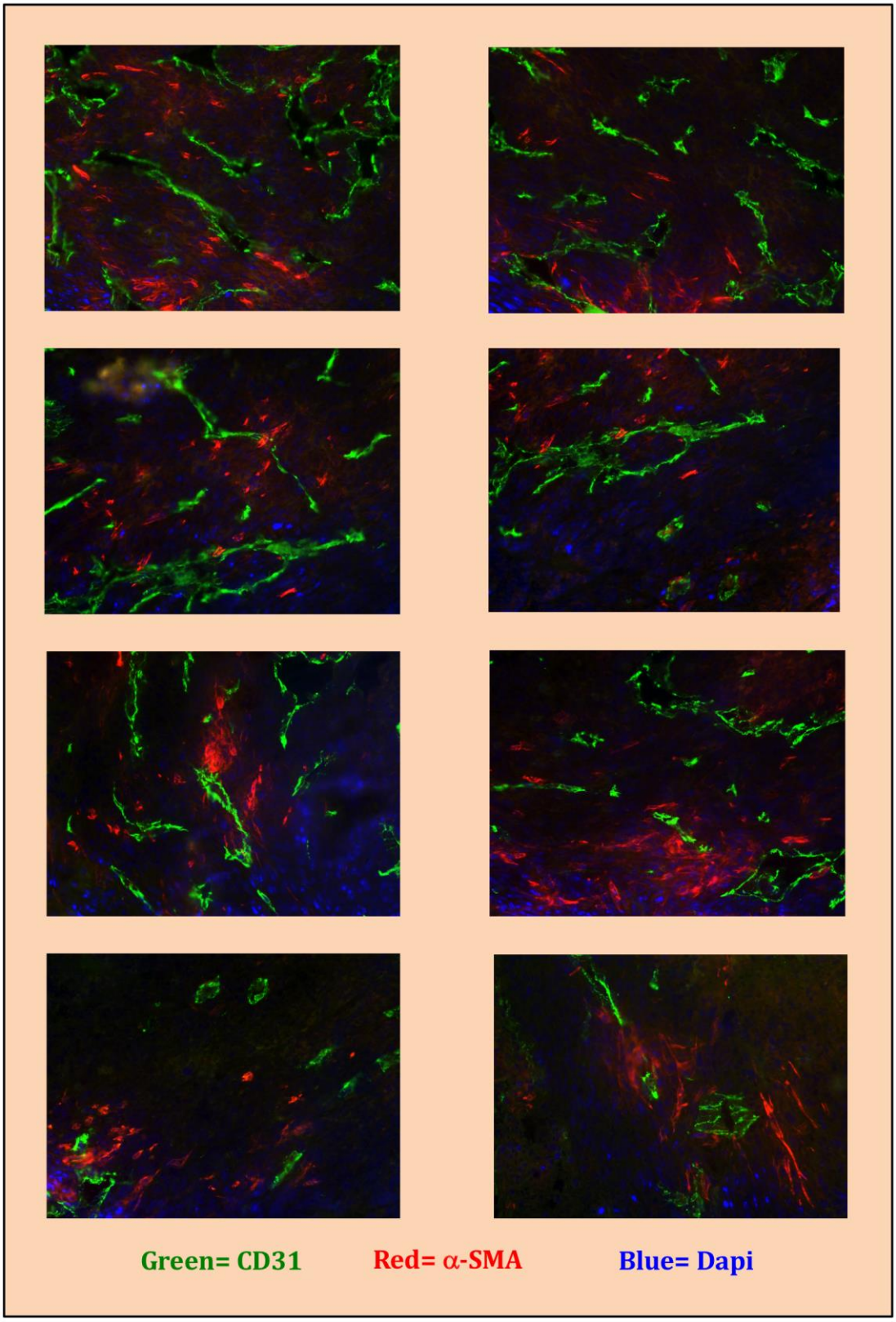
Tumour 6

FS 188 irradiated



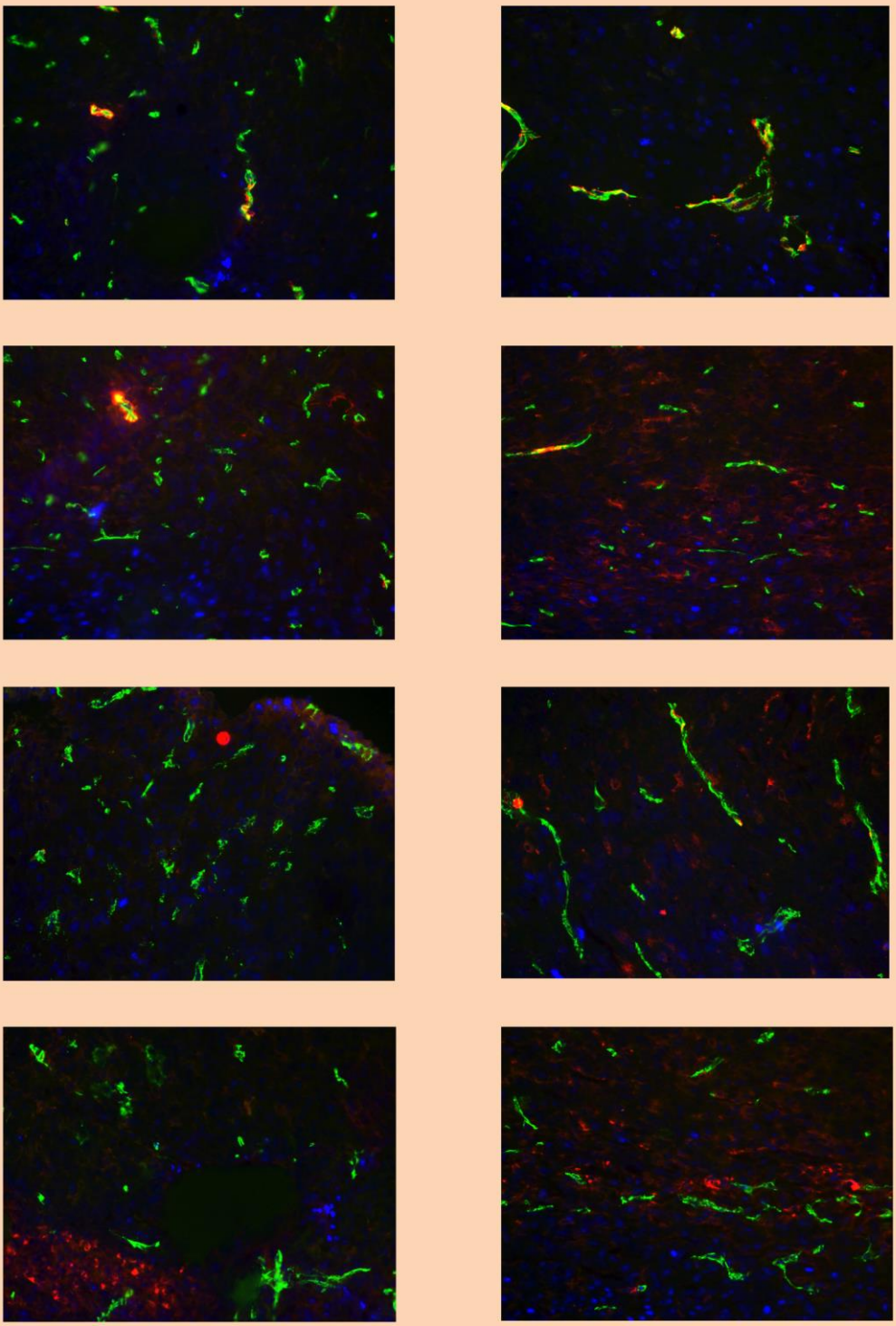
Tumour 7

FS 188 irradiated



Tumour 8

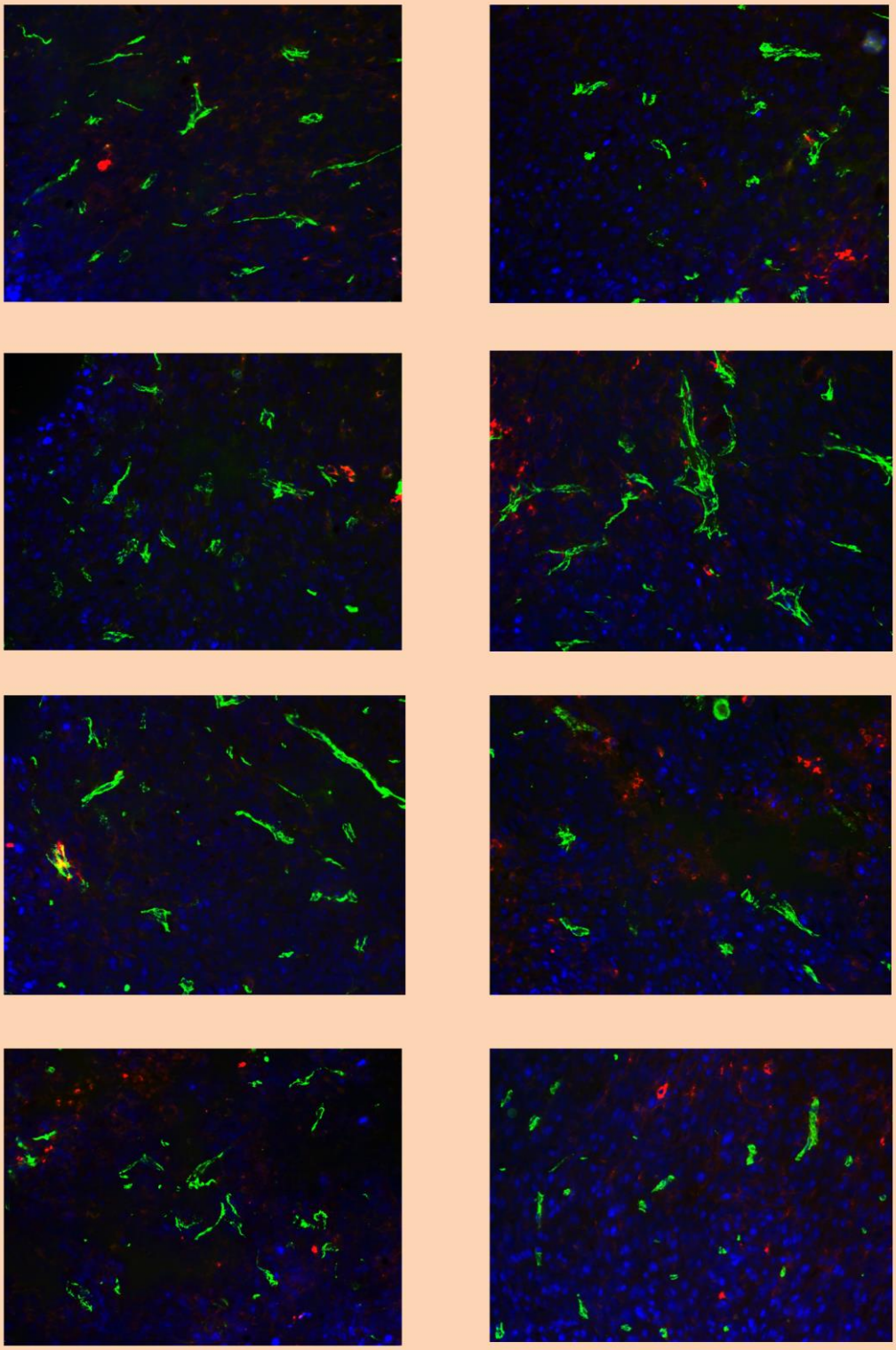
FS 120 control



Green= CD31 Red= α-SMA Blue= Dapi

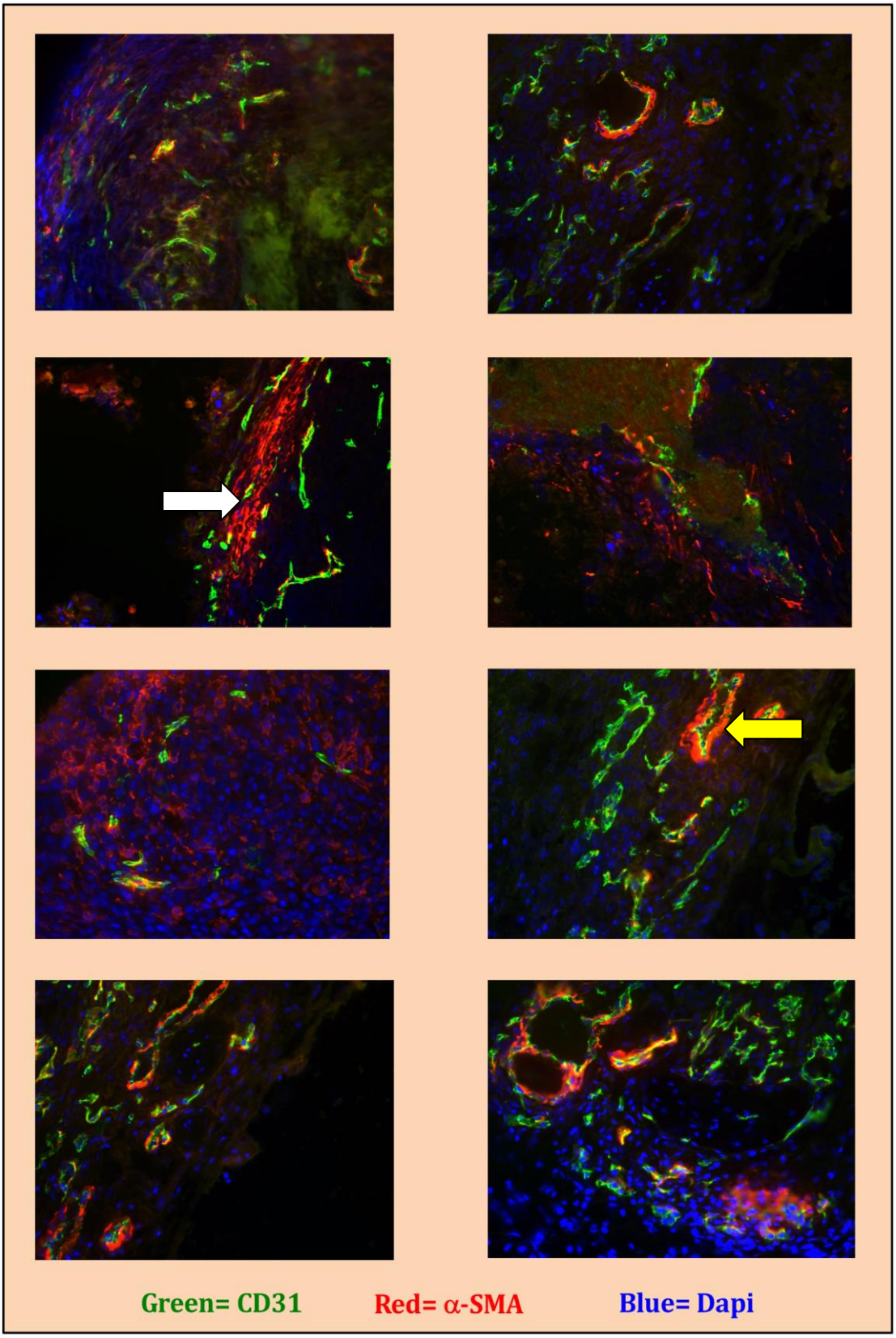
Tumour 9

FS 120 control



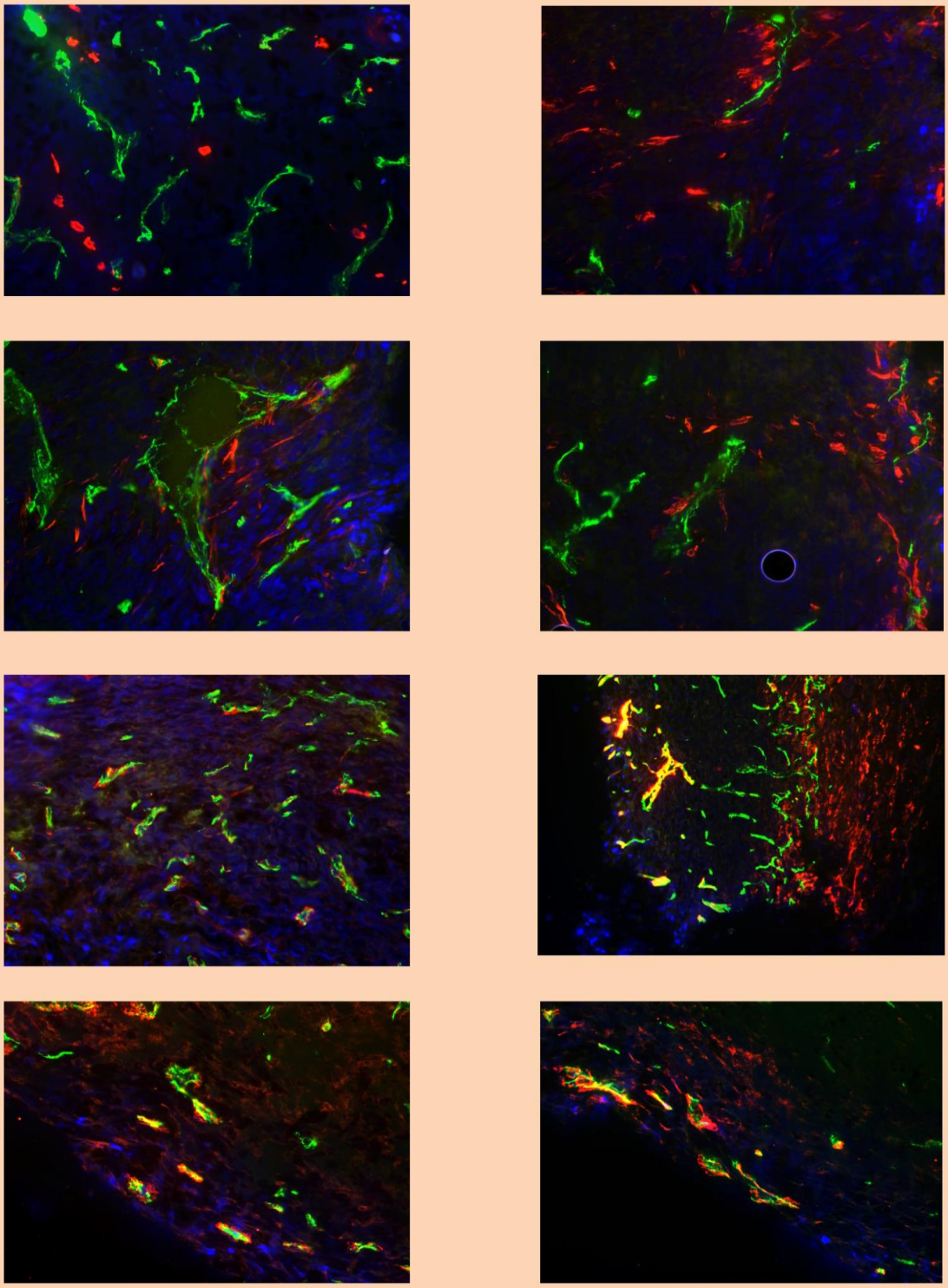
Tumour 10

FS 120 irradiated



Tumour 11

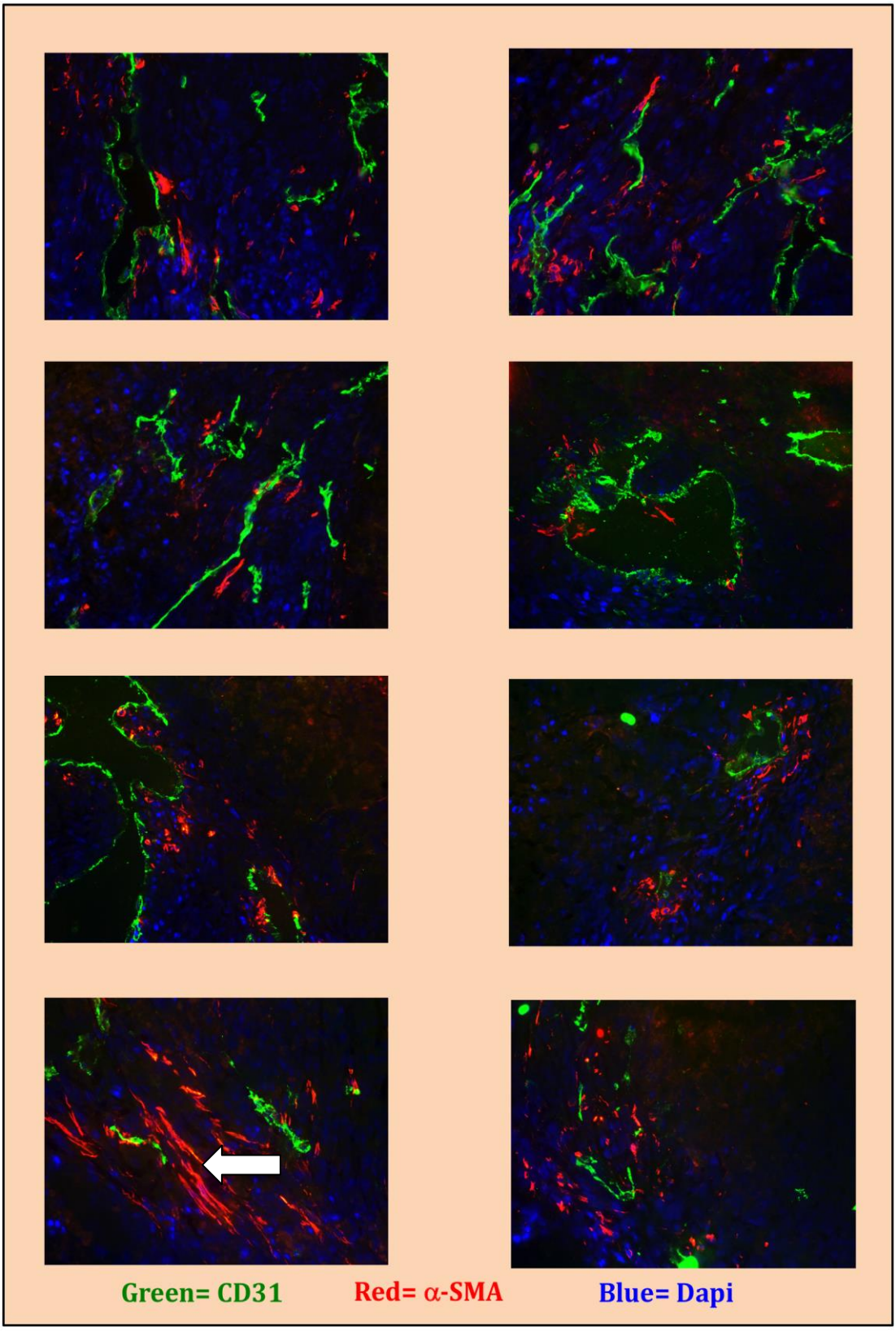
FS 120 irradiated



Green= CD31 **Red= α-SMA** **Blue= Dapi**

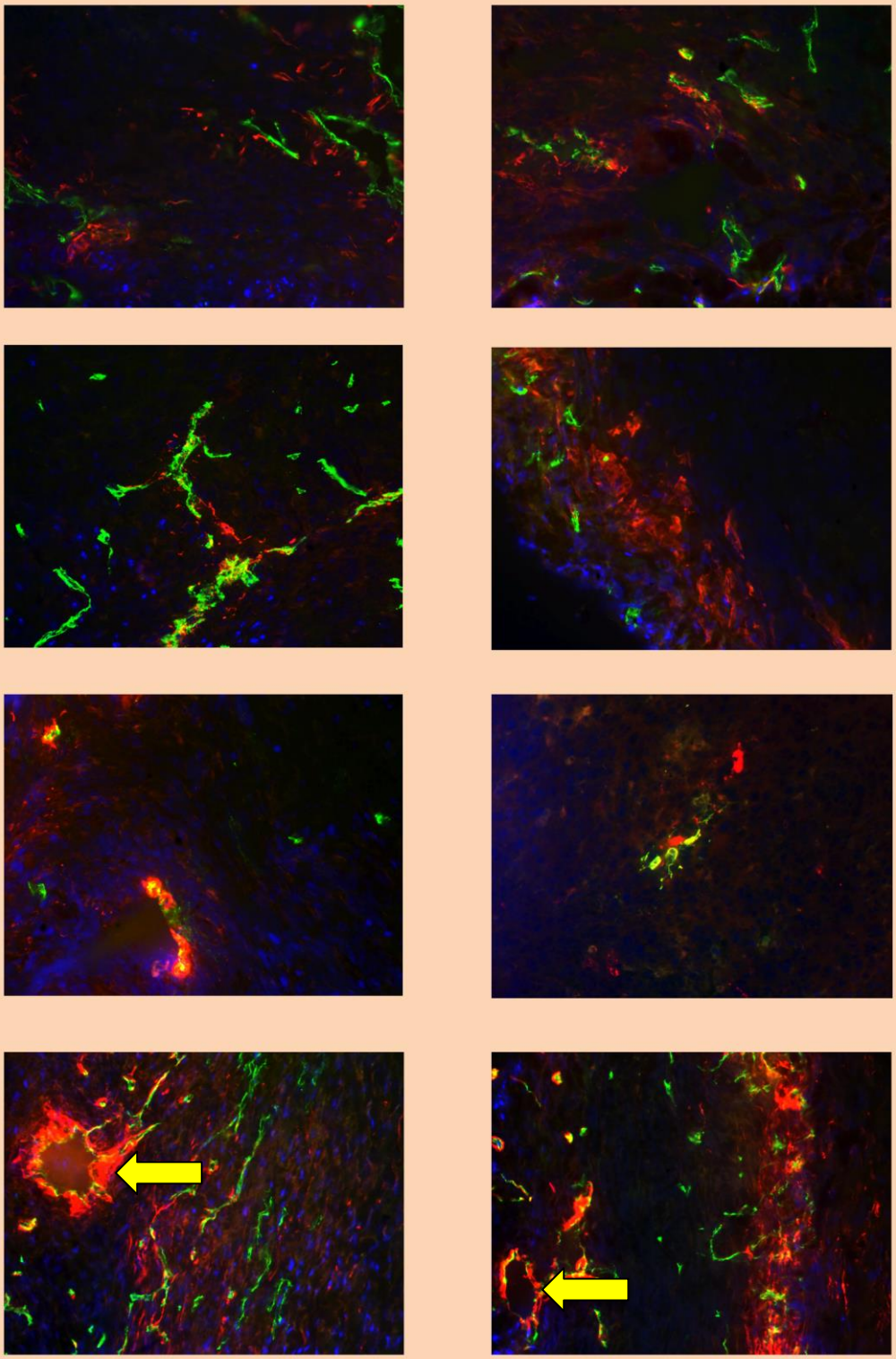
Tumour 12

FS 120 irradiated



Tumour 13

FS 120 irradiated



Tumour 14

FS 120 irradiated

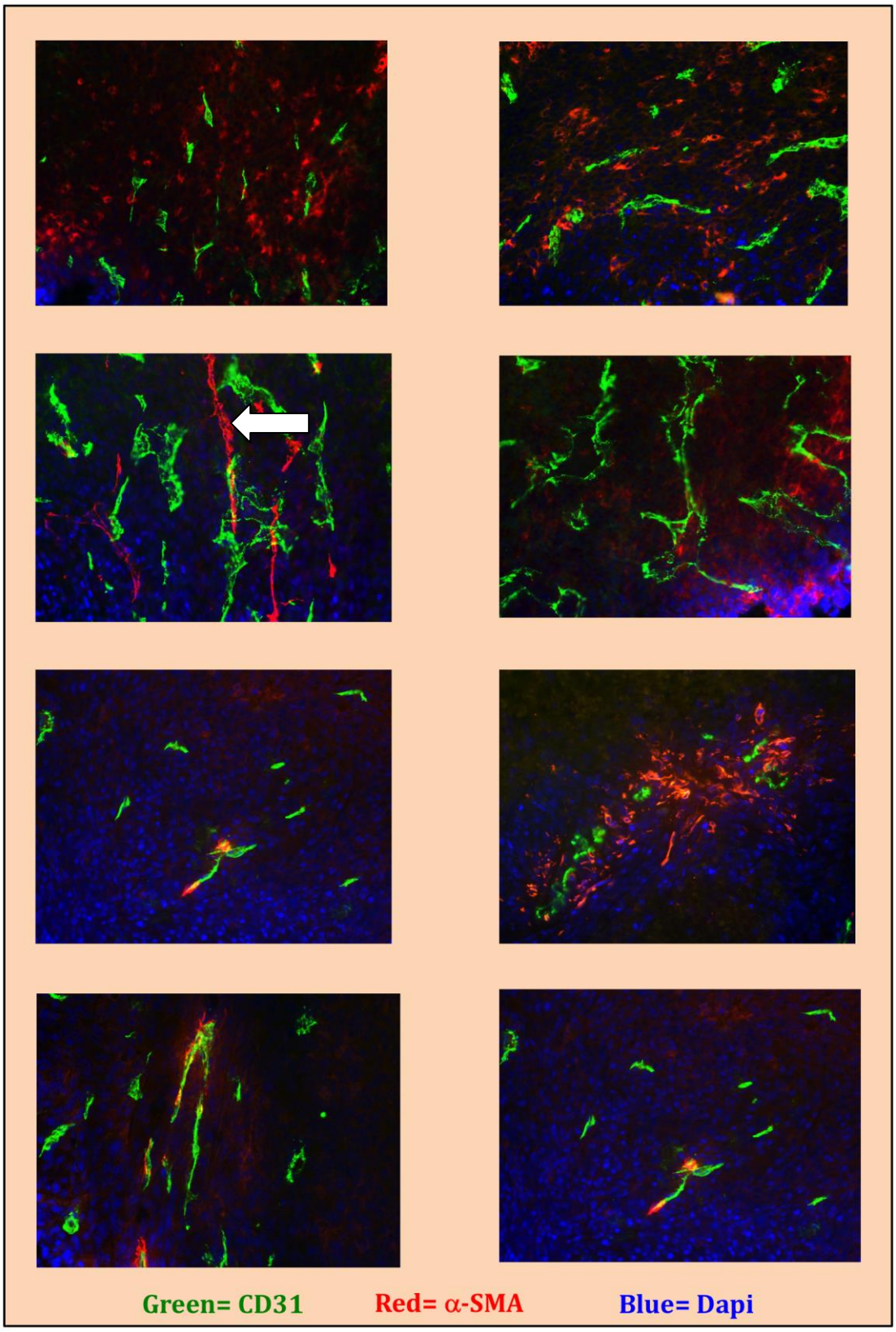
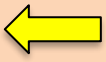
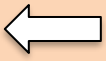


Figure 5.6 Immunofluorescence staining for mouse fibrosarcoma frozen tumour sections (control and irradiated) using CD31, and α-SMA stain.

Blood vessels were stained for CD31 (green), CAFs or pericytes were stained for α-SMA (red), and nuclei stained with DAPI (blue). In some but not all tumour sections we can see that irradiated FS188 tumors expressed more α-SMA than irradiated FS120 tumor sections, some of which were associated with CD31-positive blood vessels (see tumour sections 4, 5, and 6). Moreover, irradiation of FS188 and FS120 tumours appeared to increase the number of pericytes and CAF-like cells in the tumours. Radiation seems to enhance the perivascular pericytes cells especially in FS188 tumours compared to FS120 tumours (see tumour sections 4, 5, 6, 10, and 13).

 Represent pericytes, and  represent CAF-like cells.

5.3 Summary of the results

After showing *in vitro* that radiation enhanced MSC migration and their differentiation into CAF-like cells, it was important to study these radiation effects *in vivo*. Paraffin tumour sections were stained for Masson's trichrome staining while frozen tumour sections were stained for immunofluorescence with CD31 and α-SMA. Results from analysis of the stained images showed that radiation enhanced collagen content within viable and necrotic areas of the tumours. In addition, collagen content within irradiated FS188 was more than within FS120 tumours. Unfortunately, results from immunofluorescence staining were inconclusive, although they were suggestive of an increase in CAF-like and pericyte-like cells post-irradiation in both tumour types.

5.4 Discussion

It has been observed in previous chapters that radiation caused differentiation of MSCs into CAF-like cells *in vitro* in response to growth factors secreted by tumour cells. Furthermore, FS188 tumour cells secreted more MCP-1 than FS120 cells which were responsible for migration of MSCs toward TME *in vitro*. MCP-1 has been shown to cause expression of collagen through enhancing TGF-β1 or IL-4 endogenous signalling (Distler et al., 2006).

In order to confirm that radiation could modify TME and increase ECM protein deposition within the tumour-stromal tissue, paraffin and frozen tumour sections from an *in vivo* experiment were stained and analysed for ECM protein. Since the most common marker used to detect CAFs is α-SMA (Park et al., 1999, Sugimoto et al., 2006, Kim et al., 2015), IHC staining for α-SMA was performed for paraffin tumour sections. Unfortunately, although some slides showed a positive reaction to staining, there was a lot of background for most of the slides. Efforts were made to decrease the background but failed (see **section 5.2.1**). One explanation for this background could be because the tumours were excised from mice that have been treated with lectin and pimonidazole (as a marker for hypoxia).

As a suggestion from Dr William English, a research fellow, I asked Mr Matthew Fisher, our technician, to optimise the protocol for future use by pre-adsorbing the secondary

antibody with mouse serum. Although other members of the team tested this, again the problem was not resolved and the background remained.

As previously reported, many types of collagen are present in fibrotic conditions that make their quantifications difficult by immunohistochemical staining (Katsuda et al., 1992, Mostaco-Guidolin et al., 2013). Therefore, staining that works for different collagen types, namely Masson's trichrome were used. Masson's trichrome staining was used to stain perivascular and interstitial collagen within fibrosarcoma tumours. The protocol was optimised until clear staining was achieved (see **Figure 5.2**). The key steps were, firstly, to use Weigert's iron haematoxylin staining instead of Mayer's haematoxylin (advice from colleagues on the ResearchGate website), in which Weigert's has iron that is mordant and resistant to acid. Secondly, to decrease the incubation time with phosphomolybdic acid-phosphotungstic acid to 2 minutes insteade of 5 minutes. Many studies have used this staining technique to detect collagen fibres in fibrotic conditions such as liver, renal or cardiac fibrosis (Lo and Kim, 2017, Zhou et al., 2013, de Jong et al., 2012).

The difficulty in analysing or quantification tissues stained with Masson's trichrome stain is due to three colours of the stain that co-localise or overlap to the same areas (Miot and Brianezi, 2010). An Aperio Imagescope and HistoQuest software were used to analyse images of tumour sections stained with Masson's trichrome. The results are in the agreement with our previous results *in vitro* in which radiation significantly increased the expression of ECM proteins such as collagen and fibronectin by MSCs (see **chapter 3**). The high collagen contents of FS188 tumours compared to FS120 reflect the ECM differences between the two tumours *in vivo*, which could be due to the ability of this tumour to recruit more CAF-like cells than FS120 tumours (Tozer et al., 2008, Kalluri and Zeisberg, 2006). This result, also supports a recent study by English et al showed that FS188 tumour expressed more collagen-I compared to FS120 tumours, both *in vitro* and *in vivo* while it was the reverse for laminin (English et al., 2017).

Failure to quantify α-SMA staining due to background staining led to testing an immunofluorescence method on frozen sections. To examine FS188 and FS120 tumours for vascularity differences and distribution of CAF-like cells (α-SMA +ve cells), immunofluorescent staining with CD31 and α-SMA was done. Dual staining for

both CD31 and α-SMA indicated the presence of pericyte-like cells. No cross-reactivity with primary antibody occurred; otherwise, the whole tissue would have stained with each antibody. Presence of dim red fluorescence in certain tumour sections could be due to poor washing after incubation with the secondary antibody. Although the plan was to scan the stained slides and quantify images, time constraints and problems with some slides breaking during scanning meant that images could only be assessed by eye. However, the results were in agreement with the Masson's trichrome staining results, in which radiation increased the deposition of α-SMA positive cells (CAF-like cells) in the tumour stroma. IF staining showed, in both tumour types, that α-SMA positive cells lining blood vessels (pericytes) was more intense with FS188 tumours than with FS120 tumours (**Figure 5.6**, tumours 2, 3, 4, 6, 9,13). This was also in agreement with the English et al study, where IF for laminin staining was more intense for FS188 tumours than for FS120 tumours regarding α-SMA positive cells lining blood vessels (pericytes) (English et al., 2017). For future work, using different markers for ECM protein expression like laminin, FSP-1 and FAB-α is mandatory as recently Dr English showed that both fibrosarcoma tumours expressed α-SMA (unpublished data). This could be also a cause for background staining of α-SMA in IHC for tumour sections done in this project.

In summary, our results represent progress in understanding the role of radiation in mouse fibrosarcoma tumours that expressed VEGF 120, and VEGF 188. This study was able to show that FS188 tumours expressed more CAF-like cells than FS120 tumours *in vivo* using different laboratory techniques. Furthermore, our results suggest that radiation increased the ECM content of fibrosarcoma tumours significantly. This result has an important impact on the future treatment outcome of sarcoma with anti-VEGF drugs as presence of CAF-like cells lead to fibrosis and make vessels impermeable to macromolecules and resistant to further treatment with drugs and chemotherapy. Furthermore, these results suggest that blocking CCR2, a receptor for MCP-1 expressed on the MSCs could decrease the recruitment and/ or differentiation of MSCs into CAF-like cells that cause fibrosis and make the tumour more aggressive.

CHAPTER SIX:

General discussion, future work and conclusion

Table 6.1 Outline of the approach used in this PhD. study with the limitations and future work.

In vitro

Laboratory techniques	Aim	cells	Limitation and future work
Clonogenic assay	-To select an appropriate radiation doses for the cells.	-Tumour cells -MSCs	- To do it for endothelial cells (HDMEC) -Do it in hypoxic condition.
Western blotting	-To study CAFs differentiation markers expressed by MSCs 3-4 days after irradiation.	-Tumour cells -MSCs -Endothelial cells - Tumour cells + endothelial cells	-To increase the time post irradiation. -Culture same numbers of tumour cells and endothelial cells for co-culture assay to exactly study their cytokines and chemokines secretion in response to radiation. -To repeate experiment in hypoxic condition.
Transwell migration assay	-To study whether direct or indirect radiation cause recruitment of the MSCs.	-Tumour cells -MSCs -Endothelial cells - Tumour cells + endothelial cells	- To study why C3H10 did not cause migration as Balb/c did.
ELISA	-To detect and quantify mouse MCP-1 chemokine in the serum-free	- Serum-free CM from both, control (un-irradiated) and	-To test other cytokines and chemokines such as, SDF-1α and TGF-β1 in the serum-free CM from irradiated tumour cells.

	CM from irradiated tumour cells.	irradiated FS188 and FS120 cells.	
Transwell migration assay with blocking antibody	-To determine whether MCP-1 is a potential mediator of Balb/c MSCs migration.	-Serum-free CM from un-irradiated and irradiated (4 Gy) FS 188 cells.	-To test serum-free CM from FS120 cells (un-irradiated and irradiated). -To test other cytokines and chemokines such as, SDF-1α and TGF-β1 in the serum-free CM from tumour cells.

In vivo

Laboratory techniques	**Aim**	**Tumour**	**Limitation and future work**
IHC	-To Study whether α-SMA content of the tumours increased in response to irradiation that reflect more CAFs cells.	-Control (un-irradiated) and irradiated FS 188 and FS120 tumours.	-Need to repeat the *in vivo* experiment to get fresh tumour sections that can stained easily with better expectation.
Mason's trichrome staining	-To detect collagen fibres in tissues.	- Control (un-irradiated) and irradiated FS 188 and FS120 tumours.	-As above.
IF	-To study whether α-SMA content of the tumours increased in response to irradiation that reflect more CAFs cells.	-Control (un-irradiated) and irradiated FS 188 and FS120 tumours.	-As above -Needs more sections to stain and analyse. -Analysing staining using proper slide scanning with proper image analysis.

6.1 General discussion

Radiotherapy is one of the standard treatments for patients with intermediate and high-grade soft tissue sarcoma. One of the major complications from radiation therapy in the treatment of STS is fibrosis (Delanian and Lefaix, 2004, Martin et al., 2000). Radiation-induced fibrosis is life-threatening and a very complicated process that involves many growth factors, inflammatory cytokines, differentiation of fibroblasts and remodelling of ECM (Wynn, 2008). Fibrosis is represented by the presence of CAFs (activated myofibroblasts), which are responsible for secretion of extracellular matrix proteins such as collagen and fibronectin. The origin of CAFs in irradiated tumours has not been clearly established. Some studies showed that MSCs were recruited to irradiated tumours (Klopp et al., 2007, Fenton and Paoni, 2007). MSCs can differentiate into pericytes and CAF-like cells but the effects of radiation on their recruitment or differentiation remain unclear. CAFs can be derived from circulating BM-derived MSCs according to animal models and human breast cancer studies (Ishii et al., 2003, Direkze et al., 2004, Direkze et al., 2006, Allinen et al., 2004, Chauhan et al., 2003). Moreover, a study by LaRue et al, using mice that had been transplanted with cells derived from a single enhanced green fluorescent protein (EGFP)-positive hematopoietic stem cells, supports that theory that CAFs can originate from circulating BM-derived MSCs (LaRue et al., 2006). The focus of this project was to investigate whether radiation influences MSCs and triggers their differentiation into CAF-like cells either directly or indirectly via effects on other cell types present within the TME. Moreover, the radiation effects on recruitment/ migration of MSC towards the TME were also investigated. These alterations of MSCs in response to radiation have the potential to play a significant role in radiation-induced changes within the tumour microenvironment linked to CAF-induced tumour progression as well as the development of fibrosis.

Using *in vitro* models, and on the basis of western blotting analysis, this project showed that direct irradiation of MSCs produced some small effects in terms of the induction of expression of proteins such as collagen-I, which are associated with differentiated CAFs. Similarly, direct irradiation of MSCs had no effect on their migration. However, the indirect effects of radiation were prominent suggesting that it is the interactions between components of the TME and radiation that are important in recruiting and differentiating MSCs in tumours. This project was able to demonstrate that

fibrosarcoma cells are induced to secrete factors in response to irradiation, such as MCP-1, SDF-1 and TGF-β1, that can work in a paracrine manner to cause the migration and differentiation of MSCs into cells with CAF-like characteristics. These factors were identified by western blotting analysis of FS188 and FS120 CM. Direct evidence for a specific involvement of MCP-1 in MSC recruitment was obtained from migration experiments where MCP-1 was neutralised in the CM of fibrosarcoma cells. These experiments showed that MSC migration was inhibited by blocking MCP1 activity, suggesting that MCP-1 produced by fibrosarcoma cells was responsible, at least in part, for triggering MSC migration. These experiments point to potentially a key role for tumour-derived MCP-1 in radiation-mediated recruitment of MSCs *in vivo*. Further studies are needed to confirm if MCP1 is involved in the recruitment of MSCs towards solid fibrosarcomas following radiotherapy.

In this project, the MCP-1 receptor CCR2 was upregulated by MSCs exposed to irradiated FS188 tumour cells or their concentrated CM. Klopp et al showed that CCR2 was expressed by MSCs and its levels of expression increased after irradiation (Klopp et al., 2007). Many factors, including growth factors produced from tumour cells and inflammatory cytokines like TNF-α can up-regulate CCR2 (Ringe et al., 2007, Ponte et al., 2007). Klopp et al confirmed the involvement of the CCR2 receptor in the recruitment of MSCs towards irradiated tumour cells by using an anti-CCR2 that decreased MSC migration markedly. It will be interesting to see if blocking CCR2 could inhibit migration of MSCs induced by irradiated fibrosarcoma cells and their CM.

In this project, when MSCs were incubated with irradiated fibrosarcoma tumour cells or their CM for 3-4 days, some changes in expression of proteins characteristic of differentiated CAFs were noted but these changes were small. Mishra et al showed that human MSCs exposed to tumour-conditioned medium for long periods of time (30 days), enhanced their CAF-like myofibroblast phenotype (Mishra et al., 2008). It is possible therefore that if MSCs were incubated for longer with CM from irradiated fibrosarcoma cells more prominent effects might have been observed. The biological differences between FS188 and FS120 tumours cells and solid tumours (i.e. *in vitro* and *in vivo*) in response to radiation that could potentially affect migration and differentiation of MSCs were studied. Irradiated FS188 cells have a tendency to cause MSCs to expressed higher levels of differentiation markers and to recruit more MSCs

than irradiated FS120 cells (although not significant). However, the *in vivo* analysis established that radiation enhanced fibrosis in FS188 tumours more than FS120 tumours, which could potentially derive from more MSCs differentiated into CAF-like cells. The behavioural difference between two tumour cells (Kanthou et al., 2014) and the ability of FS188 cells to produce more CAF-like cells *in vivo* (Tozer et al., 2008) could be explained on the basis that FS188 cells secreted more cytokines such as MCP-1 than FS120. This was evident from ELISA quantification of MCP-1 in CM from control and irradiated FS188 and FS120 cells. Moreover, some studies showed, both *in vitro* and *in vivo*, that MCP-1 enhanced the migration of MSCs (Boomsma and Geenen, 2012, Dwyer et al., 2007, Belema-Bedada et al., 2008). Also MCP-1 has been shown to be expressed by human ovarian cancer cells (Furukawa et al., 2013), human endometrial cancer cells (Wang et al., 2006) and triple-negative breast cancer cells (Dutta et al., 2018).

Although MCP-1 was identified as a factor capable of inducing MSC migration, other cytokines are also likely to be involved. The migration of MSCs in response to other cytokines and chemokines was also studied in this project. TGF-β1, and SDF-1α significantly enhanced the migration of MSCs *in vitro*. The RNA sequencing of solid tumours by NGS confirmed that several factors that are known to be involved in differentiating fibroblasts and CAFs were differentially expressed by the fibrosarcomas. FS188 cells were found to express TGF-β2 transcripts more abundantly than FS120 cells. Similar to TGF-β1, TGF-β2 or TGF-β3 could also be involved in the recruitment of MSCs to tumours (Deng et al., 2017). However, FS120 cells produced more CTGF than FS188 cells which might suggest that FS120 cells had more capacity to induce fibroblast/CAF differentiation. It is likely that *in vivo* the recruitment of MSCs/CAF-like cells by fibrosarcoma cells is dependent on a complex array of different growth factors and chemokines and not a single factor, thus reflecting the cross-talk between cells within the TME.

Another important and novel finding of this study, was that irradiated endothelial cells induced the migration of MSCs in a radiation dose-dependent manner. Most studies in the literature refer to effects of MSCs on endothelial cells (Haubner et al., 2013a, Luu et al., 2013, Liang et al., 2017, Burlacu et al., 2013, Chen et al., 2015). To my knowledge, this is the first work establishing that endothelial cells induced the

migration of MSCs *in vitro* and furthermore radiation enhanced their migration further. Many studies showed that irradiation of endothelial cells affected their cytokine production (Schroder et al., 2018, Haubner et al., 2013a) that could therefore drive the effects on MSC migration seen here.

To translate this work into the *in vivo* setting, analysis of solid fibrosarcomas was also performed. In this thesis, α-SMA, the most common CAF marker (Park et al., 1999, Sugimoto et al., 2006, Kim et al., 2015), was used to stain sections of control and irradiated fibrosarcomas. Masson's trichrome staining was used also to stain perivascular and interstitial collagen within fibrosarcoma tumours. Many studies have used this staining technique to detect collagen fibres in fibrotic conditions such as liver, renal or cardiac fibrosis (Lo and Kim, 2017, Zhou et al., 2013, de Jong et al., 2012). This analysis showed more abundant collagen production within the FS188 tumours than FS120 tumours, which is in accordance to previous findings showing more abundant α-SMA stained cells in FS188 tumours. Furthermore, in irradiated tumours the levels of collagen were significantly higher compared to controls (especially in the FS188 tumours). Although analysis of α-SMA staining was inconclusive, the data taken together imply that radiation resulted in recruitment and/or activation of more CAFs producing more collagen within the tumour.

Together, *in vivo* data presented in this thesis suggest that radiation enhanced the ECM protein deposition within the stroma and perivascular areas of the tumours (especially FS188 tumours). Recently, English et al showed, both *in vitro* and *in vivo*, that FS188 tumours expressed more collagen-I than FS 120 tumours while the reverse was so for laminin (English et al., 2017).

The *in vitro* models in this project were done in two-dimensional (2D) models. This means that cells plated and growing directly on a flat , adherent surfaces (glass or plastic) with coated substrate to enhance their adhesion and behaviour like differentiation and proliferation (Morrison et al., 2011). 2D models are important, especially they are reasonably cheap, homogenous culture and reproducible tool to be used in similar with animal models. The problems in 2D models are they do not mimic real *in vivo* microenvironment (real microenvironment is more complex, hypoxic and organised), limitation in cell-cell interaction (only side-by-side contact), lack of interactions between cells and ECM and the oxygen diffusion with waste removal dynamics is

lacked (Antoni et al., 2015, Morrison et al., 2011). The limitations of the 2D models can affect cell survival, morphology, differentiation and proliferation that make three - dimensional (3D) models more important. A 3D cell culture models play an important role in cell and cancer biology as it offers an effective way to study the dynamics of cell in *in vivo*-mimicking conditions (Centeno et al., 2018). Moreover, 3D models allow cells to interact with each other and with ECM, provide better spatial organisation and more relevant *in vivo* environment models (Edmondson et al., 2014, Knight and Przyborski, 2015). However, 3D models have some disadvantages such as they are an expensive method, need special technique for visualisation/ microscopy, problems in homogenous distribution of the oxygen and nutrients (leads to necrosis and cell death); and require an expensive equipment (such as bioreactors) and expert optimisation and handling (Breslin and O'Driscoll, 2013, Rimann and Graf-Hausner, 2012).

6.2 Future work

While this thesis has established several effects of radiation on MSC biology and behaviour using a fibrosarcoma model, opportunities to extend the scope of this work remain.

6.2.1 *In vitro*

It will be important in the future to analyse in detail the factors secreted from endothelial cells in response to radiation that enhanced migration of MSCs *in vitro*. As highlighted in this project, MSCs migrated towards endothelial cells (**Figure 3.16**) as well as endothelial cells co-cultured with fibrosarcoma cells (**Figure 3.17**). Furthermore, irradiation of endothelial cells further increased migration of MSCs in a dose-dependent manner. Some studies showed that endothelial cells produce cytokines such as PDGF, TGF-β1, TNF-α, bFGF, and VEGF (Wachsberger and Burd, 2004, McBride et al., 2004) which could potentially induce recruitment of MSCs to tumours. It will also be important to investigate the role of factors other than MCP-1 in the recruitment of MSCs towards the fibrosarcomas. Both TGF-β1 and SDF-1α were found to induce the migration of MSCs (as shown in **Figure 3.18**) and were produced by irrdiated fiborasrcomas. It will be interesting to study the role of these cytokines in MSC migration in similar experiments to those performed with MCP-1 and using neutralizing anti-TGF-β1 or anti-SDF-1α antibodies.

As stated above, CCR2 expression was increased after co-culture of MSCs with irradiated tumour cells or their concentrated CM (**Figure 4.4**). To confirm that MCP-1 and its receptor was responsible for MSCs migration *in vitro* and not other inflammatory cytokines (Ringe et al., 2007, Ponte et al., 2007), blocking of CCR2 using anti-CCR2 antibody can also be done (Klopp et al., 2007).

6.2.2 *In vivo*

RNA sequencing of control non-irradiated fibrosarcoma tumours revealed differences in the expression of genes that are involved in fibroblast and CAF differentiation. To study the differences between FS188 tumours and FS120 tumours in response to radiation *in vivo*, it will be important for similar analyses to be performed using irradiated tumours. The information from such a study will be extremely useful in identifying factors that might be involved in radiation-mediated fibrosis and radiation mediated MSC recruitment into tumours.

6.3 Conclusion

The results of this study increased our understanding of the interactions between radiation and cells within the TME and their consequences on MSC biology. The results represent progress in understanding the role of radiation in a model of fibrosarcoma. The project was able to demonstrate that fibrosarcomas are influenced by radiation to recruit MSCs that can potentially acquire CAF-like characteristics and increase the ECM content of the tumour leading to fibrosis. This result has an important impact on the future treatment outcome of sarcoma, as presence of CAF can make tumours more aggressive and trigger fibrosis potentially making vessels impermeable to macromolecules and resistant to further treatment with drugs and chemotherapy. A better understanding of the interaction between MSCs and cells within tumour stroma will help to develop strategies to improve tumour therapies taking into account radiation effects on TME that influence tumour proliferation and metastasis. Further studies are needed to establish the best way to deliver radiation therapy to tumours with fewer negative consequences and taking into account patient survival and quality of life.

REFERENCES

ABBOTT, J. D., HUANG, Y., LIU, D., HICKEY, R., KRAUSE, D. S. & GIORDANO, F. J. 2004. Stromal cell-derived factor-1 alpha plays a critical role in stem cell recruitment to the heart after myocardial infarction but is not sufficient to induce homing in the absence of injury. *Circulation,* 110**,** 3300-3305.

ABRAHAM, J., SALAMA, N. N. & AZAB, A. K. 2015. The role of P-glycoprotein in drug resistance in multiple myeloma. *Leukemia & Lymphoma,* 56**,** 26-33.

ACOSTA, J. C., BANITO, A., WUESTEFELD, T., GEORGILIS, A., JANICH, P., MORTON, J. P., ATHINEOS, D., KANG, T. W., LASITSCHKA, F., ANDRULIS, M., PASCUAL, G., MORRIS, K. J., KHAN, S., JIN, H., DHARMALINGAM, G., SNIJDERS, A. P., CARROLL, T., CAPPER, D., PRITCHARD, C., INMAN, G. J., LONGERICH, T., SANSOM, O. J., BENITAH, S. A., ZENDER, L. & GIL, J. 2013. A complex secretory program orchestrated by the inflammasome controls paracrine senescence. *Nature Cell Biology,* 15**,** 978-U221.

AEBERSOLD, D. M., BURRI, P., BEER, K. T., LAISSUE, J., DJONOV, V., GREINER, R. H. & SEMENZA, G. L. 2001. Expression of hypoxia-inducible factor-1 alpha: A novel predictive and prognostic parameter in the radiotherapy of oropharyngeal cancer. *Cancer Research,* 61**,** 2911-2916.

AGULNIK, M., YARBER, J. L., OKUNO, S. H., VON MEHREN, M., JOVANOVIC, B. D., BROCKSTEIN, B. E., EVENS, A. M. & BENJAMIN, R. S. 2013. An open-label, multicenter, phase II study of bevacizumab for the treatment of angiosarcoma and epithelioid hemangioendotheliomas(dagger). *Annals of Oncology,* 24**,** 257-263.

AHN, G. O. & BROWN, J. M. 2009. Influence of bone marrow-derived hematopoietic cells on the tumor response to radiotherapy Experimental models and clinical perspectives. *Cell Cycle,* 8**,** 970-976.

AL-REFAIE, W. B., HABERMANN, E. B., JENSEN, E. H., TUTTLE, T. M., PISTERS, P. W. T. & VIRNIG, B. A. 2010. Surgery alone is adequate treatment for early stage soft tissue sarcoma of the extremity. *British Journal of Surgery,* 97**,** 707-713.

ALDRIDGE, V., GARG, A., DAVIES, N., BARTLETT, D. C., YOUSTER, J., BEARD, H., KAVANAGH, D. P., KALIA, N., FRAMPTON, J., LALOR, P. F. & NEWSOME, P. N. 2012. Human mesenchymal stem cells are recruited to injured liver in a beta 1-integrin and CD44 dependent manner. *Hepatology,* 56**,** 1063-1073.

ALLINEN, M., BEROUKHIM, R., CAI, L., BRENNAN, C., LAHTI-DOMENICI, J., HUANG, H. Y., PORTER, D., HU, M., CHIN, L., RICHARDSON, A., SCHNITT, S., SELLERS, W. R. & POLYAK, K. 2004. Molecular characterization of the tumor microenvironment in breast cancer. *Cancer Cell,* 6**,** 17-32.

ALPHONSO, A. & ALAHARI, S. K. 2009. Stromal Cells and Integrins: Conforming to the Needs of the Tumor Microenvironment. *Neoplasia,* 11**,** 1264-1271.

ALQATHAMI, M., BLENCOWE, A., YEO, U. J., DORAN, S. J., QIAO, G. & GESO, M. 2012. Novel Multicompartment 3-Dimensional Radiochromic Radiation Dosimeters for Nanoparticle-Enhanced Radiation Therapy Dosimetry. *International Journal of Radiation Oncology Biology Physics,* 84**,** E549-E555.

ALVAREZ, R. H., KANTARJIAN, H. M. & CORTES, J. E. 2006. Biology of platelet-derived growth factor and its involvement in disease. *Mayo Clinic Proceedings,* 81**,** 1241-1257.

ANDERBERG, C., LI, H., FREDRIKSSON, L., ANDRAE, J., BETSHOLTZ, C., LI, X., ERIKSSON, U. & PIETRAS, K. 2009. Paracrine Signaling by Platelet-Derived Growth Factor-CC Promotes Tumor Growth by Recruitment of Cancer-Associated Fibroblasts. *Cancer Research,* 69**,** 369-378.

ANDERBERG, C. & PIETRAS, K. 2009. On the origin of cancer-associated fibroblasts. *Cell Cycle,* 8**,** 1461-1462.

ANDERS, H. J., VIELHAUER, V., FRINK, M., LINDE, Y., COHEN, C. D., BLATTNER, S. M., KRETZLER, M., STRUTZ, F., MACK, M., GRONE, H. J., ONUFFER, J., HORUK, R., NELSON, P. J. & SCHLONDORFF, D. 2002. A chemokine receptor CCR-1 antagonist reduces renal fibrosis after unilateral ureter ligation. *Journal of Clinical Investigation,* 109**,** 251-259.

ANSCHER, M. S., CROCKER, I. R. & JIRTLE, R. L. 1990. TRANSFORMING GROWTH FACTOR-BETA-1 EXPRESSION IN IRRADIATED LIVER. *Radiation Research,* 122**,** 77-85.

ANTONI, D., BURCKEL, H., JOSSET, E. & NOEL, G. 2015. Three-Dimensional Cell Culture: A Breakthrough in Vivo. *International Journal of Molecular Sciences,* 16**,** 5517-5527.

ARMULIK, A., GENOVE, G. & BETSHOLTZ, C. 2011. Pericytes: Developmental, Physiological, and Pathological Perspectives, Problems, and Promises. *Developmental Cell,* 21**,** 193-215.

ARTAVANIS-TSAKONAS, S., RAND, M. D. & LAKE, R. J. 1999. Notch signaling: Cell fate control and signal integration in development. *Science,* 284**,** 770-776.

ATTISANO, L. & WRANA, J. L. 2002. Signal transduction by the TGF-beta superfamily. *Science,* 296**,** 1646-1647.

AUGSTEN, M., SJOBERG, E., FRINGS, O., VORRINK, S. U., FRIJHOFF, J., OLSSON, E., BORG, A. & OSTMAN, A. 2014. Cancer-Associated Fibroblasts Expressing CXCL14 Rely upon NOS1-Derived Nitric Oxide Signaling for Their Tumor-Supporting Properties. *Cancer Research,* 74**,** 2999-3010.

AZAB, A. K., HU, J. S., QUANG, P., AZAB, F., PITSILLIDES, C., AWWAD, R., THOMPSON, B., MAISO, P., SUN, J. D., HART, C. P., ROCCARO, A. M., SACCO, A., NGO, H. T., LIN, C. P., KUNG, A. L., CARRASCO, R. D., VANDERKERKEN, K. & GHOBRIAL, I. M. 2012. Hypoxia promotes dissemination of multiple myeloma through acquisition of epithelial to mesenchymal transition-like features. *Blood,* 119**,** 5782-5794.

AZORIN-VEGA, E. P., ZAMBRANO-RAMIREZ, O. D., ROJAS-CALDERON, E. L., OCAMPO-GARCIA, B. E. & FERRO-FLORES, G. 2015. Tumoral fibrosis effect on the radiation absorbed dose of (177)Lu-Tyr(3)-octreotate and (177)Lu-Tyr(3)-octreotate conjugated to gold nanoparticles. *Applied radiation and isotopes : including data, instrumentation and methods for use in agriculture, industry and medicine,* 100**,** 96-100.

BABIC, A. M., CHEN, C. C. & LAU, L. F. 1999. Fisp12/mouse connective tissue growth factor mediates endothelial cell adhesion and migration through integrin alpha(v)beta(3), promotes endothelial cell survival, and induces angiogenesis in vivo. *Molecular and Cellular Biology,* 19**,** 2958-2966.

BAEK, S. J., KANG, S. K. & RA, J. C. 2011. In vitro migration capacity of human adipose tissue-derived mesenchymal stem cells reflects their expression of receptors for chemokines and growth factors. *Experimental and Molecular Medicine,* 43**,** 596-603.

BAGGIOLINI, M. 1998. Chemokines and leukocyte traffic. *Nature,* 392**,** 565-568.

BAGULEY, B. C. 2003. Antivascular therapy of cancer: DMXAA. *Lancet Oncology,* 4**,** 141-148.

BALKWILL, F. R., CAPASSO, M. & HAGEMANN, T. 2012. The tumor microenvironment at a glance. *Journal of Cell Science,* 125**,** 5591-5596.

BALL, S. G., SHUTTLEWORTH, A. C. & KIELTY, C. M. 2004. Direct cell contact influences bone marrow mesenchymal stem cell fate. *International Journal of Biochemistry & Cell Biology,* 36**,** 714-727.

BARCELLOS-DE-SOUZA, P., COMITO, G., PONS-SEGURA, C., TADDEI, M. L., GORI, V., BECHERUCCI, V., BAMBI, F., MARGHERI, F., LAURENZANA, A., DEL ROSSO, M. & CHIARUGI, P. 2016. Mesenchymal Stem Cells are Recruited and Activated into

Carcinoma-Associated Fibroblasts by Prostate Cancer Microenvironment-Derived TGF-1. *Stem Cells,* 34**,** 2536-2547.
BARKER, H. E., PAGET, J. T. E., KHAN, A. A. & HARRINGTON, K. J. 2015. The tumour microenvironment after radiotherapy: mechanisms of resistance and recurrence. *Nature Reviews Cancer,* 15**,** 409-425.
BARRIENTOS, S., STOJADINOVIC, O., GOLINKO, M. S., BREM, H. & TOMIC-CANIC, M. 2008. Growth factors and cytokines in wound healing. *Wound Repair and Regeneration,* 16**,** 585-601.
BEACH, T. A., JOHNSTON, C. J., GROVES, A. M., WILLIAMS, J. P. & FINKELSTEIN, J. N. 2017. Radiation induced pulmonary fibrosis as a model of progressive fibrosis: Contributions of DNA damage, inflammatory response and cellular senescence genes. *Experimental Lung Research,* 43**,** 134-149.
BELEMA-BEDADA, F., UCHIDA, S., MARTIRE, A., KOSTIN, S. & BRAUN, T. 2008. Efficient homing of multipotent adult mesenchymal stem cells depends on FROUNT-mediated clustering of CCR2. *Cell Stem Cell,* 2**,** 566-575.
BELPERIO, J. A., KEANE, M. P., BURDICK, M. D., LYNCH, J. P., XUE, Y. Y., BERLIN, A., ROSS, D. J., KUNKEL, S. L., CHARO, I. F. & STRIETER, R. M. 2001. Critical role for the chemokine MCP-1/CCR2 in the pathogenesis of bronchiolitis obliterans syndrome. *Journal of Clinical Investigation,* 108**,** 547-556.
BENTOVIM, L., AMARILIO, R. & ZELZER, E. 2013. HIF1 alpha is a central regulator of collagen hydroxylation and secretion under hypoxia during bone development (vol 139, pg 4473, 2012). *Development,* 140**,** 248-248.
BENTZEN, S. M. 2006. Preventing or reducing late side effects of radiation therapy: radiobiology meets molecular pathology. *Nature Reviews Cancer,* 6**,** 702-713.
BERGERS, G. & BENJAMIN, L. E. 2003. Tumorigenesis and the angiogenic switch. *Nature Reviews Cancer,* 3**,** 401-410.
BERGERS, G., BREKKEN, R., MCMAHON, G., VU, T. H., ITOH, T., TAMAKI, K., TANZAWA, K., THORPE, P., ITOHARA, S., WERB, Z. & HANAHAN, D. 2000. Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. *Nature Cell Biology,* 2**,** 737-744.
BERGERS, G. & SONG, S. 2005. The role of pericytes in blood-vessel formation and maintenance. *Neuro-Oncology,* 7**,** 452-464.
BERNATCHEZ, P. N., SOKER, S. & SIROIS, M. G. 1999. Vascular endothelial growth factor effect on endothelial cell proliferation, migration, and platelet-activating factor synthesis is Flk-1-dependent. *Journal of Biological Chemistry,* 274**,** 31047-31054.
BICKELHAUPT, S., ERBEL, C., TIMKE, C., WIRKNER, U., DADRICH, M., FLECHSIG, P., TIETZ, A., PFOHLER, J., GROSS, W., PESCHKE, P., HOELTGEN, L., KATUS, H. A., GRONE, H. J., NICOLAY, N. H., SAFFRICH, R., DEBUS, J., STERNLICHT, M. D., SEELEY, T. W., LIPSON, K. E. & HUBER, P. E. 2017. Effects of CTGF Blockade on Attenuation and Reversal of Radiation-Induced Pulmonary Fibrosis. *Jnci-Journal of the National Cancer Institute,* 109.
BIELEFELD, K. A., AMINI-NIK, S. & ALMAN, B. A. 2013. Cutaneous wound healing: recruiting developmental pathways for regeneration. *Cellular and Molecular Life Sciences,* 70**,** 2059-2081.
BISSELL, M. J. & HINES, W. C. 2011. Why don't we get more cancer? A proposed role of the microenvironment in restraining cancer progression. *Nature Medicine,* 17**,** 320-329.
BLAY, J.-Y., PAPAI, Z., TOLCHER, A. W., ITALIANO, A., CUPISSOL, D., LOPEZ-POUSA, A., CHAWLA, S. P., BOMPAS, E., BABOVIC, N., PENEL, N., ISAMBERT, N., STADDON, A. P., SAADA-BOUZID, E., SANTORO, A., FRANKE, F. A., COHEN, P., LE-GUENNEC, S. & DEMETRI, G. D. 2015. Ombrabulin plus cisplatin versus placebo plus cisplatin in patients with advanced soft-tissue sarcomas after failure of anthracycline and

ifosfamide chemotherapy: a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet Oncology,* 16**,** 531-540.
BLOUIN, C. C., PAGE, E. L., SOUCY, G. M. & RICHARD, D. E. 2004. Hypoxic gene activation by lipopolysaccharide in macrophages: Implication of hypoxia-inducible factor 1 alpha. *Blood,* 103**,** 1124-1130.
BOIRE, A., COVIC, L., AGARWAL, A., JACQUES, S., SHERIFL, S. & KULIOPULOS, A. 2005. PAR1 is a matrix metalloprotease-1 receptor that promotes invasion and tumorigenesis of breast cancer cells. *Cell,* 120**,** 303-313.
BONNIAUD, P., MARTIN, G., MARGETTS, P. J., ASK, K., ROBERTSON, J., GAULDIE, J. & KOLB, M. 2004. Connective tissue growth factor is crucial to inducing a profibrotic environment in "fibrosis-resistant" Balb/c mouse lungs. *American Journal of Respiratory Cell and Molecular Biology,* 31**,** 510-516.
BONUCCELLI, G., AVNET, S., GRISENDI, G., SALERNO, M., GRANCHI, D., DOMINICI, M., KUSUZAKI, K. & BALDINI, N. 2014. Role of mesenchymal stem cells in osteosarcoma and metabolic reprogramming of tumor cells. *Oncotarget,* 5**,** 7575-7588.
BOOMSMA, R. A. & GEENEN, D. L. 2012. Mesenchymal Stem Cells Secrete Multiple Cytokines That Promote Angiogenesis and Have Contrasting Effects on Chemotaxis and Apoptosis. *Plos One,* 7.
BORSIG, L., WOLF, M. J., ROBLEK, M., LORENTZEN, A. & HEIKENWALDER, M. 2014. Inflammatory chemokines and metastasis-tracing the accessory. *Oncogene,* 33**,** 3217-3224.
BOYDEN, S. 1962. CHEMOTACTIC EFFECT OF MIXTURES OF ANTIBODY AND ANTIGEN ON POLYMORPHONUCLEAR LEUCOCYTES. *Journal of Experimental Medicine,* 115**,** 453-&.
BRADHAM, D. M., IGARASHI, A., POTTER, R. L. & GROTENDORST, G. R. 1991. CONNECTIVE-TISSUE GROWTH-FACTOR - A CYSTEINE-RICH MITOGEN SECRETED BY HUMAN VASCULAR ENDOTHELIAL-CELLS IS RELATED TO THE SRC-INDUCED IMMEDIATE EARLY GENE-PRODUCT CEF-10. *Journal of Cell Biology,* 114**,** 1285-1294.
BRAMWELL, V., ROUESSE, J., STEWARD, W., SANTORO, A., SCHRAFFORDTKOOPS, H., BUESA, J., RUKA, W., PRIARIO, J., WAGENER, T., BURGERS, M., VANUNNIK, J., CONTESSO, G., THOMAS, D., VANGLABBEKE, M., MARKHAM, D. & PINEDO, H. 1994. ADJUVANT CYVADIC CHEMOTHERAPY FOR ADULT SOFT-TISSUE SARCOMA-REDUCED LOCAL RECURRENCE BUT NO IMPROVEMENT IN SURVIVAL - A STUDY OF THE EUROPEAN ORGANIZATION FOR RESEARCH AND TREATMENT OF CANCER SOFT-TISSUE AND BONE SARCOMA GROUP. *Journal of Clinical Oncology,* 12**,** 1137-1149.
BRANCO-PRICE, C., ZHANG, N., SCHNELLE, M., EVANS, C., KATSCHINSKI, D. M., LIAO, D., ELLIES, L. & JOHNSON, R. S. 2012. Endothelial Cell HIF-1 alpha and HIF-2 alpha Differentially Regulate Metastatic Success. *Cancer Cell,* 21**,** 52-65.
BRESLIN, S. & O'DRISCOLL, L. 2013. Three-dimensional cell culture: the missing link in drug discovery. *Drug Discovery Today,* 18**,** 240-249.
BRITTAN, M., HUNT, T., JEFFERY, R., POULSOM, R., FORBES, S. J., HODIVALA-DILKE, K., GOLDMAN, J., ALISON, M. R. & WRIGHT, N. A. 2002. Bone marrow derivation of pericryptal myofibroblasts in the mouse and human small intestine and colon. *Gut,* 50**,** 752-757.
BROOKE, G., TONG, H., LEVESQUE, J. P. & ATKINSON, K. 2008. Molecular Trafficking Mechanisms of Multipotent Mesenchymal Stem Cells Derived from Human Bone Marrow and Placenta. *Stem Cells and Development,* 17**,** 929-940.
BROWN, J. M. & WILLIAM, W. R. 2004. Exploiting tumour hypoxia in cancer treatment. *Nature Reviews Cancer,* 4**,** 437-447.

BUCALA, R., SPIEGEL, L. A., CHESNEY, J., HOGAN, M. & CERAMI, A. 1994. CIRCULATING FIBROCYTES DEFINE A NEW LEUKOCYTE SUBPOPULATION THAT MEDIATES TISSUE-REPAIR. *Molecular Medicine,* 1**,** 71-81.

BURGER, J. A., STEWART, D. J., WALD, O. & PELED, A. 2011. Potential of CXCR4 antagonists for the treatment of metastatic lung cancer. *Expert Review of Anticancer Therapy,* 11**,** 621-630.

BURLACU, A., GRIGORESCU, G., ROSCA, A. M., PREDA, M. B. & SIMIONESCU, M. 2013. Factors Secreted by Mesenchymal Stem Cells and Endothelial Progenitor Cells Have Complementary Effects on Angiogenesis In Vitro. *Stem Cells and Development,* 22**,** 643-653.

CAICEDO, A., FRITZ, V., BRONDELLO, J. M., AYALA, M., DENNEMONT, I., ABDELLAOUI, N., DE FRAIPONT, F., MOISAN, A., PROUTEAU, C. A., BOUKHADDAOUI, H., JORGENSEN, C. & VIGNAIS, M. L. 2015. MitoCeption as a new tool to assess the effects of mesenchymal stem/stromal cell mitochondria on cancer cell metabolism and function. *Scientific Reports,* 5.

CAIRNS, R. A., KALLIOMAKI, T. & HILL, R. P. 2001. Acute (cyclic) hypoxia enhances spontaneous metastasis of KHT murine tumors. *Cancer Research,* 61**,** 8903-8908.

CANIGGIA, I., MOSTACHFI, H., WINTER, J., GASSMANN, M., LYE, S. J., KULISZEWSKI, M. & POST, M. 2000. Hypoxia-inducible factor-1 mediates the biological effects of oxygen on human trophoblast differentiation through TGF beta(3). *Journal of Clinical Investigation,* 105**,** 577-587.

CANNON, C. P., BALLO, M. T., ZAGARS, G. K., MIRZA, A. N., LIN, P. P., LEWIS, V. O., YASKO, A. W., BENJAMIN, R. S. & PISTERS, P. W. T. 2006. Complications of combined modality treatment of primary lower extremity soft-tissue sarcomas. *Cancer,* 107**,** 2455-2461.

CAPLAN, A. I. 1991. MESENCHYMAL STEM-CELLS. *Journal of Orthopaedic Research,* 9**,** 641-650.

CARMELIET, P. 2000. Mechanisms of angiogenesis and arteriogenesis. *Nature Medicine,* 6**,** 389-395.

CARMELIET, P. 2005. Angiogenesis in life, disease and medicine. *Nature,* 438**,** 932-936.

CARMELIET, P. & JAIN, R. K. 2011. Molecular mechanisms and clinical applications of angiogenesis. *Nature,* 473**,** 298-307.

CARULLI, M. T., ONG, V. H., PONTICOS, M., XU, S. W., ABRAHAM, D. J., BLACK, C. M. & DENTON, C. P. 2005. Chemokine receptor CCR2 expression by systemic sclerosis fibroblasts - Evidence for autocrine regulation of myofibroblast differentiation. *Arthritis and Rheumatism,* 52**,** 3772-3782.

CENTENO, E. G. Z., CIMAROSTI, H. & BITHELL, A. 2018. 2D versus 3D human induced pluripotent stem cell-derived cultures for neurodegenerative disease modelling. *Molecular Neurodegeneration,* 13.

CHAMBERS, A. F., GROOM, A. C. & MACDONALD, I. C. 2002. Dissemination and growth of cancer cells in metastatic sites. *Nature Reviews Cancer,* 2**,** 563-572.

CHANG, C. L., MARRA, G., CHAUHAN, D. P., HA, H. T., CHANG, D. K., RICCIARDIELLO, L., RANDOLPH, A., CARETHERS, J. M. & BOLAND, C. R. 2002. Oxidative stress inactivates the human DNA mismatch repair system. *American Journal of Physiology-Cell Physiology,* 283**,** C148-C154.

CHAUHAN, H., ABRAHAM, A., PHILLIPS, J. R. A., PRINGLE, J. H., WALKER, R. A. & JONES, J. L. 2003. There is more than one kind of myofibroblast: analysis of CD34 expression in benign, in situ, and invasive breast lesions. *Journal of Clinical Pathology,* 56**,** 271-276.

CHEN, C. A., CHUNG, W. C., CHIOU, Y. Y., YANG, Y. J., LIN, Y. C., OCHS, H. D. & SHIEH, C. C. 2016. Quantitative analysis of tissue inflammation and responses to treatment in immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome,

and review of literature. *Journal of Microbiology Immunology and Infection,* 49**,** 775-782.
CHEN, M. F., LIN, C. T., CHEN, W. C., YANG, C. T., CHEN, C. C., LIAO, S. K., LIU, J. M., LU, C. H. & LEE, K. D. 2006. The sensitivity of human mesenchymal stem cells to ionizing radiation. *International Journal of Radiation Oncology Biology Physics,* 66**,** 244-253.
CHEN, Q. H., LIU, A. R., QIU, H. B. & YANG, Y. 2015. Interaction between mesenchymal stem cells and endothelial cells restores endothelial permeability via paracrine hepatocyte growth factor in vitro. *Stem Cell Research & Therapy,* 6.
CHEN, Y. Y., WILLIAMS, J., DING, I., HERNADY, E., LIU, W. M., SMUDZIN, T., FINKELSTEIN, J. N., RUBIN, P. & OKUNIEFF, P. 2002. Radiation pneumonitis and early circulatory cytokine markers. *Seminars in Radiation Oncology,* 12**,** 26-33.
CHERESH, P., KIM, S. J., TULASIRAM, S. & KAMP, D. W. 2013. Oxidative stress and pulmonary fibrosis. *Biochimica Et Biophysica Acta-Molecular Basis of Disease,* 1832**,** 1028-1040.
CHOI, J. Y., JANG, Y. S., MIN, S. Y. & SONG, J. Y. 2011. Overexpression of MMP-9 and HIF-1 alpha in Breast Cancer Cells under Hypoxic Conditions. *Journal of Breast Cancer,* 14**,** 88-95.
CHOWDHURY, R., WEBBER, J. P., GURNEY, M., MASON, M. D., TABI, Z. & CLAYTON, A. 2015. Cancer exosomes trigger mesenchymal stem cell differentiation into pro-angiogenic and pro-invasive myofibroblasts. *Oncotarget,* 6**,** 715-731.
CIRRI, P. & CHIARUGI, P. 2012. Cancer-associated-fibroblasts and tumour cells: a diabolic liaison driving cancer progression. *Cancer and Metastasis Reviews,* 31**,** 195-208.
CLARK, M. A., FISHER, C., JUDSON, I. & THOMAS, J. M. 2005. Medical progress: Soft-tissue sarcomas in adults. *New England Journal of Medicine,* 353**,** 701-711.
CLARKE, M. R., IMHOFF, F. M. & BAIRD, S. K. 2015. Mesenchymal Stem Cells Inhibit Breast Cancer Cell Migration and Invasion Through Secretion of Tissue Inhibitor of Metalloproteinase-1 and-2. *Molecular Carcinogenesis,* 54**,** 1214-1219.
CLARKLEWIS, I., KIM, K. S., RAJARATHNAM, K., GONG, J. H., DEWALD, B., MOSER, B., BAGGIOLINI, M. & SYKES, B. D. 1995. STRUCTURE-ACTIVITY-RELATIONSHIPS OF CHEMOKINES. *Journal of Leukocyte Biology,* 57**,** 703-711.
CLAVIN, N. W., FERNANDEZ, J., SCHONMEYR, B. H., SOARES, M. A. & MEHRARA, B. J. 2008. Fractionated doses of ionizing radiation confer protection to mesenchymal stem cell pluripotency. *Plastic and Reconstructive Surgery,* 122**,** 739-748.
COHN, M. J., IZPISUABELMONTE, J. C., ABUD, H., HEATH, J. K. & TICKLE, C. 1995. FIBROBLAST GROWTH-FACTORS INDUCE ADDITIONAL LIMB DEVELOPMENT FROM THE FLANK OF CHICK-EMBRYOS. *Cell,* 80**,** 739-746.
CONNOLLY, K. A., BELT, B. A., FIGUEROA, N. M., MURTHY, A., PATEL, A., KIM, M., LORD, E. M., LINEHAN, D. C. & GERBER, S. A. 2016. Increasing the efficacy of radiotherapy by modulating the CCR2/CCR5 chemokine axes. *Oncotarget,* 7**,** 86522-86535.
CONWAY, E. M., COLLEN, D. & CARMELIET, P. 2001. Molecular mechanisms of blood vessel growth. *Cardiovascular Research,* 49**,** 507-521.
COPPES, R. P., VAN DER GOOT, A. & LOMBAERT, I. M. A. 2009. Stem Cell Therapy to Reduce Radiation-Induced Normal Tissue Damage. *Seminars in Radiation Oncology,* 19**,** 112-121.
CORREIA, A. L. & BISSELL, M. J. 2012. The tumor microenvironment is a dominant force in multidrug resistance. *Drug Resistance Updates,* 15**,** 39-49.
CRAIG, W., KAY, R., CUTLER, R. L. & LANSDORP, P. M. 1993. EXPRESSION OF THY-1 ON HUMAN HEMATOPOIETIC PROGENITOR CELLS. *Journal of Experimental Medicine,* 177**,** 1331-1342.
CROITORU-LAMOURY, J., LAMOURY, F. M. J., ZAUNDERS, J. J., VEAS, L. A. & BREW, B. J. 2007. Human mesenchymal stem cells constitutively express chemokines and

chemokine receptors that can be upregulated by cytokines, IFN-beta, and Copaxone. *Journal of Interferon and Cytokine Research,* 27**,** 53-64.
CROSS, K. J. & MUSTOE, T. A. 2003. Growth factors in wound healing. *Surgical Clinics of North America,* 83**,** 531-+.
D'ADAMO, D. R., ANDERSON, S. E., ALBRITTON, K., YAMADA, J., RIEDEL, E., SCHEU, K., SCHWARTZ, G. K., CHEN, H. & MAKI, R. G. 2005. Phase II study of doxorubicin and bevacizumab for patients with metastatic soft-tissue sarcomas. *Journal of Clinical Oncology,* 23**,** 7135-7142.
DALAL, S. & BURCHILL, S. A. 2009. Preclinical evaluation of vascular-disrupting agents in Ewing's sarcoma family of tumours. *European Journal of Cancer,* 45**,** 713-722.
DAS, B., TSUCHIDA, R., MALKIN, D., KOREN, G., BARUCHEL, S. & YEGER, H. 2008. Hypoxia enhances tumor stemness by increasing the invasive and tumorigenic side population fraction. *Stem Cells,* 26**,** 1818-1830.
DATTO, M. B., LI, Y., PANUS, J. F., HOWE, D. J., XIONG, Y. & WANG, X. F. 1995. TRANSFORMING GROWTH-FACTOR-BETA INDUCES THE CYCLIN-DEPENDENT KINASE INHIBITOR P21 THROUGH A P53-INDEPENDENT MECHANISM. *Proceedings of the National Academy of Sciences of the United States of America,* 92**,** 5545-5549.
DAUNORAVICIUS, D., BESUSPARIS, J., ZURAUSKAS, E., LAURINAVICIENE, A., BIRONAITE, D., PANKUWEIT, S., PLANCOULAINE, B., HERLIN, P., BOGOMOLOVAS, J., GRABAUSKIENE, V. & LAURINAVICIUS, A. 2014. Quantification of myocardial fibrosis by digital image analysis and interactive stereology. *Diagnostic Pathology,* 9.
DAVALOS, A. R., COPPE, J. P., CAMPISI, J. & DESPREZ, P. Y. 2010. Senescent cells as a source of inflammatory factors for tumor progression. *Cancer and Metastasis Reviews,* 29**,** 273-283.
DAVIS, A. M., O'SULLIVAN, B., TURCOTTE, R., BELL, R., CATTON, C., CHABOT, P., WUNDER, J., HAMMOND, A., BENK, V., KANDEL, R., GODDARD, K., ZEE, B., DAY, A., TU, D. S., PATER, J., CANADIAN SARCOMA, G. & GRP, N. C. I. C. C. T. 2005. Late radiation morbidity following randomization to preoperative versus postoperative radiotherapy in extremity soft tissue sarcoma. *Radiotherapy and Oncology,* 75**,** 48-53.
DE BECKER, A. & VAN RIET, I. 2016. Homing and migration of mesenchymal stromal cells: How to improve the efficacy of cell therapy? *World Journal of Stem Cells,* 8**,** 73-87.
DE JONG, S., VAN VEEN, T. A. B., DE BAKKER, J. M. T. & VAN RIJEN, H. V. M. 2012. Monitoring cardiac fibrosis: a technical challenge. *Netherlands Heart Journal,* 20**,** 44-48.
DE LA PUENTE, P., MUZ, B., AZAB, F. & AZAB, A. K. 2013. Cell Trafficking of Endothelial Progenitor Cells in Tumor Progression. *Clinical Cancer Research,* 19**,** 3360-3368.
DE WEVER, O., DEMETTER, P., MAREEL, M. & BRACKE, M. 2008. Stromal myofibroblasts are drivers of invasive cancer growth. *International Journal of Cancer,* 123**,** 2229-2238.
DELANEY, G., JACOB, S., FEATHERSTONE, C. & BARTON, M. 2006. The role of radiotherapy in cancer treatment: estimating optimal utilization from a review of evidence-based clinical guidelines (vol 104, pg 1129, 2005). *Cancer,* 107**,** 660-660.
DELANEY, T. F., TROFIMOV, A. V., ENGELSMAN, M. & SUIT, H. D. 2005. Advanced-technology radiation therapy in the management of bone and soft tissue sarcomas. *Cancer control : journal of the Moffitt Cancer Center,* 12**,** 27-35.
DELANIAN, S. & LEFAIX, J. L. 2004. The radiation-induced fibroatrophic process: therapeutic perspective via the antioxidant pathway. *Radiotherapy and Oncology,* 73**,** 119-131.

DEMETRI, G. D., VAN OOSTEROM, A. T., GARRETT, C. R., BLACKSTEIN, M. E., SHAH, M. H., VERWEIJ, J., MCARTHUR, G., JUDSON, I. R., HEINRICH, M. C., MORGAN, J. A., DESAI, J., D FLETCHER, C., GEORGE, S., BELLO, C. L., HUANG, X., BAUM, C. M. & CASALI, P. G. 2006. Efficacy and safety of sunitinib in patients with advanced gastrointestinal stromal tumour after failure of imatinib: a randomised controlled trial. *Lancet,* 368**,** 1329-1338.

DEMETRI, G. D., VON MEHREN, M., BLANKE, C. D., VAN DEN ABBEELE, A. D., EISENBERG, B., ROBERTS, P. J., HEINRICH, M. C., TUVESON, D. A., SINGER, S., JANICEK, M., FLETCHER, J. A., SILVERMAN, S. G., SILBERMAN, S. L., CAPDEVILLE, R., KIESE, B., PENG, B., DIMITRIJEVIC, S., DRUKER, B. J., CORLESS, C., FLETCHER, C. D. M. & JOENSUU, H. 2002. Efficacy and safety of imatinib mesylate in advanced gastrointestinal stromal tumors. *New England Journal of Medicine,* 347**,** 472-480.

DENEKAMP, J. 1982. ENDOTHELIAL CELL-PROLIFERATION AS A NOVEL-APPROACH TO TARGETING TUMOR-THERAPY. *British Journal of Cancer,* 45**,** 136-139.

DENEKAMP, J. 1993. ANGIOGENESIS, NEOVASCULAR PROLIFERATION AND VASCULAR PATHOPHYSIOLOGY AS TARGETS FOR CANCER-THERAPY. *British Journal of Radiology,* 66**,** 181-196.

DENEKAMP, J. 1999. The tumour microcirculation as a target in cancer therapy: a clearer perspective. *European Journal of Clinical Investigation,* 29**,** 733-736.

DENEKAMP, J. & HILL, S. 1991. ANGIOGENIC ATTACK AS A THERAPEUTIC STRATEGY FOR CANCER. *Radiotherapy and Oncology,* 20**,** 103-112.

DENG, M. Y., MEI, T. N., HOU, T. Y., LUO, K. Y., LUO, F., YANG, A. J., YU, B., PANG, H., DONG, S. W. & XU, J. Z. 2017. TGF beta 3 recruits endogenous mesenchymal stem cells to initiate bone regeneration. *Stem Cell Research & Therapy,* 8.

DESAI, V. D., HSIA, H. C. & SCHWARZBAUER, J. E. 2014. Reversible Modulation of Myofibroblast Differentiation in Adipose-Derived Mesenchymal Stem Cells. *Plos One,* 9.

DESMOULIERE, A., GEINOZ, A., GABBIANI, F. & GABBIANI, G. 1993. TRANSFORMING GROWTH-FACTOR-BETA-1 INDUCES ALPHA-SMOOTH MUSCLE ACTIN EXPRESSION IN GRANULATION-TISSUE MYOFIBROBLASTS AND IN QUIESCENT AND GROWING CULTURED FIBROBLASTS. *Journal of Cell Biology,* 122**,** 103-111.

DEVALIA, H. L. & MANSFIELD, L. 2008. Radiotherapy and wound healing. *International Wound Journal,* 5**,** 40-44.

DICKEY, J. S., BAIRD, B. J., REDON, C. E., SOKOLOV, M. V., SEDELNIKOVA, O. A. & BONNER, W. M. 2009. Intercellular communication of cellular stress monitored by gamma-H2AX induction. *Carcinogenesis,* 30**,** 1686-1695.

DINGS, R. P. M., WILLIAMS, B. W., SONG, C. W., GRIFFIOEN, A. W., MAYO, K. H. & GRIFFIN, R. J. 2005. Anginex synergizes with radiation therapy to inhibit tumor growth by radiosensitizing endothelial cells. *International Journal of Cancer,* 115**,** 312-319.

DIOMEDI-CAMASSEI, F., MCDOWELL, H. P., DE LORIS, M. A., UCCINI, S., ALTAVISTA, P., RASCHELLA, G., VITALI, R., MANNARINO, O., DE SIO, L., COZZI, D. A., DONFRANCESCO, A., INSERRA, A., CALLEA, F. & DOMINICI, C. 2008. Clinical significance of CXC chemokine receptor-4 and c-Met in childhood rhabdomyosarcoma. *Clinical Cancer Research,* 14**,** 4119-4127.

DIREKZE, N. C., HODIVALA-DILKE, K., JEFFERY, R., HUNT, T., POULSOM, R., OUKRIF, D., ALISON, M. R. & WRIGHT, N. A. 2004. Bone marrow contribution to tumor-associated myofibroblasts and fibroblasts. *Cancer Research,* 64**,** 8492-8495.

DIREKZE, N. C., JEFFERY, R., HODIVALA-DILKE, K., HUNT, T., PLAYFORD, R. J., ELIA, G., POULSOM, R., WRIGHT, N. A. & ALISON, M. R. 2006. Bone marrow-derived stromal cells express lineage-related messenger RNA species. *Cancer Research,* 66**,** 1265-1269.

DISTLER, J. H. W., AKHMETSHINA, A., SCHETT, G. & DISTLER, O. 2009. Monocyte chemoattractant proteins in the pathogenesis of systemic sclerosis. *Rheumatology,* 48**,** 98-103.

DISTLER, J. H. W., JUNGEL, A., CARETTO, D., SCHULZE-HORSEL, U., KOWAL-BIELECKA, O., GAY, R. E., MICHEL, B. A., MULLER-LADNER, U., KALDEN, J. R., GAY, S. & DISTLER, O. 2006. Monocyte chemoattractant protein 1 released from glycosaminoglycans mediates its profibrotic effects in systemic sclerosis via the release of interleukin-4 from T cells. *Arthritis and Rheumatism,* 54**,** 214-225.

DOMANSKA, U. M., TIMMER-BOSSCHA, H., NAGENGAST, W. B., MUNNINK, T. H. O., KRUIZINGA, R. C., ANANIAS, H. J. K., KLIPHUIS, N. M., HULS, G., DE VRIES, E. G. E., DE JONG, I. J. & WALENKAMP, A. M. E. 2012. CXCR4 Inhibition with AMD3100 Sensitizes Prostate Cancer to Docetaxel Chemotherapy. *Neoplasia,* 14**,** 709-718.

DUMONT, N., LIU, B., DEFILIPPIS, R. A., CHANG, H., RABBAN, J. T., KARNEZIS, A. N., TJOE, J. A., MARX, J., PARVIN, B. & TLSTY, T. D. 2013. Breast Fibroblasts Modulate Early Dissemination, Tumorigenesis, and Metastasis through Alteration of Extracellular Matrix Characteristics. *Neoplasia,* 15**,** 249-+.

DUNCAN, M. R., FRAZIER, K. S., ABRAMSON, S., WILLIAMS, S., KLAPPER, H., HUANG, X. F. & GROTENDORST, G. R. 1999. Connective tissue growth factor mediates transforming growth factor beta-induced collagen synthesis: downregulation by cAMP. *Faseb Journal,* 13**,** 1774-1786.

DUTTA, P., SARKISSYAN, M., PAICO, K., WU, Y. Y. & VADGAMA, J. V. 2018. MCP-1 is overexpressed in triple-negative breast cancers and drives cancer invasiveness and metastasis. *Breast Cancer Research and Treatment,* 170**,** 477-486.

DVORAK, H. F. 2000. VPF/VEGF and the angiogenic response. *Seminars in Perinatology,* 24**,** 75-78.

DWYER, R. M., POTTER-BEIRNE, S. M., HARRINGTON, K. A., LOWERY, A. J., HENNESSY, E., MURPHY, J. M., BARRY, F. P., O'BRIEN, T. & KERIN, M. J. 2007. Monocyte chemotactic protein-1 secreted by primary breast tumors stimulates migration of mesenchymal stem cells. *Clinical Cancer Research,* 13**,** 5020-5027.

DZIADZIO, M., SMITH, R. E., ABRAHAM, D. J., BLACK, C. M. & DENTON, C. P. 2005. Circulating levels of active transforming growth factor beta 1 are reduced in diffuse cutaneous systemic sclerosis and correlate inversely with the modified Rodnan skin score. *Rheumatology,* 44**,** 1518-1524.

EBIHARA, Y., MASUYA, M., LARUE, A. C., FLEMING, P. A., VISCONTI, R. P., MINAMIGUCHI, H., DRAKE, C. J. & OGAWA, M. 2006. Hematopoietic origins of fibroblasts: II. In vitro studies of fibroblasts, CFU-F, and fibrocytes. *Experimental Hematology,* 34**,** 219-229.

EBRAHEM, Q., CHAURASIA, S. S., VASANJI, A., QI, J. H., KLENOTIC, P. A., CUTLER, A., ASOSINGH, K., ERZURUM, S. & ANAND-APTE, B. 2010. Cross-Talk between Vascular Endothelial Growth Factor and Matrix Metalloproteinases in the Induction of Neovascularization in Vivo. *American Journal of Pathology,* 176**,** 496-503.

EDDINGTON, K. M., DOLCOS, F., CABEZA, R., KRISHNAN, K. R. R. & STRAUMAN, T. J. 2007. Neural correlates of promotion and prevention goal activation: An fMRI study using an idiographic approach. *Journal of Cognitive Neuroscience,* 19**,** 1152-1162.

EDMONDSON, R., BROGLIE, J. J., ADCOCK, A. F. & YANG, L. J. 2014. Three-Dimensional Cell Culture Systems and Their Applications in Drug Discovery and Cell-Based Biosensors. *Assay and Drug Development Technologies,* 12**,** 207-218.

EHRHART, E. J., SEGARINI, P., TSANG, M. L. S., CARROLL, A. G. & BARCELLOSHOFF, M. H. 1997. Latent transforming growth factor beta 1 activation in situ: quantitative and functional evidence after low-dose gamma-irradiation. *Faseb Journal,* 11**,** 991-1002.

EISINGER-MATHASON, T. S. K., ZHANG, M. S., QIU, Q., SKULI, N., NAKAZAWA, M. S., KARAKASHEVA, T., MUCAJ, V., SHAY, J. E. S., STANGENBERG, L., SADRI, N., PURE, E., YOON, S. S., KIRSCH, D. G. & SIMON, M. C. 2013. Hypoxia-Dependent Modification of Collagen Networks Promotes Sarcoma Metastasis. *Cancer Discovery,* 3**,** 1190-1205.

EL-BARED, N., WONG, P. & WANG, D. 2015. Soft tissue sarcoma and radiation therapy advances, impact on toxicity. *Current treatment options in oncology,* 16**,** 335-335.

ELIAS, A., RYAN, L., SULKES, A., COLLINS, J., AISNER, J. & ANTMAN, K. H. 1989. RESPONSE TO MESNA, DOXORUBICIN, IFOSFAMIDE, AND DACARBAZINE IN 108 PATIENTS WITH METASTATIC OR UNRESECTABLE SARCOMA AND NO PRIOR CHEMOTHERAPY. *Journal of Clinical Oncology,* 7**,** 1208-1216.

ENGLISH, W. R., LUNT, S. J., FISHER, M., LEFLEY, D. V., DHINGRA, M., LEE, Y. C., BINGHAM, K., HURRELL, J. E., LYONS, S. K., KANTHOU, C. & TOZER, G. M. 2017. Differential Expression of VEGFA Isoforms Regulates Metastasis and Response to Anti-VEGFA Therapy in Sarcoma. *Cancer Research,* 77**,** 2633-2646.

ERIC, J. H. & HALL, E. J. 2006. *Radiobiology for the radiologist,* Philadelphia, Pa. ; London, Philadelphia, Pa. ; London : Lippincott Williams & Wilkins, c2006.

EVANS, R. A., TIAN, Y. C., STEADMAN, R. & PHILLIPS, A. O. 2003. TGF-beta 1-mediated fibroblast-myofibroblast terminal differentiation - the role of Smad proteins. *Experimental Cell Research,* 282**,** 90-100.

EWAN, K. B., HENSHALL-POWELL, R. L., RAVANI, S. A., PAJARES, M. J., ARTEAGA, C., WARTERS, R., AKHURST, R. J. & BARCELLOS-HOFF, M. H. 2002. Transforming growth factor-beta 1 mediates cellular response to DNA damage in situ. *Cancer Research,* 62**,** 5627-5631.

FAJARDO, L. F. 2005. The pathology of ionizing radiation as defined by morphologic patterns. *Acta Oncologica,* 44**,** 13-22.

FANG, L., YAN, Y. B., KOMUVES, L. G., YONKOVICH, S., SULLIVAN, C. M., STRINGER, B., GALBRAITH, S., LOKKER, N. A., HWANG, S. S., NURDEN, P., PHILLIPS, D. R. & GIESE, N. A. 2004. PDGF C is a selective alpha platelet-derived growth factor receptor agonist that is highly expressed in platelet alpha granules and vascular smooth muscle. *Arteriosclerosis Thrombosis and Vascular Biology,* 24**,** 787-792.

FENG, X. H. & DERYNCK, R. 2005. Specificity and versatility in TGF-beta signaling through Smads. *Annual Review of Cell and Developmental Biology,* 21**,** 659-693.

FENTON, B. M. & PAONI, S. F. 2007. The addition of AG-013736 to fractionated radiation improves tumor response without functionally normalizing the tumor vasculature. *Cancer Research,* 67**,** 9921-9928.

FERRARA, N. 2010. Pathways mediating VEGF-independent tumor angiogenesis. *Cytokine & Growth Factor Reviews,* 21**,** 21-26.

FERRARA, N., GERBER, H. P. & LECOUTER, J. 2003. The biology of VEGF and its receptors. *Nature Medicine,* 9**,** 669-676.

FIDLER, I. J. 2003. Timeline - The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. *Nature Reviews Cancer,* 3**,** 453-458.

FIEDLER, J., ETZEL, N. & BRENNER, R. E. 2004. To go or not to go: Migration of human mesenchymal progenitor cells stimulated by isoforms of PDGF. *Journal of Cellular Biochemistry,* 93**,** 990-998.

FLETCHER, C. D. M. 2014. The evolving classification of soft tissue tumours - an update based on the new 2013 WHO classification. *Histopathology,* 64**,** 2-11.

FLETCHER, C. D. M., UNNI, K. K., MERTENS, F., WORLD HEALTH, O. & INTERNATIONAL ACADEMY OF, P. 2002. *Pathology and genetics of tumours of soft tissue and bone,* Lyon, Lyon : IARC Press, 2002.

FLIER, S. N., TANJORE, H., KOKKOTOU, E. G., SUGIMOTO, H., ZEISBERG, M. & KALLURI, R. 2010. Identification of Epithelial to Mesenchymal Transition as a Novel Source of

Fibroblasts in Intestinal Fibrosis. *Journal of Biological Chemistry,* 285**,** 20202-20212.
FOLKERT, M. R., SINGER, S., BRENNAN, M. F., KUK, D., QIN, L.-X., KOBAYASHI, W. K., CRAGO, A. M. & ALEKTIAR, K. M. 2014. Comparison of Local Recurrence With Conventional and Intensity-Modulated Radiation Therapy for Primary Soft-Tissue Sarcomas of the Extremity. *Journal of Clinical Oncology,* 32**,** 3236-+.
FOLKMAN, J. 1986. HOW IS BLOOD-VESSEL GROWTH REGULATED IN NORMAL AND NEOPLASTIC TISSUE - GHA CLOWES MEMORIAL AWARD LECTURE. *Cancer Research,* 46**,** 467-473.
FOLKMAN, J. 1995. ANGIOGENESIS IN CANCER, VASCULAR, RHEUMATOID AND OTHER DISEASE. *Nature Medicine,* 1**,** 27-31.
FORBES, S. J., RUSSO, F. P., REY, V., BURRA, P., RUGGE, M., WRIGHT, N. A. & ALISON, M. R. 2004. A significant proportion of myofibroblasts are of bone marrow origin in human liver fibrosis. *Gastroenterology,* 126**,** 955-963.
FOX, S. B., GASPARINI, G. & HARRIS, A. L. 2001. Angiogenesis: pathological, prognostic, and growth-factor pathways and their link to trial design and anticancer drugs. *The Lancet. Oncology,* 2**,** 278-89.
FRANCO, O. E., SHAW, A. K., STRAND, D. W. & HAYWARD, S. W. 2010. Cancer associated fibroblasts in cancer pathogenesis. *Seminars in Cell & Developmental Biology,* 21**,** 33-39.
FRANSES, J. W., BAKER, A. B., CHITALIA, V. C. & EDELMAN, E. R. 2011. Stromal Endothelial Cells Directly Influence Cancer Progression. *Science Translational Medicine,* 3.
FRAZIER, K., WILLIAMS, S., KOTHAPALLI, D., KLAPPER, H. & GROTENDORST, G. R. 1996. Stimulation of fibroblast cell growth, matrix production, and granulation tissue formation by connective tissue growth factor. *Journal of Investigative Dermatology,* 107**,** 404-411.
FRIEDENSTEIN, A. J., CHAILAKHJAN, R. K. & LALYKINA, K. S. 1970. DEVELOPMENT OF FIBROBLAST COLONIES IN MONOLAYER CULTURES OF GUINEA-PIG BONE MARROW AND SPLEEN CELLS. *Cell and Tissue Kinetics,* 3**,** 393-+.
FRIEDL, P. & ALEXANDER, S. 2011. Cancer Invasion and the Microenvironment: Plasticity and Reciprocity. *Cell,* 147**,** 992-1009.
FRUSTACI, S., GHERLINZONI, F., DE PAOLI, A., BONETTI, M., AZZARELLI, A., COMANDONE, A., OLMI, P., BUONADONNA, A., PIGNATTI, G., BARBIERI, E., APICE, G., ZMERLY, H., SERRAINO, D. & PICCI, P. 2001. Adjuvant chemotherapy for adult soft tissue sarcomas of the extremities and girdles: Results of the Italian randomized cooperative trial. *Journal of Clinical Oncology,* 19**,** 1238-1247.
FURUKAWA, S., SOEDA, S., KIKO, Y., SUZUKI, O., HASHIMOTO, Y., WATANABE, T., NISHIYAMA, H., TASAKI, K., HOJO, H., ABE, M. & FUJIMORI, K. 2013. MCP-1 Promotes Invasion and Adhesion of Human Ovarian Cancer Cells. *Anticancer Research,* 33**,** 4785-4790.
GAENGEL, K., GENOVE, G., ARMULIK, A. & BETSHOLTZ, C. 2009. Endothelial-Mural Cell Signaling in Vascular Development and Angiogenesis. *Arteriosclerosis Thrombosis and Vascular Biology,* 29**,** 630-638.
GAO, H., PRIEBE, W., GLOD, J. & BANERJEE, D. 2009. Activation of Signal Transducers and Activators of Transcription 3 and Focal Adhesion Kinase by Stromal Cell-Derived Factor 1 Is Required for Migration of Human Mesenchymal Stem Cells in Response to Tumor Cell-Conditioned Medium. *Stem Cells,* 27**,** 857-865.
GARLANDA, C., PARRAVICINI, C., SIRONI, M., DE ROSSI, M., WAINSTOK DE CALMANOVICI, R., CAROZZI, F., BUSSOLINO, F., COLOTTA, F., MANTOVANI, A. & VECCHI, A. 1994. Progressive growth in immunodeficient mice and host cell recruitment by mouse endothelial cells transformed by polyoma middle-sized T

antigen: implications for the pathogenesis of opportunistic vascular tumors. *Proceedings of the National Academy of Sciences,* 91**,** 7291-7295.
GAUGLER, M. H., VEREYCKEN-HOLLER, V., SQUIBAN, C., VANDAMME, M., VOZENIN-BROTONS, M. C. & BENDERITTER, M. 2005. Pravastatin limits endothelial activation after irradiation and decreases the resulting inflammatory and thrombotic responses. *Radiation Research,* 163**,** 479-487.
GELING, A., STEINER, H., WILLEM, M., BALLY-CUIF, L. & HAASS, C. 2002. A gamma-secretase inhibitor blocks Notch signaling in vivo and causes a severe neurogenic phenotype in zebrafish. *Embo Reports,* 3**,** 688-694.
GERHARDT, H. & SEMB, H. 2008. Pericytes: gatekeepers in tumour cell metastasis? *Journal of Molecular Medicine-Jmm,* 86**,** 135-144.
GILKES, D. M., SEMENZA, G. L. & WIRTZ, D. 2014. Hypoxia and the extracellular matrix: drivers of tumour metastasis. *Nature Reviews Cancer,* 14**,** 430-439.
GILLITZER, R. & GOEBELER, M. 2001. Chemokines in cutaneous wound healing. *Journal of Leukocyte Biology,* 69**,** 513-521.
GOMES, F. G., NEDEL, F., ALVES, A. M., NOR, J. E. & TARQUINIO, S. B. C. 2013. Tumor angiogenesis and lymphangiogenesis: Tumor/endothelial crosstalk and cellular/microenvironmental signaling mechanisms. *Life Sciences,* 92**,** 101-107.
GOODWIN, A. & JENKINS, G. 2009. Role of integrin-mediated TGF beta activation in the pathogenesis of pulmonary fibrosis. *Biochemical Society Transactions,* 37**,** 849-854.
GORELIK, L. & FLAVELL, R. A. 2002. Transforming growth factor-beta in T-cell biology. *Nature Reviews Immunology,* 2**,** 46-53.
GORSKI, D. H., BECKETT, M. A., JASKOWIAK, N. T., CALVIN, D. P., MAUCERI, H. J., SALLOUM, R. M., SEETHARAM, S., KOONS, A., HARI, D. M., KUFE, D. W. & WEICHSELBAUM, R. R. 1999. Blockade of the vascular endothelial growth factor stress response increases the antitumor effects of ionizing radiation. *Cancer Research,* 59**,** 3374-3378.
GOTTLOBER, P., STEINERT, M., BAHREN, W., WEBER, L., GERNGROSS, H. & PETER, R. U. 2001. Interferon-gamma in 5 patients with cutaneous radiation syndrome after radiation therapy. *International Journal of Radiation Oncology Biology Physics,* 50**,** 159-166.
GRIMER, R., JUDSON, I., PEAKE, D. & SEDDON, B. 2010. Guidelines for the Management of Soft Tissue Sarcomas. *Sarcoma***,** 1-15.
GRISENDI, G., BUSSOLARI, R., CAFARELLI, L., PETAK, I., RASINI, V., VERONESI, E., DE SANTIS, G., SPANO, C., TAGLIAZZUCCHI, M., BARTI-JUHASZ, H., SCARABELLI, L., BAMBI, F., FRASSOLDATI, A., ROSSI, G., CASALI, C., MORANDI, U., HORWITZ, E. M., PAOLUCCI, P., CONTE, P. & DOMINICI, M. 2010. Adipose-Derived Mesenchymal Stem Cells as Stable Source of Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand Delivery for Cancer Therapy. *Cancer Research,* 70**,** 3718-3729.
GRIVENNIKOV, S. I., GRETEN, F. R. & KARIN, M. 2010. Immunity, Inflammation, and Cancer. *Cell,* 140**,** 883-899.
GRONCHI, A. & CASALI, P. G. 2013. Adjuvant Therapy for High-Risk Soft Tissue Sarcoma in the Adult. *Current Treatment Options in Oncology,* 14**,** 415-424.
GROTENDORST, G. R., RAHMANIE, H. & DUNCAN, M. R. 2004. Combinatorial signaling pathways determine fibroblast proliferation and myofibroblast differentiation. *Faseb Journal,* 18**,** 469-479.
GRUNSTEIN, J., ROBERTS, W. G., MATHIEU-COSTELLO, O., HANAHAN, D. & RANDALL, S. 1999. Tumor-derived expression of vascular endothelial growth factor is a critical factor in tumor expansion and vascular function. *Cancer Research,* 59**,** 1592-1598.

GUO, X. Y., OSHIMA, H., KITMURA, T., TAKETO, M. M. & OSHIMA, M. 2008. Stromal fibroblasts activated by tumor cells promote angiogenesis in mouse gastric cancer. *Journal of Biological Chemistry,* 283**,** 19864-19871.
GURKE, S., BARROSO, J. F. V. & GERDES, H. H. 2008. The art of cellular communication: tunneling nanotubes bridge the divide. *Histochemistry and Cell Biology,* 129**,** 539-550.
HAASE, O. & RODEMANN, H. P. 2004. Fibrosis and cytokine mechanisms: Relevant in hadron therapy? *Radiotherapy and Oncology,* 73**,** S144-S147.
HAERYFAR, S. M. M. & HOSKIN, D. W. 2004. Thy-1: More than a mouse pan-T cell marker. *Journal of Immunology,* 173**,** 3581-3588.
HALBERG, N., KHAN, T., TRUJILLO, M. E., WERNSTEDT-ASTERHOLM, I., ATTIE, A. D., SHERWANI, S., WANG, Z. V., LANDSKRONER-EIGER, S., DINEEN, S., MAGALANG, U. J., BREKKEN, R. A. & SCHERER, P. E. 2009. Hypoxia-Inducible Factor 1 alpha Induces Fibrosis and Insulin Resistance in White Adipose Tissue. *Molecular and Cellular Biology,* 29**,** 4467-4483.
HANAHAN, D. & COUSSENS, L. M. 2012. Accessories to the Crime: Functions of Cells Recruited to the Tumor Microenvironment. *Cancer Cell,* 21**,** 309-322.
HANAHAN, D. & WEINBERG, R. A. 2000. The hallmarks of cancer. *Cell,* 100**,** 57-70.
HANAHAN, D. & WEINBERG, R. A. 2011. Hallmarks of Cancer: The Next Generation. *Cell,* 144**,** 646-674.
HANNON, G. J. & BEACH, D. 1994. P15(INK4B) IS A POTENTIAL EFFECTOR OF TGF-BETA-INDUCED CELL-CYCLE ARREST. *Nature,* 371**,** 257-261.
HARDING, S. M., COACKLEY, C. & BRISTOW, R. G. 2011. ATM-dependent phosphorylation of 53BP1 in response to genomic stress in oxic and hypoxic cells. *Radiotherapy and Oncology,* 99**,** 307-312.
HASEGAWA, M. & SATO, S. 2008. The roles of chemokines in leukocyte recruitment and fibrosis in systemic sclerosis. *Frontiers in Bioscience-Landmark,* 13**,** 3637-3647.
HASS, R. & OTTE, A. 2012. Mesenchymal stem cells as all-round supporters in a normal and neoplastic microenvironment. *Cell Communication and Signaling,* 10.
HAUBNER, F., LEYH, M., OHMANN, E., POHL, F., PRANTL, L. & GASSNER, H. G. 2013a. Effects of external radiation in a co-culture model of endothelial cells and adipose-derived stem cells. *Radiation Oncology,* 8.
HAUBNER, F., OHMANN, E., POHL, F., PRANTL, L., STRUTZ, J. & GASSNER, H. G. 2013b. Effects of radiation on the expression of adhesion molecules and cytokines in a static model of human dermal microvascular endothelial cells. *Clinical Hemorheology and Microcirculation,* 54**,** 371-379.
HAWSAWI, N. M., GHEBEH, H., HENDRAYANI, S. F., TULBAH, A., AL-EID, M., AL-TWEIGERI, T., AJARIM, D., ALAIYA, A., DERMIME, S. & ABOUSSEKHRA, A. 2008. Breast carcinoma - Associated fibroblasts and their counterparts display neoplastic-specific changes. *Cancer Research,* 68**,** 2717-2725.
HAYDONT, V., RISER, B. L., AIGUEPERSE, J. & VOZENIN-BROTONS, M. C. 2008. Specific signals involved in the long-term maintenance of radiation-induced fibrogenic differentiation: a role for CCN2 and low concentration of TGF-beta 1. *American Journal of Physiology-Cell Physiology,* 294**,** C1332-C1341.
HECKMANN, D., MAIER, P., LAUFS, S., WENZ, F., ZELLER, W. J., FRUEHAUF, S. & ALLGAYER, H. 2013. CXCR4 Expression and Treatment with SDF-1 alpha or Plerixafor Modulate Proliferation and Chemosensitivity of Colon Cancer Cells. *Translational Oncology,* 6**,** 124-132.
HEISSIG, B., RAFII, S., AKIYAMA, H., OHKI, Y., SATO, Y., RAFAEL, T., ZHU, Z. P., HICKLIN, D. J., OKUMURA, K., OGAWA, H., WERB, Z. & HATTORI, K. 2005. Low-dose irradiation promotes tissue revascularization through VEGF release from mast cells and MMP-9-mediated progenitor cell mobilization. *Journal of Experimental Medicine,* 202**,** 739-750.

HELLEVIK, T., PETTERSEN, I., BERG, V., WINBERG, J. O., MOE, B. T., BARTNES, K., PAULSSEN, R. H., BUSUND, L. T., BREMNES, R., CHALMERS, A. & MARTINEZ-ZUBIAURRE, I. 2012. Cancer-associated fibroblasts from human NSCLC survive ablative doses of radiation but their invasive capacity is reduced. *Radiation Oncology,* 7.

HENSCHLER, R., DEAK, E. & SEIFRIED, E. 2008. Homing of mesenchymal stem cells. *Transfusion Medicine and Hemotherapy,* 35**,** 306-312.

HERNANDEZ-MORERA, P., CASTANO-GONZALEZ, I., TRAVIESO-GONZALEZ, C. M., MOMPEO-CORREDERA, B. & ORTEGA-SANTANA, F. 2016. Quantification and Statistical Analysis Methods for Vessel Wall Components from Stained Images with Masson's Trichrome. *Plos One,* 11.

HIGGINS, D. F., KIMURA, K., BERNHARDT, W. M., SHRIMANKER, N., AKAI, Y., HOHENSTEIN, B., SAITO, Y., JOHNSON, R. S., KRETZLER, M., COHEN, C. D., ECKARDT, K. U., IWANO, M. & HAASE, V. H. 2007. Hypoxia promotes fibrogenesis in vivo via HIF-1 stimulation of epithelial-to-mesenchymal transition. *Journal of Clinical Investigation,* 117**,** 3810-3820.

HILL, C., FLYVBJERG, A., RASCH, R., BAK, M. & LOGAN, A. 2001. Transforming growth factor-beta 2 antibody attenuates fibrosis in the experimental diabetic rat kidney. *Journal of Endocrinology,* 170**,** 647-651.

HINZ, B., PHAN, S. H., THANNICKAL, V. J., GALLI, A., BOCHATON-PIALLAT, M. L. & GABBIANI, G. 2007. The myofibroblast - One function, multiple origins. *American Journal of Pathology,* 170**,** 1807-1816.

HIRATA, E., GIROTTI, M. R., VIROS, A., HOOPER, S., SPENCER-DENE, B., MATSUDA, M., LARKIN, J., MARAIS, R. & SAHAI, E. 2015. Intravital Imaging Reveals How BRAF Inhibition Generates Drug-Tolerant Microenvironments with High Integrin beta 1/FAK Signaling. *Cancer Cell,* 27**,** 574-588.

HO, I. A. W., TOH, H. C., NG, W. H., TEO, Y. L., GUO, C. M., HUI, K. M. & LAM, P. Y. P. 2013. Human Bone Marrow-Derived Mesenchymal Stem Cells Suppress Human Glioma Growth Through Inhibition of Angiogenesis. *Stem Cells,* 31**,** 146-155.

HOLASH, J., WIEGAND, S. J. & YANCOPOULOS, G. D. 1999. New model of tumor angiogenesis: dynamic balance between vessel regression and growth mediated by angiopoietins and VEGF. *Oncogene,* 18**,** 5356-5362.

HONCZARENKO, M., LE, Y., SWIERKOWSKI, M., GHIRAN, I., GLODEK, A. M. & SILBERSTEIN, L. E. 2006. Human bone marrow stromal cells express a distinct set of biologically functional chemokine receptors. *Stem Cells,* 24**,** 1030-1041.

HSU, M. Y., ANDL, T., LI, G., MEINKOTH, J. L. & HERLYN, M. 2000. Cadherin repertoire determines partner-specific gap junctional communication during melanoma progression. *Journal of Cell Science,* 113**,** 1535-1542.

HU, S. Y., CHEN, Y. D., LI, L. B., CHEN, J. L., WU, B., ZHOU, X., ZHI, G., LI, Q. F., WANG, R. L., DUAN, H. F., GUO, Z. K., YANG, Y. F., XIAO, F. J., WANG, H. & WANG, L. S. 2009. EFFECTS OF ADENOVIRUS-MEDIATED DELIVERY OF THE HUMAN HEPATOCYTE GROWTH FACTOR GENE IN EXPERIMENTAL RADIATION-INDUCED HEART DISEASE. *International Journal of Radiation Oncology Biology Physics,* 75**,** 1537-1544.

HUANG, S. Y., VAN ARSDALL, M., TEDJARATI, S., MCCARTY, M., WU, W. J., LANGLEY, R. & FIDLER, I. J. 2002. Contributions of stromal metalloproteinase-9 to angiogenesis and growth of human ovarian carcinoma in mice. *Journal of the National Cancer Institute,* 94**,** 1134-1142.

HURWITZ, H., FEHRENBACHER, L., NOVOTNY, W., CARTWRIGHT, T., HAINSWORTH, J., HEIM, W., BERLIN, J., BARON, A., GRIFFING, S., HOLMGREN, E., FERRARA, N., FYFE, G., ROGERS, B., ROSS, R. & KABBINAVAR, F. 2004. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *New England Journal of Medicine,* 350**,** 2335-2342.

HUSSEY, G. S., CHAUDHURY, A., DAWSON, A. E., LINDNER, D. J., KNUDSEN, C. R., WILCE, M. C. J., MERRICK, W. C. & HOWE, P. H. 2011. Identification of an mRNP Complex Regulating Tumorigenesis at the Translational Elongation Step. *Molecular Cell,* 41**,** 419-431.

IGARASHI, A., OKOCHI, H., BRADHAM, D. M. & GROTENDORST, G. R. 1993. REGULATION OF CONNECTIVE-TISSUE GROWTH-FACTOR GENE-EXPRESSION IN HUMAN SKIN FIBROBLASTS AND DURING WOUND REPAIR. *Molecular Biology of the Cell,* 4**,** 637-645.

IHN, H. 2002. Pathogenesis of fibrosis: role of TGF-beta and CTGF. *Current Opinion in Rheumatology,* 14**,** 681-685.

IOZZO, R. V., ZOELLER, J. J. & NYSTROM, A. 2009. Basement membrane proteoglycans: Modulators Par Excellence of cancer growth and angiogenesis. *Molecules and Cells,* 27**,** 503-513.

ISHII, G., SANGAI, T., ODA, T., AOYAGI, Y., HASEBE, T., KANOMATA, N., ENDOH, Y., OKUMURA, C., OKUHARA, Y., MAGAE, J., EMURA, M., OCHIYA, T. & OCHIAI, A. 2003. Bone-marrow-derived myofibroblasts contribute to the cancer-induced stromal reaction. *Biochemical and Biophysical Research Communications,* 309**,** 232-240.

ISLAM, M. S., STEMIG, M. E., TAKAHASHI, Y. & HUI, S. K. 2015. Radiation response of mesenchymal stem cells derived from bone marrow and human pluripotent stem cells. *Journal of Radiation Research,* 56**,** 269-277.

JAIN, R., SHAUL, P. W., BOROK, Z. & WILLIS, B. C. 2007. Endothelin-1 induces alveolar epithelial-mesenchymal transition through endothelin type a receptor-mediated production of TGF-beta 1. *American Journal of Respiratory Cell and Molecular Biology,* 37**,** 38-47.

JAIN, R. K. 2003. Molecular regulation of vessel maturation. *Nature Medicine,* 9**,** 685-693.

JI, J. F., HE, B. P., DHEEN, S. T. & TAY, S. S. W. 2004. Interactions of chemokines and chemokine receptors mediate the migration of mesenchymal stem cells to the impaired site in the brain after hypoglossal nerve injury. *Stem Cells,* 22**,** 415-427.

JIMENEZ, S. A. 2016. ROLE OF ENDOTHELIAL TO MESENCHYMAL TRANSITION IN THE PATHOGENESIS OF THE VASCULAR ALTERATIONS IN SYSTEMIC SCLEROSIS. *Annals of the Rheumatic Diseases,* 75**,** 43-43.

JOHANSSON, A. C., ANSELL, A., JERHAMMAR, F., LINDH, M. B., GRENMAN, R., MUNCK-WIKLAND, E., OSTMAN, A. & ROBERG, K. 2012. Cancer-Associated Fibroblasts Induce Matrix Metalloproteinase-Mediated Cetuximab Resistance in Head and Neck Squamous Cell Carcinoma Cells. *Molecular Cancer Research,* 10**,** 1158-1168.

JU, J. L. A., GODET, I., YE, I. C., BYUN, J., JAYATILAKA, H., LEE, S. J., XIANG, L. S., SAMANTA, D., LEE, M. H., WU, P. H., WIRTZ, D., SEMENZA, G. L. & GILKES, D. M. 2017. Hypoxia Selectively Enhances Integrin alpha(5)beta(1) Receptor Expression in Breast Cancer to Promote Metastasis. *Molecular Cancer Research,* 15**,** 723-734.

KABASHIMA-NIIBE, A., HIGUCHI, H., TAKAISHI, H., MASUGI, Y., MATSUZAKI, Y., MABUCHI, Y., FUNAKOSHI, S., ADACHI, M., HAMAMOTO, Y., KAWACHI, S., AIURA, K., KITAGAWA, Y., SAKAMOTO, M. & HIBI, T. 2013. Mesenchymal stem cells regulate epithelial-mesenchymal transition and tumor progression of pancreatic cancer cells. *Cancer Science,* 104**,** 157-164.

KALLURI, R. 2016. The biology and function of fibroblasts in cancer. *Nature Reviews Cancer,* 16**,** 582-598.

KALLURI, R. & NEILSON, E. G. 2003. Epithelial-mesenchymal transition and its implications for fibrosis. *Journal of Clinical Investigation,* 112**,** 1776-1784.

KALLURI, R. & ZEISBERG, M. 2006. Fibroblasts in cancer. *Nature Reviews Cancer,* 6**,** 392-401.

KAMPROM, W., KHEOLAMAI, P., U-PRATYA, Y., SUPOKAWEJ, A., WATTANAPANITCH, M., LAOWTAMMATHRON, C., ROYTRAKUL, S. & ISSARAGRISIL, S. 2016. Endothelial

Progenitor Cell Migration-Enhancing Factors in the Secretome of Placental-Derived Mesenchymal Stem Cells. *Stem Cells International*.
KANDOUZ, M. & BATIST, G. 2010. Gap junctions and connexins as therapeutic targets in cancer. *Expert Opinion on Therapeutic Targets,* 14**,** 681-692.
KANG, Y. B., SIEGEL, P. M., SHU, W. P., DROBNJAK, M., KAKONEN, S. M., CORDON-CARDO, C., GUISE, T. A. & MASSAGUE, J. 2003. A multigenic program mediating breast cancer metastasis to bone. *Cancer Cell,* 3**,** 537-549.
KANTHOU, C., DACHS, G. U., LEFLEY, D. V., STEELE, A. J., CORALLI-FOXON, C., HARRIS, S., GRECO, O., DOS SANTOS, S. A., REYES-ALDASORO, C. C., ENGLISH, W. R. & TOZER, G. M. 2014. Tumour Cells Expressing Single VEGF Isoforms Display Distinct Growth, Survival and Migration Characteristics. *Plos One,* 9.
KAPOOR, G. & DAS, K. 2012. Soft Tissue Sarcomas in Children. *Indian Journal of Pediatrics,* 79**,** 936-942.
KARAGIANNIS, G. S., POUTAHIDIS, T., ERDMAN, S. E., KIRSCH, R., RIDDELL, R. H. & DIAMANDIS, E. P. 2012. Cancer-Associated Fibroblasts Drive the Progression of Metastasis through both Paracrine and Mechanical Pressure on Cancer Tissue. *Molecular Cancer Research,* 10**,** 1403-1418.
KARAM, S. D. & BHATIA, S. 2015. The radiobiological targets of SBRT: tumor cells or endothelial cells? *Annals of Translational Medicine,* 3.
KARNOUB, A. E., DASH, A. B., VO, A. P., SULLIVAN, A., BROOKS, M. W., BELL, G. W., RICHARDSON, A. L., POLYAK, K., TUBO, R. & WEINBERG, R. A. 2007. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature,* 449**,** 557-U4.
KARP, J. M. & TEOL, G. S. L. 2009. Mesenchymal Stem Cell Homing: The Devil Is in the Details. *Cell Stem Cell,* 4**,** 206-216.
KATSUDA, S., OKADA, Y., MINAMOTO, T., ODA, Y., MATSUI, Y. & NAKANISHI, I. 1992. COLLAGENS IN HUMAN ATHEROSCLEROSIS - IMMUNOHISTOCHEMICAL ANALYSIS USING COLLAGEN TYPE-SPECIFIC ANTIBODIES. *Arteriosclerosis and Thrombosis,* 12**,** 494-502.
KEPKA, L., DELANEY, T. F., SUIT, H. D. & GOLDBERG, S. I. 2005. Results of radiation therapy for unresected soft-tissue sarcomas. *International Journal of Radiation Oncology Biology Physics,* 63**,** 852-859.
KERBEL, R. S. 1997. A cancer therapy resistant to resistance. *Nature,* 390**,** 335-336.
KHARAISHVILI, G., SIMKOVA, D., BOUCHALOVA, K., GACHECHILADZE, M., NARSIA, N. & BOUCHAL, J. 2014. The role of cancer-associated fibroblasts, solid stress and other microenvironmental factors in tumor progression and therapy resistance. *Cancer Cell International,* 14.
KHARAZIHA, P., RODRIGUEZ, P., LI, Q., RUNDQVIST, H., BJORKLUND, A. C., AUGSTEN, M., ULLEN, A., EGEVAD, L., WIKLUND, P., NILSSON, S., KROEMER, G., GRANDER, D. & PANARETAKIS, T. 2012. Targeting of distinct signaling cascades and cancer-associated fibroblasts define the efficacy of Sorafenib against prostate cancer cells. *Cell Death & Disease,* 3.
KIM, H. M., JUNG, W. H. & KOO, J. S. 2015. Expression of cancer-associated fibroblast related proteins in metastatic breast cancer: an immunohistochemical analysis. *Journal of Translational Medicine,* 13.
KIM, K., LU, Z. F. & HAY, E. D. 2002. Direct evidence for a. role of beta-catenin/LEF-1 signaling pathway in induction of EMT. *Cell Biology International,* 26**,** 463-476.
KITAORI, T., ITO, H., SCHWARZ, E. A., TSUTSUMI, R., YOSHITOMI, H., OISHI, S., NAKANO, M., FUJII, N., NAGASAWA, T. & NAKAMURA, T. 2009. Stromal Cell-Derived Factor 1/CXCR4 Signaling Is Critical for the Recruitment of Mesenchymal Stem Cells to the Fracture Site During Skeletal Repair in a Mouse Model. *Arthritis and Rheumatism,* 60**,** 813-823.

KLOPP, A. H., GUPTA, A., SPAETH, E., ANDREEFF, M. & MARINI, F. 2011. Concise Review: Dissecting a Discrepancy in the Literature: Do Mesenchymal Stem Cells Support or Suppress Tumor Growth? *Stem Cells,* 29**,** 11-19.

KLOPP, A. H., SPAETH, E. L., DEMBINSKI, J. L., WOODWARD, W. A., MUNSHI, A., MEYN, R. E., COX, J. D., ANDREEFF, M. & MARINI, F. C. 2007. Tumor irradiation increases the recruitment of circulating mesenchymal stem cells into the tumor microenvironment. *Cancer Research,* 67**,** 11687-11695.

KNIGHT, E. & PRZYBORSKI, S. 2015. Advances in 3D cell culture technologies enabling tissue-like structures to be created in vitro. *Journal of Anatomy,* 227**,** 746-756.

KOLIOPANOS, A., FRIESS, H., DI MOLA, F. F., TANG, W. H., KUBULUS, D., BRIGSTOCK, D., ZIMMERMANN, A. & BUCHLER, M. W. 2002. Connective tissue growth factor gene expression alters tumor progression in esophageal cancer. *World Journal of Surgery,* 26**,** 420-427.

KONG, P., CHRISTIA, P. & FRANGOGIANNIS, N. G. 2014. The pathogenesis of cardiac fibrosis. *Cellular and Molecular Life Sciences,* 71**,** 549-574.

KOPAN, R. & ILAGAN, M. X. G. 2009. The Canonical Notch Signaling Pathway: Unfolding the Activation Mechanism. *Cell,* 137**,** 216-233.

KORPELA, E. & LIU, S. K. 2014. Endothelial perturbations and therapeutic strategies in normal tissue radiation damage. *Radiation Oncology,* 9.

KOTURBASH, I., BAKER, M., LOREE, J., KUTANZI, K., HUDSON, D., POGRIBNY, I., SEDELNIKOVA, O., BONNER, W. & KOVALCHUK, O. 2006. Epigenetic dysregulation underlies radiation-induced transgenerational genome instability in vivo. *International Journal of Radiation Oncology Biology Physics,* 66**,** 327-330.

KOUKOURAKIS, M. I., GIATROMANOLAKI, A., SIVRIDIS, E., SIMOPOULOS, K., PISSAKAS, G., GATTER, K. C. & HARRIS, A. L. 2001. Squamous cell head and neck cancer: Evidence of angiogenic regeneration during radiotherapy. *Anticancer Research,* 21**,** 4301-4309.

KRAMER, N., WALZL, A., UNGER, C., ROSNER, M., KRUPITZA, G., HENGSTSCHLAGER, M. & DOLZNIG, H. 2013. In vitro cell migration and invasion assays. *Mutation Research-Reviews in Mutation Research,* 752**,** 10-24.

KRENNING, G., ZEISBERG, E. M. & KALLURI, R. 2010. The Origin of Fibroblasts and Mechanism of Cardiac Fibrosis. *Journal of Cellular Physiology,* 225**,** 631-637.

KRISHNAMACHARY, B., BERG-DIXON, S., KELLY, B., AGANI, F., FELDSER, D., FERREIRA, G., IYER, N., LARUSCH, J., PAK, B., TAGHAVI, P. & SEMENZA, G. L. 2003. Regulation of colon carcinoma cell invasion by hypoxia-inducible factor 1. *Cancer Research,* 63**,** 1138-1143.

KRIZHANOVSKY, V., YON, M., DICKINS, R. A., HEARN, S., SIMON, J., MIETHING, C., YEE, H., ZENDER, L. & LOWE, S. W. 2008. Senescence of activated stellate cells limits liver fibrosis (vol 134, pg 657, 2008). *Cell,* 135**,** 190-190.

KRUSE, J., FLOOT, B. G. J., TE POELE, J. A. M., RUSSELL, N. S. & STEWART, F. A. 2009. Radiation-Induced Activation of TGF-beta Signaling Pathways in Relation to Vascular Damage in Mouse Kidneys. *Radiation Research,* 171**,** 188-197.

KRYCZEK, I., WEI, S., KELLER, E., LIU, R. & ZOU, W. P. 2007. Stroma-derived factor (SDF-1/CXCL12) and human tumor pathogenesis. *American Journal of Physiology-Cell Physiology,* 292**,** C987-C995.

KUHMANN, C., WEICHENHAN, D., REHLI, M., PLASS, C., SCHMEZER, P. & POPANDA, O. 2011. DNA methylation changes in cells regrowing after fractioned ionizing radiation. *Radiotherapy and Oncology,* 101**,** 116-121.

KULTERER, B., FRIEDL, G., JANDROSITZ, A., SANCHEZ-CABO, F., PROKESCH, A., PAAR, C., SCHEIDELER, M., WINDHAGER, R., PREISEGGER, K. H. & TRAJANOSKI, Z. 2007. Gene expression profiling of human mesenchymal stem cells derived from bone marrow during expansion and osteoblast differentiation. *Bmc Genomics,* 8.

LAGRANGE, J. L., RAMAIOLI, A., CHATEAU, M. C., MARCHAL, C., RESBEUT, M., RICHAUD, P., LAGARDE, P., RAMBERT, P., TORTECHAUX, J., SENG, S. H., DE LA FONTAN, B., REME-SAUMON, M., BOF, J., GHNASSIA, J. P., COINDRE, J. M. & FEDERATION NATL CTR LUTTE CONTRE, C. 2000. Sarcoma after radiation therapy: Retrospective multiinstitutional study of 80 histologically confirmed cases. *Radiology,* 216**,** 197-205.

LAHAT, G., LAZAR, A. & LEV, D. 2008. Sarcoma epidemiology and etiology: Potential environmental and genetic factors. *Surgical Clinics of North America,* 88**,** 451-+.

LAMA, V. N., SMITH, L., BADRI, L., FLINT, A., ANDREI, A. C., MURRAY, S., WANG, Z., LIAO, H., TOEWS, G. B., KREBSBACH, P. H., PETERS-GOLDEN, M., PINSKY, D. J., MARTINEZ, F. J. & THANNICKAL, V. J. 2007. Evidence for tissue-resident mesenchymal stem cells in human adult lung from studies of transplanted allografts. *Journal of Clinical Investigation,* 117**,** 989-996.

LAMOUILLE, S., XU, J. & DERYNCK, R. 2014. Molecular mechanisms of epithelial-mesenchymal transition. *Nature Reviews Molecular Cell Biology,* 15**,** 178-196.

LAN, H. Y. 2003. Tubular epithelial-myofibroblast transdifferentiation mechanisms in proximal tubule cells. *Current Opinion in Nephrology and Hypertension,* 12**,** 25-29.

LANZEN, J., BRAUN, R. D., KLITZMAN, B., BRIZEL, D., SECOMB, T. W. & DEWHIRST, M. W. 2006. Direct demonstration of instabilities in oxygen concentrations within the extravascular compartment of an experimental tumor. *Cancer Research,* 66**,** 2219-2223.

LARUE, A. C., MASUYA, M., EBIHARA, Y., FLEMING, P. A., VISCONTI, R. P., MINAMIGUCHI, H., OGAWA, M. & DRAKE, C. J. 2006. Hematopoietic origins of fibroblasts: I. In vivo studies of fibroblasts associated with solid tumors. *Experimental Hematology,* 34**,** 208-218.

LAVERY, K., SWAIN, P., FALB, D. & ALAOUI-ISMAILI, M. H. 2008. BMP-2/4 and BMP-6/7 differentially utilize cell surface receptors to induce osteoblastic differentiation of human bone marrow-derived mesenchymal stem cells. *Journal of Biological Chemistry,* 283**,** 20948-20958.

LAWRENCE, D. A. 2001. Latent-TGF-beta: An overview. *Molecular and Cellular Biochemistry,* 219**,** 163-170.

LAZENNEC, G. & JORGENSEN, C. 2008. Concise review: Adult multipotent stromal cells and cancer: Risk or benefit? *Stem Cells,* 26**,** 1387-1394.

LEASK, A. & ABRAHAM, D. J. 2004. TGF-beta signaling and the fibrotic response. *Faseb Journal,* 18**,** 816-827.

LEE, C. H., SHAH, B., MOIOLI, E. K. & MAO, J. J. 2010. CTGF directs fibroblast differentiation from human mesenchymal stem/stromal cells and defines connective tissue healing in a rodent injury model. *Journal of Clinical Investigation,* 120**,** 3340-3349.

LEE, Y., EL ANDALOUSSI, S. & WOOD, M. J. A. 2012. Exosomes and microvesicles: extracellular vesicles for genetic information transfer and gene therapy. *Human Molecular Genetics,* 21**,** R125-R134.

LERMAN, O. Z., GREIVES, M. R., SINGH, S. P., THANIK, V. D., CHANG, C. C., SEISER, N., BROWN, D. J., KNOBEL, D., SCHNEIDER, R. J., FORMENTI, S. C., SAADEH, P. B. & LEVINE, J. P. 2010. Low-dose radiation augments vasculogenesis signaling through HIF-1-dependent and -independent SDF-1 induction. *Blood,* 116**,** 3669-3676.

LEVENTAL, K. R., YU, H. M., KASS, L., LAKINS, J. N., EGEBLAD, M., ERLER, J. T., FONG, S. F. T., CSISZAR, K., GIACCIA, A., WENINGER, W., YAMAUCHI, M., GASSER, D. L. & WEAVER, V. M. 2009. Matrix Crosslinking Forces Tumor Progression by Enhancing Integrin Signaling. *Cell,* 139**,** 891-906.

LEWIS, M. P., LYGOE, K. A., NYSTROM, M. L., ANDERSON, W. P., SPEIGHT, P. M., MARSHALL, J. F. & THOMAS, G. J. 2004. Tumour-derived TGF-beta 1 modulates myofibroblast differentiation and promotes HGF/SF-dependent invasion of squamous carcinoma cells. *British Journal of Cancer,* 90**,** 822-832.
LI, B. & WANG, J. H. C. 2011. Fibroblasts and myofibroblasts in wound healing: Force generation and measurement. *Journal of Tissue Viability,* 20**,** 108-120.
LI, H. C., FAN, X. L. & HOUGHTON, J. 2007. Tumor microenvironment: The role of the tumor stroma in cancer. *Journal of Cellular Biochemistry,* 101**,** 805-815.
LI, H. H., FU, X. B., OUYANG, Y. S., CAI, C. L., WANG, J. & SUN, T. Z. 2006. Adult bone-marrow-derived mesenchymal stem cells contribute to wound healing of skin appendages. *Cell and Tissue Research,* 326**,** 725-736.
LI, H. J., REINHARDT, F., HERSCHMAN, H. R. & WEINBERG, R. A. 2012. Cancer-Stimulated Mesenchymal Stem Cells Create a Carcinoma Stem Cell Niche via Prostaglandin E-2 Signaling. *Cancer Discovery,* 2**,** 840-855.
LI, X., XU, Q., WU, Y. H., LI, J. Y., TANG, D. S., HAN, L. & FAN, Q. Q. 2014. A CCL2/ROS autoregulation loop is critical for cancer-associated fibroblasts-enhanced tumor growth of oral squamous cell carcinoma. *Carcinogenesis,* 35**,** 1362-1370.
LI, X. Y., HU, S. Q. & XIAO, L. 2015. The cancer-associated fibroblasts and drug resistance. *European Review for Medical and Pharmacological Sciences,* 19**,** 2112-2119.
LIANG, T. Z., ZHU, L., GAO, W. L., GONG, M., REN, J. H., YAO, H., WANG, K. & SHI, D. H. 2017. Coculture of endothelial progenitor cells and mesenchymal stem cells enhanced their proliferation and angiogenesis through PDGF and Notch signaling. *Febs Open Bio,* 7**,** 1722-1736.
LIN, G. T., YANG, R., BANIE, L., WANG, G. F., NING, H. X., LI, L. C., LUE, T. F. & LIN, C. S. 2010. Effects of Transplantation of Adipose Tissue-Derived Stem Cells on Prostate Tumor. *Prostate,* 70**,** 1066-1073.
LIU, C. H., GACA, M. D. A., SWENSON, E. S., VELLUCCI, V. F., REISS, M. & WELLS, R. G. 2003. Smads 2 and 3 are differentially activated by transforming growth factor-beta (TGF-beta) in quiescent and activated hepatic stellate cells - Constitutive nuclear localization of Smads in activated cells is TGF-beta-independent. *Journal of Biological Chemistry,* 278**,** 11721-11728.
LIU, D. & HORNSBY, P. J. 2007. Senescent human fibroblasts increase the early growth of xenograft tumors via matrix metalloproteinase secretion. *Cancer Research,* 67**,** 3117-3126.
LIU, F. Y., XIAO, L., PENG, Y. M., DUAN, S. B., LIU, H., LIU, Y. H., LING, G. H., YUAN, F., CHEN, J. X., FU, X. & ZHU, J. L. 2007. Inhibition effect of small interfering RNA of connective tissue growth factor on the expression of vascular endothelial growth factor and connective tissue growth factor in cultured human peritoneal mesothelial cells. *Chinese Medical Journal,* 120**,** 231-236.
LIU, Y., LIU, H., MEYER, C., LI, J., NADALIN, S., KONIGSRAINER, A., WENG, H. L., DOOLEY, S. & TEN DIJKE, P. 2013. Transforming Growth Factor-beta (TGF-beta)-mediated Connective Tissue Growth Factor (CTGF) Expression in Hepatic Stellate Cells Requires Stat3 Signaling Activation. *Journal of Biological Chemistry,* 288**,** 30708-30719.
LLOYD, G. M., MINTO, A. W., DORF, M. E., PROUDFOOT, A., WELLS, T. N. C., SALANT, D. J. & GUTIERREZRAMOS, J. C. 1997. RANTES and monocyte chemoattractant protein-1 (MCP-1) play an important role in the inflammatory phase of crescentic nephritis, but only MCP-1 is involved in crescent formation and interstitial fibrosis. *Journal of Experimental Medicine,* 185**,** 1371-1380.
LO, R. C. & KIM, H. 2017. Histopathological evaluation of liver fibrosis and cirrhosis regression. *Clinical and Molecular Hepatology,* 23**,** 302-307.
LOCHTER, A., GALOSY, S., MUSCHLER, J., FREEDMAN, N., WERB, Z. & BISSELL, M. J. 1997. Matrix metalloproteinase stromelysin-1 triggers a cascade of molecular

alterations that leads to stable epithelial-to-mesenchymal conversion and a premalignant phenotype in mammary epithelial cells. *Journal of Cell Biology,* 139**,** 1861-1872.

LOEBINGER, M. R., EDDAOUDI, A., DAVIES, D. & JANES, S. M. 2009. Mesenchymal Stem Cell Delivery of TRAIL Can Eliminate Metastatic Cancer. *Cancer Research,* 69**,** 4134-4142.

LOU, Y. M., MCDONALD, P. C., OLOUMI, A., CHIA, S., OSTLUND, C., AHMADI, A., KYLE, A., KELLER, U. A. D., LEUNG, S., HUNTSMAN, D., CLARKE, B., SUTHERLAND, B. W., WATERHOUSE, D., BALLY, M., ROSKELLEY, C., OVERALL, C. M., MINCHINTON, A., PACCHIANO, F., CARTA, F., SCOZZAFAVA, A., TOUISNI, N., WINUM, J. Y., SUPURAN, C. T. & DEDHAR, S. 2011. Targeting Tumor Hypoxia: Suppression of Breast Tumor Growth and Metastasis by Novel Carbonic Anhydrase IX Inhibitors. *Cancer Research,* 71**,** 3364-3376.

LU, P. F., TAKAI, K., WEAVER, V. M. & WERB, Z. 2011. Extracellular Matrix Degradation and Remodeling in Development and Disease. *Cold Spring Harbor Perspectives in Biology,* 3.

LUKER, K. E., LEWIN, S. A., MIHALKO, L. A., SCHMIDT, B. T., WINKLER, J. S., COGGINS, N. L., THOMAS, D. G. & LUKER, G. D. 2012. Scavenging of CXCL12 by CXCR7 promotes tumor growth and metastasis of CXCR4-positive breast cancer cells. *Oncogene,* 31**,** 4750-4758.

LUO, H., TU, G., LIU, Z. & LIU, M. 2015. Cancer-associated fibroblasts: A multifaceted driver of breast cancer progression. *Cancer Letters,* 361**,** 155-163.

LUTSENKO, S. V., KISELEV, S. M. & SEVERIN, S. E. 2003. Molecular mechanisms of tumor angiogenesis. *Biochemistry-Moscow,* 68**,** 286-300.

LUU, N. T., MCGETTRICK, H. M., BUCKLEY, C. D., NEWSOME, P. N., RAINGER, G. E., FRAMPTON, J. & NASH, G. B. 2013. Crosstalk Between Mesenchymal Stem Cells and Endothelial Cells Leads to Downregulation of Cytokine-Induced Leukocyte Recruitment. *Stem Cells,* 31**,** 2690-2702.

MA, B., KANG, Q. Y., QIN, L., CUI, L. J. & PEI, C. 2014. TGF-beta 2 induces transdifferentiation and fibrosis in human lens epithelial cells via regulating gremlin and CTGF. *Biochemical and Biophysical Research Communications,* 447**,** 689-695.

MAEDA, A., CHEN, Y. H., BU, J. C., MUJCIC, H., WOUTERS, B. G. & DACOSTA, R. S. 2017. In Vivo Imaging Reveals Significant Tumor Vascular Dysfunction and Increased Tumor Hypoxia-Inducible Factor-1 alpha Expression Induced by High Single-Dose Irradiation in a Pancreatic Tumor Model. *International Journal of Radiation Oncology Biology Physics,* 97**,** 184-194.

MAHMOOD, S. T., AGRESTA, S., VIGIL, C. E., ZHAO, X., HAN, G., D'AMATO, G., CALITRI, C. E., DEAN, M., GARRETT, C., SCHELL, M. J., ANTONIA, S. & CHIAPPORI, A. 2011. Phase II study of sunitinib malate, a multitargeted tyrosine kinase inhibitor in patients with relapsed or refractory soft tissue sarcomas. focus on three prevalent histologies: leiomyosarcoma, liposarcoma and malignant fibrous histiocytoma. *International Journal of Cancer,* 129**,** 1963-1969.

MAKI, R. G., D'ADAMO, D. R., KEOHAN, M. L., SAULLE, M., SCHUETZE, S. M., UNDEVIA, S. D., LIVINGSTON, M. B., COONEY, M. M., HENSLEY, M. L., MITA, M. M., TAKIMOTO, C. H., KRAFT, A. S., ELIAS, A. D., BROCKSTEIN, B., BLACHERE, N. E., EDGAR, M. A., SCHWARTZ, L. H., QIN, L.-X., ANTONESCU, C. R. & SCHWARTZ, G. K. 2009. Phase II Study of Sorafenib in Patients With Metastatic or Recurrent Sarcomas. *Journal of Clinical Oncology,* 27**,** 3133-3140.

MANDEL, K., YANG, Y. Y., SCHAMBACH, A., GLAGE, S., OTTE, A. & HASS, R. 2013. Mesenchymal Stem Cells Directly Interact with Breast Cancer Cells and Promote Tumor Cell Growth In Vitro and In Vivo. *Stem Cells and Development,* 22**,** 3114-3127.

MANI, S. A., GUO, W., LIAO, M. J., EATON, E. N., AYYANAN, A., ZHOU, A. Y., BROOKS, M., REINHARD, F., ZHANG, C. C., SHIPITSIN, M., CAMPBELL, L. L., POLYAK, K., BRISKEN, C., YANG, J. & WEINBERG, R. A. 2008. The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell,* 133**,** 704-715.
MANOTHAM, K., TANAKA, T., MATSUMOTO, M., OHSE, T., INAGI, R., MIYATA, T., KUROKAWA, K., FUJITA, T., INGELFINGER, J. R. & NANGAKU, M. 2004. Transdifferentiation of cultured tubular cells induced by hypoxia. *Kidney International,* 65**,** 871-880.
MANTOVANI, A., BISWAS, S. K., GALDIERO, M. R., SICA, A. & LOCATI, M. 2013. Macrophage plasticity and polarization in tissue repair and remodelling. *Journal of Pathology,* 229**,** 176-185.
MANTOVANI, A. & LOCATI, M. 2013. Tumor-Associated Macrophages as a Paradigm of Macrophage Plasticity, Diversity, and Polarization: Lessons and Open Questions. *Arteriosclerosis Thrombosis and Vascular Biology,* 33**,** 1478-1483.
MARSH, T., PIETRAS, K. & MCALLISTER, S. S. 2013. Fibroblasts as architects of cancer pathogenesis. *Biochimica Et Biophysica Acta-Molecular Basis of Disease,* 1832**,** 1070-1078.
MARTIN, M., LEFAIX, J. L. & DELANIAN, S. 2000. TGF-beta 1 and radiation fibrosis: A master switch and a specific therapeutic target? *International Journal of Radiation Oncology Biology Physics,* 47**,** 277-290.
MCBRIDE, W. H., CHIANG, C. S., OLSON, J. L., WANG, C. C., HONG, J. H., PAJONK, F., DOUGHERTY, G. J., IWAMOTO, K. S., PERVAN, M. & LIAO, Y. P. 2004. Failla Memorial Lecture - A sense of danger from radiation. *Radiation Research,* 162**,** 1-19.
MEIER, R., MUHLETHALER-MOTTET, A., FLAHAUT, M., COULON, A., FUSCO, C., LOUACHE, F., AUDERSET, K., BOURLOUD, K. B., DAUDIGEOS, E., RUEGG, C., VASSAL, G., GROSS, N. & JOSEPH, J. M. 2007. The Chemokine Receptor CXCR4 Strongly Promotes Neuroblastoma Primary Tumour and Metastatic Growth, but not Invasion. *Plos One,* 2.
MELZER, C., YANG, Y. Y. & HASS, R. 2016. Interaction of MSC with tumor cells. *Cell Communication and Signaling,* 14.
MENDENHALL, W. M., INDELICATO, D. J., SCARBOROUGH, M. T., ZLOTECKI, R. A., GIBBS, C. P., MENDENHALL, N. P., MENDENHALL, C. M. & ENNEKING, W. F. 2009. The Management of Adult Soft Tissue Sarcomas. *American Journal of Clinical Oncology-Cancer Clinical Trials,* 32**,** 436-442.
MENG, E. R., GUO, Z. K., WANG, H. X., JIN, J. D., WANG, J. S., WANG, H., WU, C. & WANG, L. S. 2008. High mobility group box 1 protein inhibits the proliferation of human mesenchymal stem cells and promotes their migration and differentiation along osteoblastic pathway. *Stem Cells and Development,* 17**,** 805-813.
MENON, P. & FISHER, E. A. 2015. Immunostaining of Macrophages, Endothelial Cells, and Smooth Muscle Cells in the Atherosclerotic Mouse Aorta. *Methods in Mouse Atherosclerosis,* 1339**,** 131-148.
MI, Z. Y., BHATTACHARYA, S. D., KIM, V. M., GUO, H. T., TALBOT, L. J. & KUO, P. C. 2011. Osteopontin promotes CCL5-mesenchymal stromal cell-mediated breast cancer metastasis. *Carcinogenesis,* 32**,** 477-487.
MILES, F. L. & SIKES, R. A. 2014. Insidious Changes in Stromal Matrix Fuel Cancer Progression. *Molecular Cancer Research,* 12**,** 297-312.
MILLER, K., WANG, M., GRALOW, J., DICKLER, M., COBLEIGH, M., PEREZ, E. A., SHENKIER, T., CELLA, D. & DAVIDSON, N. E. 2007. Paclitaxel plus bevacizumab versus paclitaxel alone for metastatic breast cancer. *New England Journal of Medicine,* 357**,** 2666-2676.
MILLIAT, F., FRANCOIS, A., ISOIR, M., DEUTSCH, E., TAMARAT, R., TARLET, G., ATFI, A., VALIDIRE, P., BOURHIS, J., SABOURIN, J. C. & BENDERITTER, M. 2006. Influence

of endothelial cells on vascular smooth muscle cells phenotype after irradiation - Implication in radiation-induced vascular damages. *American Journal of Pathology,* 169**,** 1484-1495.
MIOT, H. A. & BRIANEZI, G. 2010. Morphometric analysis of dermal collagen by color clusters segmentation. *Anais Brasileiros De Dermatologia,* 85**,** 361-364.
MISHRA, P. J., HUMENIUK, R., MEDINA, D. J., ALEXE, G., MESIROV, J. P., GANESAN, S., GLOD, J. W. & BANERJEE, D. 2008. Carcinoma-associated fibroblast-like differentiation of human mesenchymal stem cells. *Cancer Research,* 68**,** 4331-4339.
MITU, G. & HIRSCHBERG, R. 2008. Bone morphogenetic protein-7 (BMP7) in chronic kidney disease. *Frontiers in Bioscience,* 13**,** 4726-4739.
MODING, E. J., KASTAN, M. B. & KIRSCH, D. G. 2013. Strategies for optimizing the response of cancer and normal tissues to radiation. *Nature Reviews Drug Discovery,* 12**,** 526-542.
MOELLER, B. J., CAO, Y. T., LI, C. Y. & DEWHIRST, M. W. 2004. Radiation activates HIF-1 to regulate vascular radiosensitivity in tumors: Role of reoxygenation, free radicals, and stress granules. *Cancer Cell,* 5**,** 429-441.
MOISAN, F., FRANCISCO, E. B., BROZOVIC, A., DURAN, G. E., WANG, Y. C., CHATURVEDI, S., SEETHARAM, S., SNYDER, L. A., DOSHI, P. & SIKIC, B. I. 2014. Enhancement of paclitaxel and carboplatin therapies by CCL2 blockade in ovarian cancers. *Molecular Oncology,* 8**,** 1231-1239.
MOON, J. O., WELCH, T. P., GONZALEZ, F. J. & COPPLE, B. L. 2009. Reduced liver fibrosis in hypoxia-inducible factor-1 alpha-deficient mice. *American Journal of Physiology-Gastrointestinal and Liver Physiology,* 296**,** G582-G592.
MOORE, B. B., PAINE, R., CHRISTENSEN, P. J., MOORE, T. A., SITTERDING, S., NGAN, R., WILKE, C. A., KUZIEL, W. A. & TOEWS, G. B. 2001. Protection from pulmonary fibrosis in the absence of CCR2 signaling. *Journal of Immunology,* 167**,** 4368-4377.
MORI, T., KAWARA, S., SHINOZAKI, M., HAYASHI, N., KAKINUMA, T., IGARASHI, A., TAKIGAWA, M., NAKANISHI, T. & TAKEHARA, K. 1999. Role and interaction of connective tissue growth factor with transforming growth factor-beta in persistent fibrosis: A mouse fibrosis model. *Journal of Cellular Physiology,* 181**,** 153-159.
MORIGI, M., IMBERTI, B., ZOJA, C., CORNA, D., TOMASONI, S., ABBATE, M., ROTTOLI, D., ANGIOLETTI, S., BENIGNI, A., PERICO, N., ALISON, M. & REMUZZI, G. 2004. Mesenchymal stem cells are renotropic, helping to repair the kidney and improve function in acute renal failure. *Journal of the American Society of Nephrology,* 15**,** 1794-1804.
MORINAGA, Y., SUGA, Y., EHARA, S., HARADA, K., NIHEI, Y. & SUZUKI, M. 2003. Combination effect of AC-7700, a novel combretastatin A-4 derivative, and cisplatin against murine and human tumors in vivo. *Cancer Science,* 94**,** 200-204.
MORITANI, N. H., KUBOTA, S., NISHIDA, T., KAWAKI, H., KONDO, S., SUGAHARA, T. & TAKIGAWA, M. 2003. Suppressive effect of overexpressed connective tissue growth factor on tumor cell growth in a human oral squamous cell carcinoma-derived cell line. *Cancer Letters,* 192**,** 205-214.
MORRISON, B., CULLEN, D. K. & LAPLACA, M. 2011. In Vitro Models for Biomechanical Studies of Neural Tissues. *Neural Tissue Biomechanics,* 3**,** 247-+.
MOSTACO-GUIDOLIN, L. B., KO, A. C. T., WANG, F., XIANG, B., HEWKO, M., TIAN, G. H., MAJOR, A., SHIOMI, M. & SOWA, M. G. 2013. Collagen morphology and texture analysis: from statistics to classification. *Scientific Reports,* 3.
MOUISEDDINE, M., FRANCOIS, S., SEMONT, A., SACHE, A., ALLENET, B., MATHIEU, N., FRICK, J., THIERRY, D. & CHAPEL, A. 2007. Human mesenchymal stem cells home specifically to radiation-injured tissues in a non-obese diabetes/severe

combined immunodeficiency mouse model. *British Journal of Radiology,* 80**,** S49-S55.
MU, Y. B., GUDEY, S. K. & LANDSTROM, M. 2012. Non-Smad signaling pathways. *Cell and Tissue Research,* 347**,** 11-20.
MURATA, R., SIEMANN, D. W., OVERGAARD, J. & HORSMAN, M. R. 2001. Improved tumor response by combining radiation and the vascular-damaging drug 5,6-dimethylxanthenone-4-acetic acid. *Radiation Research,* 156**,** 503-509.
MURDOCH, C. & LEWIS, C. E. 2005. Macrophage migration and gene expression in response to tumor hypoxia. *International Journal of Cancer,* 117**,** 701-708.
MURDOCH, C., MUTHANA, M., COFFELT, S. B. & LEWIS, C. E. 2008. The role of myeloid cells in the promotion of tumour angiogenesis. *Nature Reviews Cancer,* 8**,** 618-631.
MUZ, B., DE LA PUENTE, P., AZAB, F., GHOBRIAL, I. M. & AZAB, A. K. 2015. Hypoxia Promotes Dissemination and Colonization in New Bone Marrow Niches in Waldenstrom Macroglobulinemia. *Molecular Cancer Research,* 13**,** 263-272.
NAGASAKI, T., HARA, M., NAKANISHI, H., TAKAHASHI, H., SATO, M. & TAKEYAMA, H. 2014. Interleukin-6 released by colon cancer-associated fibroblasts is critical for tumour angiogenesis: anti-interleukin-6 receptor antibody suppressed angiogenesis and inhibited tumour-stroma interaction. *British Journal of Cancer,* 110**,** 469-478.
NAKAMIZO, A., MARINI, F., AMANO, T., KHAN, A., STUDENY, M., GUMIN, J., CHEN, J., HENTSCHEL, S., VECIL, G., DEMBINSKI, J., ANDREEFF, M. & LANG, F. F. 2005. Human bone marrow-derived mesenchymal stem cells in the treatment of gliomas. *Cancer Research,* 65**,** 3307-3318.
NAKAO, A., AFRAKHTE, M., MOREN, A., NAKAYAMA, T., CHRISTIAN, J. L., HEUCHEL, R., ITOH, S., KAWABATA, N., HELDIN, N. E., HELDIN, C. H. & TENDIJKE, P. 1997. Identification of Smad7, a TGF beta-inducible antagonist of TGF-beta signalling. *Nature,* 389**,** 631-635.
NAKERAKANTI, S. S., BUJOR, A. M. & TROJANOWSKA, M. 2011. CCN2 Is Required for the TGF-beta Induced Activation of Smad1-Erk1/2 Signaling Network. *Plos One,* 6.
NEDEAU, A. E., BAUER, R. J., GALLAGHER, K., CHEN, H. Y., LIU, Z. J. & VELAZQUEZ, O. C. 2008. A CXCL5-and bFGF-dependent effect of PDGF-B-activated fibroblasts in promoting trafficking and differentiation of bone marrow-derived mesenchymal stem cells. *Experimental Cell Research,* 314**,** 2176-2186.
NEUSS, S., BECHER, E., WOLTJE, M., TIETZE, L. & JAHNEN-DECHENT, W. 2004. Functional expression of HGF and HGF receptor/c-met in adult human mesenchymal stem cells suggests a role in cell mobilization, tissue repair, and wound healing. *Stem Cells,* 22**,** 405-414.
NG, F., BOUCHER, S., KOH, S., SASTRY, K. S. R., CHASE, L., LAKSHMIPATHY, U., CHOONG, C., YANG, Z., VEMURI, M. C., RAO, M. S. & TANAVDE, V. 2008. PDGF, TGF-beta, and FGF signaling is important for differentiation and growth of mesenchymal stem cells (MSCs): transcriptional profiling can identify markers and signaling pathways important in differentiation of MSCs into adipogenic, chondrogenic, and osteogenic lineages. *Blood,* 112**,** 295-307.
NGUYEN, T. Q., ROESTENBERG, P., VAN NIEUWENHOVEN, F. A., BOVENSCHEN, N., LI, Z., XU, L., OLIVER, N., ATEN, J., JOLES, J. A., VIAL, C., BRANDAN, E., LYONS, K. M. & GOLDSCHMEDING, R. 2008. CTGF Inhibits BMP-7 Signaling in Diabetic Nephropathy. *Journal of the American Society of Nephrology,* 19**,** 2098-2107.
NICOLAY, N. H., SOMMER, E., PEREZ, R. L., WIRKNER, U., BOSTEL, T., HO, A. D., LAHN, M., DEBUS, J., SAFFRICH, R. & HUBER, P. E. 2014. Mesenchymal stem cells are sensitive to treatment with kinase inhibitors and ionizing radiation. *Strahlentherapie Und Onkologie,* 190**,** 1037-1045.

NIKJOO, H., O'NEILL, P., WILSON, W. E. & GOODHEAD, D. T. 2001. Computational approach for determining the spectrum of DNA damage induced by ionizing radiation. *Radiation Research,* 156**,** 577-583.

NOR, J. E., CHRISTENSEN, J., MOONEY, D. J. & POLVERINI, P. J. 1999. Vascular endothelial growth factor (VEGF)-mediated angiogenesis is associated with enhanced endothelial cell survival and induction of Bcl-2 expression. *American Journal of Pathology,* 154**,** 375-384.

O'SULLIVAN, B., DAVIS, A. M., TURCOTTE, R., BELL, R., CATTON, C., CHABOT, P., WUNDER, J., KANDEL, R., GODDARD, K., SADURA, A., PATER, J. & ZEE, B. 2002. Preoperative versus postoperative radiotherapy in soft-tissue sarcoma of the limbs: a randomised trial. *Lancet,* 359**,** 2235-2241.

O'SULLIVAN, B. & LEVIN, W. 2003. Late radiation-related fibrosis: Pathogenesis, manifestations, and current management. *Seminars in Radiation Oncology,* 13**,** 274-289.

OESTMAN, A. & AUGSTEN, M. 2009. Cancer-associated fibroblasts and tumor growth - bystanders turning into key players. *Current Opinion in Genetics & Development,* 19**,** 67-73.

OEZDEMIR, B. C., PENTCHEVA-HOANG, T., CARSTENS, J. L., ZHENG, X., WU, C.-C., SIMPSON, T. R., LAKLAI, H., SUGIMOTO, H., KAHLERT, C., NOVITSKIY, S. V., DE JESUS-ACOSTA, A., SHARMA, P., HEIDARI, P., MAHMOOD, U., CHIN, L., MOSES, H. L., WEAVER, V. M., MAITRA, A., ALLISON, J. P., LEBLEU, V. S. & KALLURI, R. 2014. Depletion of Carcinoma-Associated Fibroblasts and Fibrosis Induces Immunosuppression and Accelerates Pancreas Cancer with Reduced Survival. *Cancer Cell,* 25**,** 719-734.

OKADA, H., KIKUTA, T., KOBAYASHI, T., INOUE, T., KANNO, Y., TAKIGAWA, M., SUGAYA, T., KOPP, J. B. & SUZUKI, H. 2005. Connective tissue growth factor expressed in tubular epithelium plays a pivotal role in renal fibrogenesis. *Journal of the American Society of Nephrology,* 16**,** 133-143.

ONG, V. H., EVANS, L. A., XU, S. W., FISHER, I. B., RAJKUMAR, V., ABRAHAM, D. J., BLACK, C. M. & DENTON, C. P. 2003. Monocyte chemoattractant protein 3 as a mediator of fibrosis - Overexpression in systemic sclerosis and the type 1 tight-skin mouse. *Arthritis and Rheumatism,* 48**,** 1979-1991.

ONOUE, T., UCHIDA, D., BEGUM, N. M., TOMIZUKA, Y., YOSHIDA, H. & SATO, M. 2006. Epithelial-mesenchymal transition induced by the stromal cell-derived factor-1/CXCR4 system in oral squamous cell carcinoma cells. *International Journal of Oncology,* 29**,** 1133-1138.

ORIMO, A., GUPTA, P. B., SGROI, D. C., ARENZANA-SEISDEDOS, F., DELAUNAY, T., NAEEM, R., CAREY, V. J., RICHARDSON, A. L. & WEINBERG, R. A. 2005. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell,* 121**,** 335-348.

OSWALD, J., BOXBERGER, S., JORGENSEN, B., FELDMANN, S., EHNINGER, G., BORNHAUSER, M. & WERNER, C. 2004. Mesenchymal stem cells can be differentiated into endothelial cells in vitro. *Stem Cells,* 22**,** 377-384.

OTSU, K., DAS, S., HOUSER, S. D., QUADRI, S. K., BHATTACHARYA, S. & BHATTACHARYA, J. 2009. Concentration-dependent inhibition of angiogenesis by mesenchymal stem cells. *Blood,* 113**,** 4197-4205.

OWEN, J. B., COIA, L. R. & HANKS, G. E. 1992. RECENT PATTERNS OF GROWTH IN RADIATION-THERAPY FACILITIES IN THE UNITED-STATES - A PATTERNS OF CARE STUDY REPORT. *International Journal of Radiation Oncology Biology Physics,* 24**,** 983-986.

PAGET, S. 1989. PAGET,STEPHEN PAPER REPRODUCED FROM THE LANCET, 1889. *Cancer and Metastasis Reviews,* 8**,** 98-101.

PAPADOPOULOU, A. & KLETSAS, D. 2011. Human lung fibroblasts prematurely senescent after exposure to ionizing radiation enhance the growth of malignant lung epithelial cells in vitro and in vivo. *International Journal of Oncology,* 39**,** 989-999.

PARADA, C., LI, J. Y., IWATA, J., SUZUKI, A. & CHAI, Y. 2013. CTGF Mediates Smad-Dependent Transforming Growth Factor beta Signaling To Regulate Mesenchymal Cell Proliferation during Palate Development. *Molecular and Cellular Biology,* 33**,** 3482-3493.

PARAISO, K. H. T. & SMALLEY, K. S. M. 2013. Fibroblast-mediated drug resistance in cancer. *Biochemical Pharmacology,* 85**,** 1033-1041.

PARK, H. J., GRIFFIN, R. J., HUI, S., LEVITT, S. H. & SONG, C. W. 2012. Radiation-Induced Vascular Damage in Tumors: Implications of Vascular Damage in Ablative Hypofractionated Radiotherapy (SBRT and SRS). *Radiation Research,* 177**,** 311-327.

PARK, J. E., LENTER, M. C., ZIMMERMANN, R. N., GARIN-CHESA, P., OLD, L. J. & RETTIG, W. J. 1999. Fibroblast activation protein, a dual specificity serine protease expressed in reactive human tumor stromal fibroblasts. *Journal of Biological Chemistry,* 274**,** 36505-36512.

PAVLIDES, S., VERA, I., GANDARA, R., SNEDDON, S., PESTELL, R. G., MERCIER, I., MARTINEZ-OUTSCHOORN, U. E., WHITAKER-MENEZES, D., HOWELL, A., SOTGIA, F. & LISANTI, M. P. 2012. Warburg Meets Autophagy: Cancer-Associated Fibroblasts Accelerate Tumor Growth and Metastasis via Oxidative Stress, Mitophagy, and Aerobic Glycolysis. *Antioxidants & Redox Signaling,* 16**,** 1264-1284.

PETRIOLI, R., CORATTI, A., CORREALE, P., D'ANIELLO, C., GRIMALDI, L., TANZINI, G., CIVITELLI, S., MARSILI, S., MESSINESE, S., MARZOCCA, G., PIRTOLI, L. & FRANCINI, G. 2002. Adjuvant epirubicin with or without ifosfamide for adult soft-tissue sarcoma. *American Journal of Clinical Oncology-Cancer Clinical Trials,* 25**,** 468-473.

PHAN, S. H. 2002. The myofibroblast in pulmonary fibrosis. *Chest,* 122**,** 286S-289S.

PHIPPS, K. D., SURETTE, A. P., O'CONNELL, P. A. & WAISMAN, D. M. 2011. Plasminogen Receptor S100A10 Is Essential for the Migration of Tumor-Promoting Macrophages into Tumor Sites. *Cancer Research,* 71**,** 6676-6683.

PIRES, I. M., BENCOKOVA, Z., MILANI, M., FOLKES, L. K., LI, J. L., STRATFORD, M. R., HARRIS, A. L. & HAMMOND, E. M. 2010. Effects of Acute versus Chronic Hypoxia on DNA Damage Responses and Genomic Instability. *Cancer Research,* 70**,** 925-935.

PISTERS, P. W. T., O'SULLIVAN, B. & MAKI, R. G. 2007a. Evidence-based recommendations for local therapy for soft tissue sarcomas. *Journal of Clinical Oncology,* 25**,** 1003-1008.

PISTERS, P. W. T., POLLOCK, R. E., LEWIS, V. O., YASKO, A. W., CORMIER, J. N., RESPONDEK, P. M., FEIG, B. W., HUNT, K. K., LIN, P. P., ZAGARS, G., WEI, C. & BALLO, M. T. 2007b. Long-term results of prospective trial of surgery alone with selective use of radiation for patients with T1 extremity and trunk soft tissue sarcomas. *Annals of Surgery,* 246**,** 675-682.

PITTENGER, M. F., MOSCA, J. D. & MCINTOSH, K. R. 2000. Human mesenchymal stem cells: Progenitor cells for cartilage, bone, fat and stroma. *Lymphoid Organogenesis,* 251**,** 3-11.

PONTE, A. L., MARAIS, E., GALLAY, N., LANGONNE, A., DELORME, B., HERAULT, O., CHARBORD, P. & DOMENECH, J. 2007. The in vitro migration capacity of human bone marrow mesenchymal stem cells: Comparison of chemokine and growth factor chemotactic activities. *Stem Cells,* 25**,** 1737-1745.

POSTLETHWAITE, A. E., SHIGEMITSU, H. & KANANGAT, S. 2004. Cellular origins of fibroblasts: possible implications for organ fibrosis in systemic sclerosis. *Current Opinion in Rheumatology,* 16**,** 733-738.

PRANTL, L., MUEHLBERG, F., NAVONE, N. M., SONG, Y. H., VYKOUKAL, J., LOGOTHETIS, C. J. & ALT, E. U. 2010. Adipose Tissue-Derived Stem Cells Promote Prostate Tumor Growth. *Prostate,* 70**,** 1709-1715.

PROCKOP, D. J. 1997. Marrow stromal cells as steam cells for nonhematopoietic tissues. *Science,* 276**,** 71-74.

PUCHTLER, H. & ISLER, H. 1958. THE EFFECT OF PHOSPHOMOLYBDIC ACID ON THE STAINABILITY OF CONNECTIVE TISSUES BY VARIOUS DYES. *Journal of Histochemistry & Cytochemistry,* 6**,** 265-270.

PUGH, C. W. & RATCLIFFE, P. J. 2003. Regulation of angiogenesis by hypoxia: role of the HIF system. *Nature Medicine,* 9**,** 677-684.

PUTHAWALA, K., HADJIANGELIS, N., JACOBY, S. C., BAYONGAN, E., ZHAO, Z. C., YANG, Z. W., DEVITT, M. L., HORAN, G. S., WEINREB, P. H., LUKASHEV, M. E., VIOLETTE, S. M., GRANT, K. S., COLAROSSI, C., FORMENTI, S. C. & MUNGER, J. S. 2008. Inhibition of integrin alpha v beta 6, an activator of latent transforming growth factor-beta, prevents radiation-induced lung fibrosis. *American Journal of Respiratory and Critical Care Medicine,* 177**,** 82-90.

QIAN, B. Z., LI, J. F., ZHANG, H., KITAMURA, T., ZHANG, J. H., CAMPION, L. R., KAISER, E. A., SNYDER, L. A. & POLLARD, J. W. 2011. CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. *Nature,* 475**,** 222-U129.

QIAO, J. L., LIU, Z. J., YANG, C., GU, L. K. & DENG, D. J. 2016. SRF promotes gastric cancer metastasis through stromal fibroblasts in an SDF1-CXCR4-dependent manner. *Oncotarget,* 7**,** 46088-46099.

QUANTE, M., TU, S. P., TOMITA, H., GONDA, T., WANG, S. S. W., TAKASHI, S., BAIK, G. H., SHIBATA, W., DIPRETE, B., BETZ, K. S., FRIEDMAN, R., VARRO, A., TYCKO, B. & WANG, T. C. 2011. Bone Marrow-Derived Myofibroblasts Contribute to the Mesenchymal Stem Cell Niche and Promote Tumor Growth. *Cancer Cell,* 19**,** 257-272.

RAMOS, C., MONTANO, M., GARCIA-ALVAREZ, J., RUIZ, V., UHAL, B. D., SELMAN, M. & PARDO, A. 2001. Fibroblasts from idiopathic pulmonary fibrosis and normal lungs differ in growth rate, apoptosis, and tissue inhibitor of metalloproteinases expression. *American Journal of Respiratory Cell and Molecular Biology,* 24**,** 591-598.

READ, W. L. & WILLIAMS, F. 2016. Metastatic Alveolar Soft Part Sarcoma Responsive to Pazopanib after Progression through Sunitinib and Bevacizumab: Two Cases. *Case Reports in Oncology,* 9**,** 639-643.

REGE, T. A. & HAGOOD, J. S. 2006. Thy-1 as a regulator of cell-cell and cell-matrix interactions in axon regeneration, apoptosis, adhesion, migration, cancer, and fibrosis. *Faseb Journal,* 20**,** 1045-1054.

REN, G. W., ZHAO, X., WANG, Y., ZHANG, X., CHEN, X. D., XU, C. L., YUAN, Z. R., ROBERTS, A. I., ZHANG, L. Y., ZHENG, B., WEN, T., HAN, Y. Y., RABSON, A. B., TISCHFIELD, J. A., SHAO, C. S. & SHI, Y. F. 2012. CCR2-Dependent Recruitment of Macrophages by Tumor-Educated Mesenchymal Stromal Cells Promotes Tumor Development and Is Mimicked by TNF alpha. *Cell Stem Cell,* 11**,** 812-824.

REZNIKOF.CA, BERTRAM, J. S., BRANKOW, D. W. & HEIDELBE.C 1973. QUANTITATIVE AND QUALITATIVE STUDIES OF CHEMICAL TRANSFORMATION OF CLONED C3H MOUSE EMBRYO CELLS SENSITIVE TO POSTCONFLUENCE INHIBITION OF CELL-DIVISION. *Cancer Research,* 33**,** 3239-3249.

REZZOUG, F., SEELAN, R. S., BHATTACHERJEE, V., GREENE, R. M. & PISANO, M. M. 2011. Chemokine-mediated migration of mesencephalic neural crest cells. *Cytokine,* 56**,** 760-768.

RHEE, K. J., LEE, J. I. & EOM, Y. W. 2015. Mesenchymal Stem Cell-Mediated Effects of Tumor Support or Suppression. *International Journal of Molecular Sciences,* 16**,** 30015-30033.
RIMANN, M. & GRAF-HAUSNER, U. 2012. Synthetic 3D multicellular systems for drug development. *Current Opinion in Biotechnology,* 23**,** 803-809.
RINGE, J., STRASSBURG, S., NEUMANN, K., ENDRES, M., NOTTER, M., BURMESTER, G. R., KAPS, C. & SITTINGER, M. 2007. Towards in situ tissue repair: Human mesenchymal stem cells express chemokine receptors CXCR1, CXCR2 and CCR2. and migrate upon stimulation with CXCL8 but not CCL2. *Journal of Cellular Biochemistry,* 101**,** 135-146.
RITTER, E., PERRY, A., YU, J., WANG, T., TANG, L. & BIEBERICH, E. 2008. Breast cancer cell-derived fibroblast growth factor 2 and vascular endothelial growth factor are chemoattractants for bone marrow stromal stem cells. *Annals of Surgery,* 247**,** 310-314.
ROBBINS, M. E., BRUNSO-BECHTOLD, J. K., PEIFFER, A. M., TSIEN, C. I., BAILEY, J. E. & MARKS, L. B. 2012. Imaging Radiation-Induced Normal Tissue Injury. *Radiation Research,* 177**,** 449-466.
ROBERTS, A. B. 1998. Molecular and cell biology of TGF-beta. *Mineral and Electrolyte Metabolism,* 24**,** 111-119.
ROBERTS, A. B., TIAN, F., BYFIELD, S. D., STUELTEN, C., OOSHIMA, A., SAIKA, S. & FLANDERS, K. C. 2006. Smad3 is key to TGF-beta-mediated epithelial-to-mesenchymal transition, fibrosis, tumor suppression and metastasis. *Cytokine & Growth Factor Reviews,* 17**,** 19-27.
RODIER, F., COPPE, J. P., PATIL, C. K., HOEIJMAKERS, W. A. M., MUNOZ, D. P., RAZA, S. R., FREUND, A., CAMPEAU, E., DAVALOS, A. R. & CAMPISI, J. 2009. Persistent DNA damage signalling triggers senescence-associated inflammatory cytokine secretion (vol 11, pg 973, 2009). *Nature Cell Biology,* 11**,** 1272-1272.
ROHWER, N. & CRAMER, T. 2011. Hypoxia-mediated drug resistance: Novel insights on the functional interaction of HIFs and cell death pathways. *Drug Resistance Updates,* 14**,** 191-201.
ROY, R., YANG, J. & MOSES, M. A. 2009. Matrix Metalloproteinases As Novel Biomarkers and Potential Therapeutic Targets in Human Cancer. *Journal of Clinical Oncology,* 27**,** 5287-5297.
RUNDHAUG, J. E. 2003. Matrix metalloproteinases, angiogenesis, and cancer - Commentary re: A. C. Lockhart et al., reduction of wound angiogenesis in patients treated with BMS-275291, a broad spectrum matrix metalloproteinase inhibitor. Clin. Cancer Res., 9: 00-00, 2003. *Clinical Cancer Research,* 9**,** 551-554.
RUSTER, B., GRACE, B., SEITZ, O., SEIFRIED, E. & HENSCHLER, R. 2005. Induction and detection of human mesenchymal stem cell migration in the 48-well reusable transwell assay. *Stem Cells and Development,* 14**,** 231-235.
RUTKOWSKI, P., KAMINSKA, J., KOWALSKA, M., RUKA, W. & STEFFEN, J. 2002. Cytokine serum levels in soft tissue sarcoma patients: Correlations with clinico-pathological features and prognosis. *International Journal of Cancer,* 100**,** 463-471.
SAALBACH, A., ANDEREGG, U., BRUNS, M., SCHNABEL, E., HERRMANN, K. & HAUSTEIN, U. F. 1996. Novel fibroblast specific monoclonal antibodies: Properties and specificities. *Journal of Investigative Dermatology,* 106**,** 1314-1319.
SAALBACH, A., WETZIG, T., HAUSTEIN, U. F. & ANDEREGG, U. 1999. Detection of human soluble Thy-1 in serum by ELISA - Fibroblasts and activated endothelial cells are a possible source of soluble Thy-1 in serum. *Cell and Tissue Research,* 298**,** 307-315.

SALEH, F. A., WHYTE, M., ASHTON, P. & GENEVER, P. G. 2011. Regulation of Mesenchymal Stem Cell Activity by Endothelial Cells. *Stem Cells and Development,* 20**,** 391-403.
SANDLER, A., GRAY, R., PERRY, M. C., BRAHMER, J., SCHILLER, J. H., DOWLATI, A., LILENBAUM, R. & JOHNSON, D. H. 2006. Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. *New England Journal of Medicine,* 355**,** 2542-2550.
SATHYANARAYANAN, V. & NEELAPU, S. S. 2015. Cancer immunotherapy: Strategies for personalization and combinatorial approaches. *Molecular Oncology,* 9**,** 2043-2053.
SCHAFER, M. J., WHITE, T. A., IIJIMA, K., HAAK, A. J., LIGRESTI, G., ATKINSON, E. J., OBERG, A. L., BIRCH, J., SALMONOWICZ, H., ZHU, Y., MAZULA, D. L., BROOKS, R. W., FUHRMANN-STROISSNIGG, H., PIRTSKHALAVA, T., PRAKASH, Y. S., TCHKONIA, T., ROBBINS, P. D., AUBRY, M. C., PASSOS, J. F., KIRKLAND, J. L., TSCHUMPERLIN, D. J., KITA, H. & LEBRASSEUR, N. K. 2017. Cellular senescence mediates fibrotic pulmonary disease. *Nature Communications,* 8.
SCHFFOEFFSKI, P., CHAWLA, S., MAKI, R. G., ITALIANO, A., GELDERBLOM, H., CHOY, E., GRIGNANI, G., CAMARGO, V., BAUER, S., RHA, S. Y., BLAY, J. Y., HOHENBERGER, P., D'ADAMO, D., GUO, M., CHMIELOWSKI, B., LE CESNE, A., DEMETRI, G. D. & PATEL, S. R. 2016. Eribulin versus dacarbazine in previously treated patients with advanced liposarcoma or leiomyosarcoma: a randomised, open-label, multicentre, phase 3 trial. *Lancet,* 387**,** 1629-1637.
SCHIOPPA, T., URANCHIMEG, B., SACCANI, A., BISWAS, S. K., DONI, A., RAPISARDA, A., BERNASCONI, S., SACCANI, S., NEBULONI, M., VAGO, L., MANTOVANI, A., MELILLO, G. & SICA, A. 2003. Regulation of the chemokine receptor CXCR4 by hypoxia. *Journal of Experimental Medicine,* 198**,** 1391-1402.
SCHITO, L., REY, S., TAFANI, M., ZHANG, H. F., WONG, C. C. L., RUSSO, A., RUSSO, M. A. & SEMENZA, G. L. 2012. Hypoxia-inducible factor 1-dependent expression of platelet-derived growth factor B promotes lymphatic metastasis of hypoxic breast cancer cells. *Proceedings of the National Academy of Sciences of the United States of America,* 109**,** E2707-E2716.
SCHMIDT, A., LADAGE, D., SCHINKOTHE, T., KLAUSMANN, U., ULRICHSA, C., KLINZ, F. J., BRIXIUS, K., ARNHOLD, S., DESAI, B., MEHLHORN, U., SCHWINGER, R. H. G., STAIB, P., ADDICKS, K. & BLOCH, W. 2006. Basic fibroblast growth factor controls migration in human mesenchymal stem cells. *Stem Cells,* 24**,** 1750-1758.
SCHMIDT, T. & CARMELIET, P. 2010. BLOOD-VESSEL FORMATION Bridges that guide and unite. *Nature,* 465**,** 697-699.
SCHRODER, S., KRIESEN, S., PAAPE, D., HILDEBRANDT, G. & MANDA, K. 2018. Modulation of Inflammatory Reactions by Low-Dose Ionizing Radiation: Cytokine Release of Murine Endothelial Cells Is Dependent on Culture Conditions. *Journal of Immunology Research*.
SECCHIERO, P., ZORZET, S., TRIPODO, C., CORALLINI, F., MELLONI, E., CARUSO, L., BOSCO, R., INGRAO, S., ZAVAN, B. & ZAULI, G. 2010. Human Bone Marrow Mesenchymal Stem Cells Display Anti-Cancer Activity in SCID Mice Bearing Disseminated Non-Hodgkin's Lymphoma Xenografts. *Plos One,* 5.
SEMENZA, G. L. 2000. HIF-1: mediator of physiological and pathophysiological responses to hypoxia. *Journal of Applied Physiology,* 88**,** 1474-1480.
SEMENZA, G. L. & WANG, G. L. 1992. A NUCLEAR FACTOR INDUCED BY HYPOXIA VIA DENOVO PROTEIN-SYNTHESIS BINDS TO THE HUMAN ERYTHROPOIETIN GENE ENHANCER AT A SITE REQUIRED FOR TRANSCRIPTIONAL ACTIVATION. *Molecular and Cellular Biology,* 12**,** 5447-5454.

SERVAIS, C. & EREZ, N. 2013. From sentinel cells to inflammatory culprits: cancer-associated fibroblasts in tumour-related inflammation. *Journal of Pathology,* 229**,** 198-207.
SHAKUNAGA, T., OZAKI, T., OHARA, N., ASAUMI, K., DOI, T., NISHIDA, K., KAWAI, A., NAKANISHI, T., TAKIGAWA, M. & INOUE, H. 2000. Expression of connective tissue growth factor in cartilaginous tumors. *Cancer,* 89**,** 1466-1473.
SHARON, W. W. & WEISS, S. W. 2008. *Enzinger and Weiss' soft tissue tumors,* St. Louis, Mo.], St. Louis, Mo. : Mosby Elsevier, 2008.
SHEN, B., LIU, J., ZHANG, F., WANG, Y., QIN, Y., ZHOU, Z. H., QIU, J. X. & FAN, Y. 2016. CCR2 Positive Exosome Released by Mesenchymal Stem Cells Suppresses Macrophage Functions and Alleviates Ischemia/Reperfusion-Induced Renal Injury. *Stem Cells International*.
SHI-WEN, X., STANTON, L. A., KENNEDY, L., PALA, D., CHEN, Y. L., HOWAT, S. L., RENZONI, E. A., CARTER, D. E., BOU-GHARIOS, G., STRATTON, R. J., PEARSON, J. D., BEIER, F., LYONS, K. M., BLACK, C. M., ABRAHAM, D. J. & LEASK, A. 2006. CCN2 is necessary for adhesive responses to transforming growth factor-beta 1 in embryonic fibroblasts. *Journal of Biological Chemistry,* 281**,** 10715-10726.
SHIBUYA, M. & CLAESSON-WELSH, L. 2006. Signal transduction by VEGF receptors in regulation of angiogenesis and lymphangiogenesis. *Experimental Cell Research,* 312**,** 549-560.
SHIMO, T., NAKANISHI, T., NISHIDA, T., ASANO, R., KANYAMA, M., KUBOKI, T., TAMATANI, T., TEZUKA, K., TAKEMURA, M., MATSUMURA, T. & TAKIGAWA, M. 1999. Connective tissue growth factor induces the proliferation, migration, and tube formation of vascular endothelial cells in vitro, and angiogenesis in vivo. *Journal of Biochemistry,* 126**,** 137-145.
SHINAGAWA, K., KITADAI, Y., TANAKA, M., SUMIDA, T., KODAMA, M., HIGASHI, Y., TANAKA, S., YASUI, W. & CHAYAMA, K. 2010. Mesenchymal stem cells enhance growth and metastasis of colon cancer. *International Journal of Cancer,* 127**,** 2323-2333.
SICA, A. & MANTOVANI, A. 2012. Macrophage plasticity and polarization: in vivo veritas. *Journal of Clinical Investigation,* 122**,** 787-795.
SIEMANN, D. W., BIBBY, M. C., DARK, G. G., DICKER, A. P., ESKENS, F., HORSMAN, M. R., MARME, D. & LORUSSO, P. M. 2005. Differentiation and definition of vascular-targeted therapies. *Clinical Cancer Research,* 11**,** 416-420.
SIME, P. J. & O'REILLY, K. M. A. 2001. Fibrosis of the lung and other tissues: New concepts in pathogenesis and treatment. *Clinical Immunology,* 99**,** 308-319.
SIME, P. J., XING, Z., GRAHAM, F. L., CSAKY, K. G. & GAULDIE, J. 1997. Adenovector-mediated gene transfer of active transforming growth factor-beta 1 induces prolonged severe fibrosis in rat lung. *Journal of Clinical Investigation,* 100**,** 768-776.
SINGH, S., KLOSS, F. R., BRUNAUER, R., SCHIMKE, M., JAMNIG, A., GREIDERER-KLEINLERCHER, B., KLIMA, G., RENTENBERGER, J., AUBERGER, T., HACHL, O., RASSE, M., GASSNER, R. & LEPPERDINGER, G. 2012. Mesenchymal stem cells show radioresistance in vivo. *Journal of Cellular and Molecular Medicine,* 16**,** 877-887.
SINGH, S., SRIVASTAVA, S. K., BHARDWAJ, A., OWEN, L. B. & SINGH, A. P. 2010. CXCL12-CXCR4 signalling axis confers gemcitabine resistance to pancreatic cancer cells: a novel target for therapy. *British Journal of Cancer,* 103**,** 1671-1679.
SMITH, J. A., WILSON, L., AZARENKO, O., ZHU, X. J., LEWIS, B. M., LITTLEFIELD, B. A. & JORDAN, M. A. 2010. Eribulin Binds at Microtubule Ends to a Single Site on Tubulin To Suppress Dynamic Instability. *Biochemistry,* 49**,** 1331-1337.
SMITH, P. K., KROHN, R. I., HERMANSON, G. T., MALLIA, A. K., GARTNER, F. H., PROVENZANO, M. D., FUJIMOTO, E. K., GOEKE, N. M., OLSON, B. J. & KLENK, D. C.

1985. MEASUREMENT OF PROTEIN USING BICINCHONINIC ACID. *Analytical Biochemistry,* 150**,** 76-85.
SOLESVIK, O. V., ROFSTAD, E. K. & BRUSTAD, T. 1984. VASCULAR CHANGES IN A HUMAN-MALIGNANT MELANOMA XENOGRAFT FOLLOWING SINGLE-DOSE IRRADIATION. *Radiation Research,* 98**,** 115-128.
SOLINAS, G., GERMANO, G., MANTOVANI, A. & ALLAVENA, P. 2009. Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation. *Journal of Leukocyte Biology,* 86**,** 1065-1073.
SONG, C. W., LEE, Y. J., GRIFFIN, R. J., PARK, I., KOONCE, N. A., HUI, S., KIM, M. S., DUSENBERY, K. E., SPERDUTO, P. W. & CHO, L. C. 2015. Indirect Tumor Cell Death After High-Dose Hypofractionated Irradiation: Implications for Stereotactic Body Radiation Therapy and Stereotactic Radiation Surgery. *International Journal of Radiation Oncology Biology Physics,* 93**,** 166-172.
SONG, H. Y., LEE, M. J., KIM, M. Y., KIM, K. H., LEE, I. H., SHIN, S. H., LEE, J. S. & KIM, J. H. 2010. Lysophosphatidic acid mediates migration of human mesenchymal stem cells stimulated by synovial fluid of patients with rheumatoid arthritis. *Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids,* 1801**,** 23-30.
SONVEAUX, P., BROUET, A., HAVAUX, X., GREGOIRE, V., DESSY, C., BALLIGAND, J. L. & FERON, O. 2003. Irradiation-induced angiogenesis through the up-regulation of the nitric oxide pathway: Implications for tumor radiotherapy. *Cancer Research,* 63**,** 1012-1019.
SOON, P. S. H., KIM, E., PON, C. K., GILL, A. J., MOORE, K., SPILLANE, A. J., BENN, D. E. & BAXTER, R. C. 2013. Breast cancer-associated fibroblasts induce epithelial-to-mesenchymal transition in breast cancer cells. *Endocrine-Related Cancer,* 20**,** 1-12.
SORDI, V., MALOSIO, M. L., MARCHESI, F., MERCALLI, A., MELZI, R., GIORDANO, T., BELMONTE, N., FERRARI, G., LEONE, B. E., BERTUZZI, F., ZERBINI, G., ALLAVENA, P., BONIFACIO, E. & PIEMONTI, L. 2005. Bone marrow mesenchymal stem cells express a restricted set of functionally active chemokine receptors capable of promoting migration to pancreatic islets. *Blood,* 106**,** 419-427.
SORRENTINO, A., FERRACIN, M., CASTELLI, G., BIFFONI, M., TOMASELLI, G., BAIOCCHI, M., FATICA, A., NEGRINI, M., PESCHLE, C. & VALTIERI, M. 2008. Isolation and characterization of CD146(+) multipotent mesenchymal stromal cells. *Experimental Hematology,* 36**,** 1035-1046.
SPERK, E., WELZEL, G., KELLER, A., KRAUS-TIEFENBACHER, U., GERHARDT, A., SUETTERLIN, M. & WENZ, F. 2012. Late radiation toxicity after intraoperative radiotherapy (IORT) for breast cancer: results from the randomized phase III trial TARGIT A. *Breast Cancer Research and Treatment,* 135**,** 253-260.
STACCHIOTTI, S., NEGRI, T., ZAFFARONI, N., PALASSINI, E., MOROSI, C., BRICH, S., CONCA, E., BOZZI, F., CASSINELLI, G., GRONCHI, A., CASALI, P. G. & PILOTTI, S. 2011. Sunitinib in advanced alveolar soft part sarcoma: evidence of a direct antitumor effect. *Annals of Oncology,* 22**,** 1682-1690.
STEEG, P. S. 2006. Tumor metastasis: mechanistic insights and clinical challenges. *Nature Medicine,* 12**,** 895-904.
STETLERSTEVENSON, W. G., AZNAVOORIAN, S. & LIOTTA, L. A. 1993. TUMOR-CELL INTERACTIONS WITH THE EXTRACELLULAR-MATRIX DURING INVASION AND METASTASIS. *Annual Review of Cell Biology,* 9**,** 541-573.
SUGIMOTO, H., MUNDEL, T. M., KIERAN, M. W. & KALLURI, R. 2006. Identification of fibroblast heterogeneity in the tumor microenvironment. *Cancer Biology & Therapy,* 5**,** 1640-1646.
SUGRUE, T., LOWNDES, N. F. & CEREDIG, R. 2014. Hypoxia Enhances the Radioresistance of Mouse Mesenchymal Stromal Cells. *Stem Cells,* 32**,** 2188-2200.

SULTAN, I., CASANOVA, M., AL-JUMAILY, U., MEAZZA, C., RODRIGUEZ-GALINDO, C. & FERRARI, A. 2010. Soft tissue sarcomas in the first year of life. *European Journal of Cancer,* 46**,** 2449-2456.
SUN, Y. 2015. Translational Horizons in the Tumor Microenvironment: Harnessing Breakthroughs and Targeting Cures. *Medicinal Research Reviews,* 35**,** 408-436.
SUN, Z., WANG, S. H. & ZHAO, R. C. 2014. The roles of mesenchymal stem cells in tumor inflammatory microenvironment. *Journal of Hematology & Oncology,* 7.
SUNG, B., JHURANI, S., AHN, K. S., MASTUO, Y., YI, T. F., GUHA, S., LIU, M. Y. & AGGARWAL, B. B. 2008. Zerumbone Down-regulates Chemokine Receptor CXCR4 Expression Leading to Inhibition of CXCL12-Induced Invasion of Breast and Pancreatic Tumor Cells. *Cancer Research,* 68**,** 8938-8944.
SUTHERLAND, R. M. 1998. Tumor hypoxia and gene expression - Implications for malignant progression and therapy. *Acta Oncologica,* 37**,** 567-574.
SUZUKI, K., SUN, R. W., ORIGUCHI, M., KANEHIRA, M., TAKAHATA, T., ITOH, J., UMEZAWA, A., KIJIMA, H., FUKUDA, S. & SAIJO, Y. 2011. Mesenchymal Stromal Cells Promote Tumor Growth through the Enhancement of Neovascularization. *Molecular Medicine,* 17**,** 579-587.
TACHIIRI, S., KATAGIRI, T., TSUNODA, T., OYA, N., HIRAOKA, M. & NAKAMURA, Y. 2006. Analysis of gene-expression profiles after gamma irradiation of normal human fibroblasts. *International Journal of Radiation Oncology Biology Physics,* 64**,** 272-279.
TADDEI, M. L., GIANNONI, E., COMITO, G. & CHIARUGI, P. 2013. Microenvironment and tumor cell plasticity: An easy way out. *Cancer Letters,* 341**,** 80-96.
TAKANO, T., LI, Y. J., KUKITA, A., YAMAZA, T., AYUKAWA, Y., MORIYAMA, K., UEHARA, N., NOMIYAMA, H., KOYANO, K. & KUKITA, T. 2014. Mesenchymal stem cells markedly suppress inflammatory bone destruction in rats with adjuvant-induced arthritis. *Laboratory Investigation,* 94**,** 286-296.
TANG, H. D., QIAO, J. & FU, Y. X. 2016. Immunotherapy and tumor microenvironment. *Cancer Letters,* 370**,** 85-90.
THIERY, J. P. & SLEEMAN, J. P. 2006. Complex networks orchestrate epithelial-mesenchymal transitions. *Nature Reviews Molecular Cell Biology,* 7**,** 131-142.
THORPE, P. E. 2004. Vascular targeting agents as cancer therapeutics. *Clinical Cancer Research,* 10**,** 415-427.
TOKUDA, A., ITAKURA, M., ONAI, N., KIMURA, H., KURIYAMA, T. & MATSUSHIMA, K. 2000. Pivotal role of CCR1-positive leukocytes in bleomycin-induced lung fibrosis in mice. *Journal of Immunology,* 164**,** 2745-2751.
TOMCHUCK, S. L., ZWEZDARYK, K. J., COFFELT, S. B., WATERMAN, R. S., DANKA, E. S. & SCANDURRO, A. B. 2008. Toll-like receptors on human mesenchymal stem cells drive their migration and immunomodulating responses. *Stem Cells,* 26**,** 99-107.
TOMULEASA, C., SORITAU, O., BRIE, I., PALL, E., FORIS, V., DICU, T., VIRAG, P., IRIMIE, A. & KACSO, G. 2010. Mesenchymal stem cell irradiation in culture engages differential effect of hyperfractionated radiotherapy for head and neck cancers. *Journal of Buon,* 15**,** 348-356.
TORRES, M. A., BALLO, M. T., BUTLER, C. E., FEIG, B. W., CORMIER, J. N., LEWIS, V. O., POLLOCK, R. E., PISTERS, P. W. & ZAGARS, G. K. 2007. Management of locally recurrent soft-tissue sarcoma after prior surgery and radiation therapy. *International Journal of Radiation Oncology Biology Physics,* 67**,** 1124-1129.
TOZER, G. M., AKERMAN, S., CROSS, N. A., BARBER, P. R., BJORNDAHL, M. A., GRECO, O., HARRIS, S., HILL, S. A., HONESS, D. J., IRESON, C. R., PETTYJOHN, K. L., PRISE, V. E., REYES-ALDASORO, C. C., RUHRBERG, C., SHIMA, D. T. & KANTHOU, C. 2008. Blood vessel maturation and response to vascular-disrupting therapy in single vascular endothelial growth factor-A isoform-producing tumors. *Cancer Res,* 68**,** 2301-11.

TOZER, G. M., PRISE, V. E., WILSON, J., CEMAZAR, M., SHAN, S. Q., DEWHIRST, M. W., BARBER, P. R., VOJNOVIC, B. & CHAPLIN, D. J. 2001. Mechanisms associated with tumor vascular shut-down induced by combretastatin A-4 phosphate: Intravital microscopy and measurement of vascular permeability. *Cancer Research,* 61**,** 6413-6422.

TRIPATHI, C., TEWARI, B. N., KANCHAN, R. K., BAGHEL, K. S., NAUTIYAL, N., SHRIVASTAVA, R., KAUR, H., BHATT, M. L. B. & BHADAURIA, S. 2014. Macrophages are recruited to hypoxic tumor areas and acquire a Pro-Angiogenic M2-Polarized phenotype via hypoxic cancer cell derived cytokines Oncostatin M and Eotaxin. *Oncotarget,* 5**,** 5350-5368.

TSUYADA, A., CHOW, A., WU, J., SOMLO, G., CHU, P. G., LOERA, S., LUU, T. H., LI, A. X. J., WU, X. W., YE, W., CHEN, S. A., ZHOU, W. Y., YU, Y., WANG, Y. Z., REN, X. B., LI, H., SCHERLE, P., KUROKI, Y. & WANG, S. E. 2012. CCL2 Mediates Cross-talk between Cancer Cells and Stromal Fibroblasts That Regulates Breast Cancer Stem Cells. *Cancer Research,* 72**,** 2768-2779.

UCCELLI, A., MORETTA, L. & PISTOIA, V. 2008. Mesenchymal stem cells in health and disease. *Nature Reviews Immunology,* 8**,** 726-736.

VALA, I. S., MARTINS, L. R., IMAIZUMI, N., NUNES, R. J., RINO, J., KUONEN, F., CARVALHO, L. M., RUEGG, C., GRILLO, I. M., BARATA, J. T., MAREEL, M. & SANTOS, S. C. R. 2010. Low Doses of Ionizing Radiation Promote Tumor Growth and Metastasis by Enhancing Angiogenesis. *Plos One,* 5.

VALKOVIC, T., DOBRILA, F., MELATO, M., SASSO, F., RIZZARDI, C. & JONJIC, N. 2002. Correlation between vascular endothelial growth factor, angiogenesis, and tumor-associated macrophages in invasive ductal breast carcinoma. *Virchows Archiv,* 440**,** 583-588.

VAN DER GRAAF, W. T. A., BLAY, J.-Y., CHAWLA, S. P., KIM, D.-W., BINH, B.-N., CASALI, P. G., SCHOFFSKI, P., AGLIETTA, M., STADDON, A. P., BEPPU, Y., LE CESNE, A., GELDERBLOM, H., JUDSON, I. R., ARAKI, N., OUALI, M., MARREAUD, S., HODGE, R., DEWJI, M. R., COENS, C., DEMETRI, G. D., FLETCHER, C. D., TOS, A. P. D., HOHENBERGER, P., TISSUE, E. S. & BONE SARCOMA, G. 2012. Pazopanib for metastatic soft-tissue sarcoma (PALETTE): a randomised, double-blind, placebo-controlled phase 3 trial. *Lancet,* 379**,** 1879-1886.

VANDEN BERG-FOELS, W. S. 2014. In Situ Tissue Regeneration: Chemoattractants for Endogenous Stem Cell Recruitment. *Tissue Engineering Part B-Reviews,* 20**,** 28-39.

VAUPEL, P. 2004. Tumor microenvironmental physiology and its implications for radiation oncology. *Seminars in Radiation Oncology,* 14**,** 198-206.

VAUPEL, P. & HARRISON, L. 2004. Tumor hypoxia: Causative factors, compensatory mechanisms, and cellular response. *Oncologist,* 9**,** 4-9.

VELARDE, M. C., DEMARIA, M. & CAMPISI, J. 2013. Senescent Cells and Their Secretory Phenotype as Targets for Cancer Therapy. *Cancer and Aging: from Bench to Clinics,* 38**,** 17-27.

VERRECCHIA, F. & MAUVIEL, A. 2002. Transforming growth factor-beta signaling through the Smad pathway: Role in extracellular matrix gene expression and regulation. *Journal of Investigative Dermatology,* 118**,** 211-215.

VERRECCHIA, F. & MAUVIEL, A. 2007. Transforming growth factor-beta and fibrosis. *World Journal of Gastroenterology,* 13**,** 3056-3062.

VIRTANEN, A., PUKKALA, E. & AUVINEN, A. 2006. Incidence of bone and soft tissue sarcoma after radiotherapy: A cohort study of 295,712 Finnish cancer patients. *International Journal of Cancer,* 118**,** 1017-1021.

VRANCEANU, A. R., TARNICERIU, C. C., JITARU, D., TERINTE, C., ZUGUN-ELOAE, F. & CARASEVICI, E. 2014. Optimisation of the quantitative analysis of inflammatory

cell infiltrates in breast cancer. *Revista Romana De Medicina De Laborator,* 22**,** 335-345.
WACHSBERGER, P. & BURD, R. 2004. Improving tumor response to radiotherapy by targeting angiogenesis signaling pathways. *Hematology-Oncology Clinics of North America,* 18**,** 1039-+.
WAHAB, N. A. & MASON, R. M. 2006. A critical look at growth factors and epithelial-to-mesenchymal transition in the adult kidney - Interrelationships between growth factors that regulate EMT in the adult kidney. *Nephron Experimental Nephrology,* 104**,** E129-e134.
WALSH, S., JEFFERISS, C., STEWART, K., JORDAN, G. R., SCREEN, J. & BERESFORD, J. N. 2000. Expression of the developmental markers STRO-1 and alkaline phosphatase in cultures of human marrow stromal cells: Regulation by fibroblast growth factor (FGF)-2 and relationship to the expression of FGF receptors 1-4. *Bone,* 27**,** 185-195.
WANG, G. L., JIANG, B. H., RUE, E. A. & SEMENZA, G. L. 1995. HYPOXIA-INDUCIBLE FACTOR-1 IS A BASIC-HELIX-LOOP-HELIX-PAS HETERODIMER REGULATED BY CELLULAR O-2 TENSION. *Proceedings of the National Academy of Sciences of the United States of America,* 92**,** 5510-5514.
WANG, J. R., ZHENG, H. E., SUNG, C. C., RICHTER, K. K. & HAUER-JENSEN, M. 1998. Cellular sources of transforming growth factor-beta isoforms in early and chronic radiation enteropathy. *American Journal of Pathology,* 153**,** 1531-1540.
WANG, L., LI, Y., GAUTAM, S. C., ZHANG, Z. G., LU, M. & CHOPP, M. 2002. Ischemic cerebral tissue and MCP-1 enhance rat bone marrow stromal cell migration in interface culture. *Experimental Hematology,* 30**,** 831-836.
WANG, L., ZHENG, W., ZHANG, S., CHEN, X. & HORNUNG, D. 2006. Expression of monocyte chemotactic protein-1 in human endometrial cancer cells and the effect of treatment with tamoxifen or buserelin. *Journal of International Medical Research,* 34**,** 284-290.
WANG, L. M., CAO, L. M., WANG, H. M., LIU, B. N., ZHANG, Q. C., MENG, Z. W., WU, X., ZHOU, Q. H. & XU, K. 2017. Cancer-associated fibroblasts enhance metastatic potential of lung cancer cells through IL-6/STAT3 signaling pathway. *Oncotarget,* 8**,** 76116-76128.
WANG, Y., LIU, L. B., PAZHANISAMY, S. K., LI, H. L., MENG, A. & ZHOU, D. H. 2010. Total body irradiation causes residual bone marrow injury by induction of persistent oxidative stress in murine hematopoietic stem cells. *Free Radical Biology and Medicine,* 48**,** 348-356.
WANG, Y., LIU, L. B. & ZHOU, D. H. 2011. Inhibition of p38 MAPK Attenuates Ionizing Radiation-Induced Hematopoietic Cell Senescence and Residual Bone Marrow Injury. *Radiation Research,* 176**,** 743-752.
WANG, Y. Y., BOERMA, M. & ZHOU, D. H. 2016. Ionizing Radiation-Induced Endothelial Cell Senescence and Cardiovascular Diseases. *Radiation Research,* 186**,** 153-161.
WARTERS, R. L. & HOFER, K. G. 1977. RADIONUCLIDE TOXICITY IN CULTURED MAMMALIAN-CELLS - ELUCIDATION OF PRIMARY SITE FOR RADIATION-INDUCED DIVISION DELAY. *Radiation Research,* 69**,** 348-358.
WATT, F. M. & HOGAN, B. L. M. 2000. Out of Eden: Stem cells and their niches. *Science,* 287**,** 1427-1430.
WEEKES, C. D., SONG, D. W., ARCAROLI, J., WILSON, L. A., RUBIO-VIQUEIRA, B., CUSATIS, G., GARRETT-MAYER, E., MESSERSMITH, W. A., WINN, R. A. & HIDALGO, M. 2012. Stromal Cell-Derived Factor 1 alpha Mediates Resistance to mTOR-Directed Therapy in Pancreatic Cancer. *Neoplasia,* 14**,** 690-+.
WENGER, C., ELLENRIEDER, V., ALBER, B., LACHER, U., MENKE, A., HAMEISTER, H., WILDA, M., IWAMURA, T., BEGER, H. G., ADLER, G. & GRESS, T. M. 1999.

Expression and differential regulation of connective tissue growth factor in pancreatic cancer cells. *Oncogene,* 18**,** 1073-1080.
WESTBURY, C. B., HAVILAND, J., DAVIES, S., GOTHARD, L., ABDI, B. A., SYDENHAM, M., BOWEN, J., STRATTON, R., SHORT, S. C. & YARNOLD, J. R. 2014. Cytokine levels as biomarkers of radiation fibrosis in patients treated with breast radiotherapy. *Radiation Oncology,* 9.
WESTBURY, C. B. & YARNOLD, J. R. 2012. Radiation Fibrosis - Current Clinical and Therapeutic Perspectives. *Clinical Oncology,* 24**,** 657-672.
WILLIAMS, K. J., TELFER, B. A., DIA, X., SHERIDAN, M. R., DESBAILLETS, I., PETERS, H. J. W., HONESS, D., HARRIS, A. L., DACHS, G. U., VAN DER KOGEL, A. & STRATFORD, I. J. 2005. Enhanced response to radiotherapy in tumours deficient in the function of hypoxia-inducible factor-1. *Radiotherapy and Oncology,* 75**,** 89-98.
WIRTZ, D., KONSTANTOPOULOS, K. & SEARSON, P. C. 2011. The physics of cancer: the role of physical interactions and mechanical forces in metastasis. *Nature Reviews Cancer,* 11**,** 512-522.
WOLL, P. J., REICHARDT, P., LE CESNE, A., BONVALOT, S., AZZARELLI, A., HOEKSTRA, H. J., LEAHY, M., VAN COEVORDEN, F., VERWEIJ, J., HOGENDOORN, P. C. W., OUALI, M., MARREAUD, S., BRAMWELL, V. H. C., HOHENBERGER, P., GRP, E. S. T. B. S. & D, N. C. T. G. S. 2012. Adjuvant chemotherapy with doxorubicin, ifosfamide, and lenograstim for resected soft-tissue sarcoma (EORTC 62931): a multicentre randomised controlled trial. *Lancet Oncology,* 13**,** 1045-1054.
WONG, P., HOUGHTON, P., KIRSCH, D. G., FINKELSTEIN, S. E., MONJAZEB, A. M., XU-WELLIVER, M., DICKER, A. P., AHMED, M., VIKRAM, B., TEICHER, B. A., COLEMAN, C. N., MACHTAY, M., CURRAN, W. J. & WANG, D. 2014. Combining Targeted Agents With Modern Radiotherapy in Soft Tissue Sarcomas. *Jnci-Journal of the National Cancer Institute,* 106.
WU, G. D., NOLTA, J. A., JIN, Y. S., BARR, M. L., YU, H., STARNES, V. A. & CRAMER, D. V. 2003. Migration of mesenchymal stem cells to heart allografts during chronic rejection. *Transplantation,* 75**,** 679-685.
WU, S., JU, G. Q., DU, T., ZHU, Y. J. & LIU, G. H. 2013. Microvesicles Derived from Human Umbilical Cord Wharton's Jelly Mesenchymal Stem Cells Attenuate Bladder Tumor Cell Growth In Vitro and In Vivo. *Plos One,* 8.
WYNN, T. A. 2008. Cellular and molecular mechanisms of fibrosis. *Journal of Pathology,* 214**,** 199-210.
XIAO, C. Y., WANG, R. H., LAHUSEN, T. J., PARK, O., BERTOLA, A., MARUYAMA, T., REYNOLDS, D., CHEN, Q., XU, X. L., YOUNG, H. A., CHEN, W. J., GAO, B. & DENG, C. X. 2012. Progression of Chronic Liver Inflammation and Fibrosis Driven by Activation of c-JUN Signaling in Sirt6 Mutant Mice. *Journal of Biological Chemistry,* 287**,** 41903-41913.
XING, F., SAIDOU, J. & WATABE, K. 2010. Cancer associated fibroblasts (CAFs) in tumor microenvironment. *Frontiers in Bioscience-Landmark,* 15**,** 166-179.
XU, J., LAMOUILLE, S. & DERYNCK, R. 2009. TGF-beta-induced epithelial to mesenchymal transition. *Cell Research,* 19**,** 156-172.
YANG, H. W., HUANG, Y. G., CHEN, X. L., LIU, J., LU, Y., BU, L. M., XIA, L. K., XIAO, W., CHEN, M., NIE, Q. Z. & LIU, Z. L. 2010. The role of CTGF in the diabetic rat retina and its relationship with VEGF and TGF-beta(2), elucidated by treatment with CTGFsiRNA. *Acta Ophthalmologica,* 88**,** 652-659.
YANG, J. C., CHANG, A. E., BAKER, A. R., SINDELAR, W. F., DANFORTH, D. N., TOPALIAN, S. L., DELANEY, T., GLATSTEIN, E., STEINBERG, S. M., MERINO, M. J. & ROSENBERG, S. A. 1998. Randomized prospective study of the benefit of adjuvant radiation therapy in the treatment of soft tissue sarcomas of the extremity. *Journal of Clinical Oncology,* 16**,** 197-203.

YANG, Y. Y., BUCAN, V., BAEHRE, H., VON DER OHE, J., OTTE, A. & HASS, R. 2015a. Acquisition of new tumor cell properties by MSC-derived exosomes. *International Journal of Oncology,* 47**,** 244-252.
YANG, Y. Y., OTTE, A. & HASS, R. 2015b. Human Mesenchymal Stroma/Stem Cells Exchange Membrane Proteins and Alter Functionality During Interaction with Different Tumor Cell Lines. *Stem Cells and Development,* 24**,** 1205-1222.
YARNOLD, J. & BROTONS, M.-C. V. 2010. Pathogenetic mechanisms in radiation fibrosis. *Radiotherapy and Oncology,* 97**,** 149-161.
YUAN, X. N., ENG, G. M., ARKONAC, D. E., CHAO, P. H. G. & VUNJAK-NOVAKOVIC, G. 2015. Endothelial Cells Enhance the Migration of Bovine Meniscus Cells. *Arthritis & Rheumatology,* 67**,** 182-192.
YUAN, Y., LU, X., TAO, C. L., CHEN, X., SHAO, H. W. & HUANG, S. L. 2013. Forced expression of indoleamine-2,3-dioxygenase in human umbilical cord-derived mesenchymal stem cells abolishes their anti-apoptotic effect on leukemia cell lines in vitro. *In Vitro Cellular & Developmental Biology-Animal,* 49**,** 752-758.
ZAGARS, G. K. & BALLO, M. T. 2003. Significance of dose in postoperative radiotherapy for soft tissue sarcoma. *International Journal of Radiation Oncology Biology Physics,* 56**,** 473-481.
ZAGARS, G. K., BALLO, M. T., PISTERS, P. W. T., POLLOCK, R. E., PATEL, S. R. & BENJAMIN, R. S. 2003. Preoperative vs. postoperative radiation therapy for soft tissue sarcoma: A retrospective comparative evaluation of disease outcome. *International Journal of Radiation Oncology Biology Physics,* 56**,** 482-488.
ZEISBERG, E. M., TARNAVSKI, O., ZEISBERG, M., DORFMAN, A. L., MCMULLEN, J. R., GUSTAFSSON, E., CHANDRAKER, A., YUAN, X. L., PU, W. T., ROBERTS, A. B., NEILSON, E. G., SAYEGH, M. H., IZUMO, S. & KALLURI, R. 2007a. Endothelial-to-mesenchymal transition contributes to cardiac fibrosis. *Nature Medicine,* 13**,** 952-961.
ZEISBERG, M., YANG, C. Q., MARTINO, M., DUNCAN, M. B., RIEDER, F., TANJORE, H. & KALLURI, R. 2007b. Fibroblasts derive from hepatocytes in liver fibrosis via epithelial to mesenchymal transition. *Journal of Biological Chemistry,* 282**,** 23337-23347.
ZENG, Q. H., LI, S. L., CHEPEHA, D. B., GIORDANO, T. J., LI, J., ZHANG, H. L., POLVERINI, P. J., NOR, J., KITAJEWSKI, J. & WANG, C. Y. 2005. Crosstalk between tumor and endothelial cells promotes tumor angiogenesis by MAPK activation of Notch signaling. *Cancer Cell,* 8**,** 13-23.
ZHANG, J. & LIU, J. S. 2013. Tumor stroma as targets for cancer therapy. *Pharmacology & Therapeutics,* 137**,** 200-215.
ZHANG, J., PATEL, L. & PIENTA, K. J. 2010. CC chemokine ligand 2 (CCL2) promotes prostate cancer tumorigenesis and metastasis. *Cytokine & Growth Factor Reviews,* 21**,** 41-48.
ZHANG, K., REKHTER, M. D., GORDON, D. & PHAN, S. H. 1994. MYOFIBROBLASTS AND THEIR ROLE IN LUNG COLLAGEN GENE-EXPRESSION DURING PULMONARY FIBROSIS - A COMBINED IMMUNOHISTOCHEMICAL AND IN-SITU HYBRIDIZATION STUDY. *American Journal of Pathology,* 145**,** 114-125.
ZHANG, Y. E. 2009. Non-Smad pathways in TGF-beta signaling. *Cell Research,* 19**,** 128-139.
ZHOU, T. B., QIN, Y. H., LEI, F. Y., HUANG, W. F. & DRUMMEN, G. P. C. 2013. Prohibitin Attenuates Oxidative Stress and Extracellular Matrix Accumulation in Renal Interstitial Fibrosis Disease. *Plos One,* 8.
ZVAIFLER, N. J. 2006. Relevance of the stroma and epithelial-mesenchymal transition (EMT) for the rheumatic diseases. *Arthritis Research & Therapy,* 8.