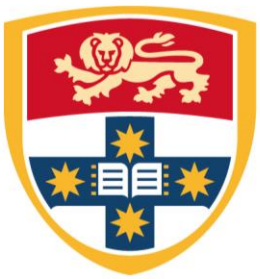

THE UNIVERSITY OF SYDNEY

Complex Interactions Between Osteosarcoma Cells and Fibroblasts

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A thesis submitted in fulfilment of the

requirements for the degree of Doctor of Philosophy

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Declaration

The work presented in this thesis is wholly the work of Manu Susan David and was performed in the Cellular and Molecular Pathology Research Unit, Department of Oral Pathology and Oral Medicine, Faculty of Dentistry at the Westmead Centre for Oral Health, between August 2007 and August 2011 towards the degree of Doctor of Philosophy in Dentistry, The University of Sydney. To the best knowledge of the student, this work is original and has not been published by other workers unless stated.

Signature

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List of Abbrevations

ArKr	Argon Krypton
ATCC	American Type Culture Collection
BCS	Bovine Calf Serum
BMDC	Bone Marrow Derived Cells
BMF	Bone Marrow Fibroblasts
BSA	Bovine Serum Albumin
CAF	Carcinoma Associated Fibroblasts
CAM	Cell Adhesion Molecule
CCL2	Cystein-Cystein Chemokine Ligand 2
CFSE	5-(6)-Carboxyfluorescein Diacetate Succinimidyl Ester
CLSM	Confocal Laser Scanning Microscopy
CM	Complete Media
CO_2	Carbondioxide
CXCL12	Cystein-X-Cystein Ligand 12
Cy5	Cyanine Dye 5
Cyst-hsc70	Cytosolic Heat Shock Cognate70
DDAO-SE	7-hydroxy-9*H* (I, 3-dichloro-9, 9-dimethylacridin-2-one) succinimidyl ester
DiD	1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindodicarbocyanine Perchlorate
DiO	3,3'-Dioctadecyloxacarbocyanine Perchlorate
DMSO	Dimethyl Sulfoxide

DNA	Deoxyribonucleic Acid
DU-145	Human Prostate Cancer Cell Line
ECGS	Endothelial Cell Growth Supplement
ECM	Extracellular Matrix
EDTA	Ethylene Diamine Tetraacetic Acid
eff	Epithelial Fusion Failure
ELAM	Endothelial Leukocyte Adhesion Molecule
ELISA	Enzyme Linked Immunoassay
EMT	Epithelial Mesenchymal Transition
FACS	Fluorescence Activated Cell Sorting
FAP	Fibroblast Activation Protein
FGF	Fibroblast Growth Factor
FITC	Fluorescence Isothiocyanate
FM	Fluorescence Microscopy
FRP	Fusion Regulatory Protein
Fsp	Fibroblast Specific Protein
G-418	Geneticin
G-CSF	Granulocyte Colony Stimulating Factor
GFP	Green Fluorescent Protein
GLTA	Glutaraldehyde
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
HaCaT	Human Keratinocyte Cell Line
HBSS	Hanks Balanced Salt Solution
Hcl	Hydrochloric Acid

HeLa	Human Cervical Cancer Cell Line
HeNe	Helium Neon
HGF	Human Gingival Fibroblasts
HIV-1 Env	Human Immunodeficiency Virus Type-1 Envelope
HL-60	Human Promyelocytic Leukemia Cells
HLA	Human Leukocyte Antigen
HREC	Human Research Ethics Committee
HUASMC	Human Umbilical Artery Smooth Muscle Cells
HUVEC	Human Umbilical Vein Endothelial Cells
I-B	Nuclear Factor Kappa B Inhibitor
ICAM	Intercellular Adhesion Molecule
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
LC3	Microtubule Associated Protein1 Light Chain 3
LK-63	Acute Lymphoblastic Leukemic Cell Lines
LNCap	Human Prostate Cancer Cell Line
LPS	Lipopolysaccharide
M199	Medium 199
MALM6	Acute Lymphoblastic Leukemic Cell Lines
MAPK	Mitogen Activated Protein Kinase
MCF-7	Human Breast Cancer Cell Line
M-CSF	Macrophage Colony Stimulating Factor
MethA	Sarcoma Cell Line

MHC	Major Histocompatibilty Complex
MMP	Matrix Metalloproteinase
MNNG	Human Osteosarcoma Cells
MOLT-4	Human Acute Lymphoblastic Leukemia Cell Line
N_2	Nitrogen
NaOH	Sodium Hydroxide
NCAM	Neural Cell Adhesion Molecule
NF-κB	Nuclear Factor Kappa B
NSF	N-Ethylmelemide Sensitive Fusion
OST	Human Osteosarcoma Cells
PBS	Phosphate Buffered Saline
PC-3	Human Prostate Cancer Cell Line
PDGF	Platelet Derived Growth Factor
PFA	Paraformaldehyde
PGE	Prostaglandin E
REH	Acute Lymphoblastic Leukemic Cell Lines
RNA	Ribonucleic Acid
RPMI 1640	Roswell Park Memorial Institute Medium 1640
RT	Room Temperature
SAOS-GFP	Green Fluorescent Protein Expressing Osteosarcoma Cells
SAOS-2	Human Osteosarcoma Cell Line
SNAP	Soluble Nsf Attachment Protein
SNARE	Soluble Nsf Attachment Protein Receptors
SODD	Silencer Of Death Domains

Syto 59	Nuclear Marker
TACE	Tumour Necrosis Factor Alpha Converting Enzyme
TE-2	Oesophageal Squamous Carcinoma Cells
TGF	Transforming Growth Factor
Tipα	Tumour Necrosis Factor Alpha Inducing Protein
TNF	Tumour Necrosis Factor
TNF-αR	Tumour Necrosis Factor Alpha Receptor
TRADD	Tumour Necrosis Factor Alpha Receptor Associated Death Domain
TRITC	Tetramethyl Rhodamine Isothiocyanate
U-937	Human Leukemic Monocyte Lymphoma Cell Line
UEA-1	*Ulex Europaeus* Lectin-1
VCAM	Vascular Cell Adhesion Molecule
VEGF	Vascular Endothelial Growth Factor
α-SMA	Alpha Smooth Muscle Actin

Summary

This thesis investigates interactions between neoplastic cells in tumours, and the stromal-host cells with which the neoplastic cells must cooperate in order to survive. Earlier work upon which this thesis is based, established contact dependent induction of apoptosis in endothelium by SAOS-2 osteosarcoma cells (McEwen et al., 2003). This was quantitated by detecting a reduction in endothelial cell culture density (McEwen et al., 2003), but when similar experiments were performed with human gingival fibroblasts (HGF), no apoptosis was detectable despite there being an apparent reduction in HGF cell culture density (Huynh, 2007). This thesis confirms that the seeming disappearance of HGF co-cultured with SAOS-2 can be accounted for by transfer of SAOS-2 membrane marker to HGF, and explores some aspects of how the microenvironment determines specific cellular interactions between neoplastic and stromal cells.

Chapter 1 summarizes background literature relevant to the work described in the remaining thesis. Chapter 2 investigates the necessary first step of cell adhesion in interaction between SAOS-2 and HGF. An adhesion assay was established in which SAOS-2 are applied to monolayers of HGF, and gently washed prior to cell counts. SAOS-2 binding to HGF was markedly increased by pre-treatment of HGF with TNF-α in a dose dependent manner, and as was seen in Chapter 3, this was found to be mediated by ICAM-1 expressed by HGF. Surprisingly, SAOS-2 pre-treated with TNF-α had reduced binding to TNF-α treated HGF, indicating that for this interaction, SAOS-2 must have a 'permissive cellular history' in which they have not been stimulated by TNF-α, and this was recognized as having implications regarding similar binding events in-vivo, where

neoplastic cells at the invading front of tumours migrate from one micro-environment to another.

Chapter 3 examines the effect of TNF-α pre-treatment upon the ability of SAOS-2 to reduce the apparent culture density of HGF. Reduction in the apparent culture density of HGF was seen in broad proportion to the increased binding of SAOS-2 to HGF mediated by TNF-α. Also, work in this chapter excluded the possible contribution of autophagy to loss of HGF in co-culture with SAOS-2.

Chapter 4 describes studies in which HGF and SAOS-2 were pre-labelled with a range of fluorescent markers for plasma membranes, cytoplasm and nuclear material. Co-culture of these cells was accompanied by the appearance of dual labelled cells, while quantitative analysis was performed to assess patterns of label exchange across cell types. Exchange of both plasma membrane and cytoplasmic markers was observed in these cultures, although plasma membrane marker exchange was more prevalent as judged by the proportion of dual labelled cells seen. No cells with both nuclear labels were found. The direction of label exchange was strongly influenced by the specific labels used in given experiments, so that it was not possible be certain whether bulk transfer of membrane or cytoplasm had occurred. Nonetheless, it was possible to conclude with reasonable confidence that continuity in plasma membranes and cytoplasm was established between HGF and SAOS-2, sufficient for the exchange of significant quantities of labelled proteins in the case of cytoplasm, as well as for membrane embedded alkaline phosphatase from SAOS-2 to HGF and lipophilic membrane dyes across both cell types. This 'cellular sipping' by SAOS-2, was suggested as a potential mechanism overcoming the effects of a progressively deranged neoplastic genome. It was also recognized that the generation of

populations of cells expressing proteins of mixed origin would increase tumour diversity, with potential to affect tumour progression. In addition, quantitative morphological assessment was performed of cell circularity and cell surface area profile, as an indirect measure for phenotypic state, and the finding that dual labelled cells expressed morphological properties intermediate to HGF and SAOS-2 suggested phenotypic impact of cellular sipping. The influence of TNF-α was also studied in this chapter, which was found to increase the extent of cellular sipping.

In Chapter 5, the effect of co-culture of SAOS-2 upon HGF pre-treated with TNF-α with regard to cytokine synthesis was examined in both: direct co-culture; and also in co-cultures where SAOS-2 and HGF were separated by transwell membranes. SAOS-2 made negligible contribution to levels of any of the cytokines studied (IL-6, GM-CSF, G-CSF, FGF). However, SAOS-2 did increase production by TNF-α pre-treated HGF of IL-6, GM-CSF, and G-CSF in transwell co-cultures, despite having the opposite effect in direct co-cultures where SAOS-2 were in intimate contact with HGF.

In summary, results in this thesis demonstrate that SAOS-2 and HGF interact intimately, through the establishment of continuity between plasma membranes and cytoplasm, and that the resulting transfer of membrane and cytoplasmic material produces four ultimate populations of cells in co-culture being: HGF; SAOS-2; HGF with SAOS-2 membrane and cytoplasmic elements; and SAOS-2 with HGF cytoplasmic and membrane elements. Furthermore, a role for TNF-α in facilitating HGF and SAOS-2 binding via ICAM-1, as well as in mediating exchange of material between cells is demonstrated. The complexity of these interactions is underscored by the opposing effect upon SAOS-2 binding to HGF of pre-treatment of either cell type with TNF-α, as well as by the opposite cytokine

synthetic profile of HGF co-cultured in direct contact with SAOS-2, as opposed to separated by a transwell membrane. These observations support the idea of there being 'permissive microenvironments' for a range of interactions between stromal and neoplastic cells, as well as the idea of a 'permissive cellular history' for some responses. The paracrine and contact dependent interactions identified support subversion of stromal cell function in support of neoplastic cells. Also, cellular sipping by neoplastic cells seems to provide a novel mechanism through which malignant neoplastic cells with irreparably damaged genomes may be able to gain survival advantage by capturing normal cytoplasmic and membrane elements from surrounding stromal cells. In addition, findings relating to permissive microenvironments, permissive cellular histories, and the generation of complex cell populations through cellular sipping, provide further explanation of architectural and cytological pleomorphism in malignant neoplasms, till now attributed primarily to the emergence of divergent sub-clones of neoplastic cells through the accumulation of genetic lesions.

Chapter 1: General Introduction

1.1 CANCER, INVASION, METASTASIS AND TUMOUR STROMA

The latin term ‘tumour’, means quite literally ‘swelling’, and although originally used to describe any cause of swelling including inflammation, it has now become convention for this word to apply only to neoplasms. Neoplasms are discrete lesions arising as poorly controlled growths within the individual, and are understood as being clonal proliferations of cells arising through accumulation of somatic mutations (Nowell, 1978, Nowell, 1976, Kumar et al., 2005).

Neoplasms are broadly classified into two main categories, being benign or malignant, such that benign neoplasms remain localized to their site of origin, while malignant neoplasms have a clinically spreading behaviour. In benign neoplasms, local growth is by expansion such that adjacent tissues are pushed aside, with remnant connective tissues forming a well defined capsule about the benign lesion.

Malignant neoplasms are quite different in that there is invasion of surrounding tissues by cells, and this results in steady destruction and replacement of normal tissues by malignant tumour mass. In metastasis, this locally spreading behaviour is extended to invasion into lymphatic or blood vessels, followed by carriage of malignant cells to distant sites in lymph or blood. Malignant cells spread in this way may be able to establish new tumour masses which comprise metastatic lesions (Kumar et al., 2005). The term ‘cancer’, refers only to malignant neoplasms, and is derived from the way in which the margins of invasive malignancies extend like adherent crab pincers into adjacent normal tissue, to create the

clinical sign of tumour ‘fixation’ to surrounding tissue. This is notably opposed to the ‘mobile’ clinical presentation of encapsulated benign neoplasms (Kumar et al., 2005).

Malignant neoplasms are currently thought to develop as a consequence of three distinct processes: initiation, promotion and progression (Kumar et al., 2005, Braun and Anderson, 2007). Firstly, normal cells experience an initial genetic lesion in a tumour associated gene, and in so doing are considered to be ‘initiated’. Few initiated cells actually develop into neoplasms, because a single genetic injury is typically insufficient for tumour development. However, if initiated cells are driven to proliferate, usually together with uninitiated neighbouring cells, the emergence of a clone of cells bearing the initial genetic defect together with the potential for DNA copy error during cell division, significantly increases the likelihood of initiated cells accumulating still further genetic injuries. Carcinogenesis is thus promoted by general cell proliferation, and it is from this that the term ‘tumour promotion’ is derived. Tumour promotion has clear clinical significance, and accounts for the high incidence of malignancy in cells repeatedly driven to proliferate such as in the breast, prostate and colon. The increased opportunity for accumulation of still further genetic lesions afforded by tumour promotion may result in the final stage of carcinogenesis which is ‘tumour progression’, in which the emergence of clones with ever more genetic lesions leads firstly to benign established neoplasms, and eventually to malignancy and a run-away loss of differentiated behaviour (Braun and Anderson, 2007, Yokota and Sugimura, 1993, Ruddon, 2007, Kumar et al., 2005).

Development of neoplasms is understood to depend upon establishment of a tumour vasculature through angiogenesis, as well as: evasion of the immune system; growth autonomy; phenotypic variation; adhesion; intercellular communication; and the secretion

of cytokines and enzymes, while this is enabled by different molecular mechanisms that act in cohesion enabling tumour survival, growth and progression (Kumar et al., 2005, De Wever and Mareel, 2003, Tlsty and Coussens, 2006, Witz, 2008, Wernert, 1997). In order to implement effective treatment, a more complete knowledge and understanding of both invasion and metastasis at the molecular level seems required.

It is important for this thesis, to appreciate the distinction between tumour parenchymal cells, which are the actual clonal neoplastic cells bearing genetic lesions, and the supporting tumour stromal cells. Stromal cells are coopted by the parenchymal cells to support tumour growth by providing the necessary extracellular matrix and vascular supply. Unlike the parenchymal tumour cells, stromal cells do not bear genetic lesions or proliferate in an uncontrolled clonal manner, but are instead enslaved by neoplastic cells to the benefit of tumour growth. The stromal response includes angiogenesis, which is widely accepted as a requirement for neoplasms to become established (Hanahan and Weinberg, 2000, Folkman et al., 1989, Kumar et al., 2005), while in addition there is also often a prominent fibrotic stromal response described as ‘desmoplasia’ (De Wever and Mareel, 2003, Kiaris et al., 2004, Mueller and Fusenig, 2004, Tlsty, 2001, Kumar et al., 2005). Stromal cell interactions with parenchymal neoplastic cells occur not only at the interface between surrounding normal tissues and invading malignant or expanding benign neoplasms, but also within the tumour mass itself. Despite the importance of tumour stroma in supporting and maintaining neoplastic parenchymal cells, the nature of interactions between these two cell populations is poorly understood, and this thesis addresses some aspects of these complex interactions.

1.1.1 Mechanisms in Invasion and Metastasis

Invasion and metastasis involve a cascade of processes with an intricate interplay of biochemical and biophysical interactions between the tumour and stromal cells. Malignant cells must first separate from the primary tumour and this process may occasionally be in some ways passive, such that tumour cells simply 'break away' from the tumour mass, although more usually there is an active process involving directed migration (Condeelis and Segall, 2003, Wyckoff et al., 2004, Kumar et al., 2005). Depending on the source tissue, tumour cells can migrate singly as is seen in many fibrosarcomas, or collectively in groups as in most carcinomas and melanomas (Kumar et al., 2005, Friedl and Wolf, 2003). There is a tendency for invasion to proceed increasingly by single tumour cells with time, presumably due to the accumulation of progressively more genetic lesions and the ensuing loss of cellular coordination (Thiery, 2002). For a tumour to spread and invade into the surrounding tissue the tumour cells actively lead the invasion and migratory process while the stromal cells within the tumour environment passively contribute by producing growth factors, cytokines and proteases that aid in degradation of the ECM. Besides exhibiting a supporting role, it has recently been demonstrated that stromal cells can actively be involved during tumour invasion, for example squamous carcinoma cells follow carcinoma associated fibroblasts as they take the leading edge during invasion as they remodel the matrix and make a path for the cells (Gaggioli, 2008, Gaggioli et al., 2007).

A range of cell surface proteins contribute to invasion and metastasis, while the lack of regulation or dysfunction of adhesion and other proteins may help account for detachment of cells from the primary tumour mass (Kumar et al., 2005, Hanahan and Weinberg, 2000). With the loss or down regulation of adhesion proteins such as cadherins and catenins, individual tumour cells become more motile, and this facilitates dissemination. Loss of

cell-cell contact likely plays a role in altered tissue architecture together with altered intra and intercellular signalling favouring tumour cell migration. E-cadherin is an adhesion molecule that exhibits homotypic binding and thus opposes invasion and metastasis by tethering adjacent tumour cells together (Behrens et al., 1992, Hanahan and Weinberg, 2000). While E-cadherin expression is lost in many epithelial malignancies, E-cadherin negative clones of TE-2 oesophageal cancer cells demonstrate a consistent level of cell detachment compared with E-cadherin positive clones, indicating the importance of regulated expression of one such adhesion molecule (Behrens et al., 1992).

Detachment of malignant cells from the tumour mass requires that they encroach through any basement membrane that may be present, into the extracellular matrix (ECM). Expression of lytic enzymes such as proteases by both the tumour parenchymal cells and supporting stromal cells, facilitates degradation of the extra cellular matrix as part of the invasive process. Matrix Metalloproteinases (MMP) are a broad group of lytic enzymes capable of degrading many ECM proteins, and are associated with increased invasiveness in breast, ovarian, lung, prostrate and colon cancers (Hagemann et al., 2004, Crawford and Matrisian, 1994, Westerlund et al., 1997, Stetler-Stevenson et al., 1993, Mueller, 1996). Serine proteinases also play a role, and there is extensive literature describing altered production of plasminogen activators, plasminogen activator inhibitors, and receptors for plasminogen activators in malignant cells (Mueller, 1996, Dano et al., 2005, Andreasen et al., 2000, Pappot et al., 1995, Ulisse et al., 2010). Similarly, hyaluronidase and heparinase have been demonstrated as increased in some malignant cell lines, consistent with a role in invasion and metastasis (Kovar et al., 2006, Delpech et al., 2002, Tamakoshi et al., 1997, Yang et al., 2005, Tang et al., 2002).

In metastasis, invading neoplastic cells penetrate either the lymphatic or vascular endothelium, to be carried to a distant site by blood or lymph (Morgan-Parkes, 1995, Bohle and Kalthoff, 1999, Woodhouse et al., 1997). Adhesive molecules are thought to play an important role at this stage, by assisting in specific interaction between the endothelium and parenchymal tumour cells. Increased expression of adhesion molecules such as Vascular Cell Adhesion Molecule -1 (VCAM-1) and Intercellular Adhesion Molecule -1 (ICAM-1) is associated with increased adhesiveness and invasiveness of tumour cells including colon carcinoma and melanoma cells (Zhu et al., 2002, Yanase et al., 1995).

For neoplasms to grow and metastasize, angiogenesis resulting in the formation of new blood vessels is required (Folkman, 1971, Folkman et al., 1989, Kumar et al., 2005). Avascularised tumours are reported to be limited to a maximum size of 1 to 2 mm in diameter, this size being determined by the maximum diffusion distance between surrounding vasculature and inner tumour mass, consistent with neoplastic cell survival (Kerbel, 2000), so that progression beyond this size requires angiogenesis. Of interest is that the proximal endothelial cells of the sprouting new blood vessels have the capacity to directly stimulate the growth of neoplastic cells (Kumar et al., 2005), while it has also been suggested that vascular channels traversing neoplasms provide the most convenient route for metastasis to distant sites (Woodhouse et al., 1997, Kumar et al., 2005). Malignant cells surviving passage through either the lymphatic or blood vasculature may become anchored in the microvascular beds of distant tissues, and if able to establish viable tumour vasculature of their own may develop into clinically significant metastatic tumours.

The tendency for tumour cells to metastasise to specific tissues has been accounted for by two separate main concepts. One view, is often described in terms of there being a 'fertile soil' required for the 'malignant seed', such that some tissues provide a uniquely favourable environment that encourages the adhesion, migration and growth of some specific malignant cells (Paget, 1989, Kumar et al., 2005). Consistent with this model is the tendency of breast and prostate cancers to metastasise to bone. With specific adhesion molecules, infiltrating primary tumour cells can selectively lodge in capillaries and further metastasize and colonise proximal and distant tissues (Chirivi et al., 1994, Daniel et al., 2003, Hosono et al., 1998, Xie et al., 1997, Zetter, 1993).

The second main concept is essentially anatomical, in that there is effective 'filtering out' of malignant cells from the lymph or blood in the first microcirculation encountered by the draining tissues (Kumar et al., 2005). This is supported by the spread of carcinomas along chains of lymph nodes draining primary lesions, as well as by the common appearance of metastasis in the lungs receiving blood from the entire body, and the liver receiving the portal circulation (Morgan-Parkes, 1995, Weiss et al., 1986, Kumar et al., 2005). Both these competing concepts seem supportable on first principles as well as on clinical observation, and the lack of any clear conflict or inconsistency between the two ideas suggests that both mechanisms likely play a role.

1.1.2 Progressive Disruption of Genetic Function with Cancer Progression

The types of genes involved with development of neoplasms include proto-oncogenes, tumour suppressor genes, anti- and pro- apoptotic genes, and genes associated with DNA repair, and while there are a number of heritable syndromes which predispose to a variety

of neoplasms, most cancers are caused by exposure to factors such as radiation, oncogenic viruses and carcinogenic chemicals (Farber, 1984, Weinberg, 1989, Kumar et al., 2005).

Of importance for this thesis, is that the steady accumulation of genetic lesions corresponds with increasing susceptibility for further genetic injury, and this is particularly so when there is failure in the mechanisms responsible for normal chromosome segregation together with development of chromosomal aneuploidy. Failure of chromosomal segregation together with consequent chromosomal: tangling; fragmentation; loss; and acquisition of excess copies of chromosomes in daughter cells, and besides instability occurring at the chromosomal level, instability occurring at the nucleotide level due to faulty DNA repair pathways such as base excision repair and nucleotide excision repair as well as defects of mismatched repair provide a clear pathway through which additional genetic lesions may be acquired over and above those which initiated tumour development (Kumar et al., 2005, Loeb and Loeb, 2000, Albertson et al., 2003, Beckman and Loeb, 2005, Lengauer et al., 1998). Amidst the ensuing genetic chaos, it is clear that the function of many genes will be involved, and it seems reasonable to note that amongst these will be many house keeping genes, quite separate to those genes recognized as contributing to carcinogenesis itself (Waxman and Wurmbach, 2007, Kumar et al., 2005). The progressive loss of cellular control is reflected by the histological features of tumour progression, with increasing variability in cell size and shape coupled with decreasing differentiation of progressively more bizarre malignant cells (Houghton et al., 1987, Brabletz et al., 2005, Kumar et al., 2005).

1.1.3 Complex Parenchymal – Stromal Interactions in the Tumour Microenvironment

As briefly mentioned above, tumours are complex and are comprised of not only the neoplastic parenchymal cells, but also the stromal network containing the extracellular matrix, cells of the connective tissues such as fibroblasts and endothelial cells, as well as cells mediating inflammatory and immune responses. In addition, cytokines and growth factors produced by the diverse populations of stromal cells have opportunity to not only influence behaviour of the stromal elements, but also of parenchymal tumour cells as well (Mueller and Fusenig, 2004, Kiaris et al., 2004, Bhowmick et al., 2004). One effect of this complex intermeshing of parenchymal with stromal cellular elements, is that the predominant but often unexpressed view of tumours in terms of the behaviour of neoplastic cells alone seems insupportable. Instead tumours should perhaps be thought of in terms of their inherently complex multicellularity (Lorusso and Ruegg, 2008).

Tumour cells can initiate or activate changes in stromal cells that result in altered stromal cell phenotype facilitating tumour progression. For example, stromal cells are observed to increase expression of type IV collagenases in response to infiltrating tumour cells, and this is suggested as aiding the degradation of the ECM (Pyke et al., 1992, Pyke et al., 1993). Involvement of stromal cells appears to occur at most stages of tumour invasion and metastasis, with the stroma supporting neoplastic cell proliferation, nutrition via angiogenesis, invasion and metastasis (Mahadevan and Von Hoff, 2007, Stetler-Stevenson et al., 1993, Wernert, 1997, Proia and Kuperwasser, 2005, Tlsty and Hein, 2001).

Similarity has been drawn with chronic inflammatory conditions, where there is also often reactive fibrosis comparable to the activation of stroma associated with solid tumours (Bhowmick et al., 2004, Kalluri and Zeisberg, 2006). As briefly mentioned above, the

initial indication of the involvement of reactive stroma is by manifestation of the "desmoplastic response", which is characterised by increased fibroblast proliferation associated with changes in ECM components and the presence of inflammatory and immune cells (De Wever and Mareel, 2003, Kiaris et al., 2004, Kumar et al., 2005, Mueller and Fusenig, 2004, Tlsty and Hein, 2001). Changes in the stromal microenvironment, including enhanced angiogenesis, altered ECM expression, increased inflammatory cell infiltration, and altered protease activity, have been recognized as elemental regulatory factors for tumour growth and invasion (Bhowmick et al., 2004).

Also briefly mentioned above, was the role of angiogenesis in tumour growth, and this represents perhaps the best characterized of interactions between tumour parenchyma and stroma (Bergers and Benjamin, 2003, Carmeliet and Jain, 2000, Hanahan and Folkman, 1996, Kumar et al., 2005). However, despite the importance of tumour angiogenesis, of significance for this thesis are separate reports indicating that additional to a proliferative angiogenic activity, at least some neoplastic cells including SAOS-2 osteosarcoma cells induce apoptosis in endothelium in a contact dependent manner (Heyder et al., 2002, Holash et al., 1999, Kebers et al., 1998, Chen et al., 2005, McEwen et al., 2003). It has been argued that such contact dependent induction of endothelial apoptosis may contribute to metastasis by facilitating metastatic cell entry to the circulation (McEwen et al., 2003, Lin et al., 2010). Further supporting complex simultaneous pro-angiogenic and apoptotic anti-endothelial activities in-vivo, is that vessels within the tumour mass are usually few in number and structurally defective, when compared with the normal vasculature of the surrounding tissues that have been replaced by malignant tumour mass (Fox et al., 1996, Kumar et al., 2005).

Direct contact of neoplastic epithelial cells with tumour associated fibroblasts induces a change in neoplastic epithelial phenotype to increase invasiveness, proliferation and differentiation (Atula et al., 1997, Tlsty, 2001). Related to this is an increased growth of epithelial neoplastic cells in association with fibroblasts both in vivo as well as in vitro (Camps et al., 1990, Olumi et al., 1999). Soluble growth factors released by fibroblasts augment prostrate cancer cell growth (Gleave et al., 1991), while myofibroblasts induce invasiveness of E-cadherin expressing murine colon cancer cells (Dimanche-Boitrel et al., 1994). A varied effect on the microcirculation of colorectal tumours has been noted upon exposure to different microenvironments (Fukumura et al., 1997). Myofibroblasts at the tumour-stroma interface modify the infiltration of immune cells, bringing about a decrease in, or even prevention of contact between tumour and immune cells considered necessary for immune dependent tumour cell destruction, so that in this context the stroma appears to provide immune protection in aid of tumour survival (Lieubeau et al., 1999). By these varied means, the stroma clearly provides significant support to neoplastic cells, and thus plays a role in carcinogenesis (Tlsty, 2001).

1.1.4 Stromal Fibroblasts in Tumour Stromal Interactions

1.1.4i Fibroblast Function, Heterogeneity and Plasticity

Fibroblasts are a core component of the connective tissue synthesizing the bulk of the ECM and as such play an important role in maintaining structural integrity. They are also an important source of paracrine growth factors and cytokines such as interleukin-6 (IL-6), tumour necrosis factor (TNF) and interferon-gamma (IFN-γ) (Bhowmick et al., 2004, Kalluri and Zeisberg, 2006). In addition, fibroblasts also produce a range of extracellular proteinases and other degradative enzymes, thus playing a central role in tissue remodelling and turnover. Because reparative granulation tissue is essentially a loose

fibrous connective tissue, fibroblasts are also a major cellular constituent in wound healing, while these cells are similarly the principal cell additional to vascular endothelium comprising tumour stroma (Parsonage et al., 2005, Tomasek et al., 2002, Kalluri and Zeisberg, 2006, Orimo and Weinberg, 2006).

Fibroblasts were first described with reference to their spindle shaped morphology embedded in the ECM of connective tissues. These cells do vary with regard to their cell volume, such that in some settings fibroblasts may be described as 'plump' or 'activated', but apart from this fibroblasts are essentially homogeneous in morphology. Despite morphological similarity across different connective tissues, however, fibroblasts derived from different tissues are heterogenous in their basal and responsive behaviour (Chang et al., 2002). Heterogeneity among fibroblasts has been shown by differences in cytokine expression, synthesis of ECM, growth rate, (Kotaru et al., 2006, Sempowski et al., 1996), response to cytokines (Verardi et al., 2007) and adhesion molecule expression (Brannigan et al., 2002).

Unfortunately, unique markers specific for all fibroblasts, as well as from individual specific tissues, have not yet been identified, and one consequence of this is that cultured fibroblasts are defined primarily in terms of their origin and isolation procedure. Nonetheless, there are some markers shared across fibroblasts from several tissue sources as well as with other cell types (Kalluri and Zeisberg, 2006). Fibroblast-specific protein (FSP1), also known as S100/A4, is an intermediate filament-associated calcium binding protein belonging to the S100 superfamily, expressed in multiple types of fibroblasts, and this is currently the most frequently used marker for fibroblasts *in vivo* (Strutz et al., 1995). Other markers include vimentin expressed throughout most mesenchymal cells, α-smooth

muscle actin (α-SMA) also expressed by smooth muscle cells, fibroblast activation protein (FAP), and desmin usually expressed by muscle (Tomasek et al., 2002, Rettig et al., 1993, Ronnov-Jessen et al., 1995). Fibroblasts also demonstrate high plasticity, and in some circumstances can differentiate into other mesodermal cell types, and have even been suggested as capable of acquiring an endothelial phenotype during angiogenesis (Kon and Fujiwara, 1994). Prolonged exposure to T cell extracts has also been reported to reprogram fibroblasts to express functions similar to that of T cells (Hakelien et al., 2002).

1.1.4ii Fibroblasts in the Tumour Microenvironment

As one of the primary cell types in tumour stroma, fibroblasts are likely to play a prominent role in interactions between stromal and parenchymal tumour cells (Kalluri and Zeisberg, 2006, De Wever and Mareel, 2003). Fibroblasts are recognized as contributing to tumour angiogenesis (Orimo et al., 2005), epithelial to mesenchymal transition (EMT) (Tlsty, 2001, Kalluri and Zeisberg, 2006), genetic instability (Kurose et al., 2001, Moinfar et al., 2000) and tumour invasiveness (Westerlund et al., 1997). Collectively, these studies suggest that during the course of tumour progression, tissue-specific fibroblasts undergo significant phenotypic change and contribute towards tumour progression (Kiaris et al., 2004, Olumi et al., 1999, Orimo and Weinberg, 2006, Silzle et al., 2004).

Cancers and wounds have long been described as sharing a large number of molecular characteristics in common. In 1972, Haddow making a functional comparison between wounds and tumours, called a wound a "tumour that heals itself" (Haddow, 1972). Later on, Dvorak making a similar comparison described tumours as wounds that never heal (Dvorak, 1986). Fibroblasts have a regulatory role in wound healing and inflammation, and the ECM secreted by fibroblasts recruited during wound healing acts as a scaffolding

cytoskeleton upon which myofibroblasts mediate wound contraction (Tettamanti et al., 2004). Besides their involvement in wound healing, normal fibroblasts inhibit the growth of some tumour cells including MCF-7 breast cancer cells (Dong-Le Bourhis et al., 1997), recurrent oral squamous cell carcinoma (Rasanen et al., 2009) and did not affect growth of T-antigen transformed epithelial cells (Olumi et al., 1999). Contrasting with this, is that fibroblasts induce epithelial outgrowths when co-engrafted with mammary epithelial organoids (Kuperwasser et al., 2004). Growth inhibitory effects have been associated with soluble factors released by fibroblasts as seen with MCF-7 tumour cells (Adams et al., 1988). But different from the environment of a wound, the tumour microenvironment remains in a state of inflammation, and fibroblasts that are recruited into the expanding stroma are constantly exposed to activation signals. Expression of α-SMA, increased collagen production, and altered growth and proliferation patterns have been found in fibroblasts surrounding tumours, similar to fibroblasts in wound healing, and this has led to the description of such altered fibroblasts as carcinoma associated fibroblasts (CAF). CAF's may promote the growth and spread of tumours such that, initiated epithelial cells can be driven to proliferate in the process of tumour promotion, with consequent tumour progression to malignancy (Olumi et al., 1999, Baglole et al., 2006). Paracrine effects of secreted platelet derived growth factors (PDGF) from non-tumourigenic immortalised human keratinocytes (HaCaT) activated fibroblasts promoting its tumourigenic conversion (Skobe and Fusenig, 1998). Separately, increased expression of MMP-2 by fibroblasts leads to an increase in human ovarian cancer cell invasion (Westerlund et al., 1997). Surprisingly, CAF's do not appear to lose their altered phenotype in the absence of continuous contact with tumour cells, suggesting a permanent change in phenotype that some findings described in the current thesis may partly explain (Orimo and Weinberg, 2006).

1.2 CELL FUSION

Cell fusion is a relatively common biological process contributing significantly to development and differentiation, in which two or more cells merge their plasma membranes to create a hybrid cell, and in which the nuclei may or may not be fused as well (Chen et al., 2007). In the development and regeneration of skeletal muscles, myoblasts fuse to form the multinucleated syncytia of skeletal muscle fibres (Horsley and Pavlath, 2004, Paululat et al., 1999). Osteoclasts responsible for bone resorption, are multinucleated cells formed by macrophage fusion, and defects in osteoclast formation can result in bone abnormalities (Vignery, 2005, Vignery, 2000). Separate to cell fusion proper, membrane fusion is essential to many cellular processes such as intracellular vesicle traffic, exocytosis, endocytosis, and also the entry of enveloped viral particles (Chen and Olson, 2005, Paululat et al., 1999).

Regarding the fate of nuclei in fused cells, there is reference to heterokaryons or synkaryons. Synkaryons are hybrid cells formed after fusion resulting in the genomes of both cells being shared in a single nucleus, whereas herterokaryons are when bi- or multinulcleated cells are formed. Despite the distinction drawn between these two forms of fusion, heterokaryons may over time fuse and form synkaryons. Fusion makes available opportunity not only for transfer of nuclear material between cells, but also for cellular contents between the fusion partners. Proteins from one fusion partner are capable of affecting gene expression in the newly fused cell with the possibility of regression or even disappearance of certain specific cellular properties. Perhaps more reasonably expected however, is maintenance in the resulting fused cell, of properties expressed by both original parental cells (Konieczny et al., 1983, Harris and Watkins, 1965, Ringertz et al.,

1971). Supporting this are observations that hybrid cells arising from fusion of chick erythrocytes with rat myogenic cells express myogenic differentiation across the two species, with chick myosin seen in the otherwise rat skeletal muscle (Carlsson et al., 1974). The incorporation of a chick erythrocyte nucleus into murine cells results in reactivation of RNA synthesis and cytoplasmic protein synthesis (Harris et al., 1969). Regression and eventual disappearance in expression of muscle proteins such as myosin, desmin and creatine kinase upon heterotypic cell fusion of myocytes with cells of non-myogenic lineage was noted (Lawrence and Coleman, 1984). A similar regression of muscle cell function was noted in heterokaryotic hybrids of myocytes and fibroblasts (Wright and Aronoff, 1983).

Much of the current understanding of membrane fusion is based on study of the fusion of enveloped viruses with host cells during infection, where the viral membrane envelope fuses with the target host cell plasma membrane. Viral glycoproteins play a key role in this process, and are broadly recognised as Class I or II viral fusion proteins. Both haemagglutinin and human immunodeficiency virus type-1 (HIV-1) envelope (Env) fusion proteins are well characterized class I proteins (Hernandez et al., 1996, Skehel and Wiley, 2002), while Flavivirus glycoprotein is a well characterised Class II protein (Rey et al., 1995). Separate to viral fusion, are the routine fusion events required for intracellular membrane trafficking, in which soluble NSF attachment protein receptors (SNAREs) play an important role by forming a complex with N-ethylmeleimide sensitive fusion (NSF) proteins and receptor soluble NSF attachment proteins (SNAPs) (Clary et al., 1990, Sollner et al., 1993). Purified SNARE is able to mediate fusion of liposomes, supporting a central role for this protein complex. Calcium ions can either induce or enhance membrane fusion of artificial as well as biological membranes. Calcium ions were demonstrated to be

required for syncytium formation during the process of cell fusion mediated by CD4-human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein interaction which was further confirmed with the inhibition of syncitia formation in the presence of calcium chelators EDTA or EGTA (Dimitrov et al., 1993).

Fusion regulatory protein-1 (FRP-1) and fusion regulatory protein-2 (FRP-2) are essential in regulating molecular mechanisms of fusion in immune cells and virus-cell fusion (Tsurudome and Ito, 2000). The expression of epithelial fusion failure (eff-1) gene drives epidermal cell fusion (Shemer et al., 2004) as seen in *Caenorhabditis elegans* which serves as a convenient model to study cell fusion because one third of their cells fuse to form syncytia (Shemer and Podbilewicz, 2003). Eff-1 is expressed shortly before fusion occurs, and inactivation blocks cell fusion. Ectopic expression of eff-1 is able to induce fusion of epithelial cells with other cells (Podbilewicz et al., 2006, Shemer et al., 2004). Compared to the detailed delineation of intracellular and viral fusion mechanisms, information regarding the mechanism of cell-cell membrane fusion is still limited. Intercellular transfer of proteins via nanotubules that can provide cytoplasmic bridges between cells have been observed as a process representing transient fusion as seen between rat cardiomyocytes and human endothelial progenitor cells (Koyanagi et al., 2005). Nanotubules can either be formed by an actin driven protrusion that can create a connection with a neighbouring cell or by temporary membrane fusion of cells that come in contact for a while and then separate (Davis and Sowinski, 2008). They have also been observed in macrophages, B cells, T cells, human peripheral blood natural killer cells and human embryonic kidney cells (Chauveau et al., 2010, Sowinski et al., 2008, Onfelt et al., 2006, Onfelt et al., 2004) . Another means of intercellular transfer is via secretory exosomes that are membrane bound vesicles containing proteins, lipids and mRNA that once released from cells, interact with

the surrounding cells and can influence the function and physiology of the recipient cell (Thery, 2011, Smalheiser, 2007).

1.2.1 Cell Fusion and Cancer

Fusion provides a mechanism through which neoplastic cells may be able to obtain phenotypic and genotypic diversity creating new cells with new functional properties. The concept of malignant neoplastic cells fusing with stromal cells to produce hybrids with an enhanced metastatic phenotype was first proposed by Aichell in 1911, who demonstrated that fusion hybrids of leukocytes and somatic cells may facilitate tumour formation, while it is separately demonstrated that tumour cell diversity results from the uneven chromosome distribution after fusion (Rachkovsky et al., 1998). Heterokaryons formed upon fusion of human cervical cancer (HeLa) cells with Ehrlich ascitis tumour cells when induced using the Sendai virus (Harris and Watkins, 1965). Heterokaryons formation has been observed in the fusion of macrophages with: melanoma cells (Chakraborty et al., 2001, Rachkovsky et al., 1998, Rupani et al., 2004); colorectal cancer cells; (Powell et al., 2011); and Meth A sarcoma cells (Busund et al., 2003); while breast cancer cells have been seen to form heterokaryons with: host stromal cells (Jacobsen et al., 2006); as well as with bone marrow derived cells (BMDCs); and there is further fusion with both transformed intestinal stem cells (Rizvi et al., 2006) and a mouse tumour cell line (Kerbel et al., 1983). The acquisition of varied phenotypes from parental cells may provide opportunities for aneuploidy (Lu and Kang, 2009, Pawelek, 2000). Fusion events may also assist in tumour evasion of the immune system, through acquisition of normal cell phenotype as seen in sarcoma cells that obtain a phenotype mimicking macrophages with which they are co-cultured (Busund et al., 2002). In addition, fusion may provide a means for chromosomal repair in neoplastic cells, by obtaining unaltered chromosomes from their

host fusion cell partner (Pawelek, 2000, Parris, 2006). Enhancement of metastatic traits (Pawelek and Chakraborty, 2008, Rachkovsky et al., 1998), and the opposite process of recession of malignant properties has also been suggested upon cell fusion (Harris, 1971).

1.3 AUTOPHAGY

Autophagy is a physiologic cellular mechanism involving the degradation of proteins and organelles, and has been observed in organisms as different as yeast and humans (Levine and Yuan, 2005). During this process, cytoplasmic proteins and organelles are sequestered into membrane limiting structures defined as autophagic vacuoles, and are subsequently degraded by lysosomal enzymes (Klionsky and Emr, 2000). Autophagy plays a role in the elimination of damaged proteins and organelles and the amino acids yielded from protein degradation are returned to the amino acid pool so that autophagy may be protective in settings of nutritional deprivation or stress (Klionsky, 2004).

Autophagy is a self limiting and strictly regulated process, and different forms of autophagy having been recognised and broadly categorised into macroautophagy, microautophagy and chaperone mediated autophagy. In macroautophagy, a double or multi- membraned structure begins to form in the cytoplasm forming an autophagosome which engulfs cytoplasmic components and transports them to the lysosome. The outer membrane of the autophagosome fuses with the lysosome and releases the inner membrane bound vesicle. The vesicular contents along with the membrane are then degraded by lysosomal enzymes (Dunn, 1994). In microautophagy, invagination of the lysosomal membrane allows direct engulfing of the cytoplasmic components. These invaginated pouches pinch off to form intravacoular vesicles that are degraded by lysosomal proteases

(Dunn, 1994). In chaperone mediated autophagy, cytosolic proteins in the lysosome are selectively degraded by the use of a chaperone protein cytosolic heat shock cognate70 (Cyst-hsc70), which recognises substrate proteins containing the signal motif KFERQ (Massey et al., 2004).

Autophagy plays a role in the removal of unwanted proteins in neurodegenerative disorders, and also in removal of intracellular pathogens in infectious diseases (Levine and Yuan, 2005). Its role in cancer, however, seems much more complex. A protective role for autophagy has been suggested by providing nutrients to neoplastic cells that are under metabolic stress (Mathew et al., 2007a). A decrease in autophagy and an increase in cell death was noted in Beclin (+/-) epithelial tumours in areas that were under stress (Degenhardt et al., 2006, Karantza-Wadsworth et al., 2007). At the same time autophagy prevents accumulation of unwanted proteins and hence limits inflammation also influencing tumour development (Tsuchihara et al., 2009). The autophagy gene beclin 1 has been shown to limit chromosomal instability (Tsuchihara et al., 2009, Mathew et al., 2007b), and it's absence is associated with an increase in the incidence of breast, ovarian and prostrate tumours (Jin and White, 2007).

1.4 CYTOKINES

Cytokines are multifunctional signalling peptide molecules that are involved in inflammatory and immunological processes. In addition to functioning as immunomodulatory proteins, cytokines also regulate cell activities in various other biological processes such as differentiation, haematopoiesis, fibrinogenesis, protein synthesis and more recently the origin and spread of tumours (Panozzo et al., 1996, Bereta

et al., 1991, Dinarello, 2000, Kumar et al., 2005). Interleukins, tumour necrosis factors, colony-stimulating factors (CSF), interferons, peptide growth factors and chemokines each comprise families of cytokines with important effects in inflammation and immunity (Negus and Balkwill, 1996, Kumar et al., 2005). The production of a cytokine is not restricted to one cell type, while these proteins are functionally pleiotropic in different cells and tissues (Bereta et al., 1991, Kumar et al., 2005). Different types of cells can biologically respond to various cytokines in a similar manner, known as "cytokine crosstalk", and this property aids in maintaining the balance of physiological functions if any one cytokine is lacking. Being able to function in a network, cytokines are able to activate cells and induce the release of other cytokines, and either function in isolation or perhaps more often in synergy or inhibition with each other.

Cytokines may act locally only in their microenvironment so that a small amount of the protein acting for short duration may be adequate to bring about a significant functional response. In some instances, however, large quantities of cytokine can initiate destructive local responses and tissue damage. The amount of cytokine production elicited by a stimulus varies amongst different individuals and appears at least partly genetically regulated (Whiteside, 1994). Inflammation or injury can trigger the production of cytokines to induce a wide variety of responses including: pro-coagulant activity; expression of cell adhesion molecules such as ICAM-1, VCAM-1, endothelial-leukocyte adhesion molecule 1 (ELAM-1); and major histocompatibility antigens (Kumar et al., 2005). Of relevance to the current work, is that some cytokines increase intercellular adhesion, particularly that of leukocytes and even neoplastic cells to endothelium (Bereta et al., 1991, Alon et al., 1994, Kumar et al., 2005). From this, cytokines likely contribute to events in tumour development.

1.5 QUESTIONS ARISING FROM LITERATURE AND EARLIER OBSERVATIONS, ADDRESSED IN THIS THESIS

For the reasons outlined above, tumour progression can be seen as involving cross talk between parenchymal and stromal cells, both within the tumour mass and at the interface between neoplasm and surrounding normal tissues. However, these interactions are as yet poorly characterized, and the current thesis investigates some aspects of these complex parenchymal-stromal interactions.

Earlier work in this laboratory identified contact dependent apoptosis of endothelium in response to SAOS-2 osteosarcoma cells to produce a reduction in endothelial cell culture density (McEwen et al., 2003), and similar activity has been reported by others (Heyder et al., 2002, Holash et al., 1999, Kebers et al., 1998, Chen et al., 2005). A further as yet unpublished observation made in this laboratory by an earlier PhD scholar, is that human gingival fibroblasts (HGF) and human umbilical artery smooth muscle cells (HUASMC) also have reduced cell culture density when co-cultured with SAOS-2, but that this is surprisingly independent of apoptosis (Huynh, 2007). Instead of SAOS-2 inducing apoptosis in HGF and HUASMC, there appeared to be uptake of SAOS-2 alkaline phosphatase membrane marker by the stromal cells, creating an appearance of reduced stromal cell culture density but in-fact generating a third population of stromal cells bearing the SAOS-2 alkaline phosphatase marker. This was further supported by additional experiments using the cytoplasmic fluorescent marker CFSE in HGF and HUASMC, and the lipophilic fluorescent marker DiD in SAOS-2 (Huynh 2007). However, only very limited characterization of this surprising finding was possible in the context of the earlier student's PhD candidature, so that there were no experiments co-

culturing stromal cells with SAOS-2 using either membrane, cytoplasmic or nuclear labels in such a way as to characterize the extent that there may be membrane, cytoplasmic and nuclear fusion in this model system. From this, an important objective of the current thesis has been to further study exchange of membrane, cytoplasm and nuclear material between SAOS-2 and HGF. In doing so, the current thesis explores the idea suggested by others, that tumour cells can fuse with stromal cells to facilitate tumour survival and progression, such that hybrid cells may acquire more normal phenotype to facilitate attachment, migration and immune evasion (Pawelek and Chakraborty, 2008, Busund et al., 2002, Parris, 2006, Pawelek, 2000, Rachkovsky et al., 1998).

Architectural and cytological pleomorphism is a prominent feature within malignant lesions, and this is generally assumed to be due to the development of multiple separate clones of neoplastic cell bearing divergent genetic lesions (Kumar et al., 2005). The extent of this pleomorphism, however, is so extreme that may at times be difficult to find any two cells near each other that have a significantly similar morphology, while clear clusters of similar looking cells are also often not seen. From this, intra-tumour variability of neoplastic cells seems incompletely explained, and this thesis addresses some mechanisms that may help account for tumour variability independent of discrete genetic lesions.

Recognizing that intercellular adhesion is a likely necessary precursor to any exchange event between cells, and that in-vivo tumour parenchymal-stromal interactions are likely to take place in an inflammatory setting, this thesis also investigates specific adhesion events between SAOS-2 and HGF, as well as the effect of tumour necrosis factor-α (TNF-α) upon such interactions. The effect of TNF-α upon exchange of cellular material between HGF

and SAOS-2 is also studied, as is the effect of HGF co-culture with SAOS-2 upon cytokine synthesis.

Chapter 2: TNF-α Increases Adhesion of SAOS-2 Osteosarcoma Cells to Human Gingival Fibroblasts

2.1 INTRODUCTION

Cell adhesion seems a minimal and essential requirement for contact dependent interactions between cells. Intercellular adhesion via integrins, ICAM-1, VCAM-1 and cadherins, is implicated in both detachment and attachment of neoplastic cells during both invasion and metastasis (Chirivi et al., 1994, Honn and Tang, 1992, LaBiche et al., 1993, Nicolson, 1988). While some studies have shown adhesion of neoplastic cells to the ECM (Lester and McCarthy, 1992, Stetler-Stevenson et al., 1993, Honn et al., 1989) and endothelial cells (Steinbach et al., 1996, Honn and Tang, 1992, Gassmann et al., 2010), separate studies have demonstrated adhesion of neoplastic cells to mesothelium (van Grevenstein et al., 2006, Klein et al., 1995). The specific interest in this thesis, however, is adhesion of neoplastic cells to fibroblasts as the major non-vascular stromal cell type.

Tumour necrosis factor-α (TNF-α) is a pleiotropic pro-inflammatory cytokine produced primarily by mononuclear cells in response to inflammation or injury. It was initially recognised for its anti-tumour activity, as it was found to induce hemorrhagic necrosis in solid tumours and also exhibited cytotoxic and cytostatic effects on some tumour cells (Desch et al., 1990, van der Merwe, 1988, Pfizenmaier et al., 1987, Malik et al., 1990). Much broader activity has been subsequently identified for TNF-α so that it is now no longer thought of as a primarily anti-neoplastic cytokine, but instead as a highly active pro-inflammatory signal (Tracey and Cerami, 1994, Barbara et al., 1996).

Several cell types can produce TNF-α, including macrophages/monocytes, natural killer cells, B cells, T cells, basophils, eosinophils, mast cells, osteoblasts, neutrophils and some

tumour cells. TNF-α elicits a wide variety of biological activities and contributes to apoptosis, cell proliferation, cell differentiation, immune and inflammatory responses (Rath and Aggarwal, 1999, Gaur and Aggarwal, 2003, Morrison et al., 2003, Vassalli, 1992, van der Merwe, 1988, Tracey and Cerami, 1994) TNF-α is produced in response to numerous stimuli including: other cytokines such as granulocyte macrophage-colony stimulating factor GM-CSF, IL-1 and TNF-α; cell surface proteins such as CD44, CD45; hypoxia; reactive oxygen species; nitric oxide; viral infection; irradiation; lipopolysaccharide (LPS); complement component 5a; enterotoxin; and toxic shock toxin-1 (Thomson and Lotze, 2003), and mediating its effects via a wide range of signalling pathways, transcription factors and genes. TNF-α induces highly divergent responses amongst which are stimulation of the immune response, increased resistance to infections, increased microvascular permeability, and induction of leukocyte adhesion molecules by endothelium (Collins et al., 1986, Pober et al., 1986), while this cytokine is also known to complicate pathological conditions (Idriss, 2000, Kunkel et al., 1989, Tracey and Cerami, 1992, Tracey, 1992, Szlosarek et al., 2006a).

Underscoring the biological importance of TNF-α is that dysregulation of this cytokine is associated with a variety of diseases (Vilcek and Lee, 1991, van der Merwe, 1988, van Deuren et al., 1992, Balkwill, 2006). TNF-α is recognised as one of the main factors responsible for the wasting syndrome known as cachexia associated with cancer, and was at one stage described as “cachectin” (Malik et al., 1990, Kumar et al., 2005, Szlosarek and Balkwill, 2003), while raised circulating TNF-α levels are detected in patients with increased metastasis (Szlosarek et al., 2006a, Szlosarek et al., 2006b). For these reasons, it seems important to consider cellular interactions in neoplasms in context of the effect of TNF-α as a key tumour associated cytokine.

The gene for human TNF-α is located on the short arm of chromosome-6. TNF-α is synthesized as a 26-kDa type II transmembrane precursor that is displayed on the plasma membrane, with the N-terminus in the cytoplasm and the C-terminus exposed to the extracellular space. Soluble TNF-α (sTNF-α) is a 17kDa protein and is produced from the membrane bound stable TNF-α precursor via proteolytic cleavage by TNF-α converting enzyme (TACE), while mature TNF-α monomers self associate to form homotrimers (Smith and Baglioni, 1987, Szlosarek and Balkwill, 2003).

Both the membrane bound and soluble forms of TNF-α are biologically active, so that TNF-α can act in both a cell contact dependent manner and as a soluble agent. TNF-α effects are mediated via two structurally distinct receptors, TNF-α receptor type 1 (TNF-α-R1, p60 or p55) and TNF-α receptor type 2 (TNF-α-R2, p80 or, p75). These two high affinity receptors are present on a wide variety of cells and mediate strong cellular responses even with low receptor occupancy. TNF-α R1 is considered responsible for most biological actions of TNF-α (Idriss and Naismith, 2000, Wiegmann et al., 1992, Neumann et al., 1996, Heller and Kronke, 1994), and while being expressed in many tissues can be activated by the membrane bound as well as the soluble form of TNF-α. TNF-α R2 can be activated only by the membrane bound form of TNF-α, and is expressed only by cells of the immune system (Balkwill, 2006). Once TNF-α binds to its receptor, conformational change results in detachment of inhibitory protein silencer of death domains (SODD), providing opportunity for the adaptor protein TNF receptor type 1 associated death domain (TRADD) to bind to the intracellular death domain. This brings about activation of nuclear factor-kappa B (NF-kB), and mitogen activated protein kinase (MAPK) pathways, or induction of death signalling (Schutze et al., 1995, Schutze et al., 1992, Heller and Kronke, 1994).

TNF-α stimulates the secretion of Prostaglandin E2 (PGE2), IL-6, IL-8, GM-CSF and synthesis of DNA by fibroblasts (Fitzgerald et al., 2003, Burch et al., 1989, Motoyama et al., 1994, Larsen et al., 1989, Pang et al., 1994, Battegay et al., 1995). Induction of MMP-2, collagen, class I human leukocyte antigens (HLA), alpha 2 beta integrins, and receptors for collagen and laminin is observed in fibroblasts stimulated with TNF- α (Migita et al., 1996, Wolchok and Vilcek, 1992, Ezoe and Horikoshi, 1993). TNF- α can also induce migration of fibroblasts to sites of injury (Postlethwaite and Seyer, 1990). TNF-α also has a proliferative effect on FS-4 fibroblasts, while a similar mitogenic effect is also reported for human embryo lung fibroblasts and human skin fibroblasts (Vilcek et al., 1986). In contrast, high levels of TNF-α expression in macrophage supernatants suppress fibroblast proliferation in sarcoidosis (Fireman et al., 1992). Induction of ICAM-1 gene in cultured dermal fibroblasts (Marlor et al., 1992), and augmented expression of other cell adhesion molecules such as VCAM-1 is seen upon stimulation with TNF-α (Pang et al., 1994). Apoptosis is also induced in fibroblasts by TNF-α in vitro as well as in vivo, via caspase-8 activation (Mohan et al., 2000, Alikhani et al., 2004). Although most fibroblast responses to TNF-α are similar regardless of fibroblast origin, there is nonetheless some variation according to tissue type (Chang et al., 2002).

Many cancer and stromal cells have detectable TNF-α mRNA, and this is often associated with poor prognosis (Balkwill, 2006, Balkwill, 2009, Balkwill and Mantovani, 2001). TNF-α in neoplasms is often associated with concomitant expression of IL-1, IL-6, macrophage colony stimulating factor M-CSF, IL-8, CCL2 and CXCL12 (Balkwill, 2002, Balkwill, 2006), and it is interesting to note that TNF-α augments the production of receptor – ligand pairs in tumour stroma and parenchyma for: vascular endothelial growth factor (VEGF) (Ohba et al., 2009, Sun et al., 2005), basic fibroblast growth factor (bFGF)

and IL-8 (Yoshida et al., 1997). In intraperitoneal ovarian cancer xenografts, TNF-α promotes adhesion to the peritoneum with solid peritoneal deposits forming from free floating tumour cells (Malik et al., 1992).

Over expression of TNF-α is associated with increased metastatic potential, with Chinese hamster ovary cells demonstrating increased invasive capacity for peritoneal surfaces upon transfection with the TNF-α gene (Malik et al., 1990). A lung carcinoma cell line known to produce versican which is an ECM proteoglycan, activates macrophages to secrete TNF-α and IL-6, which in turn promotes metastasis (Kim et al., 2009). TNF-α also induces the expression of other cytokines in stromal and tumour cells, with for example increased production of IL-6 by glioblastoma cells (Van Meir et al., 1990), osteosarcoma cells (Motoyama et al., 1993b), malignant mesothelioma cells (Motoyama et al., 1993a), and morphological change in pulmonary carcinoma cells (Motoyama et al., 1994).

In some cases, increased TNF-α mRNA stability may be responsible for continued production of the cytokine by tumour cells. The Tipα (TNF-α inducing protein) gene family members of helicobacter pylori induce TNF-α and together with Ras can drive epithelial malignancy, demonstrating at least one genetic link for the effect of TNF-α on tumourigenesis (Suganuma et al., 2006). Besides autocrine and paracrine effects, TNF-α may also have direct effects on malignant cells, as in the case of an in vitro colorectal cancer model wherein epithelial-mesenchymal transformation is induced by TNF-α (Bates and Mercurio, 2003). Prolonged exposure of carcinogen treated fibroblasts to TNF-α drives carcinogenesis in nude mice (Balkwill and Mantovani, 2001, Malik et al., 1990).

The adherence of malignant neoplastic cells to the endothelium is a crucial step in tumour metastasis. The migration of leukocytes from the vasculature in inflammation and immunity is strongly influenced by cytokines, so that a similar interaction of tumour cells with blood vessels and cytokines seems likely during metastasis (Bereta et al., 1991). Cytokines such as IL-1, IL-2, IL-6, IFN and TNF-α cause increased endothelial adhesion of a range of tumour cell types including: adenocarcinoma cells (Bertomeu et al., 1993), colorectal carcinoma cells (Dejana et al., 1988, Gangopadhyay et al., 1998, Lauri et al., 1991, ten Kate et al., 2004), melanoma cells (Rice et al., 1988, Kim et al., 1993), lymphoma (Swerlick et al., 1992) and renal carcinoma cells (Steinbach et al., 1996, Yanase et al., 1995), and this seems to have in-vivo significance with regard to metastasis (Dejana et al., 1988). It seems important to stress the striking similarity between leukocyte adhesion to endothelium, and that of metastasising malignant cells to endothelium. Cell adhesion molecules induced by cytokines mediate adhesion of leukocytes to the endothelium (Hashimoto, 1994, Antonelli et al., 2001), while the same cytokine mediated mechanism appears responsible for increased binding of neoplastic cells to endothelium. For example, cytokines including TNF-α, IL-1, IFN-γ and IL-6, induce endothelial expression of cell adhesion molecules such as VCAM-1, ICAM-1and ELAM-1 (Sawa et al., 2007, Crook et al., 2000, Dufour et al., 1998, Iademarco et al., 1995, Pang et al., 1994, Yanase et al., 1995, Steinbach et al., 1996). Although various studies have demonstrated a role for TNF-α in increasing tumour cell adhesion to endothelium via the induction of adhesion molecules, there is still only limited knowledge of the wider effects of TNF-α in facilitating interactions with other non-endothelial stromal cells. Chapter 3 of the current thesis addresses how TNF-α stimulated SAOS-2 adhesion to HGF influences interaction between the two cell types.

TNF-α induces increased invasion, motility, laminin adhesion and MMP9 production, in human osteosarcoma cell lines such as OST and MNNG. On stimulating osteosarcoma cell lines with TNF-α, there is altered association of cytoplasmic NF-κB with NF-κB inhibitor (I-B), followed by translocation to the nucleus with consequent regulation of NF-κB gene transcription. Inhibition of NF- κB activity results in inhibition of cell invasion and motility, supporting correlation between invasion and motility induced by TNF-α via NF-κB activation (Harimaya et al., 2000). TNF-α also induces the expression of cytokines such as IL-6 in osteosarcoma cells (Motoyama et al., 1993b, Nakayama et al., 2004).

The current chapter describes work examining and characterizing the binding of SAOS-2 to cytokine stimulated HGF, as a necessary prelude to further work described in Chapters 3, 4 and 5 examining the consequence of these binding events.

2.2 MATERIALS

2.2.1 Materials for Cell Culture

Hank's balanced salt solution (HBSS), cell culture medium 199 (M199), gelatine, haematoxylin and polymyxin B solution were obtained from Sigma-Aldrich (St. Louis, USA). Iron fortified bovine calf serum was from Bovogen (Victoria, Australia). Trypsin (0.25%) / EDTA (1 mM) was from JRH Biosciences (Lenexa, USA). The antibiotics penicillin and streptomycin were from CSL Biosciences (VIC, Australia). Bovine serum albumin (BSA) fraction V and amphotericin B and were from ICN Biomedicals Inc. (Ohio, USA). Phosphate buffered saline (PBS) tablets were obtained from Oxoid (Hampshire, England), dimethyl sulfoxide (DMSO) was purchased from Ajax Chemicals (NSW, Australia). Centrifuge tubes were purchased from Iwaki, Scitech Division (Chiba, Japan). Disposable membrane filters were obtained from Sartorius, Ministart (Gottingen, Germany). Cell culture flasks (25 cm^2, 75 cm^2 and 225 cm^2) and all other cell culture plastic ware used were supplied by Costar (Cambridge, USA). TNF-α was purchased from Chemicon (Billerica, USA).

2.2.2 Materials for Detecting Alkaline Phosphatase in SAOS-2

Naphthol AS-MX phosphate, fast red violet, Tris HCl were obtained from Sigma-Aldrich (St. Louis, USA).

2.3 METHODS

2.3.1 Isolation of Human Gingival Fibroblasts (HGF)

HGF were isolated by explant culture from human gingival biopsies which were obtained with informed consent from the Oral surgery unit, Westmead Centre for Oral Health, Westmead Hospital, under a protocol approved by the Sydney West Area Health Service Human Research Ethics Committee. Specimens were collected in sterile specimen jars containing HBSS with the antibiotics penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (0.25 µg/ml).

The gingival fragments were first washed with M199 with antibiotics at least 5 times. Once thoroughly washed, the tissue was cut into small pieces of about 1-2 mm in size and placed into 6 well tissue culture plates, the well plates having been previously scored in up to 5 separate places to aid attachment of separate tissue fragments. Scoring was done using a scalpel, with each score consisting of 3 curved converging near parallel lines. Explant tissues were then covered with just sufficient medium to form a delicate meniscus, the medium comprising M199 with BCS (20%) and the antibiotics penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (0.25 µg/ml). Well plates were then incubated at 37 ^{o}C under 5% CO_2. Medium was replaced every day for a week, after which explants were fed with M199 with antibiotics and a lower BCS concentration (10%) which comprised HGF complete medium (CM) every day for 3 to 4 days, followed by replacement of medium every 3 days, continuing with M199 and BCS (10%). Throughout all culture procedures and experiments, M199 contained the antibiotics penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B(0.25 µg/ml).

2.3.2 Culture of Human Gingival Fibroblasts

After the first week some fibroblasts were usually found spreading out from the edge of tissue fragments. Once sheets of sufficient numbers of the cells were observed, explant tissues were carefully removed by aspiration and the remaining cells further cultured with 1ml of CM. When HGF reached confluence, cells were released with trypsin/EDTA followed by quenching of proteinase activity by 3 ml of BCS and pelleting by centrifugation at 1500 rpm at 4^{o}C for 7 minutes. Cell pellets were then resuspended in 5 ml of M199 and BCS (10%) and transferred to gelatine coated 25 cm^2 tissue culture flasks for incubation at 37^{o}C under 5% CO_2. Once HGF reached confluence, they were split into 75 cm^2 and 225 cm^2 tissue culture flasks in a ratio of 1:3 as appropriate, using trypsin/EDTA as outlined above. HGF were cultured up to the 8^{th} passage, and all experiments were performed with cells in from 6^{th} to 8^{th} passage.

2.3.3 Isolation of Human Umbilical Vein Endothelial Cells (HUVEC)

HUVEC were isolated from umbilical cords from vaginal deliveries obtained with informed consent at the Westmead Hospital maternity unit, with ethical approval of the Sydney West Area Health Service Human Research Ethics Committee. Cords were transported in sterile jars containing HBSS with the antibiotics penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (0.25 µg/ml). Endothelial cells were then isolated by the collagenase perfusion method (Jaffe et al., 1973). Cords were wiped down using sterile gauze and inspected for clamp marks or likely leakage points before proceeding. If any defects were found, cords were cut above the defects and blood clots if present ‘milked out’. Veins were cannulated with one-way cannulae and rinsed 4 times with 20 ml volumes of HBSS using 20 ml disposable syringes. Twenty ml volumes of warmed collagenase (1 mg / ml) in HBSS were then flushed into the veins followed by

immediate clamping of the free ends of cords to retain the collagenase. Cords were then covered with gauze soaked in HBSS and the collagenase allowed to work for 20 to 30 minutes. Detached cells were then collected in 5 ml volumes of BCS by flushing twice with HBSS. Cell pellets obtained by centrifuging at 1500 rpm at 4^{o}C for 10 mins were suspended in 5ml volumes of HUVEC-complete media (HUVEC-CM) containing M199 with BCS (20%), heparin (30 U/ml), ECGS (40 µg/ml) and antibiotics, before seeding into 25 cm^{2} tissue culture flasks pre-coated with gelatine (0.1% in PBS), and incubated at 37^{o}C under 5% CO_2.

2.3.4 Culture of HUVEC

Confluent HUVEC were released from plates using trypsin/EDTA and split into gelatine coated 75 cm^{2} or 225 cm^{2} flasks as appropriate at a ratio of 1:3. The cells in primary isolates were initially washed with M199 and fed with new HUVEC-CM daily, but subsequently fed every 3-4 days. HUVEC were cultured only to 5th passage, and all experiments were performed with cells in either 4th or 5th passage.

2.3.5 Storage of HUVEC and HGF

Confluent HUVEC in 4th passage as well as confluent HGF from 5 to 7th passage were sometimes stored frozen for later use. In brief, CM was removed and the cells washed once with 10ml of HBSS prior to release with trypsin/EDTA as described in section 2.3.2. Cells were then pipetted into 5ml of BCS and centrifuged at 1500 rpm at 4^{o}C for 10 mins. Cell pellets were then resuspended in appropriate volumes of CM containing M199 with antibiotics, BCS (20% in the case of HUVEC, 10% for HGF), and 10% DMSO so that 1 ml of cell suspension represented a confluent 75 cm^{2} flask. 1 ml volumes of cell suspensions were transferred into cryovials before placement in foam containers containing

Isopropyl alcohol for slow freezing overnight to -80^{o}C. The following day, cryovials were transferred to liquid nitrogen (N_2) for long term storage.

When required, frozen cryovials of cells were removed from liquid N_2 and thawed rapidly in a 37^{o}C water bath, maintaining gentle agitation and a vertical orientation throughout. Melted suspensions were immediately transferred into 5 ml volumes of BCS and centrifuged at 1500 rpm at 4^{o}C for 10 mins before resuspending pellets in 10 ml volumes of complete culture medium for HUVEC or HGF as appropriate and seeding into 75 cm^{2} flasks for further culture.

2.3.6 Isolation and Culture of Human Umbilical Artery Smooth Muscle Cells (HUASMC)

Human umbilical cords were collected as described above in section 2.3.3. The umbilical cords were transported in sterile jars containing HBSS with the antibiotics penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (0.25 µg/ml). The cord was wiped down using sterile gauze and inspected for clamp marks or any leakages before proceeding. If any defects were found, the cord was cut above it. Blood clots if present were expressed out. Paired umbilical arteries were identified and dissected free of the surrounding cord material. Arteries were then cut open along their length using fine scissors, washed thoroughly and repeatedly with M199, and cut into small sections of 1-2 mm in size. These were then placed into scored 6 well tissue culture plates using an approach identical to that described for HGF in section 2.3.1. Tissues were then incubated at 37^{o}C under 5% CO_2, fed with M199 and 20% BCS every day for 1 week, and every 3-4 days thereafter. HUASMC were cultured and stored frozen up to 8th passage using

methods identical to those described for HGF in sections 2.3.2 and 2.3.5, while HUASMC from 6^{th} to 8^{th} passage were used in experiments.

2.3.7 Culture of Human Osteosarcoma Tumour Cell Line SAOS-2

The human osteosarcoma cell line SAOS-2 was obtained from the American Type Culture Collection (ATCC, VA, USA). Cells were grown in 75 cm^2 flasks in CM at 37^oC under 5% CO_2. When confluent they were released from the culture surface using trypsin/EDTA, pelleted by centrifugation at 1500 rpm at 4^oC for 10 mins and split into 75 cm^2 and 225 cm^2 tissue culture flasks at a ratio of 1:3.

2.3.8 Detection of Alkaline Phosphatase in SAOS-2

SAOS-2 cells express the liver-bone kidney isoenzyme of alkaline phosphatase (Murray et al., 1987), and this is convenient for the identification of SAOS-2 in co-culture. In brief, monolayers were fixed with 10% neutral buffered formalin before washing with PBS 3 times and staining for alkaline phosphatase activity with a solution comprising naphthol AS-MX phosphate (0.1 mg/ml) and fast red violet (0. 2 mg/ml) in Tris buffer (10mM, pH 8.2). Staining solution was applied to each well in volumes ranging from 2 to 3ml per well, and incubated at 37^oC for 1hr. Monolayers were then washed with Milli-Q water and counterstained with haematoxylin for 5 mins followed by Scott's blueing solution for further differentiation for up to 2 mins. The cells were observed under an Olympus CK2 phase contrast inverted microscope (Tokyo, Japan) with the phase ring removed.

2.3.9 Cell Culture Conditions for Experiments Investigating Adhesion of SAOS-2 to Stromal Cells

2.3.9i Application of SAOS-2 to Stromal Cell Cultures in Wells for Evaluation of SAOS-2 Adhesion

Confluent cultures of HGF in passage 6 and 7 were harvested with trypsin / EDTA as outlined in section 2.3.2, and pelleted cells seeded at confluence into gelatinised 12 well tissue culture plates and allowed to attach overnight at 37^{o}C under 5% CO_2. HGF were treated with TNF-α at concentrations of 0 nM, 0.029 nM, 0.039 nM, 0.058 nM, 0.12 nM, 0.58 nM or 1.16 nM from 0 to 24 hrs before washing twice with M199 alone and immediate evaluation of SAOS-2 binding. In some experiments, adhesion of SAOS-2 to HGF was evaluated after an initial 24 hr period of HGF stimulation with TNF-α (1.16 nM), and from 0 to 24 hrs of further HGF culture without TNF-α stimulation.

Confluent SAOS-2 were harvested using a method identical to that for HGF, with the exception that SAOS-2 were adjusted to a final concentration of 1.5×10^5 cells / ml in CM before application of 1 ml / well of SAOS-2 cell suspension to washed HGF in wells. After allowing 5 minutes of attachment, the non-adherent SAOS-2 were removed from wells by gentle washing with M199 and monolayers fixed with 10% neutral buffered formalin for 5 minutes followed by three washes with PBS and one last wash with Mili-Q water.

Similar adhesion assays were performed evaluating SAOS-2 binding to HUASMC using a method identical to that for HGF, while additional studies were performed with HUVEC using HUVEC-CM as the suspending medium (section 2.3.3).

In some experiments, specificity of responses to TNF-α was probed by addition of polymyxin B (1g/ml) known to bind and inactivate trace amounts of potentially contaminant lipopolysaccharide (Cavaillon and Haeffner-Cavaillon, 1986, Morrison and Jacobs, 1976, Lynn and Golenbock, 1992), and or boiling TNF-α at 100^{o}C for 30 mins. The effect of TNF-α upon SAOS-2 with regard to subsequent SAOS-2 ability to bind HGF was determined in additional experiments, where SAOS-2 were first stimulated with TNF-α (1.16 nM) for 24 hrs, prior to harvesting for adhesion assays.

2.3.9ii Evaluation of the Effect of Pre-Fixation of HGF and SAOS-2 upon Binding

To explore the role of cell viability, as well as chemical sensitivity of adhesion mechanisms, some experiments were performed with HGF fixed for 10 minutes with either 10% neutral buffered formalin, ice-cold methanol, 4% paraformaldehyde or glutaraldehyde, prior to application of SAOS-2, using unfixed HGF as controls. Note that after fixation, HGF were washed 3 times with HBSS to remove fixative before application of SAOS-2. The effect of similar fixation of SAOS-2 on binding to viable HGF was assessed in a similar manner, with the difference that SAOS-2 were fixed in suspension and washed twice with HBSS before commencing the adhesion assay.

2.3.9iii Evaluation of the Role of BCS in the Adhesion Assay

Some experiments were also performed substituting bovine serum albumin (BSA) to a final concentration of 4% w/v for the BCS used in adhesion assays (section 2.3.9.a). In addition, the effect of heat, acid and alkali treatment of BCS upon SAOS-2 binding was studied. Briefly, BCS was subjected to 4 separate heat treatments, being maintained at: 60^{o}C for 24 hrs; 80^{o}C for 24 hrs; 60^{o}C for 72 hrs; and 80^{o}C for 72 hrs. Acid treatment of BCS was by adjusting pH to a value of 2 through drop-wise addition of HCl (1M), and

storage at RT for 24 hrs before neutralising pH to 7.2 with NaOH (1M). Alkali treatment of BCS was performed using a similar approach, adding NaOH (1M) to a final pH of 11 and using HCl (1M) to return pH to 7.2.

2.3.10 Quantitation of SAOS-2 Adhesion to Stromal Cell Monolayers

Endogenous alkaline phosphatase activity in SAOS-2 was exploited to aid identification of these cells in adhesion assays, using a histochemical approach outlined in section 2.3.8, while haematoxylin was used as a convenient counterstain. Monolayers stained for alkaline phosphatase activity were observed with an Olympus CK2 phase contrast inverted microscope (Tokyo, Japan) with the phase ring removed, and photomicrographs of 4 separate fields per well recorded using a Scope photo 3.0 digital camera. Each field imaged represented a site in the mid-region between the centre and peripheral rim of an individual well, and was at a corner of what would be a square drawn within the circle of the well. The number of adherent SAOS-2 per well was determined as the total number of these cells identified in the four photomicrographs and data expressed as cells per graticule area. Average values for each treatment condition were determined across triplicate wells, while the statistical significance of individual experiments was assessed using Student's t Test, the results of multiple separate experiments with cells from different donors was evaluated using the Wilcoxon Ranked-Sign Test.

2.3.11 Variability in Bioactivity of TNF-α

Recombinant cytokines may demonstrate modest variability in bioactivity across batches. For this reason whenever a new batch of TNF-α was obtained, dose response in relation to SAOS-2 binding to TNF-α stimulated HGF was performed and maximal stimulatory

concentration determined. When TNF-α was applied, it was always used at a concentration that demonstrated maximum stimulation, ranging between 0.58nM -1.16nM.

2.4 RESULTS

2.4.1 Cells Isolated from Gingival Explants were Morphologically Characteristic of Fibroblasts

Outgrowths from gingival explants were visible within 3-4 days. By 12 to 14 days of culture, confluent monolayers of cells were formed around many explant tissue fragments. The migrating cells were homogenous in shape and had morphological features characteristic of fibroblasts including: elongated cell bodies, oval nuclei and a linear or bundle like arrangement of cells (Figure 2.1).

2.4.2 Cells Isolated from Umbilical Cord Veins were of Endothelial Origin

Cells isolated from umbilical veins initially formed small clusters of cells during the first few days post isolation. After about 7 days, monolayers formed with a cobblestone morphology characteristic of endothelium. Cells isolated by this procedure were always positive for the endothelial marker *Ulex europaeus* lectin-1 (UEA-1), confirming endothelial identity (Figure 2.1).

2.4.3 Cells Isolated from Umbilical Artery Explants were Morphologically Characteristic of Smooth Muscle Cells

Cell outgrowth from umbilical artery explants appeared within 3-4 days, with most of the explant fragments showing outgrowth within a week. After migrating out, cells began to proliferate around the explant tissues such that by 4 weeks, confluent monolayers were observed (Figure 2.1). Morphologically, the cells were elongated and acquired a ‘hill and valley’ pattern characteristic of smooth muscle cells. Cells obtained by the method

described (2.3.6) were always positive for the SMC marker smooth muscle actin (SMA), confirming identity as SMC.

2.4.4 Alkaline Phosphatase was Expressed by SAOS-2 but not by HGF, HUASMC or HUVEC

Alkaline phosphatase was strongly expressed by SAOS-2 and this helped in readily differentiating between SAOS-2 and stromal cells which were consistently alkaline phosphatase negative in isolated culture (Figure 2.2).

2.4.5 TNF-α Increased SAOS-2 Adhesion in a Dose Dependent Manner

Figure 2.3 shows that HGF increased SAOS-2 binding over that to culture plastic alone ($p<0.001$), and this was increased by HGF pre-treatment with TNF-α ($p<0.001$). Furthermore, increasing concentrations of TNF-α caused a dose dependant increase in the number of SAOS-2 binding HGF with maximum activity at 1.16nM ($p<0.05$) and loss of activity at 0.006 nM (Figure 2.4). Increased SAOS-2 binding to HGF pre-treated with TNF-α was observed in 16 separate experiments with HGF from 6 separate donors. A similar effect on SAOS-2 binding was observed with HUVEC ($p<0.05$) as well as HUAMSC ($p<0.0001$) (Figure 2.5).

2.4.6 Characterisation of SAOS-2 Binding

2.4.6i Fixation of HGF Abrogated TNF-α Stimulated Binding of SAOS-2 but not Background Binding, while Fixation of SAOS-2 Prevented all Binding

The effect of fixing HGF upon SAOS-2 binding was determined to investigate if SAOS-2 adhesion is dependent on an active HGF response. Figure 2.6A shows that HGF treatment with a range of fixatives abrogated TNF-α induced SAOS-2 binding ($p<0.05$) to levels

similar to background binding, equivalent to HGF without TNF-α. There was no effect, however, of fixation on background binding to HGF. Data suggest that the TNF-α stimulated mechanism is fixation sensitive and separate to the mechanisms responsible for background binding of SAOS-2 to HGF. Similar results were obtained in two separate experiments using HGF from two separate donors.

When SAOS-2 were fixed by the same fixatives, there was complete loss of both background and TNF-α induced binding to HGF (Figure 2.6B). Similar results were obtained in two separate experiments using HGF from two separate donors.

2.4.6ii Serum Contributed to TNF-α Stimulated SAOS-2 Binding to HGF

Figure 2.7 shows the result of an experiment in which the possible role of serum factors in facilitating TNF-α induced SAOS-2 binding to HGF was investigated, by substituting BSA for BCS. SAOS-2 cell adhesion to TNF-α treated HGF was significantly increased in the presence of both BCS ($p<0.05$) and 4% BSA ($p<0.05$), although this was less pronounced in the absence of serum. While serum also increased background binding relative to binding to HGF unstimulated with TNF-α, polymyxin B did not affect results, while TNF-α was inactivated by boiling. Similar results were obtained in 2 separate experiments using 2 different donors.

2.4.6iii Heat Treatment of Serum Inactivated the Serum Contribution to SAOS-2 Binding of HGF, but Neither Acid or Alkali Treatment of Serum had any Effect

Heat treatment of BCS at 60^{o}C abrogated the effect of TNF-α with regard to SAOS-2 binding ($p<0.03$), while treatment at 80^{o}C both abrogated the effect of TNF-α ($p<0.001$) and reduced background binding ($p<0.0001$) (Figure 2.8). No difference was seen between

heat treatment of BCS for 24 hrs and heat treatment for 72 hrs. Similar results were obtained in 6 separate experiments with BCS heated to 60^{o}C, and in 5 separate experiments where BCS was heated to 80^{o}C, while HGF were from three different donors in each case. Neither acid nor alkali treatment of BCS had any effect on SAOS-2 binding (Figure 2.9). Similar results were obtained in 2 separate experiments using 2 different donors. These data indicate that a heat labile serum factor contributes to TNF-α mediated adhesion of SAOS-2 to HGF, and that this serum activity is insensitive to both acid and alkali treatment.

2.4.6iv Time Course of TNF-α Activity in the Binding of SAOS-2 to HGF

Figure 2.10 illustrates two time course experiments for the effect of TNF-α on increasing SAOS-2 binding to HGF. An experiment performed over 24 hrs demonstrated that binding was maximal by 6 hr of cytokine stimulation ($p<0.0001$). In a separate experiment performed within a 6 hr period, increased binding occurred after 30 minutes of cytokine stimulation, with maximal binding by 1.5 hr of cytokine stimulation ($p<0.001$).

To determine if TNF-α induced binding of SAOS-2 to HGF declined over time, and the rate of any such decline, an experiment was performed in which HGF were stimulated with TNF-α for 24 hrs, before being washed and further cultured with CM for increasing times before testing for SAOS-2 binding. Figure 2.11 shows that SAOS-2 binding to TNF-α stimulated HGF was stable for at least 6 hrs, but that by 12 hr some reduction in SAOS-2 binding occurred ($p<0.05$), while this appeared to plateau by 18 hrs ($p<0.001$).

2.4.6v Pre-treatment of SAOS-2 with TNF-α Reduced Binding to TNF-α Pre-treated HGF, but not to Unstimulated HGF or Culture Plastic

To examine a potential direct response of SAOS-2 to TNF-α with regard to HGF binding, SAOS-2 were pre-treated with TNF-α for 24hrs, and subsequent binding to culture plastic, HGF and HGF pre-treated with TNF-α studied (Figure 2.12). Pre-treating SAOS-2 with TNF-α did not have any effect on subsequent adhesion to either tissue culture plastic or unstimulated HGF. While pre-treatment of HGF with TNF-α increased binding of unstimulated SAOS-2 as expected from earlier experiments ($p<0.002$), there was markedly reduced binding of SAOS-2 that had been pre-treated with TNF-α to similarly pre-treated HGF ($p<0.003$) such that binding was comparable to that seen to unstimulated HGF (Figure 2.12).

Figure 2.1 Phase contrast photomicrographs of cultured human gingival fibroblasts (HGF), human umbilical artery smooth muscle cells (HUASMC) and human umbilical vein endothelial cells (HUVEC). Confluent HGF monolayers of cells formed within 2 weeks around gingival explant tissue fragments were homogenous in shape and had morphological features typical of fibroblasts. Similarly, umbilical artery explant cultures generated cells with the fusiform morphology and a 'hill and valley' orientation typical of SMC within 4 weeks of culture. Cells isolated and cultured from the umbilical vein demonstrated the epithelioid cobblestone morphology typical of endothelium. (Bars = 50µm)

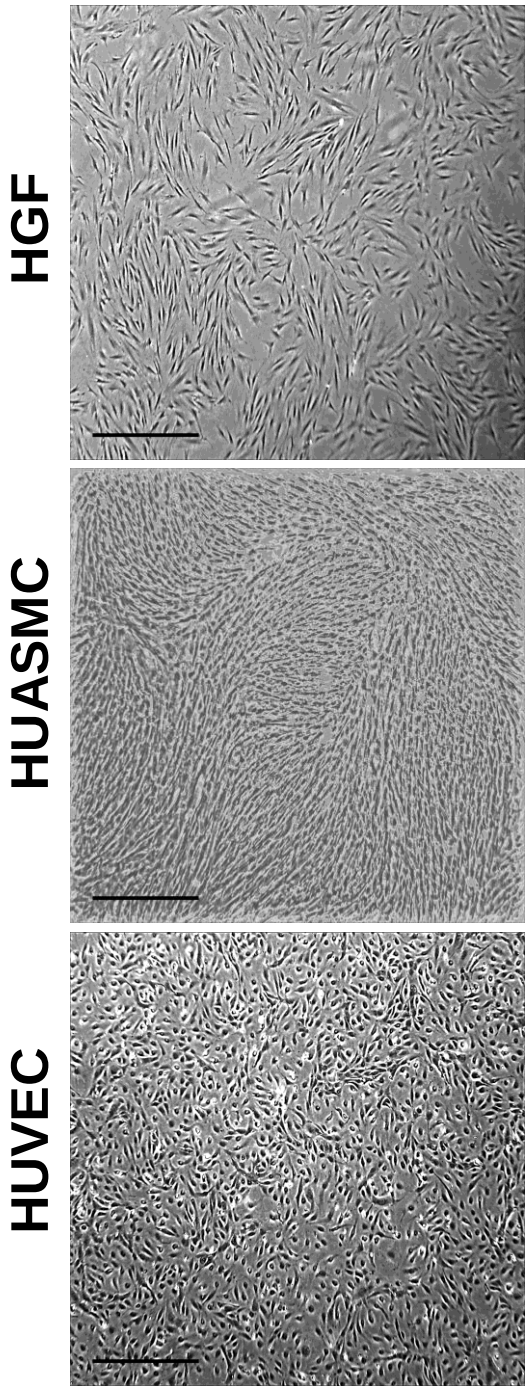
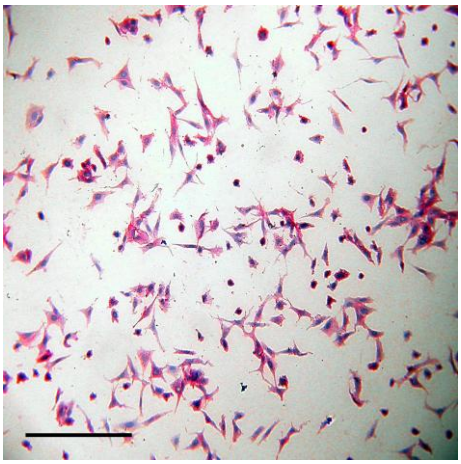
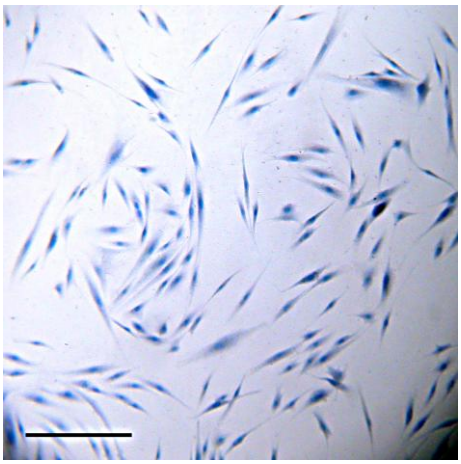

HGF
HUASMC
HUVEC

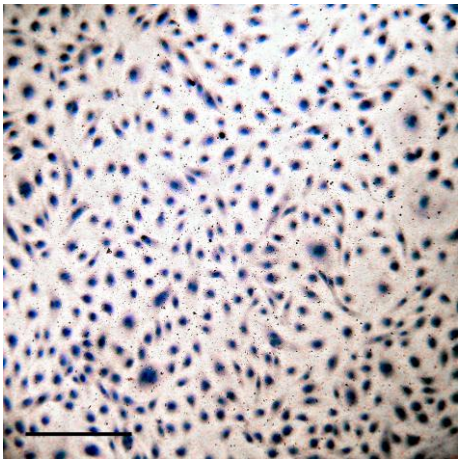
Figure 2.2 Photomicrograph of SAOS-2, HGF, HUVEC and HUASMC stained for alkaline phosphatase activity. SAOS-2 appear red as they strongly express alkaline phosphatase whereas the fibroblasts, HUVEC and HUASMC cells did not demonstrate any alkaline phosphatase activity (Bar = 50μm).



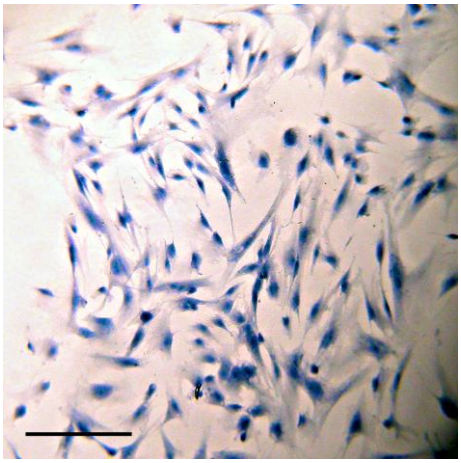
SAOS-2



HGF

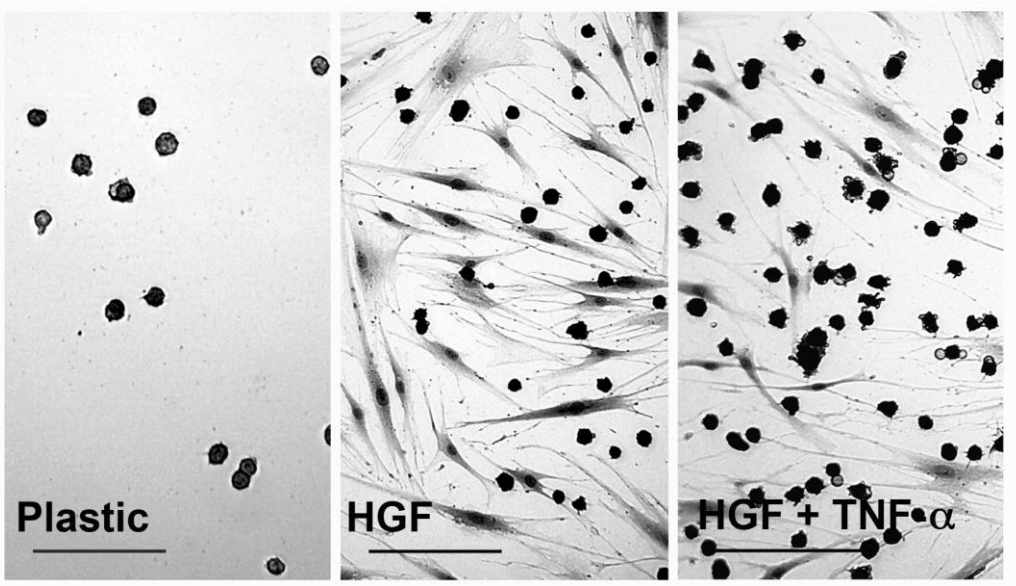


HUVEC



HUASMC

Figure 2.3 Photomicrographs of SAOS-2 bound to culture plastic, HGF and HGF pre-treated for 24 Hr with TNF-α (1.16nM), as well as a histogram demonstrating quantitative analysis of SAOS-2 binding in these culture conditions. SAOS-2 binding was greater in the presence than absence of HGF ($p<0.001$), and this was increased by TNF ($p<0.001$), ($n=16$). (Bar = 50μm)


Plastic
HGF
HGF + TNF-α

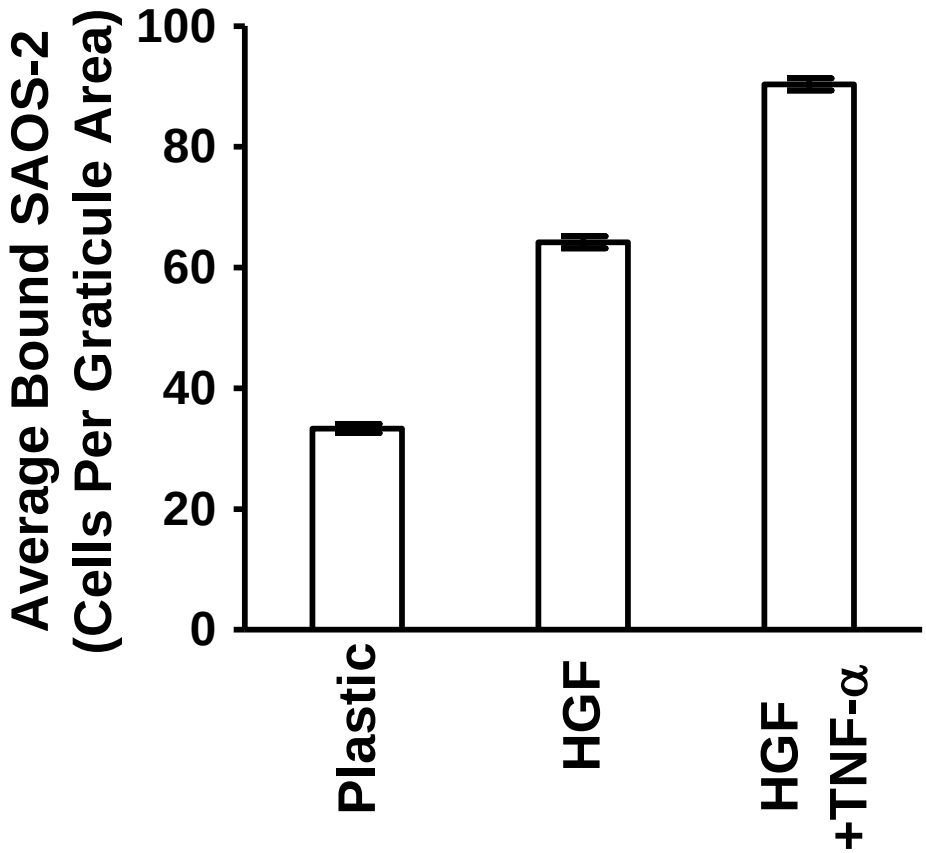

Average Bound SAOS-2
(Cells Per Graticule Area)
100
80
60
40
20
0
Plastic
HGF
HGF
+TNF-α

Figure 2.4 Graph showing the dose response for TNF-α of HGF stimulated for 24 Hr, with regard to subsequent SAOS-2 binding. The effect of TNF-α was seen by 0.06 nM of cytokine, and was maximal by 1.16 nM concentration ($p<0.05$).

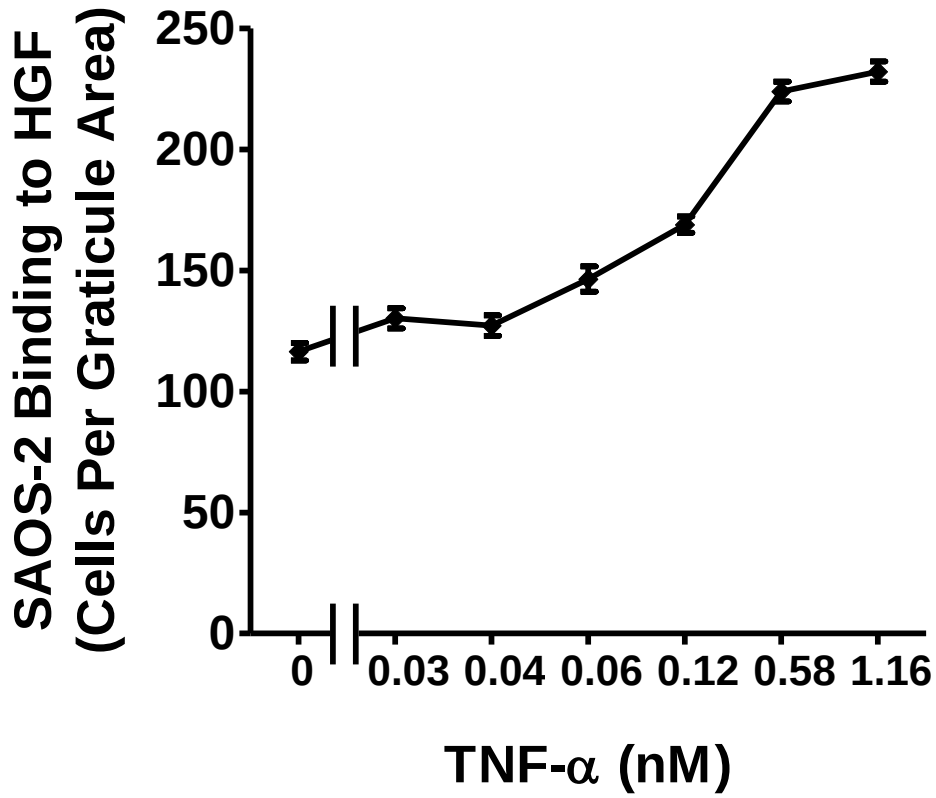

SAOS-2 Binding to HGF
(Cells Per Graticule Area)
250
200
150
100
50
0
0
0.03
0.04
0.06
0.12
0.58
1.16
TNF-α (nM)

Figure 2.5 Histograms demonstrating quantitative analysis of SAOS-2 binding to culture plastic, HUVEC (A) and HUASMC (B) with and without prior stimulation by TNF-α (1.16nM). SAOS-2 binding was greater to HUVEC ($p<0.05$) and HUASMC ($p<0.0001$) than to culture plastic surfaces, and this was increased by TNF-α pre-treatment ($p<0.05$) (A and B, n=3).

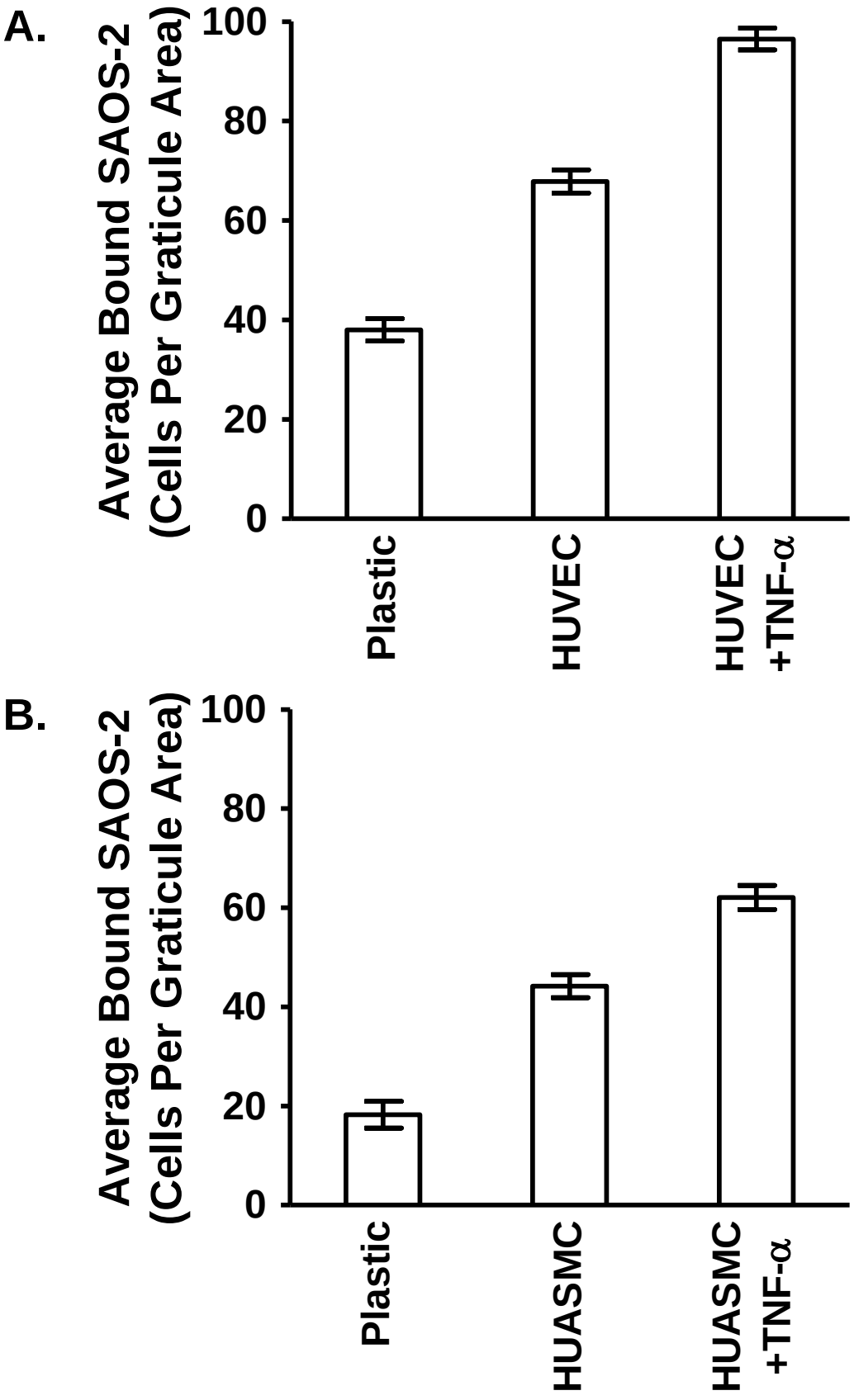

A.
Average Bound SAOS-2
(Cells Per Graticule Area)
100
80
60
40
20
0
Plastic
HUVEC
HUVEC
+TNF-α
B.
Average Bound SAOS-2
(Cells Per Graticule Area)
100
80
60
40
20
0
Plastic
HUASMC
HUASMC
+TNF-α

Figure 2.6. Histograms showing the effect of HGF (A) and SAOS-2 (B) fixation with 10% neutral buffered formalin, methanol, 4% paraformaldehyde (PFA) and glutaraldehyde (GLTA) upon SAOS-2 adhesion, with and without prior stimulation of HGF with TNF-α (1.16nM) for 24 hr. (A) TNF-α pre-treatment increased SAOS-2 binding, but all fixation protocols used reduced SAOS-2 binding to levels comparable to background levels without TNF-α stimulation ($p<0.05$). Background binding to HGF without TNF-α stimulation was not fixation sensitive. (B) Fixation of SAOS-2 prevented all SAOS-2 binding to HGF. (A and B, n=2)

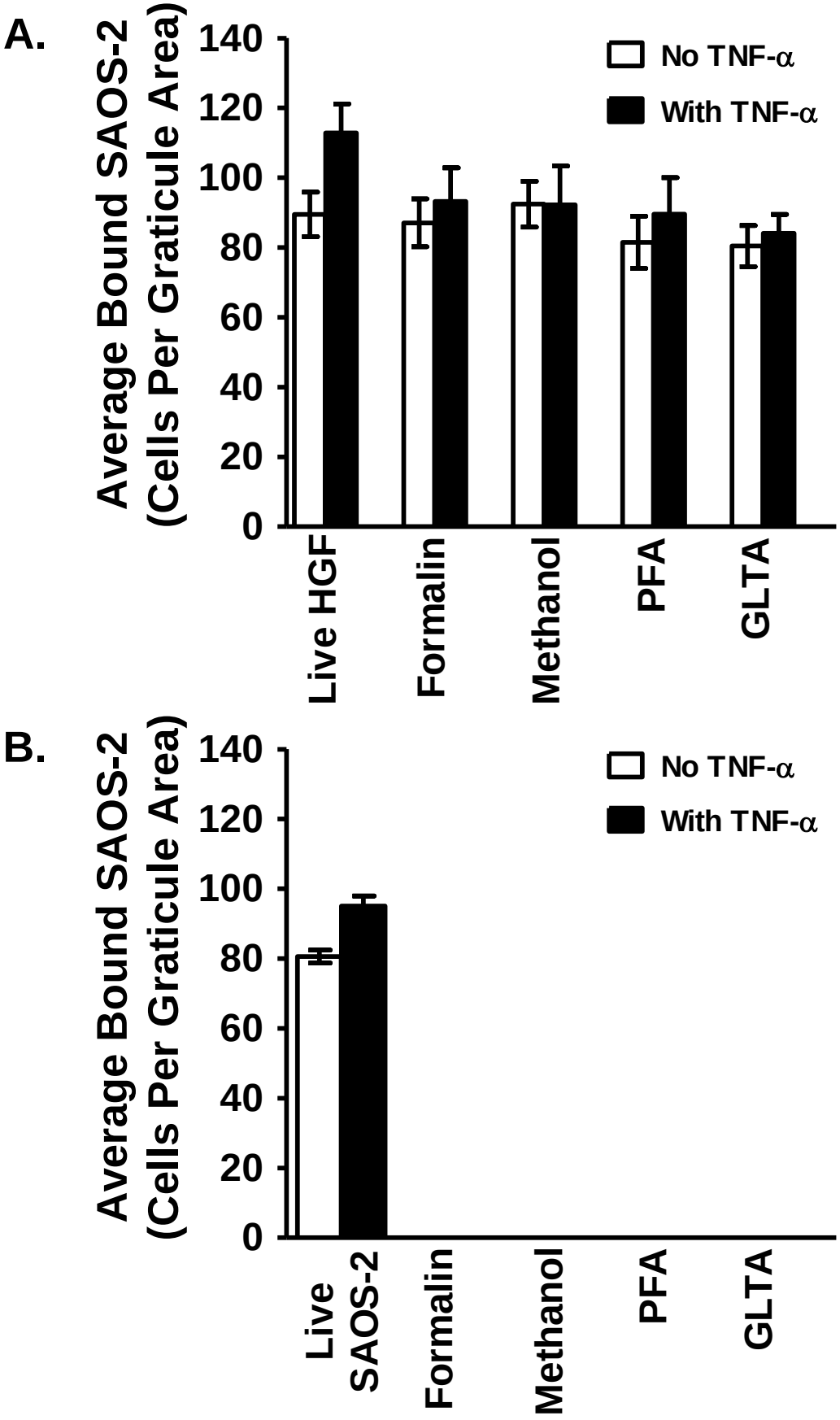

A.
Average Bound SAOS-2
(Cells Per Graticule Area)
140
120
100
80
60
40
20
0
No TNF-α
With TNF-α
Live HGF
Formalin
Methanol
PFA
GLTA
B.
Average Bound SAOS-2
(Cells Per Graticule Area)
140
120
100
80
60
40
20
0
No TNF-α
With TNF-α
Live SAOS-2
Formalin
Methanol
PFA
GLTA

Figure 2.7 Histogram showing SAOS-2 binding in the presence of either BCS (10%) or BSA (4%) to HGF cultured with or without native or boiled TNF-α (0.58nM), as well as the effect of polymyxin B (1g/ml) upon the TNF-α response. Native TNF-α significantly increased binding of SAOS-2 to HGF in the presence of BCS ($p<0.05$) and BSA (4%) ($p<0.05$) and this effect was lost when TNF-α was boiled. There was a moderate increase in the number of SAOS-2 binding to HGF in the presence of BCS relative to BSA. The inclusion of Polymyxin B with TNF-α indicated that the effect observed was due to TNF-α and not contaminating lipopolysaccharide (n=2).

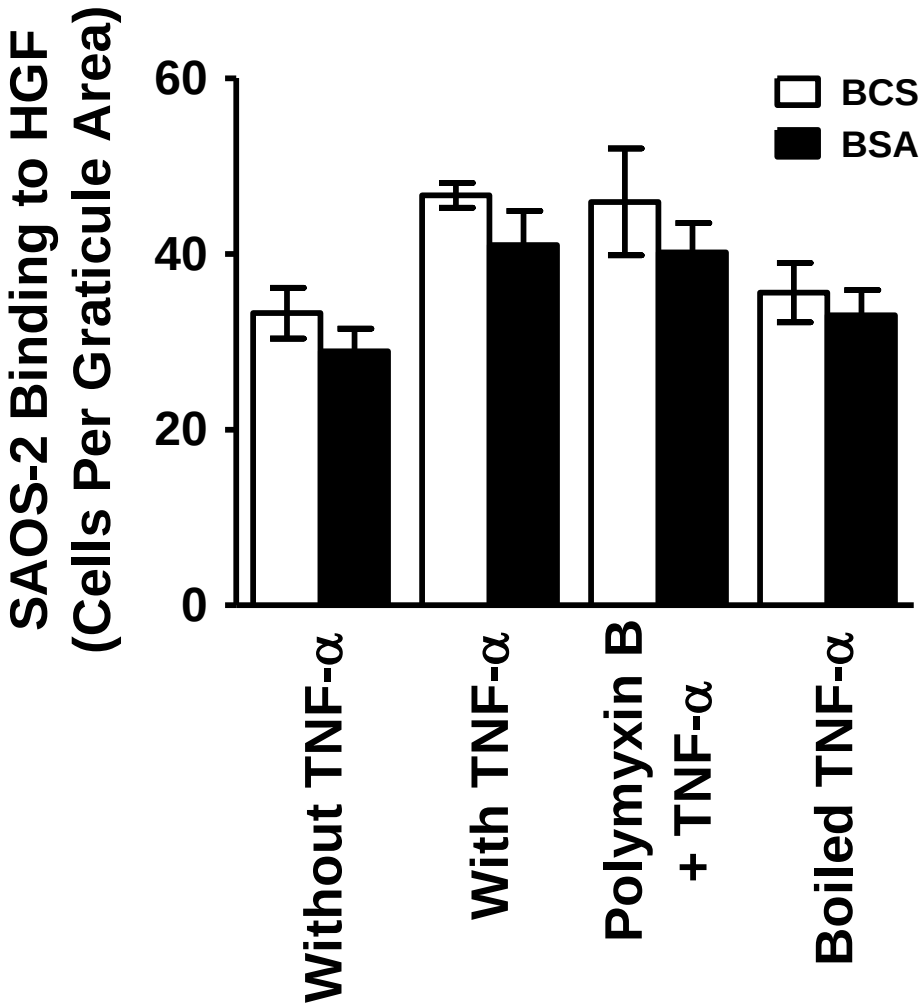

SAOS-2 Binding to HGF
(Cells Per Graticule Area)
60
40
20
0
BCS
BSA
Without TNF-α
With TNF-α
Polymyxin B
+ TNF-α
Boiled TNF-α

Figure 2.8 Histograms showing the effect on SAOS-2 adhesion to TNF-α pre-treated HGF with heat treated serum. The number of attached SAOS-2 to TNF-α treated HGF decreased in the presence of heat treated BCS, A. (60^{o}C, $p<0.03$) & B. (80^{o}C, $p<0.001$). This effect on SAOS-2 binding was greater upon heating to a higher temperature of 80^{o}C, in that treatment of serum at 60^{o}C reduced TNF-α mediated binding but not background binding, and treatment at 80^{o}C reduced both TNF-α induced and background binding. These effects were, however, indifferent to the duration of heat treatment (24 or 72 hrs), (A,n=6, B,n=5).

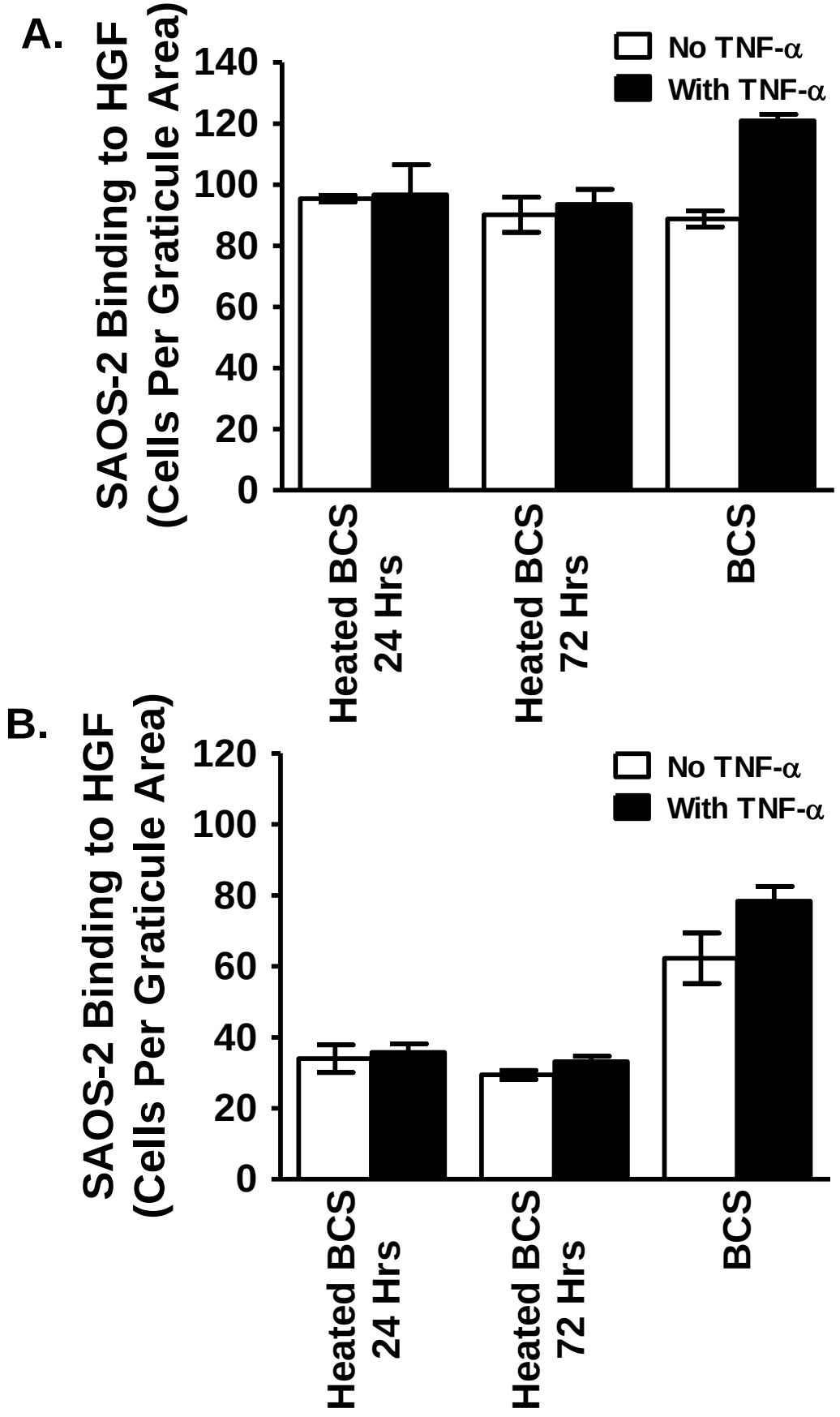

A.
SAOS-2 Binding to HGF
(Cells Per Graticule Area)
140
120
100
80
60
40
20
0
No TNF-α
With TNF-α
Heated BCS
24 Hrs
Heated BCS
72 Hrs
BCS
B.
SAOS-2 Binding to HGF
(Cells Per Graticule Area)
120
100
80
60
40
20
0
No TNF-α
With TNF-α
Heated BCS
24 Hrs
Heated BCS
72 Hrs
BCS

Figure 2.9 Histogram showing the effect of acid and alkali treatment of BCS upon the subsequent binding of SAOS-2 to HGF either pre-treated with TNF-α (1.16nM) for 24 hr or not. No effect of acid or alkali treatment was seen (n=2).

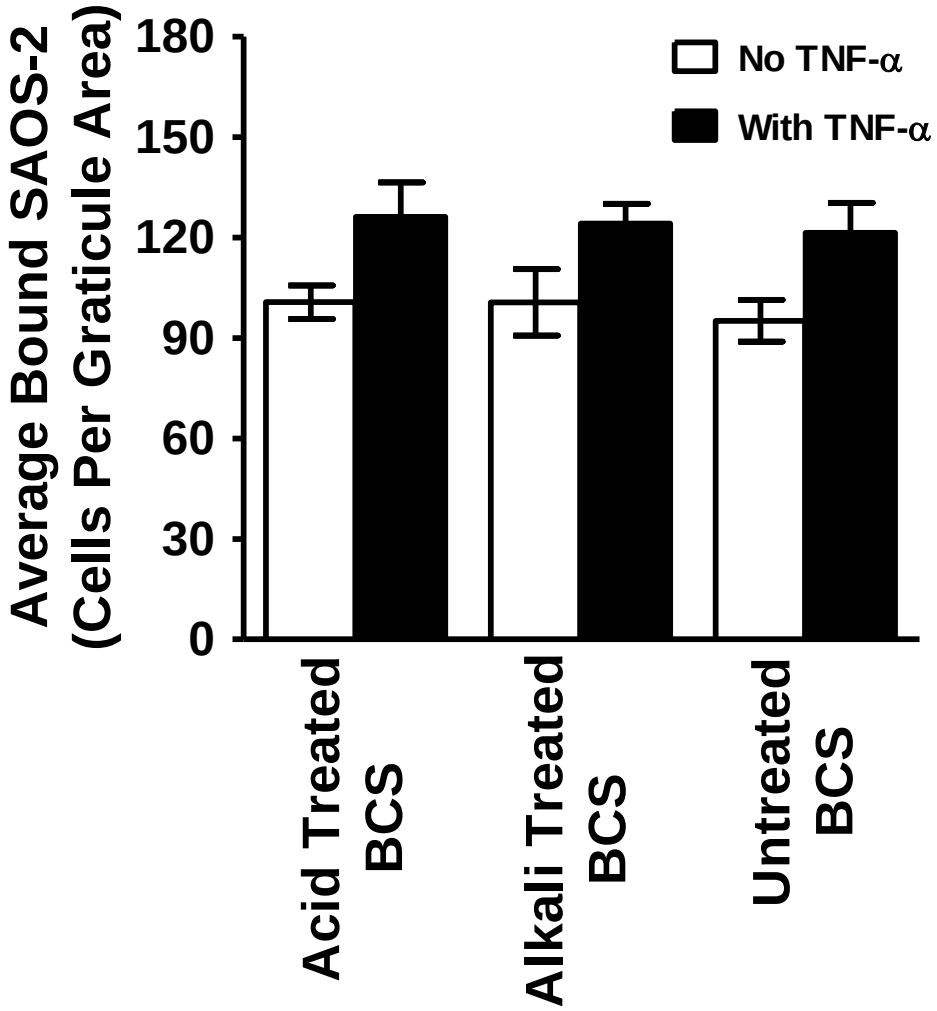
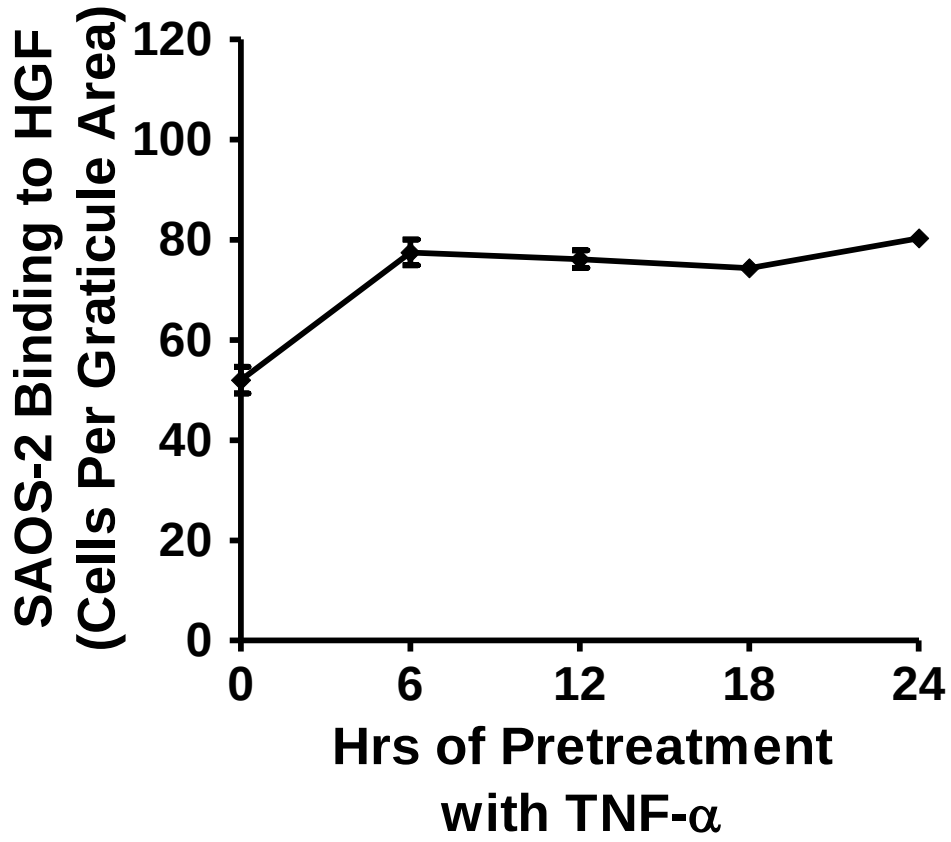

Average Bound SAOS-2
(Cells Per Graticule Area)
180
150
120
90
60
30
0
No TNF-α
With TNF-α
Acid Treated BCS
Alkali Treated BCS
Untreated BCS

Figure 2.10 Graphs showing the results of two separate time course experiments performed over 24 hr (A) and 6 hr (B) respectively, for the effect of increasing periods of HGF stimulation by TNF-α (1.16nM) with regard to SAOS-2 binding. (A) TNF-α increased SAOS-2 binding to maximal levels by 6 hrs of cytokine stimulation ($p<0.0001$). (B) A separate time course experiment examining the first six hours of cytokine stimulation revealed the absence of response by 30 minutes, but significantly increased SAOS-2 binding by 1 hr of cytokine stimulation, and maximal binding by 1.5 hrs of TNF-α stimulation ($p<0.001$).

A.


SAOS-2 Binding to HGF
(Cells Per Graticule Area)
120
100
80
60
40
20
0
0
6
12
18
24
Hrs of Pretreatment
with TNF-α

B.

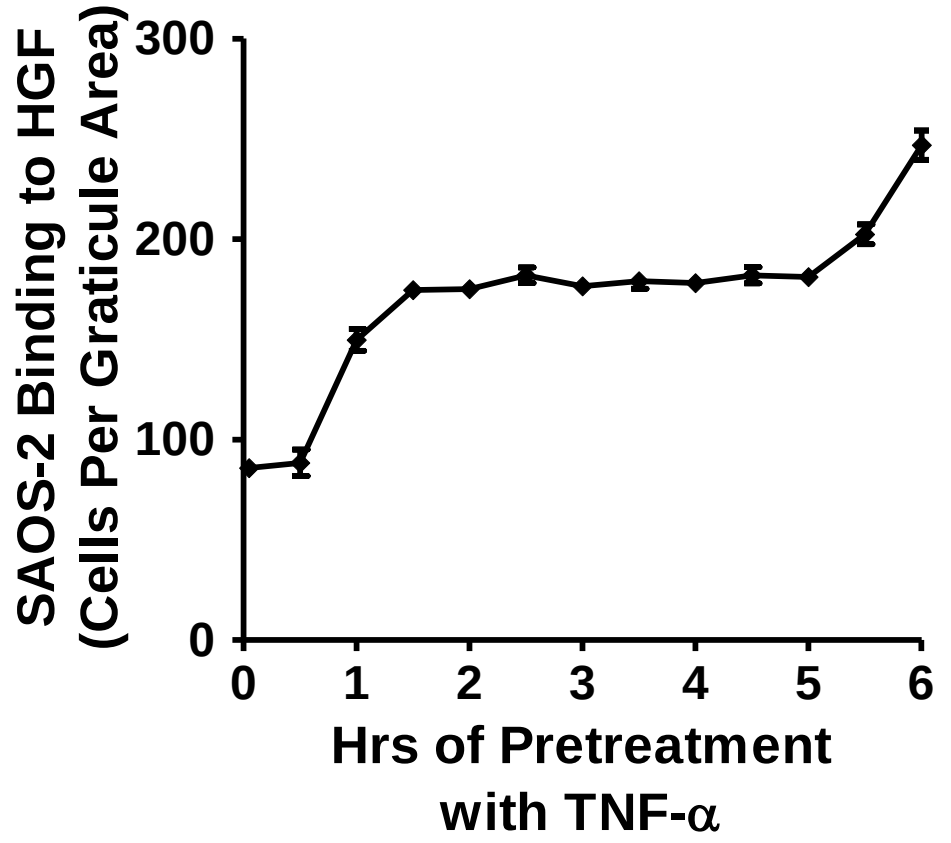

SAOS-2 Binding to HGF
(Cells Per Graticule Area)
300
200
100
0
0
1
2
3
4
5
6
Hrs of Pretreatment
with TNF-α

Figure 2.11 Graph showing SAOS-2 binding to HGF stimulated with TNF-α (1.16nM) for 24 hrs, and then washed before further culture in CM without cytokine from 0 to 24 hrs. HGF incubated with TNF-α for 24hrs showed an increase in SAOS-2 binding ($p<0.0001$). A decrease in attachment was detectable after 12 hrs post cytokine stimulation ($p<0.005$), with decay in SAOS-2 binding reaching a plateau by 18 hrs ($p<0.001$).

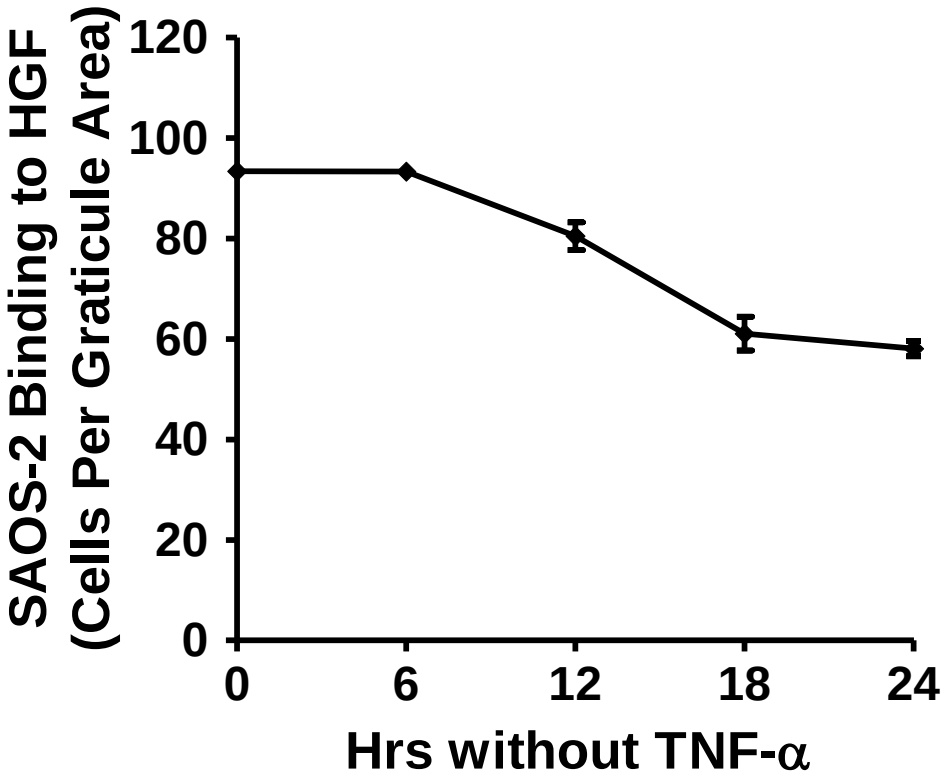
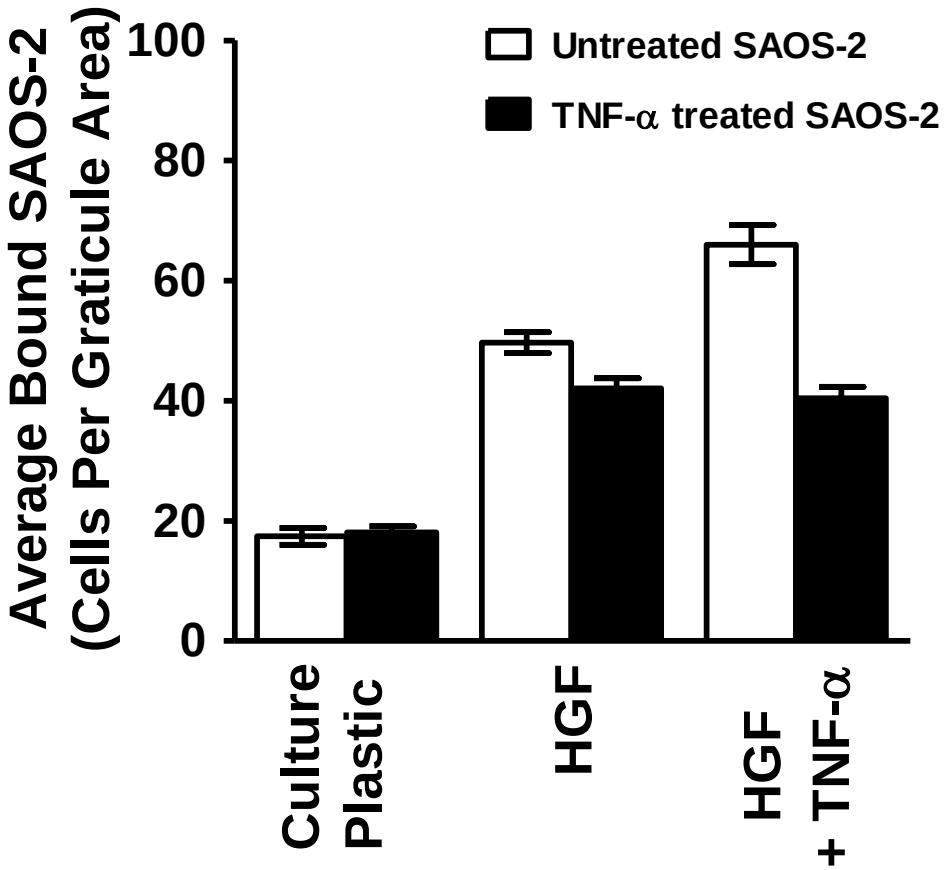

SAOS-2 Binding to HGF
(Cells Per Graticule Area)
120
100
80
60
40
20
0
0
6
12
18
24
Hrs without TNF-α

Figure 2.12 Histogram demonstrating the effect of pre-treating SAOS-2 with TNF-α (1.16nM) for 24hrs upon subsequent SAOS-2 binding to culture plastic, untreated HGF and HGF previously stimulated with TNF-α (1.16nM) for 24 hrs. TNF-α treatment of SAOS-2 did not affect SAOS-2 attachment to tissue culture plastic or unstimulated HGF. The expected increased binding of SAOS-2 to HGF pre-treated with TNF-α was seen ($p<0.002$), there was markedly reduced binding of SAOS-2 pre-treated with TNF-α to similarly pre-treated HGF ($p<0.003$) such that binding was comparable to that seen in unstimulated HGF (n=2).


Average Bound SAOS-2
(Cells Per Graticule Area)
100
80
60
40
20
0
Untreated SAOS-2
TNF-α treated SAOS-2
Culture Plastic
HGF
HGF + TNF-α

2.5 DISCUSSION

Cell adhesion is an important initiating event in contact dependent neoplastic – stromal cell interactions. Cytokines enhance expression of cell surface adhesion molecules and therefore participate in regulation of adhesion events (Tosi et al., 1992, Wong and Dorovini-Zis, 1992, Wicks et al., 1992, Sawa et al., 2007, Pang et al., 1994). The work described in this chapter addressed the important and necessary step of cell adhesion between tumour parenchymal and stromal cells while also studying the modulating effect of an important tumour associated cytokine upon these adhesive interactions.

A convenient and reliable source of fibroblasts was used in the current study, being HGF isolated from gingival tissue explants by a readily reproduced method. Fibroblasts obtained by this standard protocol are rarely contaminated by other cell types and literature accepts that these cells can be used as fibroblasts for experimentation without further characterisation (Uehara et al., 2005, Tuter et al., 2002, Kim et al., 2008, Dongari-Bagtzoglou et al., 1997). While many cells contribute to the stroma within and around tumours, including SMC and endothelium briefly studied in the current chapter, fibroblasts comprise the most prominent non-vascular stromal cell population, so that the focus of the current study on fibroblasts as opposed to other stromal cells seems reasonable. The neoplastic cells focused upon in this study were SAOS-2, on the basis that earlier work in this laboratory has identified interesting activities in these cells, the further study of which comprise this thesis (McEwen et al., 2003, Huynh, 2007).

The data presented supports the concept that the presence of a key inflammatory cytokine such as TNF-α, may encourage neoplastic cell attachment to stromal cells, and thus enhance opportunity for more complex tumour parenchymal-stromal cell interactions. As outlined in the introduction, TNF-α seems a reasonable cytokine upon which to focus for a variety of reasons. Notably, a pro-tumour activity for TNF-α has been noted in various studies (Malik et al., 1990, Mackay et al., 1993, Balkwill, 2002, Balkwill, 2009, Moore et al., 1999, Tlsty and Coussens, 2006), and this seems consistent with the increase in SAOS-2 cell attachment to HGF facilitated by this cytokine in the current study.

Recognizing the possibility that trace contaminant lipopolysaccharide in cytokine preparations may occasionally provide misleading results, the current study used polymyxin B as a well accepted inhibitor of LPS activity (Cavaillon and Haeffner-Cavaillon, 1986, Lynn and Golenbock, 1992, Morrison and Jacobs, 1976), to help control against this possibility. Similarly, inactivation of specific cytokine activity by boiling is accepted as a reasonable control to both confirm cytokine specificity and control against the contribution of potential contaminants, and this approach was also used where it seemed appropriate in the current study.

Direct morphological observation seems to provide an unambiguous and direct method for the study of adhesive interactions between stromal and neoplastic cells in cultures, so that the use of morphological quantitation of attached neoplastic cells to stromal cells in the current study seemed reasonable and reliable.

The current observation of increased SAOS-2 binding to TNF-α stimulated HGF is consistent with studies demonstrating that TNF-α has the potential to increase adhesion of

myeloid leukemic cells to bone marrow fibroblasts (Stolze et al., 1998, Bendall et al., 1997). Also consistent with the data shown, is that TNF-α upregulates adhesion of some neoplastic cells to endothelium or mesothelium (Rice et al., 1988, Steinbach et al., 1996, van Grevenstein et al., 2007, Lauri et al., 1991, Bereta et al., 1991, Bertomeu et al., 1993).

It is interesting that in the current study, TNF-α did not act upon SAOS-2 to increase adhesion to unstimulated HGF, but that there was a surprising counter response by SAOS-2 with reduced binding of TNF-α treated SAOS-2 to TNF-α treated HGF (Figure 2.12). It is tempting to interpret this startling difference in terms of differential roles of TNF-α in different parts of the tumour. Where fibroblasts and neoplastic cells inhabit the same inflammatory milieu, reduced neoplastic cell adhesion molecule expression may counter increased adhesion molecule expression by fibroblasts. At invading tumour margins, however, there may be local inflammation, but invading neoplastic cells by definition must enter from otherwise uninflamed deeper tumour areas, and as a result fibroblasts stimulated by cytokines may more likely encounter and bind invading neoplastic cells uninhibited by inflammatory cytokines. Such a model would seem consistent with the time course experiments performed, in which a rapid increase in SAOS-2 adhesion was seen by TNF-α stimulated HGF, while there was also a prolonged time following cytokine stimulation during which HGF had increased binding of SAOS-2. While it is acknowledged that this scenario is highly speculative and would require in-vivo confirmation as well as further characterization of the SAOS-2 response to TNF-α, data in this chapter do highlight the extreme complexity of interactions between neoplastic and stromal cells and suggest at least partial explanation for the great morphological variability seen within different domains of single neoplasms in-vivo (Marusyk and Polyak, 2010, Visvader, 2011, Fidler and Hart, 1982).

Experiments comparing adhesion in the presence of BCS or BSA, indicated a role for serum factors in TNF-α mediated adhesion. Adhesive serum factors along with cytokines have been shown to increase adhesiveness of cells, with for example, fibronectin bound TNF-α increasing the adhesiveness of CD4+ T lymphocytes (Alon et al., 1994, Franitza et al., 2000). Further, TNF-α in association with laminin increases binding of CD4+ T Cells (Hershkoviz et al., 1995). Based on the data obtained showing increased SAOS-2 adhesion in the presence of BCS compared to BSA, together with our observation of increased SAOS-2 binding to TNF-α stimulated HGF, it seems possible that serum factors may work in concert with TNF-α in a synergistic way to mediate increased SAOS-2 adhesion. Maintained background binding of SAOS-2 to HGF, despite loss of TNF-α induced SAOS-2 binding following heat treatment of BCS seems consistent with this proposal. Irrespective of the as yet incompletely elucidated mechanism through which serum factors contribute to TNF-α stimulated SAOS-2 binding to HGF, it is interesting to note the potential in-vivo relevance, in that HGF are normally only exposed to serum factors in-vivo in sites of inflammation, consistent with the idea of multiple complex signals being involved with permitting fibroblast – neoplastic cell interactions.

Chapter 3: TNF-α Induced Binding by Human Gingival Fibroblasts of Osteosarcoma SAOS-2 Cells is Mediated by Increased ICAM-1 Expression and Influences Apparent Culture Density of Human Gingival Fibroblasts

3.1 INTRODUCTION

As outlined in Chapter 1, interactions of neoplastic cells with stromal cells contribute to the phenotypic characteristics of malignancies, such as an unrestricted ability to replicate, self-sufficiency in growth signals, insensitivity to anti-growth signals, sustained angiogenesis and the ability to invade and metastasize. While inter-cellular interaction coordinates the normal behaviour of normal tissues, similar interactions are coopted into the malignant process to the advantage of tumour growth, invasion and metastasis, and this cross-talk may be either by direct cell to cell contact or through soluble factors (Park et al., 2000, Bhowmick and Moses, 2005, Ruiter et al., 2002, Sugiyama et al., 2005, Micke and Ostman, 2004).

Cell adhesion molecules (CAMs) are glycoproteins, located on the cell membrane or in the cytoplasm, and bind with partner CAMs on other cells or with the extracellular matrix. It is convenient to consider CAMs as belonging to one of four major families: integrins, cadherins, the immunoglobulin superfamily; and the selectins.

The involvement of CAMs in transendothelial migration of leukocytes has been established (Hashimoto, 1994, Antonelli et al., 2001), while separate studies acknowledge the involvement of CAMs in tumour cell adhesion (Swerlick et al., 1992, Kim et al., 1993). Tumour cells often appear to bind preferentially to the ECM rather than the endothelium (Liotta et al., 1984), and bind to intact stromal cells in an organ-specific manner, dependent on the cell surface specific antigens present (Lichtner et al., 1989) .

Altered expression of specific adhesion molecules and cytokines, not only by the tumour cells but also the surrounding stroma, is associated with tumour growth, invasion and metastasis (Honn and Tang, 1992, LaBiche et al., 1993, Nicolson, 1988, Chirivi et al., 1994). For example, E-cadherin is expressed at the adherens junction between epithelial cells and is necessary for epithelial intercellular adhesion, but is reduced with increased invasiveness of epithelial malignant neoplasms of the oesophagus, stomach, and breast, as well as in hepatocellular carcinomas (Shiozaki and Mori, 1991, Shimoyama and Hirohashi, 1991a, Shimoyama and Hirohashi, 1991b, Tohma et al., 1992). Similarly, decrease in neural-cell adhesion molecule (N-CAM) correlates with invasion by colonic carcinomas (Blaheta et al., 2006). Specific integrins on tumour cells facilitate adhesion, migration, metastasis (Sloan et al., 2006) and interaction with some extracellular matrix proteins including collagen, fibronectin, laminin and vitronectin (Bussemakers and Schalken, 1996). VCAM-1 has been associated with the interaction of renal cancer cells and melanoma cells with endothelium (Johnson, 1999, Steinbach et al., 1996).

Of particular relevance to the current thesis, is that cytokines such as IL-1 and TNF-α increase expression and activity of adhesion molecules by both parenchymal and stromal tumour cells (Yoneda et al., 1993, Swerlick et al., 1992, Steinbach et al., 1996, Kim et al., 1993, Guarini et al., 1990).

Intercellular adhesion molecule 1 (ICAM-1), belongs to the Immunoglobulin super family of cell adhesion molecules and facilitates the adhesion of leukocytes to the endothelium as part of the inflammatory response (Ohno et al., 1997). During this process there is clustering of ICAM-1 on the endothelial surface, and co-localisation of this molecule with

actin filaments seems to contribute to migration in addition to adhesion (Yang et al., 2006). Endothelial ICAM-1 expression is augmented upon stimulation with several cytokines including TNF-α, IL-1 and IFN-γ (Wicks et al., 1992, Sawa et al., 2007, Hashimoto, 1994, Amberger et al., 1997, Antonelli et al., 2001, Dufour et al., 1998, Wong and Dorovini-Zis, 1992). A similar effect is also reported for eosinophils (Czech et al., 1993), fibroblasts (Hosokawa et al., 2006, Pang et al., 1994, Spoelstra et al., 1999), epithelial cells (Tosi et al., 1992), synoviocytes (Wicks et al., 1992) and central nervous system tumour cells (Guarini et al., 1990).

Increased expression of ICAM-1 is linked with tumour progression and an increased risk of metastasis, and this is consistent with both enhanced expression of ICAM-1 by cytokine stimulated endothelial cells, as well as with an increase in the binding of endothelium to both T cells and melanoma cells (Kim et al., 1993, McCarron et al., 1993).

Two-dimensional co-culture models are convenient for the study of interactions between neoplastic and stromal cells. Direct contact dependent interactions can be distinguished from those dependent on soluble factors by comparing results of experiments in simple co-cultures, with those of parallel experiments using stromal or tumour cell conditioned media, as well as experiments using transwell inserts porous to culture medium but not to the two cell populations (McEwen et al., 2003, O'Connor et al., 2007, Dong-Le Bourhis et al., 1997, Heneweer et al., 2005, Fierro et al., 2004, Kebers et al., 1998, Koshida et al., 2006, Kurogi et al., 1996).

Several fibroblast responses to separate co-cultured cells have been identified including: up regulation of fibroblast cyclooxygenase-2 in response to both non-neoplastic and pancreatic malignant epithelial cells (Uenoyama et al., 2006, Sato et al., 2004); increased ECM tenascin contributing to metastasis in response to melanoma cells (Adam et al., 2006); and increased MMP-2 in response to laryngeal cancer cells (Suzuki et al., 2004).

A particular focus in this thesis, is the effect of neoplastic cells on the survival of fibroblasts in co-cultures. Related to this, is that soluble factors from MCF-7 breast cancer cells stimulate proliferation of fibroblasts (Adam et al., 1994). Proliferative interactions between fibroblasts and MCF-7 cells are bi-directional as demonstrated in experiments with transwell inserts, such that soluble factors from each cell type stimulate growth in the other. It is interesting that the effect of fibroblasts on epithelial cells is more apparent in the presence of serum, suggestive of complex interactions between serum and fibroblast derived factors (Hofland et al., 1995). A similar observation has been made with direct contact of serum activated fibroblasts on human breast cancer cells (Samoszuk et al., 2005). Separately, exposure to MCF-7 derived soluble factors, stimulates fibroblast proliferation as well as conversion of fibroblasts to a myofibroblast phenotype with increased expression of SMC α actin (Valenti et al., 2001). Fibroblast proliferation also occurs upon exposure to soluble factors from pancreatic cell lines such as LNCaP, PC-3 and DU-145, and this has been ascribed to the presence of growth factors such as PDGF, FGF and the cytokine TNF-α (Kaminski et al., 2006). Prostatic fibroblasts however manifest a growth inhibitory effect on LNCaP epithelial tumour cells, and this is supported by an in vivo observation of low apoptotic rate and formation of large tumours by LNCaP xenografts in the presence of fibroblasts (Olumi et al., 1998).

The laboratory in which work in this thesis was performed, earlier reported that SAOS-2 osteosarcoma tumour cells induce apoptosis in human umbilical vein endothelial cells (HUVEC) in a contact dependent manner, and that this is manifested in co-cultures by a reduction in HUVEC culture density (McEwen et al., 2003). Others have made similar observations for endothelium using MCF-7 breast carcinoma cells, Jar choriocarcinoma cells and bladder carcinoma cells (Kebers et al., 1998, Chen et al., 2005, Heyder et al., 2002, Holash et al., 1999).

Separate and as yet unpublished work by an earlier PhD student in the laboratory from which the current thesis derives, extended the earlier study identifying the apoptotic response of HUVEC to SAOS-2 (McEwen et al., 2003), to demonstrate a similar reduction in apparent culture density of HGF and SMC co-cultured with SAOS-2. While apoptosis had been positively identified as the mechanism whereby SAOS-2 induce reduced HUVEC cell culture density (McEwen et al 2003), no evidence for apoptosis of HGF or HUASMC could be found in co-cultures of these cells with SAOS-2 (Huynh, 2007). As will be further discussed in Chapter 4, the reduction in HGF and HUASMC culture density in co-cultures with SAOS-2 was eventually suggested as more apparent than real, in that it seemed to reflect transfer of the alkaline phosphatase marker from SAOS-2 to the stromal cells studied, rather than an actual loss of stromal cells from cultures (Huynh, 2007). Not investigated at the time, however, was the further possibility that co-cultured SAOS-2 might induce autophagy in HGF and HUASMC, and this chapter contains work examining this potential explanation for the data then obtained (Huynh, 2007).

In autophagy, cells undergo an apoptosis like self-degradation, with the eventual outcome that cells may be lost due to an inability to maintain homeostasis (Levine et al., 2011, Wu et al., 2010, Kelekar, 2005, Rabinowitz and White, 2010). One approach to detecting autophagy is immunofluorescence for LC3, a mammalian homolog of yeast Atg8 recognized as a major constituent of autophagosomal membranes (Kabeya et al., 2000, Tanida et al., 2004, Yoshioka et al., 2008), and this is used in the current chapter to investigate co-cultures of HGF with SAOS-2.

In addition to investigating autophagy, the current chapter expands upon earlier studies (McEwen et al 2003, Huynh 2007), as well as the work described in Chapter 1, by firstly investigating the involvement of HGF ICAM-1 in SAOS-2 binding, and then further exploring the effect of such adhesion upon apparent HGF culture density in subsequent co-cultures.

3.2 MATERIALS

3.2.1 Materials for Cell Culture

Materials for culture of HGF and SAOS-2 were the same as those used and described in section 2.2.1. ICAM-1 Blocking antibody was obtained from Abcam (Cambridge, USA).

3.2.2 Materials for Flow Cytometry

Anti-Human ICAM1 antibody, Anti-Human VCAM-1 antibody and FITC labelled IgG was obtained from Abcam (Cambridge, USA). Falcon FACS tubes were obtained from Becton Dickinson Labware (NJ, USA).

3.2.3 Materials for Detecting Alkaline Phosphatase in SAOS-2

Materials for detecting alkaline phosphatase in SAOS-2 were the same as those used and described in section 2.2.2.

3.2.4 Materials for Detection of Autophagy

LC3A/B and TRITC conjugated rabbit IgG and was obtained from Abcam (Cambridge, USA). Tween 20 was obtained from Sigma-Aldrich (St. Louis, USA).

3.3 METHODS

3.3.1 Isolation, Culture and Storage of HGF, HUVEC, HUASMC and SAOS-2

HGF were isolated, cultured and stored in liquid nitrogen as described in section 2.3.1, 2.3.2 and 2.3.5. HUVEC were isolated, cultured and stored in liquid nitrogen as described in section 2.3.3, 2.3.4 and 2.3.5. HUASMC were isolated, cultured and stored in liquid nitrogen as described in section 2.3.6.

3.3.2 Detection of Alkaline Phosphatase in SAOS-2

Detection of alkaline phosphatase in SAOS-2 was as described in section 2.3.8

3.3.3 Flow Cytometric (FACS) Analysis of ICAM-1 and VCAM-1 Expression by HGF

Confluent HGF in gelatine coated 6 well tissue culture plates were either stimulated with TNF-α (1.16 nM) or provided with fresh CM. After from 0 to 24 hrs, cells were washed with PBS and released with trypsin/EDTA before pelleting by centrifugation and resuspension in PBS with BCS (1%) and a further round of centrifugation at 1500 rpm for 5 min. Supernatants were discarded and pellets resuspended in PBS with BCS (1%) for 20 minutes incubation on ice to block non-specific protein binding sites, before addition of Anti ICAM-1 or VCAM-1 antibody solutions (1µg/ml) and a further 30 mins incubation on ice. Cells were then washed with PBS with BCS (1%) and following centrifugation, resuspended in 100 µl of FITC labeled anti-mouse IgG (1:100 dilution) for 30 min in ice, avoiding exposure to light. Following this, cells were further washed with PBS containing

BCS (1%) and fixed with paraformaldehyde (2% in PBS) for 15 min at RT. Following fixation, cells were washed again with PBS containing BCS (1%), resuspended in 400 µl volumes of PBS with BCS (1%) and analysed with a Becton Dickinson LSRII Flow Cytometer using BD FACS Diva software. Controls consisted of unstained cells, HGF left untreated with TNF-α or stained with only the secondary antibody. Data was analysed using Cyflogic 1.2.1 software and Student t Test.

3.3.4 Evaluation of the Effect of Blocking Antibody Directed Against ICAM-1 upon SAOS-2 Binding to HGF in Experiments Investigating Adhesion of SAOS-2 to Stromal Cells

Binding experiments were performed as described in section *2.3.9a* in which HGF were either unstimulated or stimulated with TNF-α, and further treated for 10 min with a blocking antibody against ICAM-1 prior to application of SAOS-2 in the binding assay.

3.3.5 Application of SAOS-2 to Stromal Cell Cultures in Wells for Evaluation of SAOS-2 Adhesion

Adhesion assays were performed evaluating SAOS-2 binding to HGF using a method similar to that described in section *2.3.9a* with the exception that SAOS-2 were adjusted to a final concentration of 1.5×10^5 / ml in BCS (10%) before application to HGF.

3.3.6 Quantitation of SAOS-2 Adhesion to Stromal Cell Monolayers

Quantitation of the number of attached SAOS-2 to HGF was as described in section 2.3.10.

3.3.7 Prolonged Co-Culture of SAOS-2 with Stromal Cells

Separate to brief culture of SAOS-2 with stromal cells to investigate early binding events, further experiments were performed where SAOS-2 were co-cultured with stromal cells for up to 24 hrs. Briefly, confluent cultures of HUVEC, HGF and HUASMC were seeded into 24 well tissue culture plates in their respective appropriate complete media (Sections 2.3.1, 2.3.3 and 2.3.6) at 3×10^4 cells per well, and allowed to attach overnight at 37^oC under CO_2 (5%). Stromal cell monolayers were then washed with M199 before being seeded with SAOS-2 at 5×10^4 SAOS-2 per well in M199 with BSA (4%) and the antibiotics penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (0.25 µg/ml). Please note that BSA was used to substitute for BCS in co-culture media to avoid stimulation of cells by proliferative serum factors.

In some experiments, HGF or SAOS-2 were pre-treated with TNF-α (1.16 nM), TNF-α (1.16 nM) inactivated at 100^oC for 30 mins, or TNF-α (1.16 nM) together with polymyxin B (1 mg/ml). In addition, some experiments were performed evaluating the effect of TNF-α (1.16 nM) upon culture density of SAOS-2 seeded without the presence of stromal cells.

Monolayers were co-cultured up to 24 hrs and briefly washed with M199 before fixation with 10% neutral buffered formalin followed by 3 washes with PBS and a final wash with Milli-Q water. Stromal and SAOS-2 cells cultured alone served as controls.

3.3.8 Quantitation of Stromal and Tumour Cell Apparent Culture Density

Fixed monolayers of co-cultures were stained to detect alkaline phosphatase activity endogenous to SAOS-2 as described earlier in section 2.3.8, while haematoxylin was used

as a counterstain. Four photomicrographs were taken of each well as described in section 2.3.10, and used for quantitative analysis where the number of alkaline phosphatase positive and negative cells was determined and apparent cell culture density expressed as cells per graticule area.

3.3.9 Immunofluorescence for the LC3 Autophagosome Marker

Expression of LC3 an autophagosome membrane marker on HGF cells cultured alone and in co-culture with SAOS-2 was determined by indirect immunofluorescence labelling. Briefly, HGF cultured alone and pre-labelled with cytoplasmic marker CFSE (0.674μM) for 1 hour at 37^{o}C, as well as in co-culture with SAOS-2 as outlined in section 3.3.7, were fixed at 0, 4, 8 and 24 hrs with PFA (4%, pH 7.4) for 15 mins and then washed three times with PBS. Cells were then permeabilised for 15 mins with tween 20 (0.5%) in PBS prior to which the cover slips were mounted onto glass slides. After three further washes with PBS for 5 mins each, cells were blocked with PBS containing BCS (1%) for 20-30 mins. Following this cells were washed once with tween 20 (0.5 %) in PBS and twice with PBS. The cells were then incubated for 2 hrs with 100 μl volumes of the primary antibody (LC3A/B) at a concentration of 1:50 in PBS with tween 20 (0.5%) and BCS (1%). Cells were then washed as described earlier in this section and incubated with TRITC conjugated rabbit Ig at a concentration of 1:50 in PBS with tween 20 (0.5%) and BCS (1%) for 1 hr. Subsequently the cells were given three final washes for 15 mins with tween 20 (0.5 %) in PBS. Negative controls consisted of cells labelled with only the secondary antibody. The cells were then imaged through an Olympus FV 1000 Confocal Laser Scanning Microscope under 60x oil objective and the images were processed using FV10-ASW 1.7 analysis software.

3.4 RESULTS

3.4.1 HGF Expressed ICAM-1 in a TNF-α Inducible Manner and Blocking Antibody Against ICAM-1 Inhibited SAOS-2 Binding

FACS analysis revealed that background levels of ICAM-1 expression were elevated by 24 Hr of pre-treatment with TNF-α (Figure 3.1), while the results of a separate time course experiment with HGF from a different donor are also shown, and indicate maximal expression of ICAM-1 by 6 hrs of TNF-α treatment over a 24 hr period (Figure 3.1). In a parallel experiment performed at the same time with HGF from the same donor, SAOS-2 binding to HGF was also maximal by 6 hrs of TNF-α stimulation ($p<0.001$) (Figure 3.1).

To examine the role of ICAM-1 expression in SAOS-2 binding to HGF, three experiments were performed with HGF from additional separate donors in which a blocking antibody against ICAM-1 was seen to reduce binding of SAOS-2 to untreated HGF ($p<0.04$), while similar low levels of SAOS-2 binding were seen when blocking antibody was applied to TNF-α stimulated HGF ($p<0.005$) (Figure 3.1). VCAM-1 expression was also examined, but FACS analysis from 2 experiments with 2 different donors failed to demonstrate VCAM-1 expression on HGF both prior to and after TNF-α stimulation (Figure 3.2).

3.4.2 There was a Contact Dependent Decrease in the Apparent Cell Culture Densities of HGF, HUVEC and HUASMC when in Co-Culture with SAOS-2

Since this thesis relates strongly to earlier work in this laboratory describing contact dependent reduction in apparent stromal cell culture density mediated by co-cultured SAOS-2, it was important to perform experiments reproducing the earlier observations as a

basis for further investigation. To that end, 24 hr co-culture experiments were performed of SAOS-2 with HGF, HUVEC and HUASMC.

Photomicrographs in Figure 3.3 demonstrate the strong alkaline phosphatase activity of SAOS-2, and apparent reduction in the culture density of HGF, HUVEC and HUASMC following co-culture with SAOS-2. Quantitation of these experiments is shown in Figure 3.4, where a significant reduction in apparent HGF, HUVEC and HUASMC cell culture densities was seen in SAOS-2 co-cultures compared with stromal cells cultured alone. Stromal cell culture density was reduced by 2 hrs relative to the zero hr time point ($p<0.0001$), with only modest change from 4 to 6 hrs of co-culture with SAOS-2.

SAOS-2 culture density increased modestly up to 4 hrs in all co-cultures, and it was only at the 0 time point that any difference was seen between co-cultures and SAOS-2 seeded alone, reflecting preferential binding of SAOS-2 to stromal cells as opposed to culture plastic, as seen earlier in Chapter 2 (Figures 2.3 and 2.5).

Since the focus of the current thesis is upon interactions between SAOS-2 and HGF, additional experiments confirming reduced apparent culture density of HGF co-cultured with SAOS-2 were performed. Figure 3.5 summarizes the results of 9 separate experiments with HGF from 6 separate donors, demonstrating reduction in apparent HGF culture density with SAOS-2 co-culture ($p<0.004$) and expanding upon the data shown in Figure 3.4.

3.4.3 TNF-α Enhanced the SAOS-2 Mediated Effect of Reducing HGF Apparent Culture Density in Co-Cultures

The finding that TNF-α increased SAOS-2 binding to HGF demonstrated in Figures 2.3 and 2.7, prompted investigation if TNF-α would affect SAOS-2 induced reduction in HGF apparent cell culture density. To that end, co-culture experiments were performed in which HGF were either pre-treated with TNF-α for 24 hr, or where TNF-α was also present during the subsequent co-culture period. As shown in Figure 3.6, in both instances TNF-α increased the effect of co-culture with SAOS-2 of reducing apparent HGF culture density ($p<0.01$). Boiling TNF-α abrogated this effect, while it was notable that the effect of TNF-α was similar regardless if applied as a pre-treatment alone, or in more prolonged culture.

A modest increase in apparent SAOS-2 culture density in co-cultures was observed with both TNF-α pre-treatment ($p<0.05$) and prolonged stimulation ($p<0.05$) (Figure 3.7).

3.4.4 TNF-α did not Affect HGF or SAOS-2 Apparent Culture Density in Monocultures of these Cells

Because data in these experiments was expressed in terms of cell culture density, additional experiments were performed to evaluate the potential effect of TNF-α on HGF and SAOS-2 apparent culture density. Neither 24 or 48 hrs of stimulation with TNF-α affected HGF apparent cell culture density (Figure 3.8). Similarly, as shown in Figure 3.9, there was no effect of TNF-α on SAOS-2 culture density over a 24 or 48 hr period.

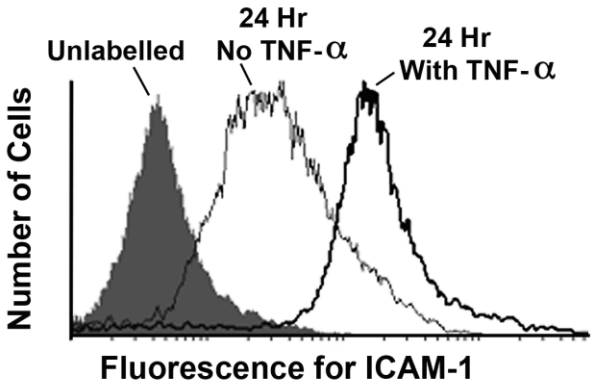
Adhesion assays carried out in parallel with the TNF-α stimulated co-cultures substantiated earlier findings seen in Chapter 2 (Figure 2.7). Native TNF-α consistently increased SAOS-2 binding to HGF ($p<0.01$) and this effect was abrogated by boiling the cytokine (Figure 3.10).

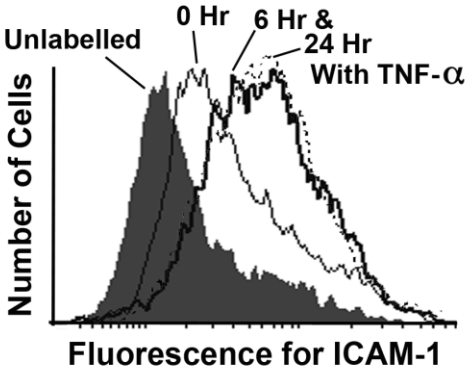
3.4.5 Immunofluorescent Analysis of Autophagic Activity in Co-Cultures of HGF with SAOS-2 Failed to Reveal a Role for Autophagy in Reduction of Apparent HGF Culture Density

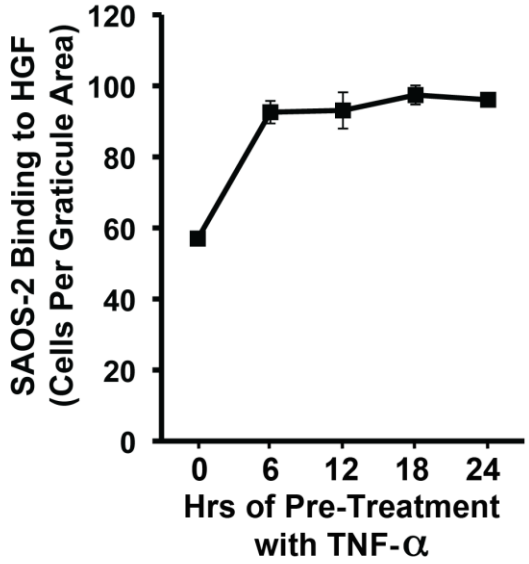
While the reduction in the cell culture density of alkaline phosphatase negative cells suggested loss of HGF in co-cultures with SAOS-2 in earlier work (Huynh, 2007), and is reproduced in this thesis (Figures 3.4 and 3.5), Huynh (2007) was unable to find any evidence for apoptotic or necrotic loss of HGF. The further possibility was examined in the current thesis, that HGF autophagy accounted for the reduction in apparent HGF cell culture density, and to test this possibility, immunofluorescence experiments were performed using the autophagosome marker LC3.

Representative CFM images are shown in Figure 3.11, which demonstrate that co-cultured fibroblasts display a small number of autophagic vesicles. On quantification, no significant difference in amount of LC3 accumulation was observed in co-culture with SAOS-2, so that there was no evidence supporting a role for autophagy to account for the reduction in apparent HGF culture density in co-cultures.

Figure 3.1 FACS analysis for ICAM-1 expression in HGF with or without TNF-α pre-treatment, as well as at increasing times over 24 Hr of TNF-α (1.16nM) treatment, compared with a graph showing the time-course of response to TNF-α (1.16nM) of HGF with regard to SAOS-2 binding, and a histogram of the effect of blocking antibody against ICAM-1 upon SAOS-2 binding to HGF with or without TNF-α (1.16nM) pre-treatment for 24 Hr. HGF expressed ICAM-1 and this was increased by TNF-α with maximal expression by 6 Hr of cytokine treatment correlating with maximal binding of SAOS-2 by 6 Hr ($p<0.001$). Blocking antibody against ICAM-1 reduced binding of SAOS-2 to both untreated and TNF-α stimulated HGF to similar levels ($p< 0.04$).


Unlabelled
24 Hr
No TNF-α
24 Hr
With TNF-α
Number of Cells
Fluorescence for ICAM-1


Unlabelled
0 Hr
6 Hr &
24 Hr
With TNF-α
Number of Cells
Fluorescence for ICAM-1


SAOS-2 Binding to HGF
(Cells Per Graticule Area)
120
100
80
60
40
20
0
0
6
12
18
24
Hrs of Pre-Treatment
with TNF-α

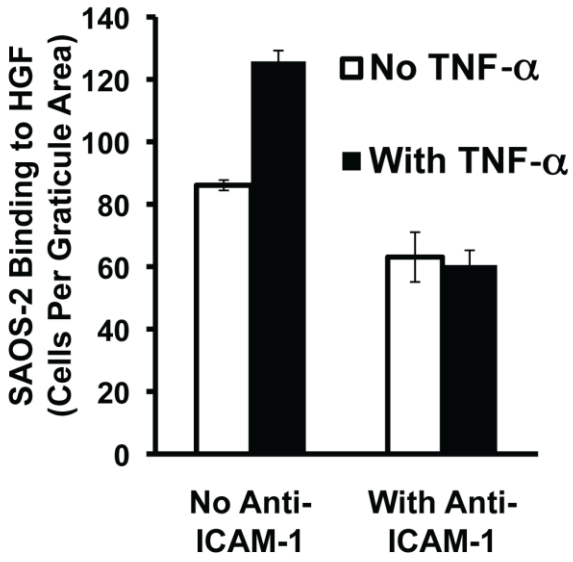

SAOS-2 Binding to HGF
(Cells Per Graticule Area)
140
120
100
80
60
40
20
0
No TNF-α
With TNF-α
No Anti-
ICAM-1
With Anti-
ICAM-1

Figure 3.2 FACS analysis for VCAM-1 expression in HGF with and without TNF-α (1.16nM) pre-treatment for 24 hrs. VCAM-1 was not detected in HGF, and TNF-α had negligible effect with only a slight and unconvincing increase in the proportion of cells with low levels of antibody binding.

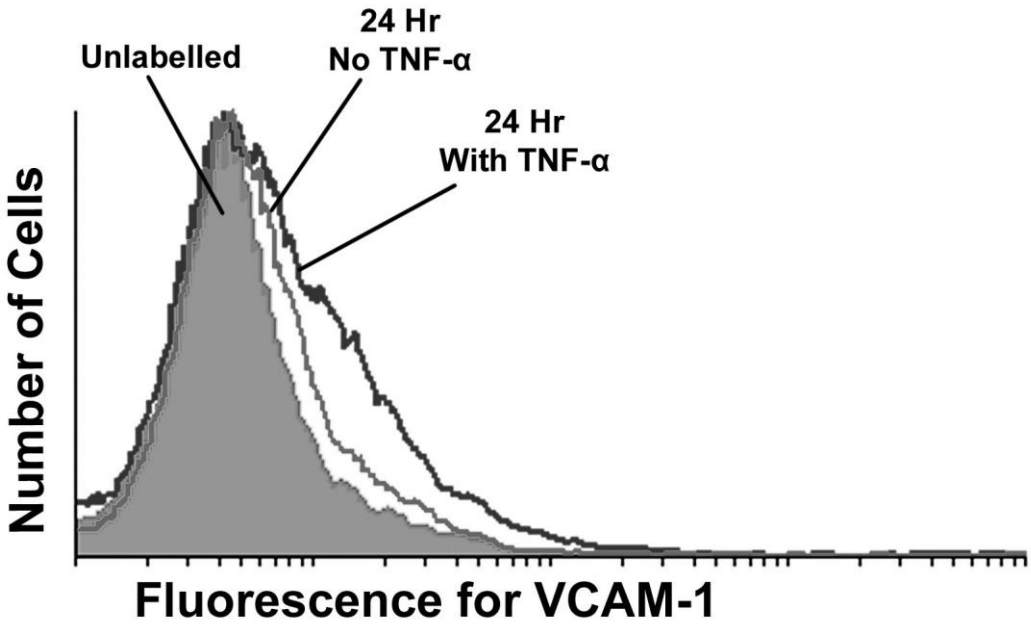

Unlabelled
24 Hr
No TNF-α
24 Hr
With TNF-α
Number of Cells
Fluorescence for VCAM-1

Figure 3.3 Photomicrographs of HGF, HUVEC and HUASMC cultured for 24hrs alone or in co-culture with SAOS-2 stained for alkaline phosphatase expression. SAOS-2 were readily identified in co-cultures on the basis of strong expression of alkaline phosphatase activity. While the HGF, HUVEC and HUASMC expressed no such activity and were detected by haematoxylin counterstain (Bar = 50μm).

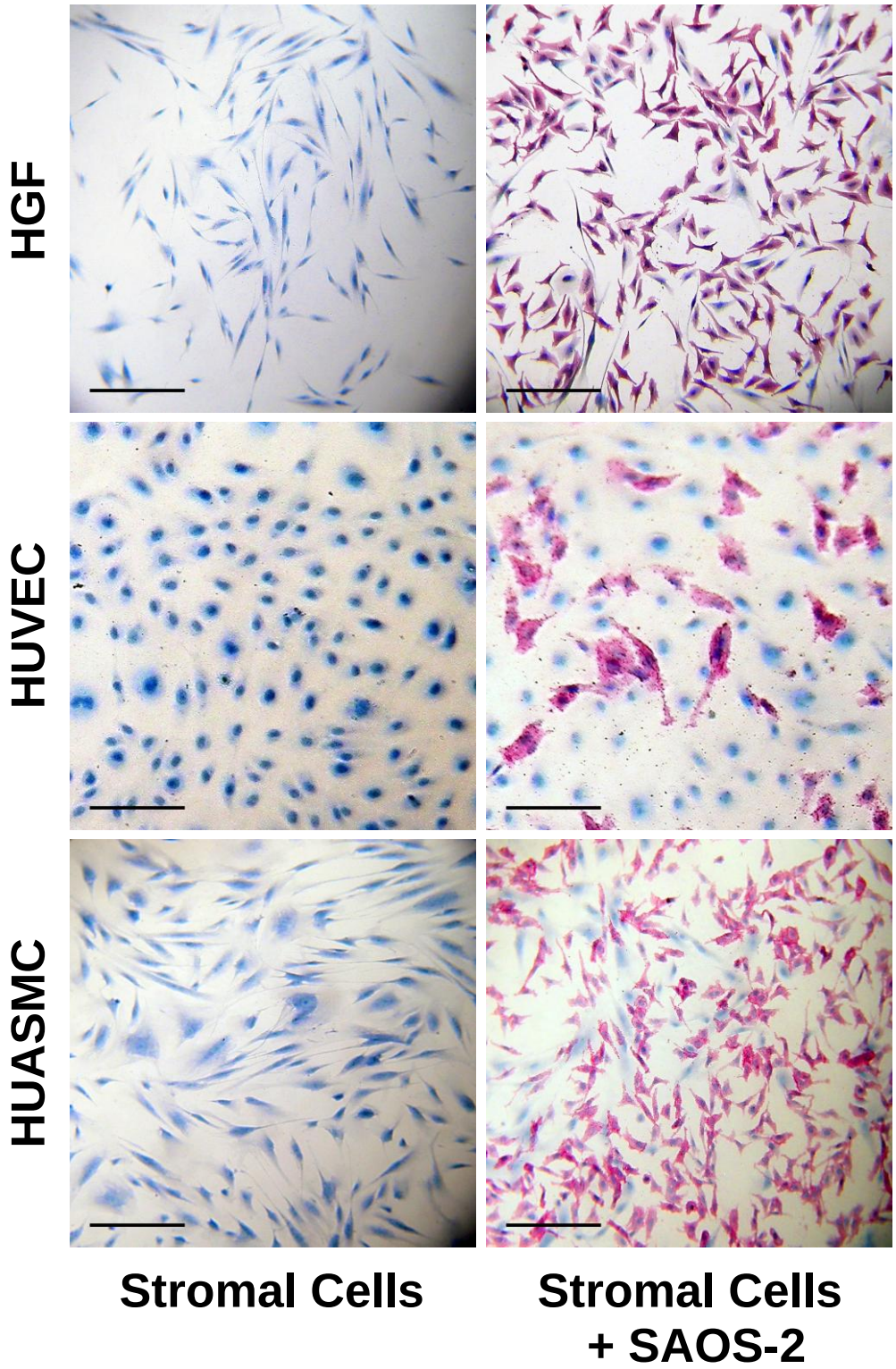

HGF
HUVEC
HUASMC
Stromal Cells
Stromal Cells
+ SAOS-2

Figure 3.4 Histograms demonstrating the effect of co-culture with SAOS-2 on the apparent culture density of HGF, HUVEC and HUASMC over a 24 hr period. Co-culture with SAOS-2 reduced the apparent culture density of all stromal cells studied, with a decrease seen by 2hrs and only modest reduction after from 4 to 6 hrs of co-culture ($p<0.0001$) . SAOS-2 culture density increased only modestly over the 24 hr experimental period in all co-cultures studied, with apparent culture density approaching a maximum by 4 hrs. Higher SAOS-2 apparent culture density at the 0 hr time point in co-cultures as opposed to SAOS-2 seeded alone is interpreted as due to increased initial binding mediated by contact with stromal cells.

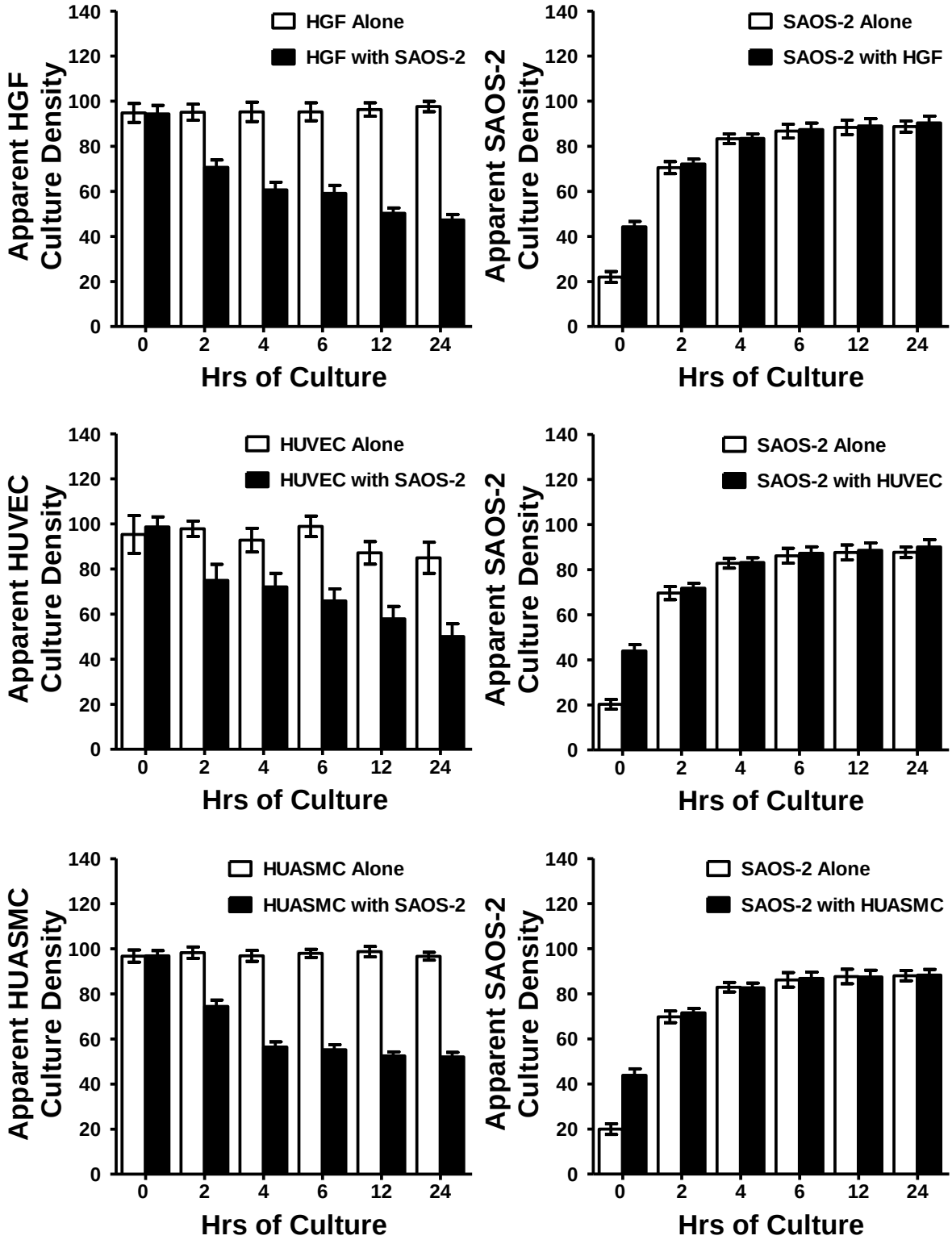

HGF Alone
HGF with SAOS-2
Apparent HGF Culture Density
Hrs of Culture
SAOS-2 Alone
SAOS-2 with HGF
Apparent SAOS-2 Culture Density
Hrs of Culture
HUVEC Alone
HUVEC with SAOS-2
Apparent HUVEC Culture Density
Hrs of Culture
SAOS-2 Alone
SAOS-2 with HUVEC
Apparent SAOS-2 Culture Density
Hrs of Culture
HUASMC Alone
HUASMC with SAOS-2
Apparent HUASMC Culture Density
Hrs of Culture
SAOS-2 Alone
SAOS-2 with HUASMC
Apparent SAOS-2 Culture Density
Hrs of Culture
0
20
40
60
80
100
120
140
0
2
4
6
12
24

Figure 3.5 Scattergram showing the effect on apparent HGF culture density of co-culture with SAOS-2 for 24 hrs relative to cells cultured without SAOS-2 indicated by the dashed line at 100%. Co-culture with SAOS-2 resulted in a decrease in apparent HGF culture density ($p<0.004$).

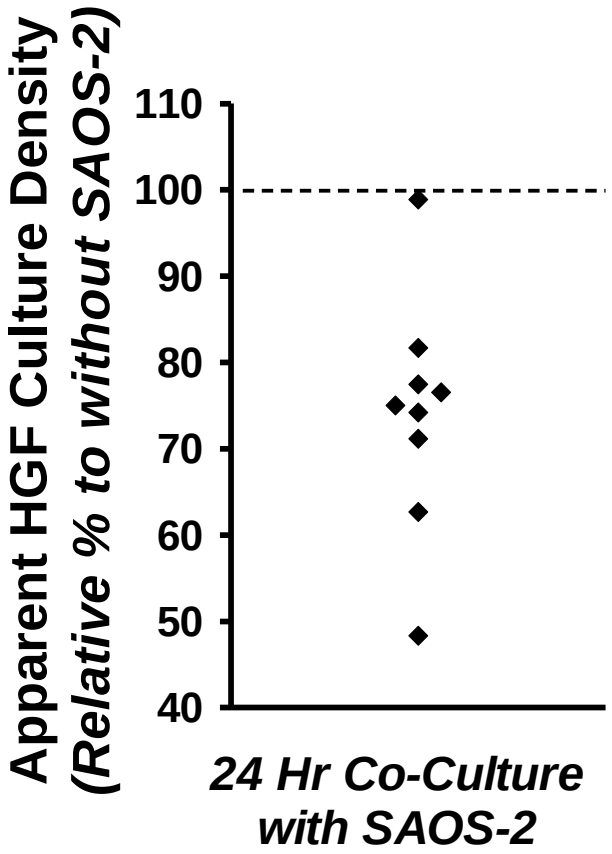

Apparent HGF Culture Density
(Relative % to without SAOS-2)
110
100
90
80
70
60
50
40
24 Hr Co-Culture
with SAOS-2

Figure 3.6 Scattergrams showing the results of separate experiments determining the apparent culture density of HGF co-cultured with SAOS-2, where HGF were treated with either native or boiled TNF-α (1.16nM) for 24 Hr prior to application of SAOS-2, or alternatively with further TNF-α stimulation during 24 Hr co-culture. Data is expressed as a percentage relative to controls not stimulated with TNF-α, indicated by the dashed line at 100%. Treatment of HGF with TNF-α resulted in further reduction in apparent HGF culture density ($p<0.01$), and this effect was abrogated by boiling the cytokine.

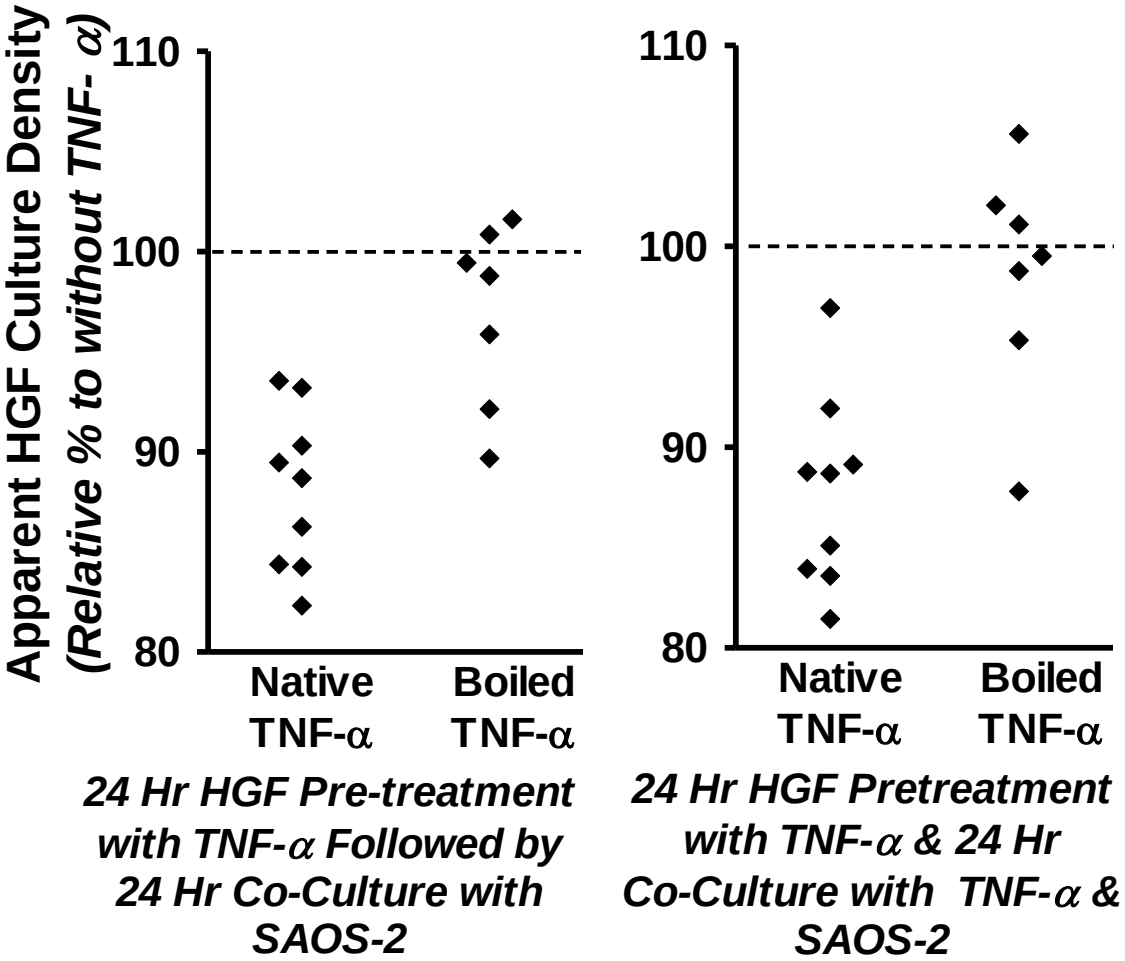

Apparent HGF Culture Density
(Relative % to without TNF- α)
110
100
90
80
Native TNF-α
Boiled TNF-α
24 Hr HGF Pre-treatment with TNF-α Followed by 24 Hr Co-Culture with SAOS-2
110
100
90
80
Native TNF-α
Boiled TNF-α
24 Hr HGF Pretreatment with TNF-α & 24 Hr Co-Culture with TNF-α & SAOS-2

Figure 3.7 Scattergrams showing the results of separate experiments determining the apparent culture density of SAOS-2 co-cultured with HGF treated with either native or boiled TNF-α (1.16nM) for 24 Hr prior to application of SAOS-2, or alternatively with further TNF-α stimulation during 24 Hr co-culture. Data is expressed as a percentage relative to controls untreated with TNF-α, indicated by the dashed line at 100%. An increase in SAOS-2 apparent culture density with cytokine pre-treatment ($p<0.05$) or with prolonged stimulation ($p<0.05$) was observed during co-culture.

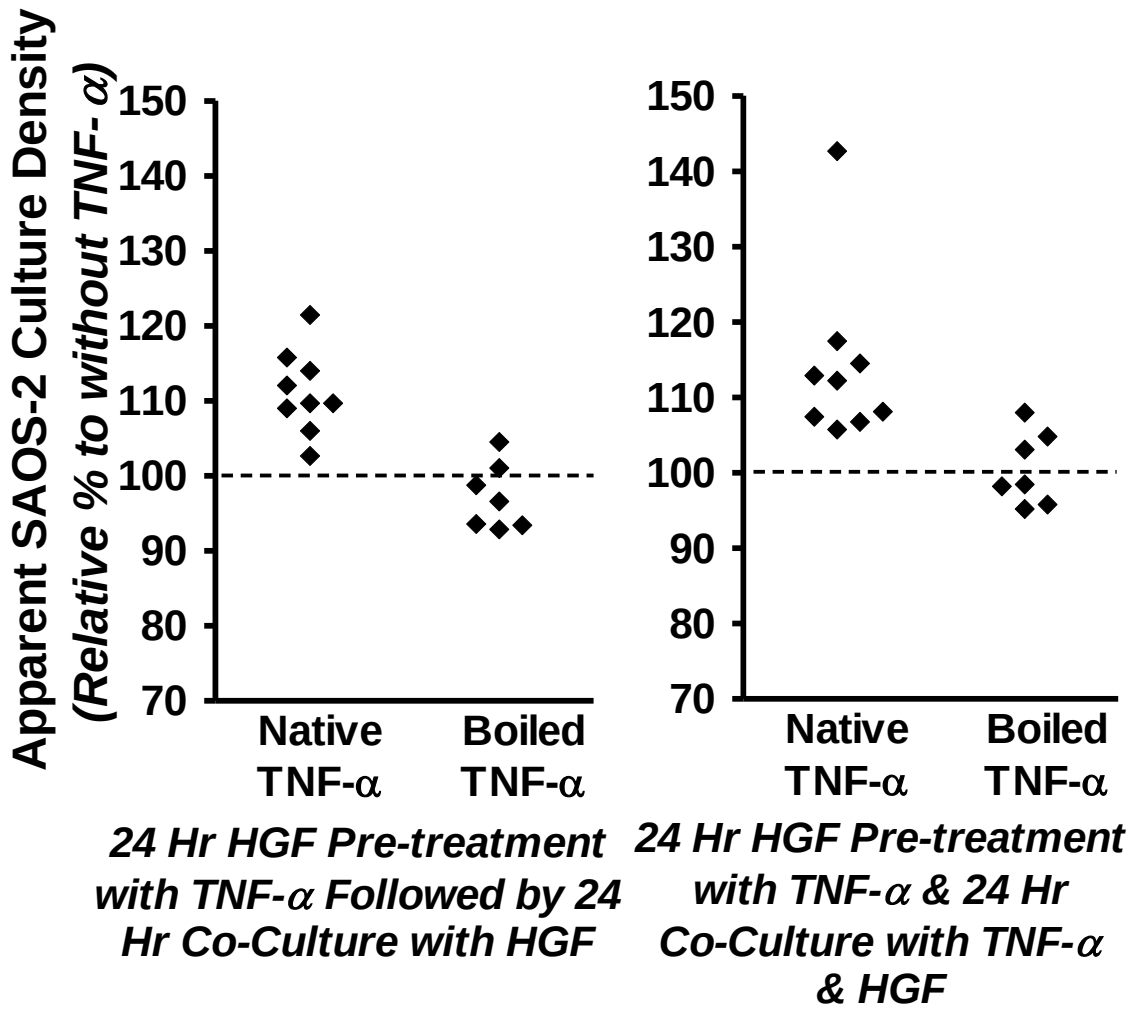

Apparent SAOS-2 Culture Density
(Relative % to without TNF-α)
150
140
130
120
110
100
90
80
70
Native TNF-α
Boiled TNF-α
24 Hr HGF Pre-treatment with TNF-α Followed by 24 Hr Co-Culture with HGF
24 Hr HGF Pre-treatment with TNF-α & 24 Hr Co-Culture with TNF-α & HGF

Figure 3.8 Scattergrams showing the apparent culture density of HGF following either 24 hr or 48 hr stimulation with native or boiled TNF-α (1.16nM). In order to parallel culture conditions used in co-culture experiments, HGF were treated initially for 24 hr with cytokine and either quantitated relative to unstimulated cells, or alternatively further treated with cytokine for an additional 24 hr and then quantitated relative to appropriate 48 hr controls. Data is expressed as a percentage relative to controls untreated with TNF-α, indicated by the dashed line at 100%. TNF-α treatment did not affect HGF culture density for either of the two treatment periods studied, simulating conditions of cytokine pre-treatment or prolonged treatment used in the current study.

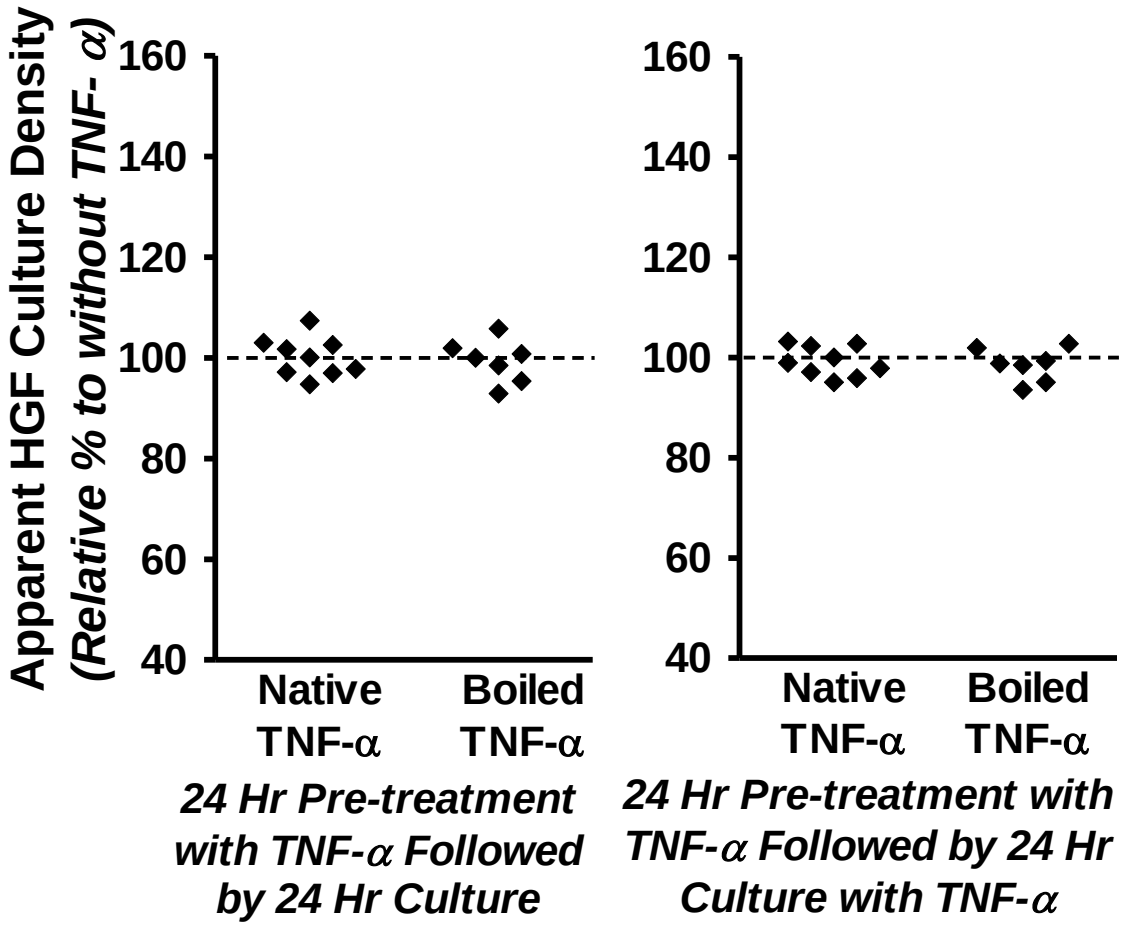

Apparent HGF Culture Density
(Relative % to without TNF- α)
160
140
120
100
80
60
40
Native TNF-α
Boiled TNF-α
24 Hr Pre-treatment with TNF-α Followed by 24 Hr Culture
160
140
120
100
80
60
40
Native TNF-α
Boiled TNF-α
24 Hr Pre-treatment with TNF-α Followed by 24 Hr Culture with TNF-α

Figure 3.9 Histogram demonstrating the effect of TNF-α on SAOS-2 apparent culture density when cultured alone. Pre-treatment for 24hrs with TNF-α as well as prolonged stimulation during 24hrs of culture did not have any effect on SAOS-2 apparent culture density.

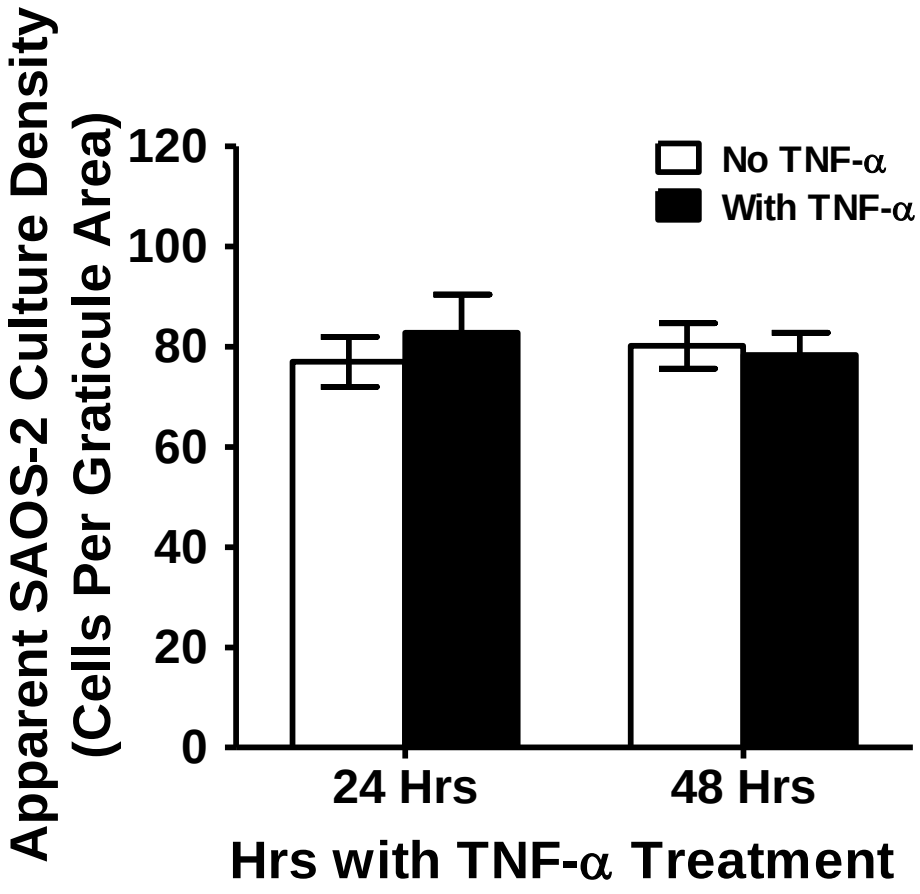

Apparent SAOS-2 Culture Density
(Cells Per Graticule Area)
120
100
80
60
40
20
0
No TNF-α
With TNF-α
24 Hrs
48 Hrs
Hrs with TNF-α Treatment

Figure 3.10 Scattergram showing the effect of treating HGF with either TNF-α (1.16nM) or inactivated boiled TNF-α (1.16nM) for 24 Hr, upon the subsequent binding of SAOS-2 relative to control cells not treated with cytokine as indicated by the dashed line at 100%) Similar to results in Figure 2.7, native TNF-α consistently increased binding of SAOS-2 to HGF ($p<0.01$) and boiled TNF-α abrogated this response across 9 experiments.

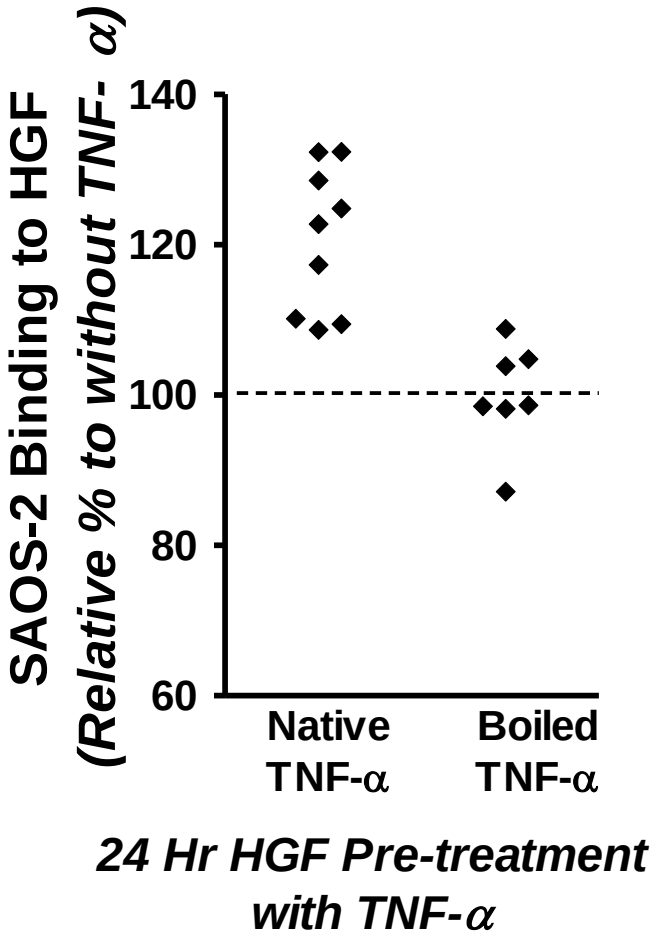
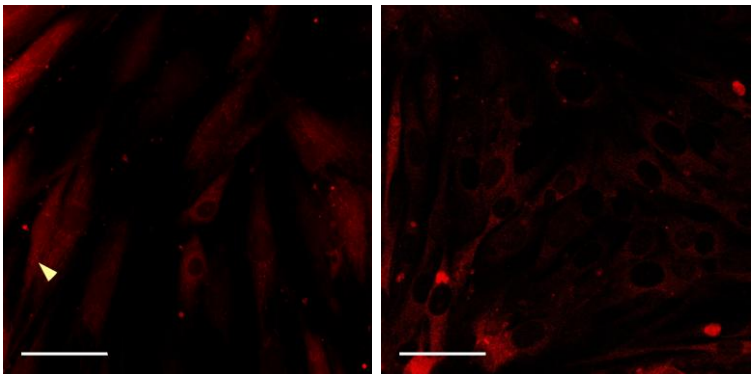
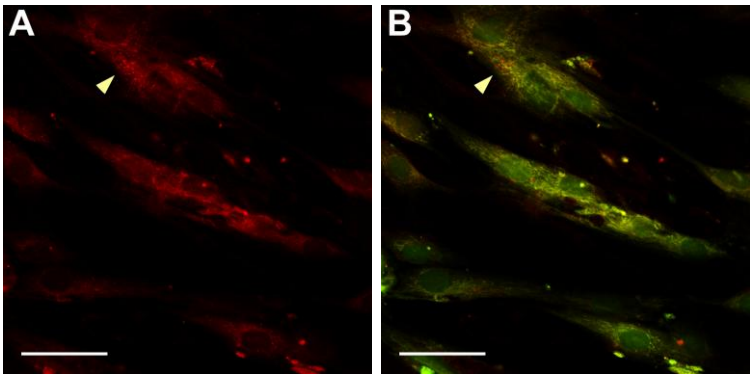

SAOS-2 Binding to HGF
(Relative % to without TNF- α)
140
120
100
80
60
Native
TNF-α
Boiled
TNF-α
24 Hr HGF Pre-treatment
with TNF-α

Figure 3.11 CLSM images of HGF and SAOS-2 cultured alone and in co-culture, stained for the autophagosome marker LC3. HGF in isolated culture displayed low levels of LC3, identified by the accumulation of LC3 positive autophagosomes (arrowheads), while similarly labelled cells in co-cultures (A) were interpreted as HGF as they were positive for CFSE (B). SAOS-2 cells did not display any LC3 positive autophagosomes. Comparatively few SAOS-2 expressed LC3, but there was no clear increase in LC3 labelling in co-cultures compared with HGF cultured alone. (Bar = 50μm)



HGF Alone

SAOS-2 Alone



HGF in Co-Culture

3.5 DISCUSSION

The complex interactions between cytokines, tumour cells and the surrounding stromal cells that contribute towards tumour invasion and metastasis are not fully understood. The work described in this chapter addressed several aspects of these complex interactions, being the issue of the role of adhesion molecules mediating intercellular adhesion, the effect of inter-cellular interaction on the apparent cell culture density of the two cell populations, and the modulating effect of an important tumour associated cytokine upon these interactions.

FACS analysis provides a simple and reliable method to rapidly measure surface markers of interest and thereby achieve objective quantification with a large number of cells. The analysis of positive and negative controls performed simultaneously along with the test sample ensures accuracy of the assay. This approach to study cell surface adhesion molecule expression has been successfully used in other studies (Brandsma et al., 2002, Koga et al., 1997, Grabner et al., 2000, Platts et al., 1995), so it seemed reasonable to use this method for the study of adhesion molecule expression in the current investigation, while care was taken to use appropriate controls to assist interpretation of FACS data.

Similar to the approach described in Chapter 2, direct morphological quantitation was used to study contact dependent interactions between neoplastic and stromal cells in co-cultures. This was extended to the study of apparent cell culture density in monocultures of HGF and SAOS-2, despite the greater ease that less direct methods such as haemocytometer

counts would have provided, because it was felt important that a consistent method for measuring this property should be applied across the same study.

While the emphasis in the current study is upon contact dependent interactions between stromal and neoplastic cells, it may occur to a reader of this thesis that the data shown in this chapter does not show conclusively that reduced apparent stromal cell culture density in SAOS-2 co-cultures is contact dependent, because neither medium transfer or trans-well experiments required to confirm this impression are presented. It is, however, important to note that these necessarily important experiments have indeed been performed by earlier students in this laboratory (Huynh, 2007, McEwen et al., 2003), as have experiments identical to those shown in Figures 3.4 and 3.5 demonstrating contact dependent reduction in apparent stromal cell culture density. The reason why new experimental data showing these already established responses is shown in this thesis, relates to this being an important starting point for further work in this thesis, and the consequent need of the current study to confirm the activity. It did not, however, seem rational for the current thesis to reproduce all aspects of the earlier work, as this would have necessarily detracted from opportunity to break new ground.

Although co-culture is of necessity only a crude approximation of the complexity seen in vivo, co-culture does provide a reasonable model that permits at least initial analysis of the types of interactions that may occur between stromal and neoplastic cells.

Coincidence in expression of ICAM-1 by HGF coupled with inhibition of SAOS-2 binding by blocking antibody against ICAM-1 strongly suggests this as an important molecule

involved with TNF-α mediated SAOS-2 binding to HGF. With reduced binding of TNF-α treated SAOS-2 to TNF-α treated HGF (Figure 2.12), it is tempting to speculate that TNF-α down-regulates expression of the SAOS-2 ICAM-1 binding partner, but without clarity regarding the identity of this ligand binding partner in SAOS-2 and necessary additional data, such speculation must be limited.

Several integrins are known binding partners for ICAM-1 (Bella and Rossmann, 2000, Ley, 2007, Springer, 1990), and while these seem primarily expressed by leukocytes, it seems reasonable to assume that SAOS-2 may express similar or related integrins. Fibrinogen is a major plasma protein which binds ICAM-1 (Pluskota and D'Souza, 2000, Gardiner and D'Souza, 1997, Gardiner and D'Souza, 1999, Tsakadze et al., 2002), and it is tempting to speculate that the heat labile serum factor identified in Chapter 2 as contributing to TNF-α stimulated binding by HGF of SAOS-2, may be trace fibrinogen that has survived the preparation of serum from plasma. Were this the case, then fibrinogen may act as a bridge between HGF ICAM-1, and an as yet unidentified fibrinogen binding molecule on the surface of SAOS-2. Were more time available, it would be interesting to pursue this question further beyond this thesis.

Data shown in this chapter regarding ICAM-1 expression and adhesion of SAOS-2 to HGF are consistent with earlier reports of cytokines enhancing the adhesion of neoplastic cells to stromal cells via modulated adhesion molecule expression. For example, there is increased adhesion between melanoma cells and HUVEC via ICAM-1 following stimulation with TNF-α, IL-1α or IFN (Kim et al., 1993). TNF-α also increases adhesion of HL60, U937, and MOLT-4 to HUVEC by the up regulation of VCAM-1, ICAM-1 and

E-selectin (Mackay et al., 1993). Also, TNF-α and IL-1β increased renal cancer cell adherence to HUVEC correlates with an increased expression of VCAM-1 and ELAM-1 (Steinbach et al., 1996). TNF-α or IL-1 treatment of endothelium augments expression of VCAM-1, which in turn increases adhesion of melanoma and colorectal cancer cells (Yanase et al., 1995), although the current study did not demonstrate significant increased expression of VCAM-1 by HGF before or after TNF-α stimulation.

As noted above and earlier in section 3.4.2, earlier studies in the laboratory where the current work was performed demonstrated that SAOS-2 mediate a contact dependent decrease in HGF apparent cell culture density (Huynh, 2007), and that this was in many ways similar to still earlier work in the same laboratory demonstrating contact dependent apoptosis with reduced cell culture density in HUVEC co-cultured with SAOS-2 (McEwen et al., 2003). Neither apoptosis (Huynh, 2007) nor autophagy (Figure 3.11) appeared responsible for the reduction in HGF density in co-culture. Chapter 4 of the current thesis addresses the question of how SAOS-2 reduce the apparent culture density of HGF despite the absence of HGF death, but the current chapter investigates first the effect of TNF-α on the interaction between SAOS-2 and HGF.

Recognizing adhesion between HGF and SAOS-2 as an important first step towards contact dependent mediated phenomena, we investigated the possibility that increased TNF-α mediated binding of SAOS-2 to HGF seen in the current work, may also increase the apparent reduction in HGF cell culture density earlier seen in co-cultures with SAOS-2 (Huynh, 2007).

Having first reproduced the model system earlier established (Huynh, 2007) and demonstrated reduced HGF apparent cell culture density with SAOS-2 co-culture, the novel observation of that TNF-α enhanced this effect was made. Findings in the time-course of ICAM-1 expression by TNF-α stimulated HGF, as well as with regard to the time course of SAOS-2 adhesion to cytokine stimulated HGF and persistence of increased SAOS-2 adhesion following withdrawal of TNF-α, are all consistent with the essentially identical effect of TNF-α on HGF apparent cell culture density in SAOS-2 co-cultures, irrespective if the cytokine was present only during HGF pre-treatment, or also throughout co-culture. Lack of effect of TNF-α on apparent cell culture density of both HGF and SAOS-2 in isolated cell culture, is interpreted as indicative that untoward proliferative effects did not affect interpretation of the co-culture data.

Regarding apparent cell culture density of SAOS-2 in co-cultures, there did appear to be a difference between TNF-α stimulated co-cultures of HGF, and co-cultures where HGF were not treated with cytokine, however, this was observed at the earliest time points which can be accounted for on the basis of increased adhesion of SAOS-2 to HGF characterized in the current study.

Alkaline phosphatase activity was confirmed as strongly expressed by SAOS-2, and not expressed by HGF, HUASMC and HUVEC, so that it seemed reasonable to use this as a discriminating criterion between stromal and neoplastic cells in SAOS-2 co-cultures. As will be further discussed in Chapter 4, however, a more complex interpretation of this data becomes necessary with the realization that the alkaline phosphatase label itself is shared from SAOS-2 to stromal cells in co-culture. Nonetheless, for the reasons outlined in the introduction, it was important to conduct the range of experiments described in this

chapter, using the co-culture and alkaline phosphatase dependent quantitive methods as described.

The two experimental models investigating on the one hand, SAOS-2 binding to HGF, and on the other hand the effect of SAOS-2 upon HGF apparent culture density, are broadly similar, but do differ in the important respect that serum was present in adhesion assays, but intentionally absent in co-cultures to avoid proliferative effects. While data in Chapter 2 indicate that initial binding of SAOS-2 to stromal cell monolayers will have been compromised by the absence of serum, data in the current chapter suggest that because SAOS-2 in culture wells are effectively captive over HGF monolayers, that the absence of serum assistance in initial binding did not have a significant bearing on the final apparent cell culture density data. Several lines of evidence support this interpretation. Firstly, TNF-α did affect ultimate apparent HGF culture density, despite the absence of serum. Also, independence of eventual SAOS-2 adhesion from initial binding mediated by ICAM-1 is evidenced by the difference seen between 0 hr binding with and without cells, compared with the disappearance of this at later time points. Similarly, the lack of difference in outcomes between experiments where HGF were pre-treated with TNF-α, as opposed to subjection to prolonged simulation with the cytokine, further supports ultimate indifference to serum in the specific experimental model used. It is stressed, however, that this does not mean that initial binding events are not biologically important *in-vivo*, because neoplastic cells in-vivo are not captive to the local environment in the way that cells in culture wells are, but can readily emigrate to other sites. For this reason, initial capture of neoplastic cells by fibroblast ICAM-1 may be important in determining the fate

of individual cells *in-vivo*, in a way that cannot be readily simulated with the simple model systems used in this study.

In the work shown in this chapter, TNF-α did not have any clear proliferative effect on HGF. However in contrast to our observation, TNF-α has been reported to have proliferative effects on fibroblasts (Vilcek et al., 1986, Battegay et al., 1995), but the culture conditions were significantly different to those in the current study, as reported fibroblast proliferation requires concomitant growth factors such as PDGF or serum, while initiation of proliferation was observed only after 2 days of culture, exceeding the times of stimulation in the current study.

The observations made in this chapter thus significantly add to those made in earlier studies, and provide a basis for suggesting that interactions between stromal and neoplastic cells in tumours can be highly regulated by both cytokines and serum factors, and that the individual history of neoplastic cells migrating from sites with differing inflammatory levels will have a profound effect upon the type of interaction that occurs with stromal cells encountered during migration. Notably, our data suggests the concept of 'permissible micro-environments', such that some stromal-parenchymal tumour cell interactions may only occur in defined areas of tumours where the inflammatory environment is suitably permissible for specific interactions, and that these may be further dependent on the individual 'permissive history' of the cells involved.

Results of the current study also support propensity in inflammation for increased invasion and metastasis by increased interaction with stromal cells, as suggested by others

(Macarthur et al., 2004, Schwartsburd, 2003, Schottenfeld and Beebe-Dimmer, 2006, Balkwill and Mantovani, 2001, Rakoff-Nahoum, 2006).

Chapter 4: Exchange of Membrane and Cytoplasmic Markers Between SAOS-2 Osteosarcoma Cells and Human Gingival Fibroblasts During Co-Culture, in a Manner Enhanced by TNF-α

4.1 INTRODUCTION

As mentioned in Chapters 2 and 3, work by an earlier student in this laboratory demonstrated that SAOS-2 induce apoptosis in HUVEC via a contact dependent mechanism (McEwen et al., 2003). Notably, quantitation of the consequent reduction in HUVEC cell culture density exploited binding of *Ulex europaeus* lectin by endothelium to identify cells in co-culture, as well as to confirm the origin of detached apoptotic cells (McEwen et al. 2003).

A similar result was expected when a later student sought to build upon the initial studies with HUVEC (McEwen et al., 2003), by searching for both reduced stromal cell culture density and stromal cell apoptosis in co-cultures of SAOS-2 with either HGF or HUASMC (Huynh, 2007).

While apoptosis had been positively identified as the mechanism whereby SAOS-2 induce reduced HUVEC cell culture density (McEwen et al., 2003), both apoptosis and autophagy have been excluded as contributing to reduction in apparent HGF culture density in similar experiments (Huynh 2007, Chapter 3 Figure 3.11). A critical difference, from the initial study with HUVEC, however, was that no convenient positive marker for HGF was available, but this was circumvented by using the strong SAOS-2 endogenous marker of alkaline phosphatase activity to distinguish neoplastic from stromal cells in co-cultures (Huynh, 2007). While the expected reduction in HGF and HUASMC cell culture density was seen in co-cultures with SAOS-2, it was surprising that there was no clear evidence for apoptosis of these stromal cells, so that

the basis for reduced stromal cell culture density remained uncertain. Particularly confounding, was that despite reproducible reduction in the cell culture density of alkaline phosphatase negative stromal cells in SAOS-2 co-culture, time-lapse photography performed in parallel and essentially identical co-cultures failed to reveal any single instance of stromal cell death or disappearance (Huynh, 2007). Neither apoptosis nor autophagy appeared able to account for cell culture density data obtained from alkaline phosphatase stained co-cultures (Huynh 2007, Figure 3.11 Chapter 3), while time-lapse photography suggested that there was no cell death phenomenon amongst HGF or HUASMC comparable to that seen in HUVEC co-cultured with SAOS-2 (Huynh, 2007). In this way, data was both reproducible and internally inconsistent, and this forced reconsideration of the assumptions made in building and interpreting data from the experimental model.

One assumption that had been made, was that the SAOS-2 alkaline phosphatase marker would remain bound to SAOS-2, and could not be transferred from the neoplastic to the stromal cells. When it was recognized, however, that this assumption may not be justified, the possibility was considered that perhaps reduction in stromal cell culture density was more apparent than real, in that data could also be potentially accounted for by the direct transfer of alkaline phosphatase from SAOS-2 to the co-cultured stromal cells, rather than due to actual disappearance of the stromal cells from cultures (Huynh, 2007). To investigate this possibility, SAOS-2 were labelled with the lipophilic membrane marker DiD, and co-incubated with HGF and HUASMC pre-labelled with the cytoplasmic label CFSE. The emergence of dual labelled populations in such co-cultures suggested that cell fusion events were responsible for the observed reduction in alkaline phosphatase negative stromal cells, and with this the internal inconsistency

could be at least partially reconciled, although it was clear that further work was required using additional membrane, cytoplasmic and nuclear labelling approaches (Huynh, 2007).

Supporting the proposed exchange of cellular material between neoplastic and stromal cells, were previous and subsequent reports of such interactions between macrophages, endothelial cells, epithelial cells and bone marrow derived cells as host fusion partners (Busund et al., 2003, Bhatia et al., 2008, Mortensen et al., 2004, Pawelek, 2008, Pawelek and Chakraborty, 2008b). In view of alkaline phosphatase being bound to the cell surface via a glycosylphosphatidylinositol group, it is interesting to note that precedent for cell contact dependent transfer of similarly bound proteins is established by the glycosylphosphatidylinositol attached co-receptor Cripto-1, which regulates responsiveness to the signalling molecule Nodal by being physically transferred from the cell surface of one cell to another (Watanabe and Salomon, 2010).

It was unfortunate that the time limitations of the previous student's PhD studies precluded further investigation of the processes involved (Huynh, 2007). Nonetheless, work in the current thesis does further examine transfer of cellular material between SAOS-2 and HGF, using more appropriate methods to distinguish between membrane, cytoplasmic and nuclear compartments of the two cell populations. Please note, that the experimental approach of using alkaline phosphatase as a SAOS-2 marker in co-cultures developed by Huynh (2007) was used in this thesis to: confirm in Chapter 3 the work of the earlier student (Huynh, 2007); establish continuity with the earlier work from which to further investigate these phenomena; and also to evaluate the effect of TNF-α on reduction in apparent stromal cell culture density (Figures 3. 6).

While most intercellular communication appears to be via soluble or cell surface bound ligands binding to specific receptors on the surface of opposing cells, it has also been recently reported that some intercellular communication is mediated by the physical transfer of membrane and or cytoplasmic components between cells (Niu et al., 2009, Watanabe and Salomon, 2010), and nanotubes have been suggested as providing a physical basis for similar exchange in some circumstances (Koyanagi et al., 2005, Onfelt et al., 2004, Rustom et al., 2004).

It seems reasonable to consider transfer of membrane and cytoplasm between cells as a limited form of cell fusion, and comprises a normal biological process in mammalian development and differentiation, such as in the formation of the syncytiotrophoblast, skeletal muscle cells and osteoclasts (Horsley and Pavlath, 2004, Paululat et al., 1999, Vignery, 2005, Huppertz and Gauster, 2011). Fusion events have also, however, been suggested as responsible for generating populations of cells within tumours that share traits of malignant as well as non-malignant cells (Pawelek, 2005). Fusion between leukocytes (Lazova et al., 2011) and lymphocytes (Mekler, 1971) with malignant cells has been noted. The tendency for neoplastic cells to fuse spontaneously forming heterokaryons in vitro and in vivo is demonstrated across several studies (Duelli and Lazebnik, 2003, Bjerkvig et al., 2005, Pawelek and Chakraborty, 2008b, Pawelek and Chakraborty, 2008a, Miller et al., 1988, Rachkovsky et al., 1998). Hybrid cells arising from fused neoplastic and stromal cells and demonstrating increased malignancy are reported for macrophage melanoma fusion hybrids (Rachkovsky et al., 1998), and macrophages with bone marrow derived cells in vivo and in vitro (Pawelek and Chakraborty, 2008b). There is progression of non-tumourigenic T-lymphocytes to cells with metastatic properties after fusion with normal lymphoreticular host cells, while

these hybrids also exhibit organ specific metastasis (De Baetselier et al., 1984a, De Baetselier et al., 1984b). Chromosomal segregation has been suggested as important in the acquisition of metastatic properties following fusion of stromal host cells with MDW4 (WGAr), a murine tumour cell line and variant of the MDAY-D2 DBA/2 mouse tumour (Kerbel et al., 1983). Similar observations of neoplastic cells gaining metastatic properties by fusion events is also noted by others (Larizza and Schirrmacher, 1984).

Of particular interest to this thesis is the fusion of neoplastic cells with fibroblasts, cells that this thesis argues are of special relevance due to their prominence as the major extravascular stromal cells. Evidence of fibroblasts fusing with human HeLa tumour cells (Lichy et al., 1996, Stanbridge et al., 1982), as well as with SEWA and TA_3 murine tumour cells (Harris et al., 1971) is noted, and interestingly these hybrids demonstrate regression in malignancy attributed to the loss of chromosomes upon fusion (Harris, 1971, Srivatsan et al., 1986). In the case of HeLa fibroblast hybrids, differential expression of the ST_5 gene is involved in suppression of malignant properties (Lichy et al., 1996). In contrast, mouse melanoma / lymphoma tumour cells and fibroblast hybrids demonstrate tumourigenicity after chromosome loss (Evans et al., 1982).

Although most studies relating to cell fusion events have focused on complete fusion between cells, the work in this thesis investigates the wider possibility of partial fusion, or perhaps what should be more appropriately considered the simple exchange of material between cells. Heterocellular communication with partial cell fusion between fibroblasts and cardiomyocytes is reported, with an increase in expression of adhesion molecules at the point of contacts and associated structural changes in the myocytes

upon contact with the fibroblasts (Driesen et al., 2006, Driesen et al., 2005). Also, SNARE which is a fusion protein involved with reconstitution into liposomes, induces complete fusion as well as hemifusion / partial fusion as an end point, and not an intermediate state prior to progression to complete fusion (Giraudo et al., 2005).

A number of methods are used to detect transfer between cells including: screening of cell surface expressed gene products, exchange of fluorescent dyes, monitoring expression of reporter genes, heterokaryon detection and tracking of double labelled cells over a period of time (White et al., 1982, Fuerst et al., 1986, Nussbaum et al., 1994, Munoz-Barroso et al., 1998, Driesen et al., 2005, Lin et al., 2010, Shirasawa et al., 2005, Cohen and Melikyan, 1998). Fluorescent dyes appear to provide the most direct approach, and enable visualisation and localisation of membrane structures, cytoplasm and nuclei. Fluorescent dyes have for example been used to study partial cell fusion by others in a model studying cardiomyocyte interaction with fibroblasts cells (Driesen et al., 2005).

The membrane fluorescent lipophilic markers 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate (DiD/DiIC_{18}(5); abbreviated in this thesis as DiD) (maximum excitation at 644nm, maximum emission at 665nm) is an analogue of DiI, and provides strong red fluorescence as opposed to 3,3'-dioctadecyloxacarbocyanine perchlorate (DiO/DiOC_{18}(3), abbreviated in this thesis as DiO) (maximum excitation at 484nm, maximum emission at 501nm) which has green fluorescence. Both DiD and DiO are convenient and effective for tracking membrane continuity both in-vitro and in-vivo, as they are known not to be transferred across even very close synaptic junctions, but do nonetheless trace the entire membrane surface of

labelled cells (Honig and Hume, 1986, Timmers et al., 2002). With time, the shuttling of membrane between surface plasma membrane and endoplasmic reticulum (Alberts, 2008), does result in widespread labelling of cells by DiD and DiO, but despite this the highly hydrophobic nature of these labels confines them to the membrane compartment (Honig and Hume, 1989, Pawley, 2006). The lateral mobility of carbocyanine dyes is reported to be in the range of 10^{-7} - 10^{-8} cm^2/s (Honig and Hume, 1989).

Although 5-(6)-carboxyfluorescein diacetate succinimidyl ester is not itself fluorescent, it does diffuse readily through the plasma membrane to be converted by intracellular esterases into much less permeable and highly fluorescent carboxyfluorescein succinimidyl ester (CFSE) (maximum excitation at 492nm, maximum emission at 517nm). CFSE binds covalently via its succinimidyl group to primary amines of proteins and other intracellular molecules, so that this fluorescent label establishes near permanent labelling of cell cytoplasm (Wang et al., 2005a, Parish, 1999, Lyons, 2000). The separate fluorescent label 7-hydroxy-9*H* (I, 3-dichloro-9, 9-dimethylacridin-2-one) succinimidyl ester (DDAO-SE) (maximum excitation 647nm, maximum emission 657nm) is also initially permeable to the cell but once activated by esterase and bound covalently to free amine groups by reaction with the succinimidyl of the dye, becomes an effective permanent marker for cell cytoplasm (Mapili et al., 2005, Malhotra et al., 2008, Couture et al., 2009).

Syto59 is a cell permeant DNA label (maximum excitation 622nm, maximum emission 645nm), which was found convenient for work described in this chapter.

Green fluorescent protein (GFP) (maximum excitation 488nm, maximum emission 507nm) is widely used as a transfected fluorescent label (Xu et al., 2004, Wang et al., 2005b, Shimomura et al., 1962, Peyruchaud et al., 2001, Luu et al., 2005, Lippincott-Schwartz and Patterson, 2003, Naumov et al., 1999). In the current study, a cloned GFP expressing SAOS-2 cell line was used in a number of experiments (SAOS-GFP), which had been stably transfected with GFP using pEGFP-N1 and selected using the cytotoxic antibiotic G-418 (Huynh, 2007). SAOS-GFP were cultured with the continued use of the selective agent to maintain GFP expression, and over 92% of cells in this cell line had strong expression of GFP in both cytoplasmic and nuclear compartments. Importantly for this study, SAOS-GFP maintained both alkaline phosphatase activity and the ability to reduce apparent HGF culture density in co-culture using alkaline phosphatase as the cell discriminatory characteristic, so that there can be confidence that the activity of interest survived the transfection and cloning process (Huynh, 2007).

The work described in this chapter explores the potential transfer of membrane, cytoplasmic and nuclear material between SAOS-2 and HGF using combinations of the above mentioned fluorescent labels. Also investigated, is the effect of TNF-α on transfer of cellular material between HGF and SAOS-2, and some structural phenotypic consequences of co-culture.

4.2 MATERIALS

4.2.1 Materials for Cell Culture and Alkaline Phosphatase Detection

Materials for culture of HGF, SAOS-2, SAOS-GFP and for detection of alkaline phosphatase were the same as those used and described in section 2.2.1 and 2.2.2 respectively. Geneticin (G-418) was purchased from Invitrogen (Oregon, USA).

4.2.2 Materials for Fluorescent Microscopy and Confocal Microscopy

CFSE, DDAO-SE, DiD, DiO, Syto 59 and slow fade anti fade kit were purchased from Invitrogen (Oregon, USA). Nail varnish was obtained from Maybelline (Woolworths). DAPI, Hoechst 33342, Dihydroethidium, Syto 17, 59, 60, 61, 62, 63 and 64 were obtained from Invitrogen (Oregon, USA), Sybr green and acridine orange were obtained from Sigma-Aldrich (St. Louis, USA), and Nuclear ID Red DNA stain and DRAQ5 were purchased from Alexis Biochemicals (Enzo Lifesciences, NY, USA).

4.2.3 Materials for Assessing Phenotypic Changes in HGF and SAOS-2

A Scope Photo 3.0 Digital camera attached to a Olympus CK2 phase contrast inverted microscope was used, as was a Zeiss Axiovert 200M fluorescent microscope and ImageJ 1.44i image analysis software.

4.2.4 Green Fluorescent Protein Expressing SAOS-2 Cell Line

GFP expressing SAOS-2 were a gift prepared by Dr. Hyunh.M (Huynh, 2007). In brief, SAOS-2 were transfected with a DNA mixture of the transfection plasmid (2 μg of pEGFP-N1 in 600 μl of Opti-MEM) combined with Lipofectamine (1:50 dilution in

Opti-MEM). SAOS-2 conditioned media with the antibiotic selective agent G-418 (500 μg/ml) was added after 48 hrs of incubation and replenished twice a week for a fortnight until isolated colonies began to form. Colonies once formed were selected using cloning rings and grown using SAOS-2 conditioned media with the antibiotic selective agent G-418 (500 μg/ml) and stored in liquid nitrogen for later use (Huynh, 2007). GFP-SAOS were characterized with regard to their expression of GFP and alkaline phosphatase, as well as their ability to cause reduced apparent cell culture density of HGF in co-culture (Huynh, 2007).

4.3 METHODS

4.3.1 Isolation, Culture and Storage of HGF

HGF were isolated, cultured and stored in liquid nitrogen as described in section 2.3.1 2.3.2 and 2.3.5 respectively.

4.3.2 Culture of SAOS-2

SAOS-2 were cultured and stained for alkaline phosphatase activity as described in 2.3.7 and 2.3.8 respectively.

4.3.3 Culture and Storage of SAOS-GFP

SAOS-GFP were cultured in T75 cm^2 tissue culture flasks using M199 with antibiotics and BCS (10%) together with G-418 (500µg/ml) as the selective agent. When confluent they were split at a ratio of 1:3 by the method described in section 2.3.7, while cells were stored in liquid nitrogen via the protocol indicated in section 2.3.5. Note that the selective agent was excluded in co-culture experiments.

4.3.4 Loading of HGF and SAOS-2 with Fluorescent Membrane, Cytoplasmic and Nuclear Labels

Confluent HGF were cultured and harvested using trypsin/EDTA as outlined in section 2.3.2, before being seeded into gelatinised (0.1% in PBS) glass cover slips in 6 well tissue culture plates. Cells were left to attach overnight at 37^oC under 5% CO_2 and then deprived of serum with or without TNF-α (0.58 nM) for 24 hrs. HGF were then labelled with membrane or cytoplasmic markers for further experimentation. The two

membrane markers used were DiD and DiO, applied as 5 μM solutions in HBSS at 37^{o} C for 1 hr . The two cytoplasmic markers used were CFSE (2.5 μM) and DDAO-SE (1.5 μM), applied in HBSS at 37 ^{o}C for 1 hour and 20 min respectively. SAOS-2 were cultured in T75 cm^{2} tissue culture flasks and labelled by an identical protocol to that described for HGF, before harvesting with trypsin/EDTA, centrifugation and resuspension in M199 with antibiotics and BSA (4%) for application to pre-labelled HGF. Controls for specificity of fluorescence signal comprised pre-labelled homotypic cultures of HGF and SAOS-2, as well as unlabelled cells.

Nuclear labels were required to study the possible fusion of nuclear material, as well as to trace the origin of individual cells in co-culture. SAOS-GFP were noted as having strongly labelled nuclei in addition to cytoplasm, and this provided a convenient and reliable means for tracing the origin of SAOS-2 nuclei in co-cultures. SAOS-GFP were cultured under the selective pressure of G-418 (500 μg/ml) as outlined in section 4.2.4, and harvested for application to HGF in co-cultures as indicated in section 2.3.2. Pre-labelling of nuclei in HGF was with Syto59, while these cells were also labelled with DDAO-SE as a cytoplasmic marker as outlined in section 4.3.4. Syto59 at a dilution of 1:5000 was applied to cells together with DDAO-SE (1.5 μm) for 20 mins at 37^{o}C under 5% CO_2 before washing with HBSS and initiation of co-culture for 24 hr with SAOS-GFP harvested with trypsin/EDTA, in a co-culture medium comprising M199 with antibiotics and BSA (4%).

Irrespective of the means of cell labelling, all cultures were fixed at the conclusion of experiments with 4% paraformaldehyde for 15 mins, and washed gently with PBS

before being mounted onto glass slides with slow fade and sealing with nail varnish to prevent drying.

4.3.5 Quantification of Membrane, Cytoplasmic and Nuclear Exchange

Images were captured using an Olympus FV1000 Confocal laser scanning microscope (CLSM) with Olympus IX-81 ZDC microscope and FV10-ASW 1.7a capture software, applying lasers ArKr 473 nm, HeNe 633 nm and bandpass filters for FITC and Cy5. Images were merged in Olympus FV10-ASW 1.7a analysis software and the number of cells labeled with DiO, DiD, CFSE, DDAO-SE or GFP, as well as dual labeled cells was quantitated by direct counting. Statistical significance was determined using the Wilcoxon Ranked Sign Test.

4.3.6 Alternative Nuclear Markers Attempted for Labelling Nuclear Material

Although Syto59 was used in final experiments, this label was chosen only after extensive preliminary experimentation. It seems important for this thesis to outline the various approaches trialled in attempts to achieve nuclear labelling in SAOS-2 and HGF. For example, 4',6-diamidino-2-phenylindole (DAPI, maximum excitation 358, maximum emission 461) was used at 300 nM with an incubation time of 5 mins, using a similar approach as described in section 4.3.4. Similarly, acridine orange (maximum excitation 502 nm, maximum emission 526 nm) was used at 5 μg/ml; Hoechst 33342 (maximum excitation 350 nm and maximum emission 460 nm) was applied at a concentration of 1 μg/ml (15 minutes); Dihydroethidium (maximum excitation 518 nm and maximum emission 605 nm) was used at 10 μM (30 minutes); DRAQ5 which is a far red nuclear marker was used at 10 μm for 15 minutes; Sybr green (maximum excitation 494 nm and maximum emission 521 nm) was trialled used at dilution 1/5000

for 15 minutes; Nuclear ID Red DNA stain (maximum excitation 566 nm and maximum emission 650 nm) was applied at a dilution of 1:2000; and finally Syto 17, 59, 60, 61, 62, 63 and 64, which are all dyes with maximum excitation ~600 nm and above were trialled at 1 μM.

4.3.7 Morphometric Analysis of HGF, SAOS-2 and Dual Labelled Cells when Cultured Alone and in Co-Culture with SAOS-2

Images were captured using a Scope photo 3.0 digital camera at 10x magnification using a Zeiss Axiovert 200M fluorescent microscope at 20x magnification. ImageJ 1.44i image analysis software was used, in which cell borders were outlined on screen to permit determination of cell surface profile area and cell circumference. Using these measures, it was possible to calculate a value for the circularity of individual cells, such that a value of 1 indicates a perfect circle, and values approach zero as cells become increasingly elliptical. At least 100 cells were studied per specimen, and statistical significance between samples was evaluated using the Mann Whitney U test.

4.4 RESULTS

4.4.1 Identification of Syto59 as an Appropriate Nuclear Label for Experimentation

Critical criteria for successful labelling related to cellular cytotoxicity, strength of labelling, ability to distinguish from an opposing partner label, and permanent capture of the label within the nucleus without diffusion across membranes to label adjacent cells in the absence of actual nuclear fusion. HGF and SAOS-2 were labelled and fixed after 24 hrs of co-culture before observation. Acridine orange showed a strong green nuclear fluorescence, but was found to suffer 'bleed through' when examined with a blue excitation light. DAPI, which can be used both for live as well as fixed cells, was not an adequately permanent marker for experiments. Hoechst 33342 showed strong blue nuclear labelling at 0hrs, but at subsequent time points appeared in unlabelled co-cultured cells, indicating leakage of the dye post labelling and its uptake and binding to the DNA of other cells in co-culture independent of actual nuclear fusion. Dihydroethidium did not demonstrate any labelling at different time points, despite increasing concentration and incubation times up to 100 μM and 4 hrs respectively. DRAQ5, stained DNA very rapidly but the staining was stable only for 1 to 4 hrs, after which the dye accumulated in the cytoplasm with the nuclear staining disappearing, so that this label was unsuitable for the current study where culture periods extended up to 24 hours. Sybr green showed strong nuclear labelling at 0, 4 and 24 hrs, but bleached out in under 1 minute upon examination with blue excitation light. Syto 17, 60, 61, 62, 63 and 64 markers demonstrated very weak nuclear labelling and were very indistinct with a lot of non-specific cytoplasmic labelling in addition to a number of cells

demonstrating nuclei that were devoid of any staining, with the exception of Syto59 which demonstrated strong and stable nuclear staining.

4.4.2 SAOS-2 and HGF Exchange Membrane and Cytoplasmic Markers During Co-Culture

When HGF and SAOS-2 were pre-labelled with the lipophilic membrane markers DiD and DiO respectively and then co-cultured for 24 hrs, numerous cells bearing both membrane markers were found by fluorescence microscopy, while CLSM confirmed this was not due to cell overlap (Figure 4.1).

Dual labelled cells were also found when similar experiments were performed where cytoplasmic and nuclear rather than membrane markers were used (Figure 4.2), in which HGF were pre-labelled with Syto59 and DDAO-SE and then co-cultured with SAOS-GFP. The intensity of HGF cytoplasmic marker was low amongst dual labelled cells when compared with that of the SAOS-2 cytoplasmic marker. No clear evidence for the possible fusion of HGF and SAOS-2 nuclei in co-cultures was seen, despite GFP being strongly expressed in labelled SAOS-2 and Syto59 effectively labelling HGF nuclei. Also, where cells expressed both cytoplasmic labels, nuclear labelling was usually characteristic of SAOS-2 rather than HGF (Figure 4.2). Fibroblasts were pre-labelled with DiO (green) and co-cultured with SAOS-2 pre-labelled with DiD (red). As can be seen in the image many cells expressing both labels are observed. The dual labelled cells have been demonstrated to have a morphology intermediate to that of fibroblasts and SAOS-2 and the cells that can be seen here through the green filter can no longer be identified entirely as fibroblasts but as cells that have undergone membrane transfer and hence appear more rounded.

4.4.3 SAOS-2 in Isolated Culture Exchanged Membrane but not Cytoplasmic Labels Amongst Themselves, but there was no Membrane or Cytoplasmic Exchange Amongst HGF Cultured Alone

By creating two populations of SAOS-2, each labelled with either DiD or DiO, and then re-combining these two for culture over 24 hrs, it was found that exchange of membrane label occurred frequently within SAOS-2 populations cultured alone (Figure 4.3). However, it was not possible to demonstrate a similar exchange of cytoplasmic label between populations of SAOS-2 which had been pre-labelled with the cytoplasmic markers CFSE and DDAO-SE (Figure 4.4). When similar experiments were performed labelling populations of HGF with membrane or cytoplasmic markers, no dual labelled cells were found either with respect to membrane or cytoplasmic markers (Figure 4.4).

4.4.4 Quantitative Analysis of Membrane and Cytoplasmic Labelling for HGF (DiO, CFSE, Syto59) and SAOS-2 (DiD) or SAOS-GFP in Co-Cultures Revealed Dual Labelled Membrane Markers Increased at the Expense of Cells with HGF Membrane Marker Alone, while TNF-A Increased Exchange of Membrane and Cytoplasmic Labels Between SAOS-2 and HGF

Additional experiments using an essentially similar approach to that described above in section 4.3.4 were performed to both quantitate exchange of membrane, cytoplasmic or nuclear labels between HGF and SAOS-2, and also to investigate the possibility that these exchange processes may be affected by TNF-α, consistent with the effect of this cytokine seen in Chapters 2 and 3. Note that in these particular experiments, HGF were labelled only for membrane, cytoplasm or nuclear material alone in any given culture,

while SAOS-2 were membrane labelled with DiD, and SAOS-GFP were used to examine both cytoplasmic and nuclear labelling.

Figure 4.5 shows quantitative analysis of six separate experiments performed with HGF isolated from 2 separate donors. Cells bearing dual labels for membranes appeared to increase in proportion primarily at the expense of cells bearing membrane marker for HGF alone, such that there were consistently fewer cells with HGF membrane marker alone than with HGF cytolasmic marker alone ($p<0.01$), and the proportion of cells with dual labelling for membrane markers was comparable to the difference between cells with HGF membrane and cytoplasmic markers alone. Fewer cells expressed the SAOS-2 cytoplasmic marker alone compared with cells bearing the SAOS-2 membrane marker alone ($p<0.01$). Amongst dual labelled cells, the reverse was observed in that a larger proportion of cells had membrane markers, as compared with those having cytoplasmic markers ($p<0.001$).

While there was no effect of TNF-α on the proportion of cells bearing membrane or cytoplasmic labels for HGF or SAOS-2 alone, there was a modest although reproducible effect of TNF-α at increasing the proportion of dual labelled cells with regard to both membrane and cytoplasmic markers ($p<0.001$).

Similar to the observations outlined in sections 4.4.2, no sharing of nuclear material was observed between SAOS-2 and HGF in co-cultures, with or without TNF-α.

4.4.5 The Direction of Predominant Membrane Marker Exchange was Critically Dependent on the Markers Used, so that DiD Passed Most Readily from HGF (DiD Membrane Marker, Syto59 Nuclear Marker) to SAOS-GFP (DiO Membrane Marker, GFP Nuclear Marker), Giving Opposite Results to when SAOS-2 were Labelled with DiD and HGF Labelled with DiO

As shown in Figure 4.2, cytoplasm exchange appeared to be exclusively from HGF to SAOS-2 in experiments with DDAO-SE labelled HGF and SAOS-GFP, while membrane markers appeared to move primarily from SAOS-2 to HGF in experiments where HGF membranes were labelled with DiO and SAOS-2 membranes labelled with DiD (Figure 4.1), as well as in experiments where SAOS-2 alkaline phosphatase was studied (Figures 3.4 and 3.5). To further examine the apparent directionality of membrane marker movement between from SAOS-2 to HGF, additional experiments were performed in which HGF were labelled with DiD and Syto59 as membrane and nuclear labels respectively, and co-cultured with SAOS-GFP expressing intrinsic GFP cytoplasmic and nuclear label, but also additionally pre-labelled with DiO as a membrane label. This series of experiments was performed on six separate occasions with HGF isolated from 3 separate donors.

Figure 4.6 shows the appearance of cells in such cultures, in which SAOS-GFP, HGF and dual labelled cells were readily identified. No cells were found in which nuclei expressed both HGF and SAOS-2 nuclear markers. Dual labelling of cells with membrane marker was, however, seen, while most dual labelled cells had nuclei indicative of SAOS-2 origin (upper panel), and only occasional dual labelled cells were found in which nuclei were of HGF origin (lower panel).

Figure 4.7 shows a quantitative analysis of the relative proportion of cells bearing nuclear and membrane markers for HGF alone, nuclear, cytoplasmic and membrane markers for SAOS-2, and cells which are dual labelled but with nuclear label for HGF or SAOS respectively. Contrasting with data shown in Figure 4.1 where HGF membranes were pre-labelled with DiO, this experimental configuration pre-labelling HGF with DiD revealed movement of membrane label primarily from HGF to SAOS-2 ($p<0.05$), and only a very small proportion of membrane dual labelled cells were found to have nuclei of HGF origin ($p<0.05$). This was also inconsistent with observations of the alkaline phosphatase marker, for which data indicated movement from SAOS-2 to HGF (Figures 3.4 and 3.5). No cells with dual labelling of nuclear material were found.

Figure 4.8 shows the summated results of quantitative analysis of cell type in each type of co-culture studied, demonstrating sensitivity of final results upon the specific labels used in co-culture experiments.

4.4.6 Dual Labelled Cells in Co-Culture had Morphological Properties Intermediate to HGF and SAOS-2 while TNF–α did not Affect Cell Morphology

The morphology of cells in co-cultures appeared different to that in isolated cell culture, and it was considered possible that the sharing of membrane and cytoplasm between HGF and SAOS-2 would have phenotypic effects. For this reason, morphological changes were characterized by image analysis comparing the surface area profile and circularity of HGF, SAOS-2 and dual labelled cells, in co-cultures as well as in isolated cell culture using either membrane or cytoplasmic labelling.

Membrane labelled HGF cultured alone were confirmed as having a larger surface area profile and being less circular than membrane labelled SAOS-2 in isolated culture ($p<0.001$) (Figure 4.9). Co-culture of the two cell types resulted in a modestly increased surface area profile for both HGF and SAOS-2, while there was also substantially increased circularity for HGF ($p<0.001$) to the extent that the difference between HGF and SAOS-2 was lost, although there was no statistically significant effect of co-culture upon the circularity of SAOS-2 considered alone (Figure 4.9). Membrane dual labelled cells had a surface area profile intermediate to that of HGF and SAOS-2 in co-culture ($p<0.001$) (Figure 4.9). While the circularity of dual labelled cells was greater than that in HGF cultured alone ($p<0.001$), there was no statistically meaningful difference in circularity between dual labelled cells and either HGF or SAOS-2 in co-culture (Figure 4.9).

Pre-treatment of HGF with TNF-α did not affect either the surface area profiles or circularity of HGF, SAOS-2 or dual labelled cells (Figures 4.10 and 4.11).

A similar analysis of cell surface area profile and circularity was performed with HGF pre-labelled with the cytoplasmic marker DDAO-SE and SAOS-GFP, and the results of this are shown in Figure 4.12. There was no difference in either surface area profile or circularity of co-cultured HGF or SAOS-2, when measured using membrane or cytoplasmic markers (Figures 4.13 and 4.14), providing confidence in the approach used. Other results were very similar to those obtained using membrane markers (Figures 4.9), although cytoplasmic dual labelled cells were significantly smaller than HGF cultured alone ($p<0.001$) (Figure 4.12). To make more direct comparison between cells in co-culture labelled with membrane as opposed to cytoplasmic markers, data in

Figures.4.9 and 4.12 were re-drawn onto the same graphs (Figures. 4.13 and 4.14) It is interesting that the only statistically meaningful difference amongst cells in co-cultures labelled with membrane as opposed to cytoplasmic markers, was that, dual labelled cells identified using cytoplasmic markers were smaller than when labelled with membrane markers ($p<0.0001$) (Figure 4.13).

Figure 4.1 Fluorescence microscopy and sequential CLSM optical sections (a, b, c) of HGF pre-labelled with the membrane marker DiO, co-cultured for 24 hrs with SAOS-2 pre-labelled with the membrane marker DiD. While many cells expressed only the membrane labels applied (green arrow heads for HGF, red arrow heads for SAOS-2), there were also many cells expressing labels for both HGF and SAOS-2 (yellow arrow heads). CLSM confirmed that dual labelling was not due to overlap of adjacent SAOS-2 and HGF. FM (Bar = 25μm) and CLSM (Bar = 50 μm)

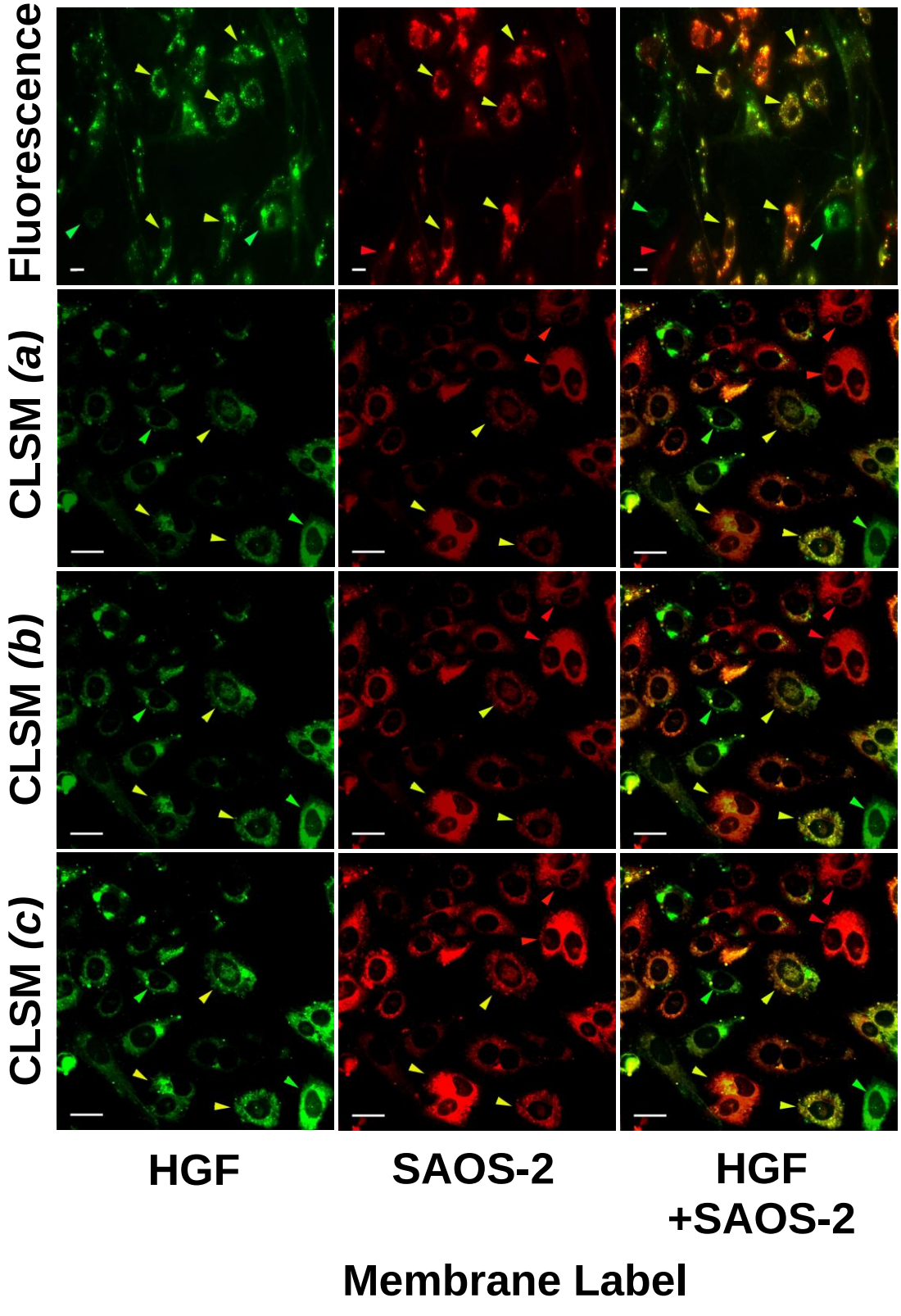

Fluorescence
CLSM (a)
CLSM (b)
CLSM (c)
HGF
SAOS-2
HGF +SAOS-2
Membrane Label

Figure 4.2 Fluorescence microscopy and sequential CLSM optical sections (a, b, c) of HGF pre-labelled with the cytoplasmic marker DDAO-SE and the nuclear marker Syto59, co-cultured for 24 hrs with SAOS-2 stably transfected with GFP expressed in both cytoplasmic and nuclear compartments. While many cells only expressed the cytoplasmic and nuclear labels applied (red arrow heads for HGF, green arrow heads for SAOS-2), some cells expressed labels for both HGF and SAOS-2 (yellow arrow heads). CLSM confirmed that dual labelling was not due to overlap of adjacent SAOS-2 and HGF.

FM (Bar = 25μm) and CLSM (Bar = 50 μm)

.

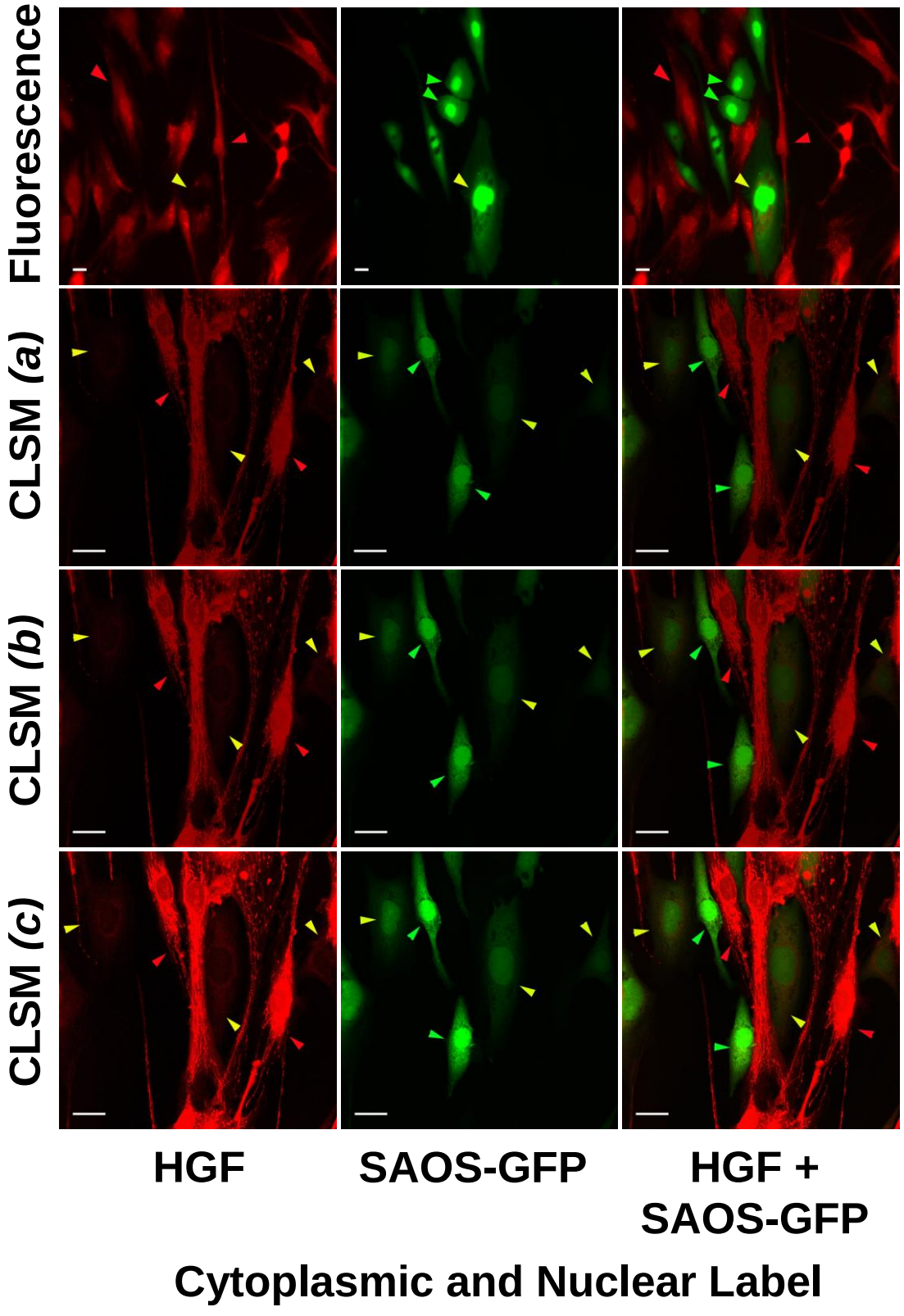

Fluorescence
CLSM (a)
CLSM (b)
CLSM (c)
HGF
SAOS-GFP
HGF + SAOS-GFP
Cytoplasmic and Nuclear Label

Figure 4.3 Fluorescence microscopy and sequential CLSM optical sections (a, b, c) of SAOS-2 pre-labelled with either the membrane marker DiO or DiD, and then co-cultured for 24 hrs. While most cells expressed only one or the other of the two membrane labels applied (green arrow heads for SAOS-2 labelled with DiO, red arrow heads for SAOS-2 labelled with DiD), some cells expressed labels for both markers (yellow arrow heads). CLSM confirmed that dual labelling was not due to overlap of adjacent cells.

FM (Bar = 25μm) and CLSM (Bar = 50 μm)

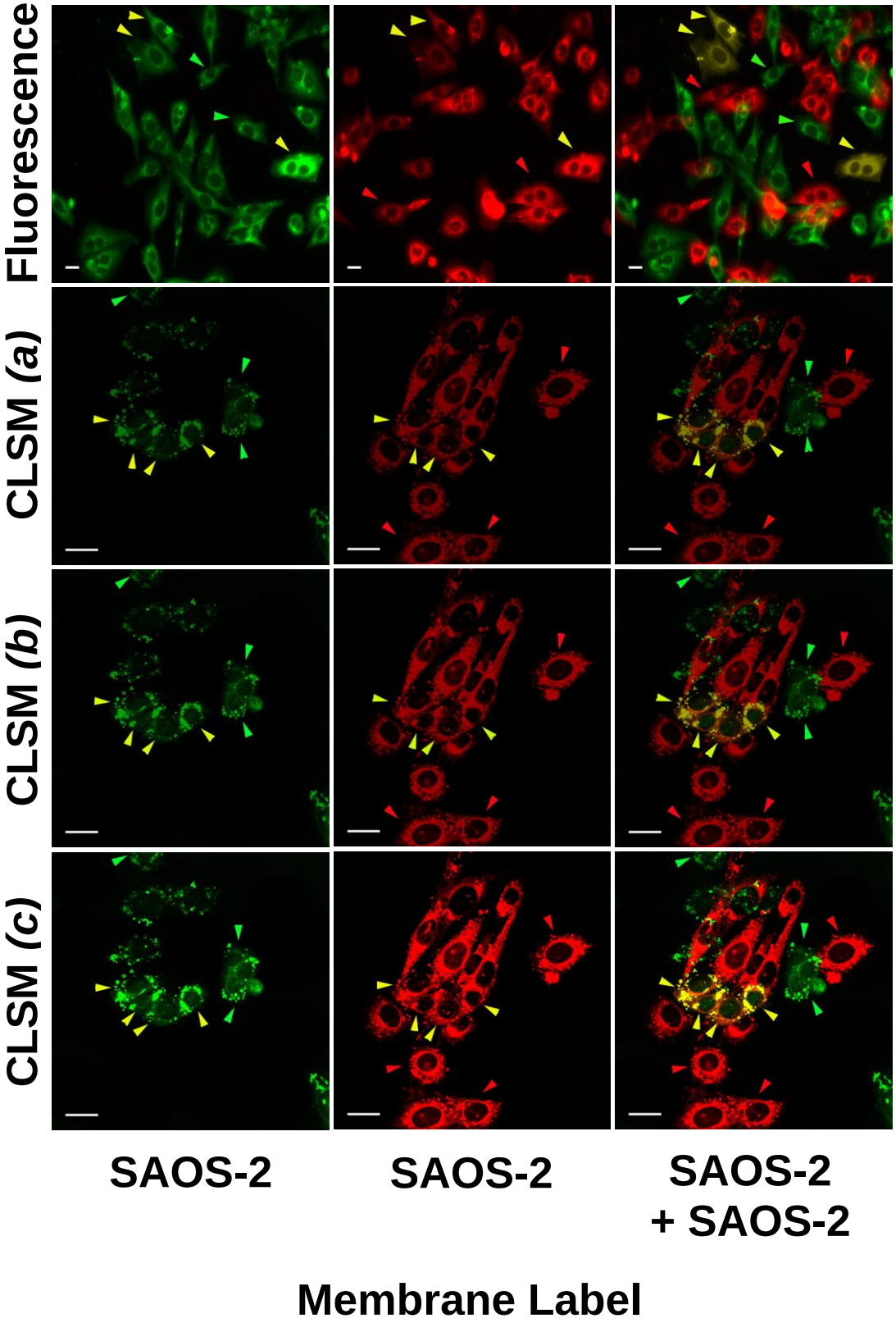

Fluorescence
CLSM (a)
CLSM (b)
CLSM (c)
SAOS-2
SAOS-2
SAOS-2
+ SAOS-2
Membrane Label

Figure 4.4 Fluorescence microscopy images of populations of HGF pre-labelled with either membrane (DiO – green arrow heads, or DiD – red arrow heads) and cytoplasmic (CFSE – green arrow heads or DDAO-SE – red arrow heads) labels before being recombined for 24 hrs culture, as well as SAOS-2 similarly pre-labelled and cultured with cytoplasmic markers. Dual labelled cells were not clearly apparent in any of these culture conditions. FM (Bar = 50 μm)

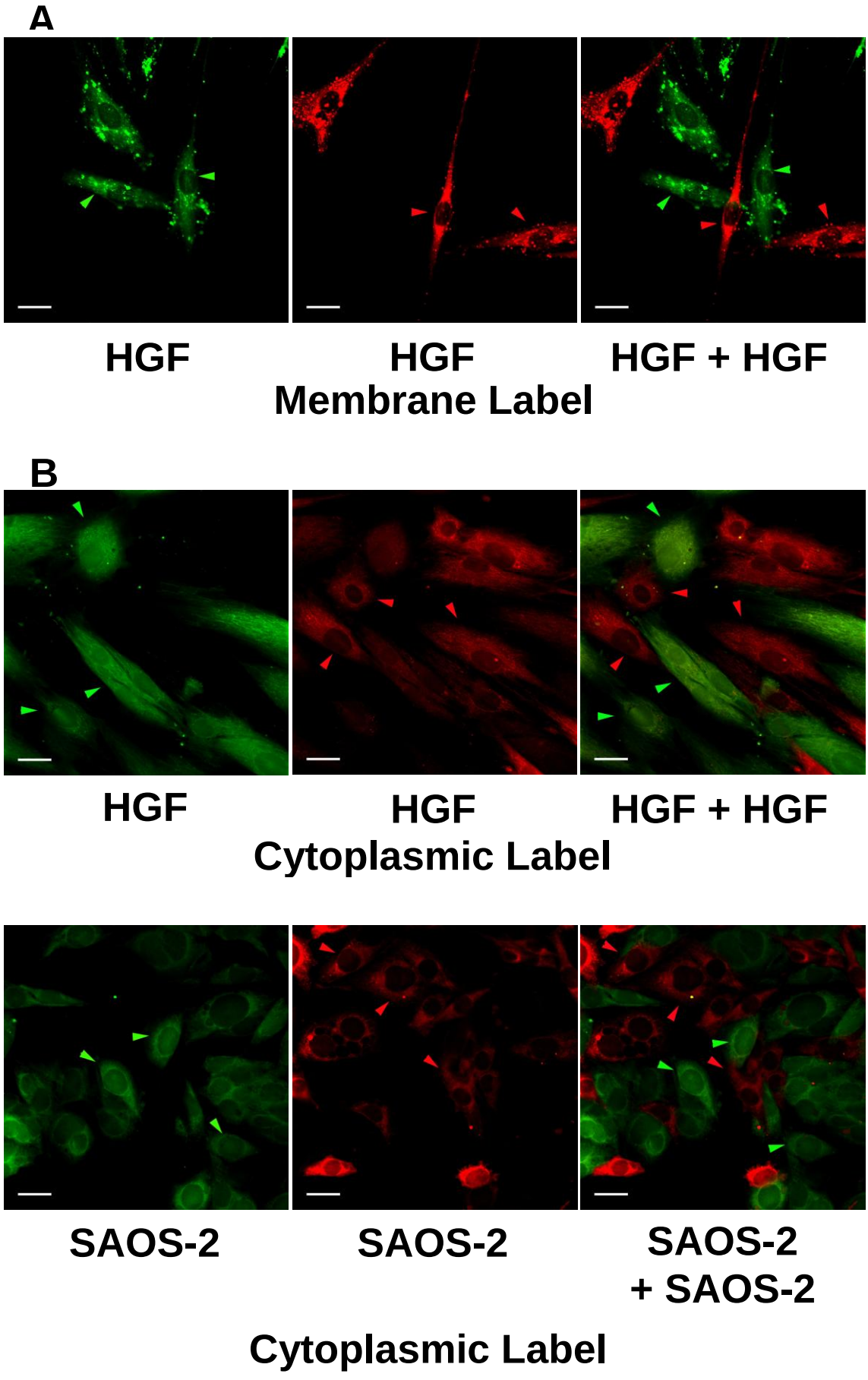

A
HGF
HGF
HGF + HGF
Membrane Label
B
HGF
HGF
HGF + HGF
Cytoplasmic Label
SAOS-2
SAOS-2
SAOS-2
+ SAOS-2
Cytoplasmic Label

Figure 4.5 Scattergram comparing the relative proportion of pre-labelled cells bearing membrane and cytoplasmic markers respectively for HGF (DiO for membrane, DDAO-SE for cytoplasm) or SAOS-2 (DiD for membrane, GFP for cytoplasm and nucleus), as well as dual labelled cells, in six separate experiments with and without TNF-α pre-treatment of HGF (1.16nM). Fewer HGF expressed the membrane marker as compared with those in which the cytoplasmic marker were seen ($p<0.001$). Similarly, fewer SAOS-2 expressed the membrane marker as compared with those in which the cytoplasmic marker was seen ($p<0.01$). Amongst dual labelled cells, the reverse was observed in that a large proportion of cells had dual membrane markers, as compared with only a small proportion of cells with dual labelling for the cytoplasmic markers ($p<0.001$). A clear difference in proportion of membrane and cytoplasmic labelling amongst SAOS-2 and dual labelled cells was noted. From this, cells with dual label membrane markers appeared to arise at the expense of HGF expressing membrane marker. TNF-α had a modest but nonetheless reproducible effect of increasing the proportion of cells with dual label for both membrane and cytoplasmic markers ($p<0.04$).

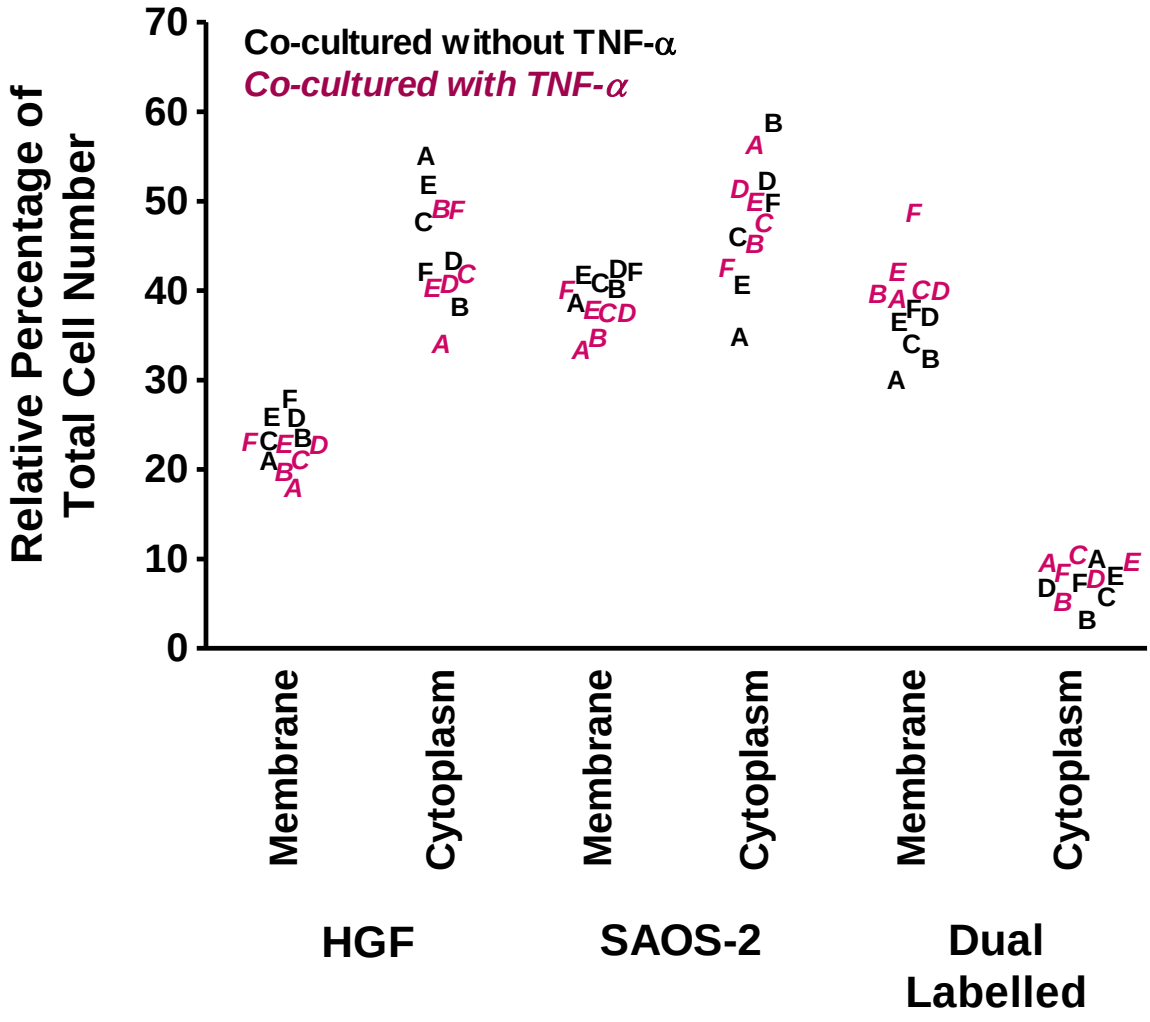

Co-cultured without TNF-α
Co-cultured with TNF-α
Relative Percentage of Total Cell Number
70
60
50
40
30
20
10
0
Membrane
Cytoplasm
Membrane
Cytoplasm
Membrane
Cytoplasm
HGF
SAOS-2
Dual Labelled

Figure 4.6 CLSM optical sections of HGF pre-labelled with the membrane marker DiD and the nuclear marker Syto59, co-cultured with SAOS-GFP pre-labelled with DiO and thus expressing nuclear, cytoplasmic and membrane labels, so that HGF were unambiguously labelled red and SAOS-GFP labelled green. HGF and SAOS-2 bearing only markers for these two cell types alone were readily identified in co-cultures. Amongst these, however, were occasional cells which expressed both HGF and SAOS-2 membrane markers, and most of these dual labelled cells had green nuclei indicative of SAOS-2 origin (green arrow heads) as seen in the upper panel. Nonetheless, there were occasional cells with dual labelling that had clearly red nuclei indicative of HGF origin (red arrow heads) as seen in the lower panel. (Bar = 50 μm)

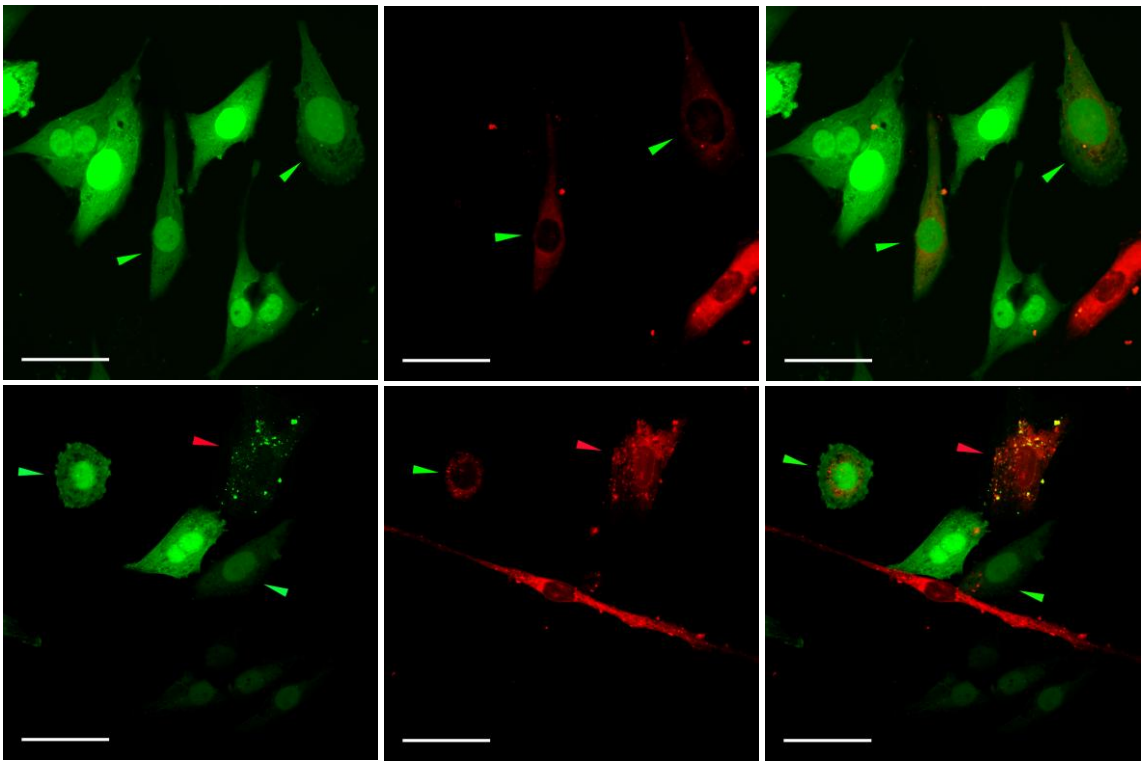

SAOS-GFP
HGF
SAOS-GFP
+ HGF

Figure 4.7 Scattergram of the relative percentage of cells expressing markers for nuclei (Nuc.), membrane (Memb.) or cytoplasm (Cyto.), as well as dual labelled membrane cells (DL) with nuclei of HGF or SAOS-2 origin, in six separate experiments where HGF were pre-labelled with the membrane marker DiD and nuclear marker Syto59, and co-cultured for 24 hrs with SAOS-GFP also pre-labelled with membrane marker DiO. While some cells with HGF nuclear labelling were found to also have dual labelling for membrane marker, most cells with dual label for membrane marker had nuclei of SAOS-GFP origin ($p<0.05$).

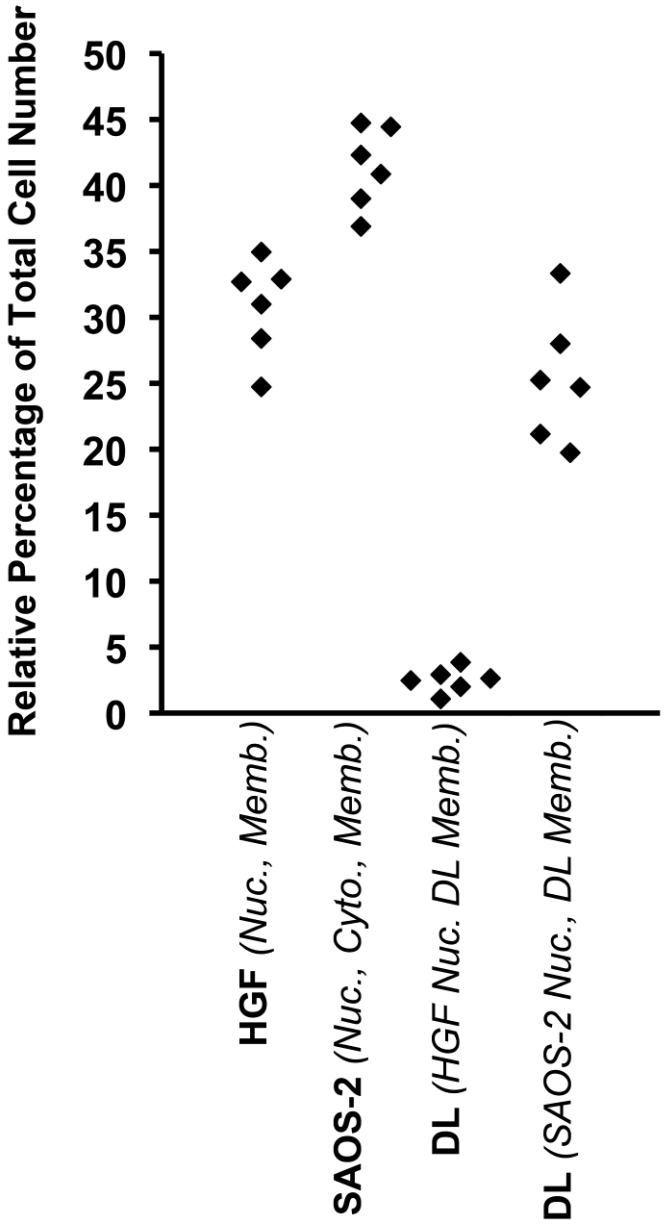

Relative Percentage of Total Cell Number
50
45
40
35
30
25
20
15
10
5
0
HGF (Nuc., Memb.)
SAOS-2 (Nuc., Cyto., Memb.)
DL (HGF Nuc. DL Memb.)
DL (SAOS-2 Nuc., DL Memb.)

Figure 4.8 Schematic quantitative representation of the proportional distribution of cells bearing markers for HGF, SAOS-2 or dual labelling at 0 hrs as well as at 24 hrs in each of the experimental co-culture configurations studied including: (i) alkaline phosphatase membrane labelling of SAOS-2; (ii) fluorescent membrane labelling of HGF with DiO opposed to DiD membrane labelling of SAOS-2; (iii) cytoplasmic labelling of SAOS cytoplasm with GFP opposed to HGF cytoplasmic labelling with DDAO-SE; and (iv) membrane labelling in co-cultures of SAOS-GFP having nuclear and cytoplasmic GFP together with membrane DiO label opposed to HGF with the nuclear and membrane labels Syto59 and DiD respectively. The proportional distribution of cells with HGF, SAOS-2 and dual labelling characteristics varied greatly with experimental configuration, indicating sensitivity of the directionality of label transfer to the specific labels used in individual experiments.

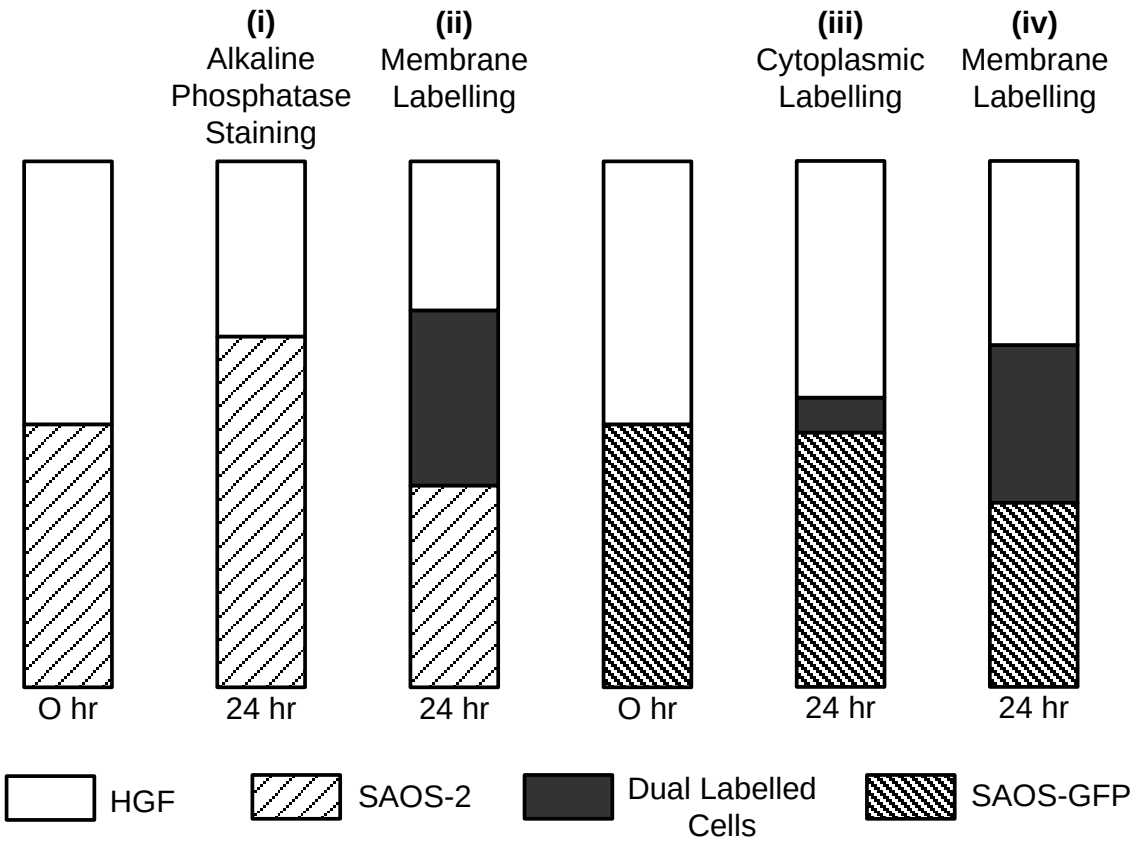

(i)
Alkaline Phosphatase Staining
(ii)
Membrane Labelling
(iii)
Cytoplasmic Labelling
(iv)
Membrane Labelling
O hr
24 hr
24 hr
O hr
24 hr
24 hr
HGF
SAOS-2
Dual Labelled Cells
SAOS-GFP

Figure 4.9 Distribution plots showing the relative percentage of total cell number according to surface area profile and circularity in 24 hr co-cultures of: HGF labelled with the membrane marker DiO; and SAOS-2 labelled with the membrane marker DiD; as well as dual labelled cells. HGF cultured alone were less circular and larger than SAOS-2 in isolated culture ($p<0.001$). Co-culture increased the surface area profiles of HGF and SAOS-2, while dual labelled cells had a surface area profile intermediate to that of both SAOS-2 and HGF ($p<0.001$). An increase in circularity of HGF in co-culture was noted, and dual labelled cells exhibited circularity values intermediate to HGF and SAOS-2 cultured alone ($p<0.001$).

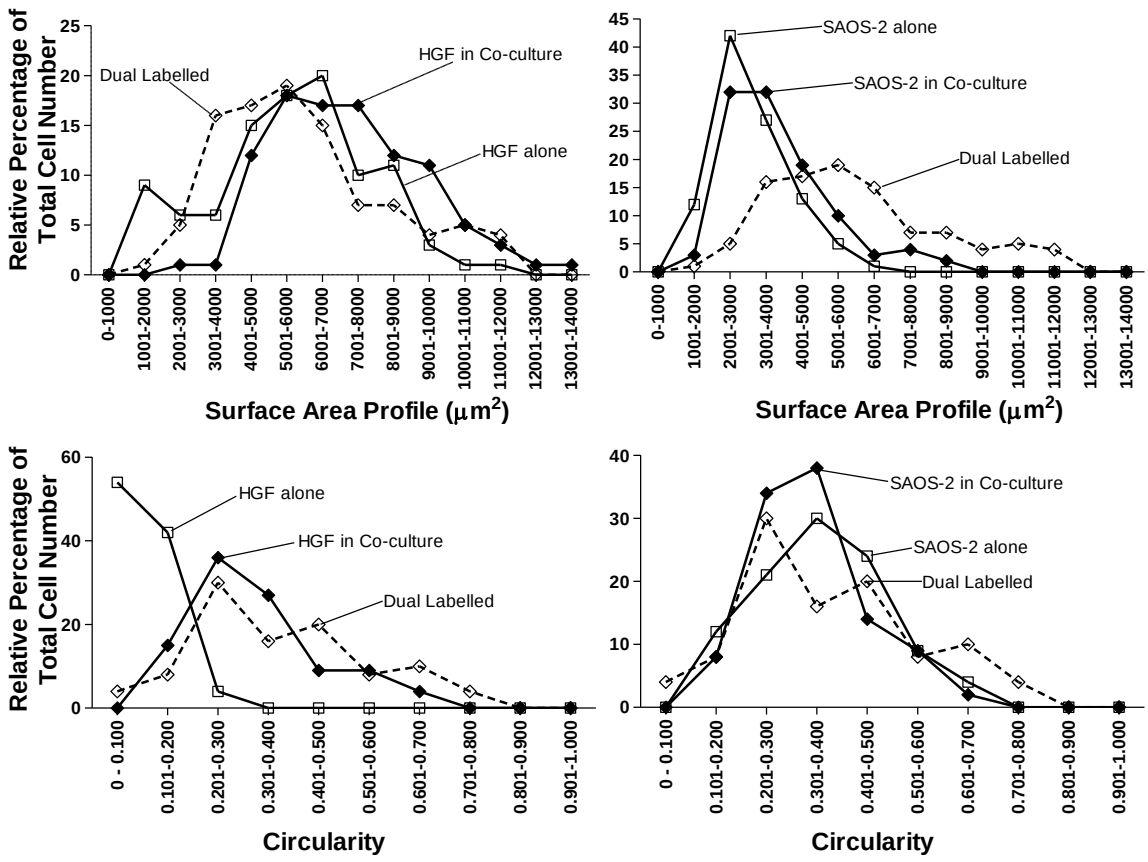

Relative Percentage of Total Cell Number
Dual Labelled
HGF in Co-culture
HGF alone
0-1000
1001-2000
2001-3000
3001-4000
4001-5000
5001-6000
6001-7000
7001-8000
8001-9000
9001-10000
10001-11000
11001-12000
12001-13000
13001-14000
Surface Area Profile (μm²)
SAOS-2 alone
SAOS-2 in Co-culture
Dual Labelled
Surface Area Profile (μm²)
Relative Percentage of Total Cell Number
HGF alone
HGF in Co-culture
Dual Labelled
0 - 0.100
0.101-0.200
0.201-0.300
0.301-0.400
0.401-0.500
0.501-0.600
0.601-0.700
0.701-0.800
0.801-0.900
0.901-1.000
Circularity
SAOS-2 in Co-culture
SAOS-2 alone
Dual Labelled
Circularity

Figure 4.10 Distribution plots comparing the relative percentage of total cell number according to surface area profile of: HGF pre-labelled with the membrane marker DiO; SAOS-2 pre-labelled with the membrane marker DiD; and dual labelled cells in 24 hrs co-culture; with and without pre-treatment of HGF with TNF-α for 24 hrs. Consistent with data in Figure 4.9, co-culture increased the surface area profile of HGF and SAOS-2, while dual labelled cells were intermediate in surface area profile to HGF and SAOS-2. Importantly, TNF-α treatment of HGF did not alter the surface area profile of cells in co-culture of any of the cell populations studied.

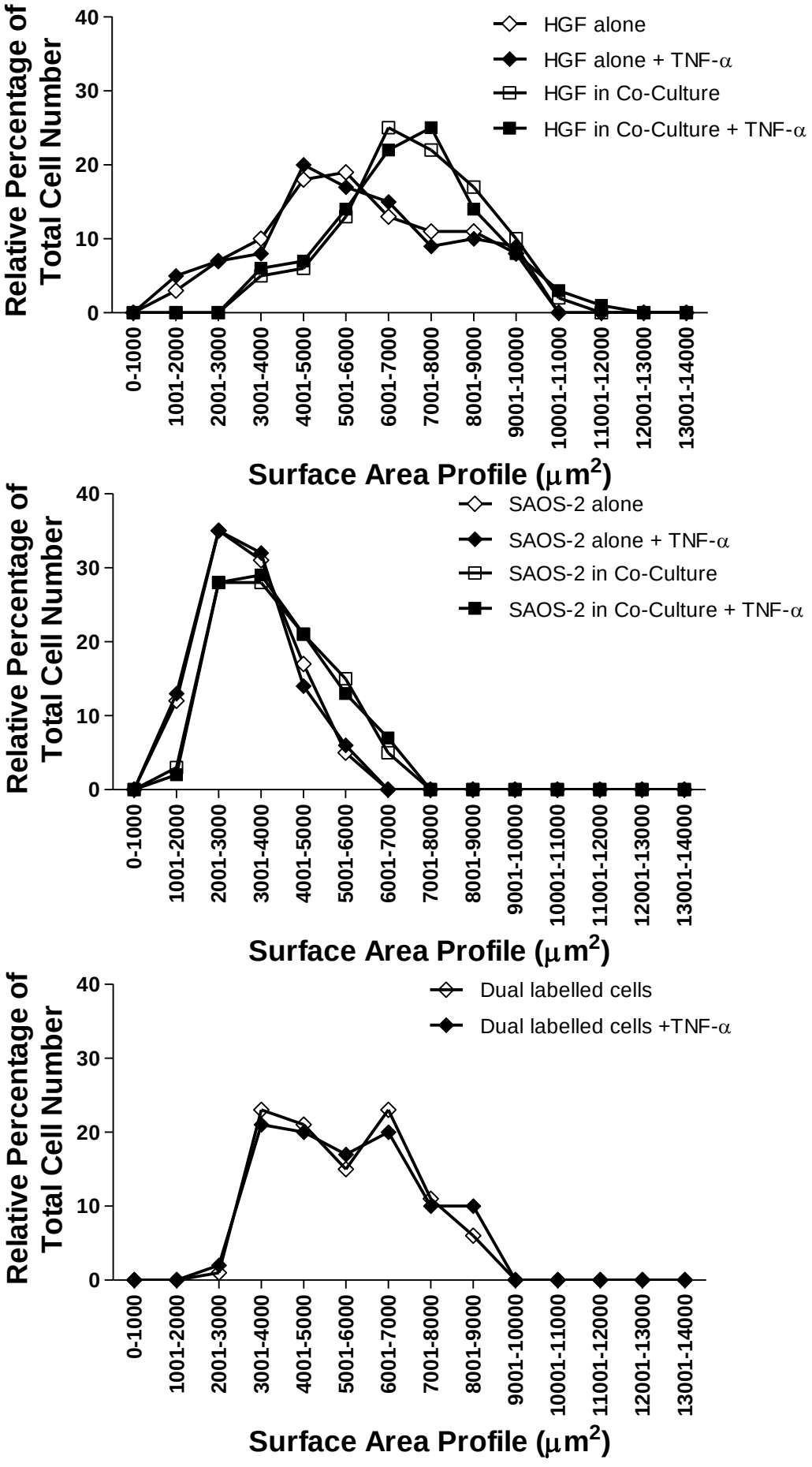

Relative Percentage of Total Cell Number
HGF alone
HGF alone + TNF-α
HGF in Co-Culture
HGF in Co-Culture + TNF-α
0-1000
1001-2000
2001-3000
3001-4000
4001-5000
5001-6000
6001-7000
7001-8000
8001-9000
9001-10000
10001-11000
11001-12000
12001-13000
13001-14000
Surface Area Profile (μm²)
Relative Percentage of Total Cell Number
SAOS-2 alone
SAOS-2 alone + TNF-α
SAOS-2 in Co-Culture
SAOS-2 in Co-Culture + TNF-α
Surface Area Profile (μm²)
Relative Percentage of Total Cell Number
Dual labelled cells
Dual labelled cells +TNF-α
Surface Area Profile (μm²)

Figure 4.11 Distribution plots comparing the relative percentage of total cell number according to circularity of: HGF pre-labelled with the membrane marker DiO; SAOS-2 pre-labelled with the membrane marker DiD; and dual labelled cells in 24 hrs co-culture; with and without pre-treatment of HGF with TNF-α for 24 hrs. Consistent with data in Figure 4.9, HGF were less circular than SAOS-2 in isolated culture ($p<0.0001$). Co-culture resulted in increased circularity of HGF ($p<0.0001$), and dual labelled cells demonstrated circularity intermediate to that of HGF ($p<0.001$) and SAOS-2 ($p<0.01$). TNF-α treatment of HGF did not affect the circularity of any of the co-cultured cell populations studied.

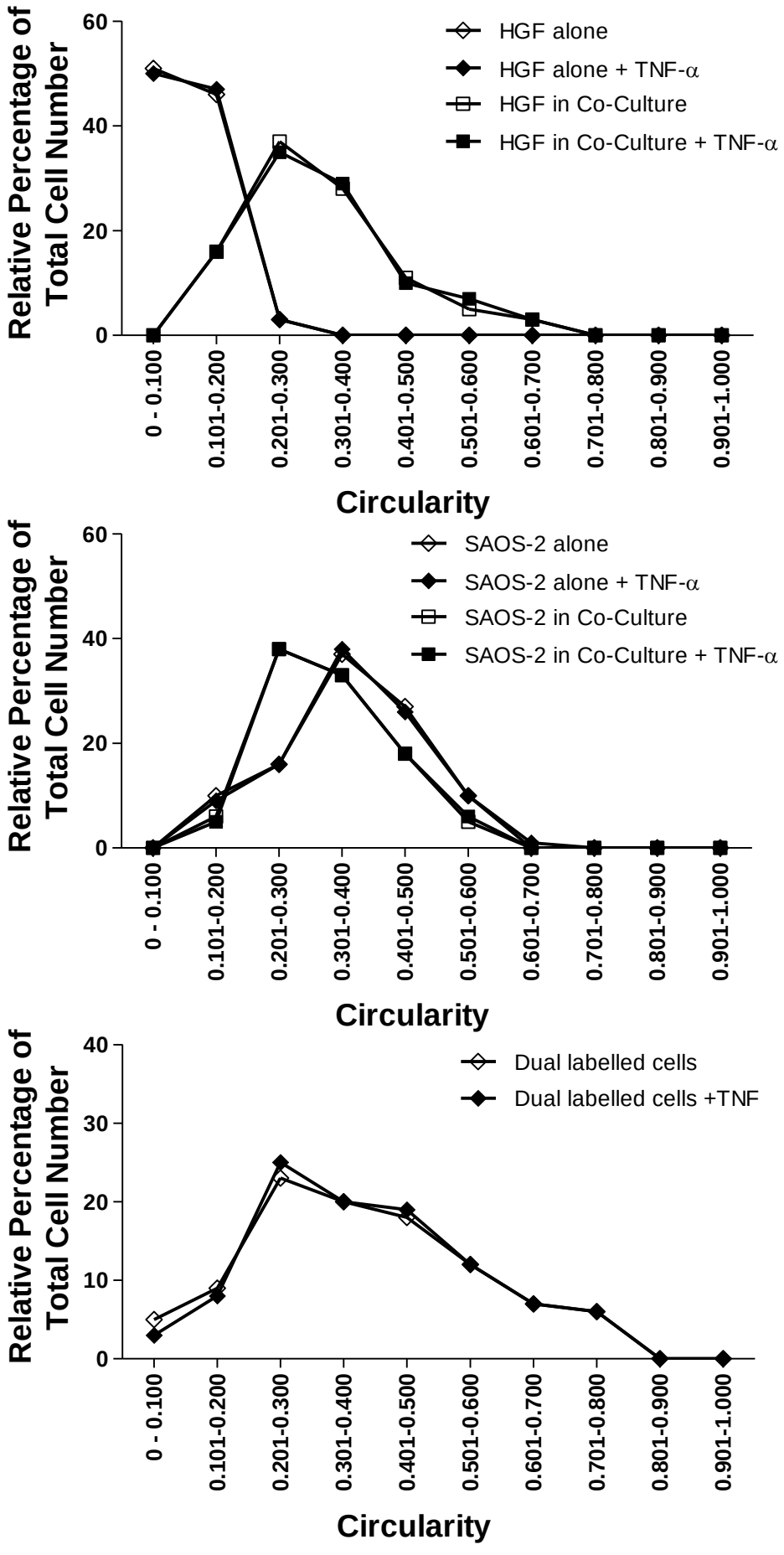

Relative Percentage of Total Cell Number
60
40
20
0
HGF alone
HGF alone + TNF-α
HGF in Co-Culture
HGF in Co-Culture + TNF-α
0 - 0.100
0.101-0.200
0.201-0.300
0.301-0.400
0.401-0.500
0.501-0.600
0.601-0.700
0.701-0.800
0.801-0.900
0.901-1.000
Circularity
Relative Percentage of Total Cell Number
60
40
20
0
SAOS-2 alone
SAOS-2 alone + TNF-α
SAOS-2 in Co-Culture
SAOS-2 in Co-Culture + TNF-α
0 - 0.100
0.101-0.200
0.201-0.300
0.301-0.400
0.401-0.500
0.501-0.600
0.601-0.700
0.701-0.800
0.801-0.900
0.901-1.000
Circularity
Relative Percentage of Total Cell Number
40
30
20
10
0
Dual labelled cells
Dual labelled cells +TNF
0 - 0.100
0.101-0.200
0.201-0.300
0.301-0.400
0.401-0.500
0.501-0.600
0.601-0.700
0.701-0.800
0.801-0.900
0.901-1.000
Circularity

Figure 4.12 Distribution plots showing the relative percentage of total cell number according to surface area profile and circularity in 24 hr co-cultures of: HGF labelled with the cytoplasmic marker DDAO-SE; and SAOS-GFP expressing the cytoplasmic marker GFP; as well as dual labelled cells. HGF cultured alone were less circular than SAOS-2 in isolated culture ($p<0.0001$). Co-culture increased the surface area profiles of HGF ($p<0.001$) and SAOS-2 ($p<0.01$), while dual labelled cells had a surface area profile intermediate to that of both SAOS-2 ($p<0.03$) and HGF ($p<0.0001$). An increase in circularity of HGF in co-culture was noted and dual labelled cells exhibited circularity values intermediate to HGF ($p<0.001$) and SAOS-2 cultured alone ($p<0.03$).

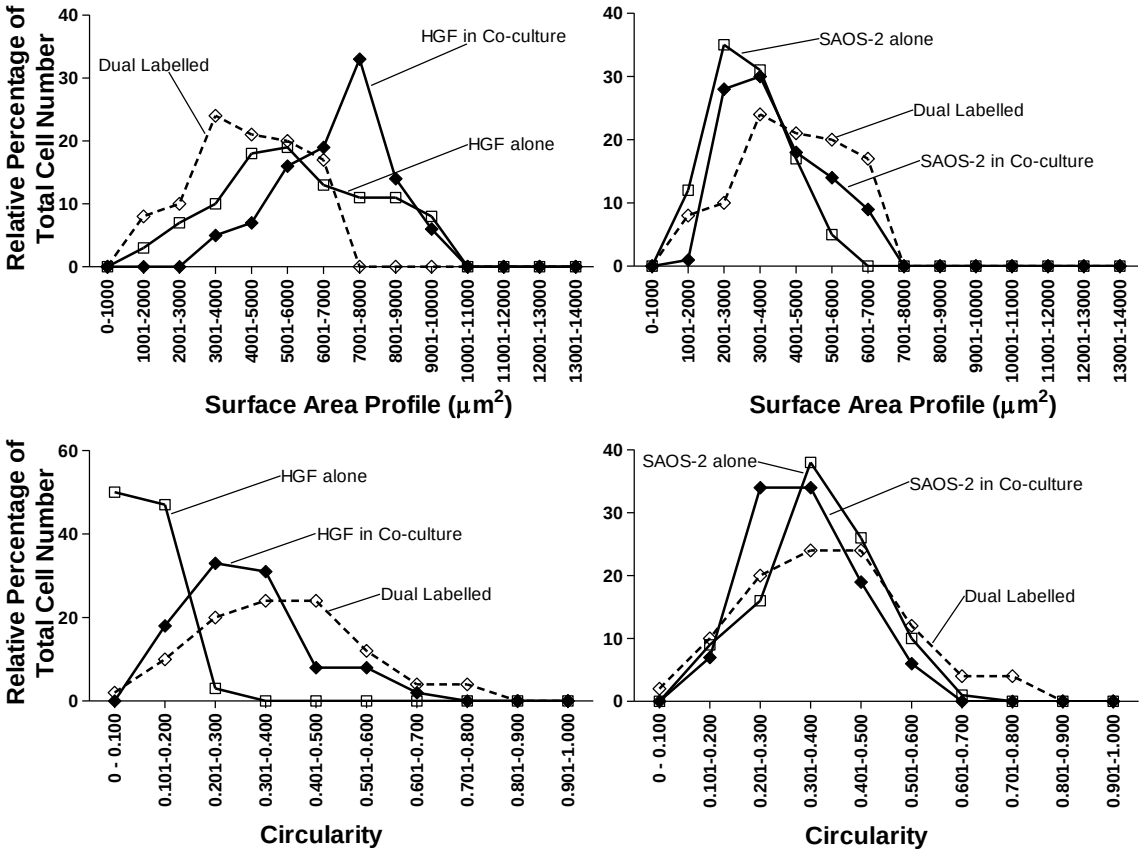
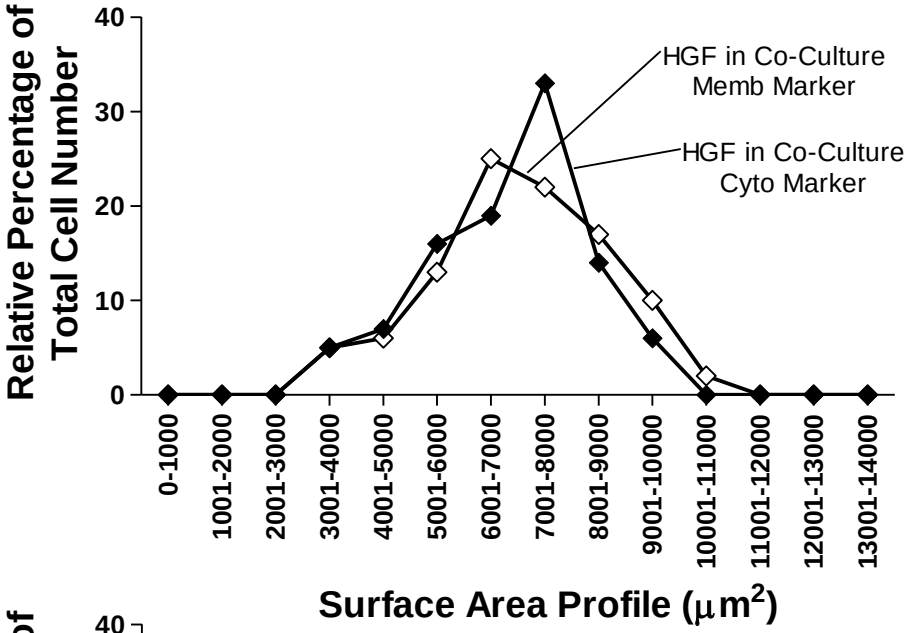
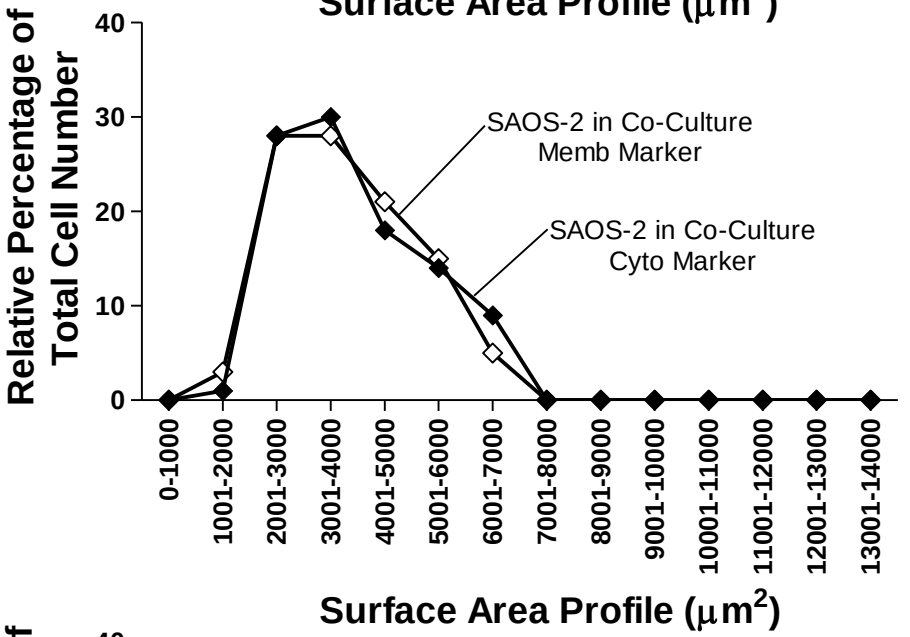

Relative Percentage of Total Cell Number
Dual Labelled
HGF in Co-culture
HGF alone
0-1000
1001-2000
2001-3000
3001-4000
4001-5000
5001-6000
6001-7000
7001-8000
8001-9000
9001-10000
10001-11000
11001-12000
12001-13000
13001-14000
Surface Area Profile (μm²)
SAOS-2 alone
Dual Labelled
SAOS-2 in Co-culture
Surface Area Profile (μm²)
Relative Percentage of Total Cell Number
HGF alone
HGF in Co-culture
Dual Labelled
0 - 0.100
0.101-0.200
0.201-0.300
0.301-0.400
0.401-0.500
0.501-0.600
0.601-0.700
0.701-0.800
0.801-0.900
0.901-1.000
Circularity
SAOS-2 alone
SAOS-2 in Co-culture
Dual Labelled
Circularity

Figure 4.13 Distribution plots comparing the relative percentage of total cell number according to surface area profile of HGF, SAOS-2 and dual labelled cells in co-culture labelled with membrane (Memb), (HGF / DiO; SAOS-2 / DiD) and cytoplasmic (Cyto), (HGF / DDAO-SE; SAOS-GFP / GFP) markers. There was no statistically meaningful difference in surface area profiles of HGF or SAOS-2 in co-culture when labelled with membrane or cytoplasmic markers. However dual labelled cells labelled with cytoplasmic markers were smaller than when labelled with membrane markers ($p<0.0001$)


Relative Percentage of Total Cell Number
40
30
20
10
0
HGF in Co-Culture Memb Marker
HGF in Co-Culture Cyto Marker
0-1000
1001-2000
2001-3000
3001-4000
4001-5000
5001-6000
6001-7000
7001-8000
8001-9000
9001-10000
10001-11000
11001-12000
12001-13000
13001-14000
Surface Area Profile (μm²)


Relative Percentage of Total Cell Number
40
30
20
10
0
SAOS-2 in Co-Culture Memb Marker
SAOS-2 in Co-Culture Cyto Marker
0-1000
1001-2000
2001-3000
3001-4000
4001-5000
5001-6000
6001-7000
7001-8000
8001-9000
9001-10000
10001-11000
11001-12000
12001-13000
13001-14000
Surface Area Profile (μm²)

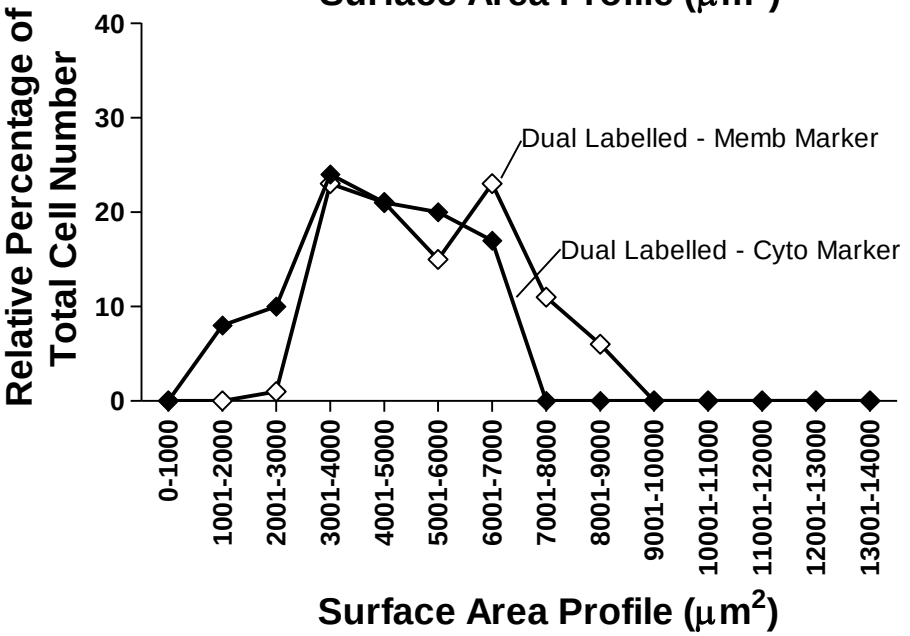
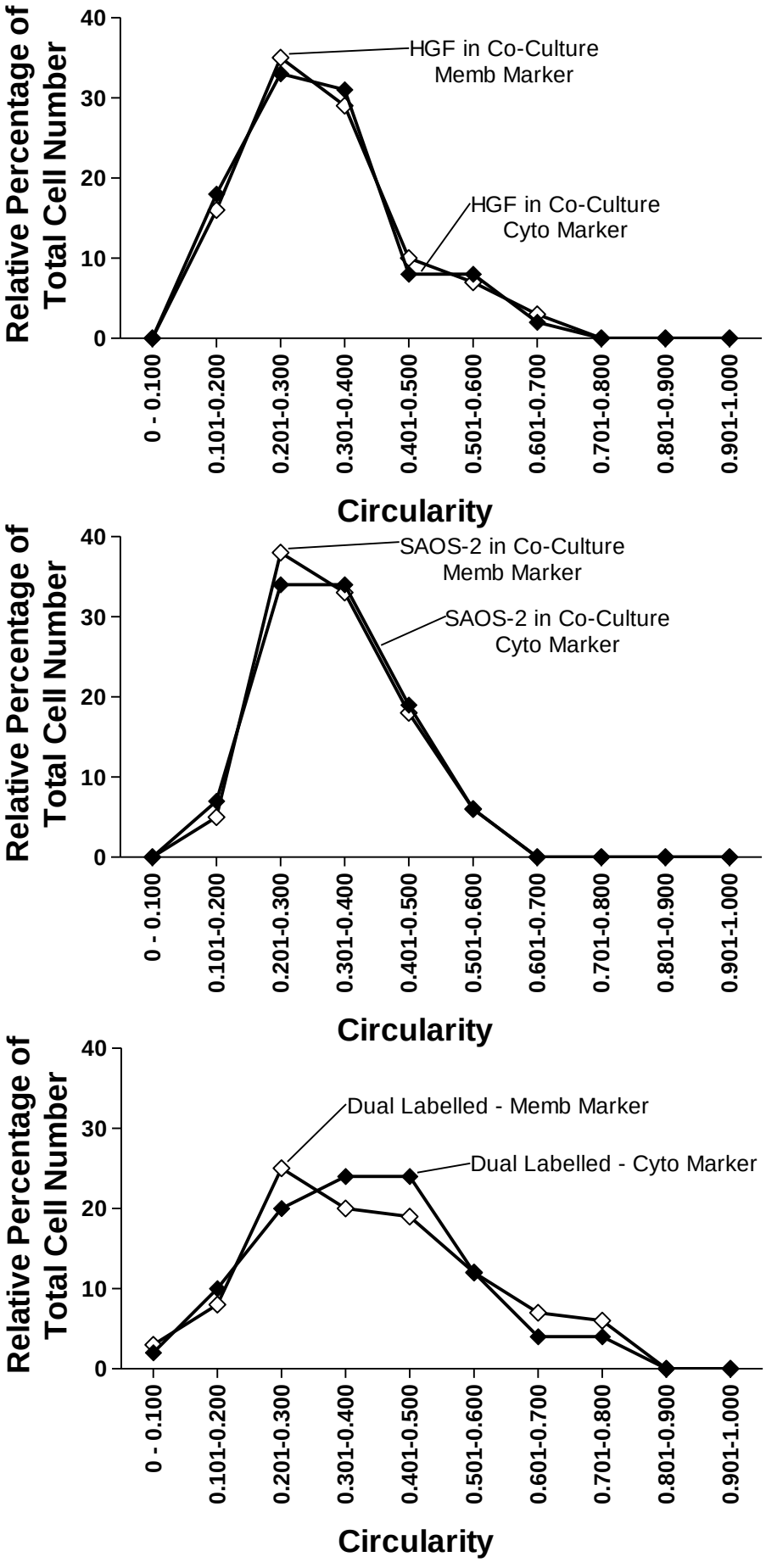

Relative Percentage of Total Cell Number
40
30
20
10
0
Dual Labelled - Memb Marker
Dual Labelled - Cyto Marker
0-1000
1001-2000
2001-3000
3001-4000
4001-5000
5001-6000
6001-7000
7001-8000
8001-9000
9001-10000
10001-11000
11001-12000
12001-13000
13001-14000
Surface Area Profile (μm²)

Figure 4.14 Distribution plots comparing the relative percentage of total cell number according to circularity of HGF, SAOS-2 and dual labelled cells in co-culture labelled with membrane (Memb), (HGF / DiO; SAOS-2 / DiD) and cytoplasmic (Cyto), (HGF / DDAO-SE; SAOS-GFP / GFP) markers. There was no statistically meaningful difference in circularity of HGF, SAOS-2 or dual labelled cells in co-culture when labelled with membrane or cytoplasmic markers.


Relative Percentage of Total Cell Number
40
30
20
10
0
HGF in Co-Culture Memb Marker
HGF in Co-Culture Cyto Marker
0 - 0.100
0.101-0.200
0.201-0.300
0.301-0.400
0.401-0.500
0.501-0.600
0.601-0.700
0.701-0.800
0.801-0.900
0.901-1.000
Circularity
Relative Percentage of Total Cell Number
40
30
20
10
0
SAOS-2 in Co-Culture Memb Marker
SAOS-2 in Co-Culture Cyto Marker
0 - 0.100
0.101-0.200
0.201-0.300
0.301-0.400
0.401-0.500
0.501-0.600
0.601-0.700
0.701-0.800
0.801-0.900
0.901-1.000
Circularity
Relative Percentage of Total Cell Number
40
30
20
10
0
Dual Labelled - Memb Marker
Dual Labelled - Cyto Marker
0 - 0.100
0.101-0.200
0.201-0.300
0.301-0.400
0.401-0.500
0.501-0.600
0.601-0.700
0.701-0.800
0.801-0.900
0.901-1.000
Circularity

4.5 DISCUSSION

It is convenient to consider cells as comprised of three major compartments, being: the plasma membrane in constant exchange with intracellular organellar membranes; the cytoplasm enclosed by the plasma membrane and separate from the third major compartment which is the nucleus (Alberts, 2008). While it is recognized that the interactions in-vivo are inevitably more complex than those seen in culture model systems such as that studied in the current thesis, it does seem necessary to use such over-simplified systems to at least determine what types of response may be possible in-vivo, while subsequent studies with explant cultures and animal experimentation may be justified if observations of sufficient novelty or interest are identified in cell culture studies.

Earlier studies with HGF and SAOS-2 co-cultured and stained for alkaline phosphatase activity, demonstrated reduced cell culture density of alkaline phosphatase negative HGF upon co-culture comparable to reduced HUVEC cell culture density seen in similar culture conditions with SAOS-2 (McEwen et al., 2003, Huynh 2007, Chapter 3). However, unlike HUVEC (McEwen et al., 2003), it was not possible to account for reduced HGF apparent cell culture density in SAOS-2 co-cultures on the basis of HGF apoptosis (Huynh, 2007) or autophagy (Chapter 3). This led to the suggestion investigated in the current chapter, that reduction in apparent HGF culture density in SAOS-2 co-cultures was not due to loss of HGF, but instead reflected sharing of the SAOS-2 alkaline phosphatase label with HGF.

To pursue this question, work in this chapter sought to examine possible exchange of plasma membrane, cytoplasm and or nuclear material between HGF and SAOS-2, and two discrete experimental approaches to this question are apparent. The first and perhaps most direct approach, is to trace the movement of membrane, cytoplasmic or nuclear contents between cell types in co-culture, and in this thesis, endogenous alkaline phosphatase activity in SAOS-2 was used as a membrane marker native to these neoplastic cells but not normally expressed by cultured HGF. A variant of the 'direct labelling' approach, is to express by transfection technology, a newly introduced and conveniently detected marker molecule to one or both of the cells, and the current study also used this approach where SAOS-2 were transfected with a GFP expressing plasmid such that a cloned stable transfected cell line with strong fluorescent GFP expression in the cytoplasm and nucleus was produced and used (Huynh, 2007). The value of this cell line for the studies shown was apparent in that alternative methods for labelling nuclear material were ultimately ineffective (section 4.4.1), with the exception of Styo59 labelling used to label HGF nuclear material.

Styo59 as a foreign fluorescent label pre-loaded into cells, comprises an example of the second main type of tool available for experiments such as those described in this chapter. Other fluorescent labels pre-loaded into HGF and SAOS-2 prior to co-culture were DiD and DiO as effective membrane labels, and both CFSE and DDAO-SE which were both good cytoplasmic labels. The use of fluorescent dyes to study movement of cellular components is well established, and permits both qualitative and quantitative analysis (Blumenthal et al., 1996, Spotl et al., 1995, Li et al., 1996, Munoz-Barroso et al., 1998). Optical confocal microscopy sectioning at 1µm distance was used in the current study to confirm co-localisation of the fluorescent markers, and exclude the

possibility that observed dual labelled cells were artefacts of overlapping cells. The possibility of dye uptake from other cells as well as from any unbound dye in the media was considered, while in preliminary experiments with Hoechst, this proved to be a problem confounding observations. The dyes used in final experiments are, however, known to be quite stable with no intercellular transfer even across very close cell associations such as in neural synapses (Dumitriu et al., 2001, Honig and Hume, 1986). In addition, to obviate the possibility of uptake of any unbound dye, the cells were repeatedly and thoroughly washed before they were co-cultured. Further, labelling was seen to be diffuse rather than punctate in dual labelled cells, inconsistent with possible phagocytic uptake of particles (Drevets and Campbell, 1991). In addition, the clearly different pattern of exchange seen when HGF were cultured with SAOS-2, as compared with when HGF were co-cultured with oppositely labelled HGF, supports specificity of the findings made. It is acknowledged, however, that the methods used in this chapter do have the possibility of being insensitive to detecting low levels of dye transfer, due to possible dilution of dye caused by the disparity in sizes of the two cell types interacting, as well as fading or bleaching out of the dye during observation. Regardless of these minor limitations, our method clearly demonstrates the presence of membrane and cytoplasmic transfer between SAOS-2 and HGF, that is increased with TNF-α stimulation.

While the experimental tools used appear to offer a reasonable strategy to study transfer of membrane, cytoplasmic or nuclear material between co-cultured cells, it must be borne in mind that experiments using these tools are ultimately only able to trace the movement of the labels used, rather than the bulk movement of membranes, cytoplasm or nuclei respectively, or even the movement of components of these three cell

compartments. Inclusion of this concern when interpreting data from experiments may at first sight appear unnecessarily cautious, however, the necessity for appropriate caution is underscored by the finding illustrated in Figures 4.5 and 4.7, where casual and less cautious interpretation of the data shown would suggest completely opposite bulk movement of membrane between SAOS-2 and HGF. It seems important to invest some time considering the implications of data in these two figures more carefully.

In Figure 4.5, the dual membrane labelled cells clearly arise at the expense of HGF, as determined by comparison of the relative proportion of cells bearing HGF cytoplasmic label CFSE relative to those with the HGF membrane label alone. We see no other reasonable interpretation of this experiment, other than to conclude that DiD moved preferentially from SAOS-2 to HGF, suggesting that bulk membrane transport would be from SAOS-2 to HGF. This interpretation is also consistent with findings of experiments using SAOS-2 alkaline phosphatase activity, where there was a clear reduction in the apparent culture density of alkaline phosphatase negative cells, presumed reasonably to be HGF in earlier studies (Figures 3.4 and 3.5). Given these data alone, using two separate experimental tools, that of endogenous alkaline phosphatase and exogenous DiD and DiO, an incautious conclusion could reasonably be reached that there is bulk-transfer of plasma membrane from SAOS-2 to HGF during co-culture of these cells. However, when DiD is used as the membrane marker in HGF, with DiO as the SAOS-2 membrane marker in the experiments summarised in Figure 4.7, the opposite finding seems apparent, that there is bulk transport of membrane from HGF to SAOS-2. These two opposing conclusions are reconciled, by recognition that DiO has a lower lateral diffusion rate when compared to other carbocyanine dyes including DiD (Derzko and Jacobson, 1980, Kobbert et al., 2000),

while the preferential migration of alkaline phosphatase from SAOS-2 to HGF is also consistent with the known very high lateral mobility of this glycosylphosphatidyl inositol bound membrane ectoenzyme (Noda et al., 1987).

From the above, data presented do not allow statements to be made regarding the bulk transport of either HGF or SAOS-2 membrane between the two cell types in co-culture. Data do, however, permit legitimate conclusion that during co-culture, physical continuity between SAOS-2 and HGF plasma membranes must be established, and that this permits lateral diffusion of plasma membrane components between the two cell types, including membrane markers DiD, alkaline phosphatase and also to a lesser extent DiO. Additional experiments transferring medium that has been pre-conditioned by pre-labelled cells have been performed since conclusion of the experimental work detailed in this thesis, and have confirmed that there is no detectable leakage of label either from disrupted cells or via exosomes, that might account for transfer of label between cells. Instead, contact dependency for the transfer seen in the current thesis has been confirmed.

The membrane marker DiO is known to have a lower lateral diffusion rate when compared to DiI ($DiIC_{18}(3)$) (Derzko and Jacobson, 1980, Kobbert et al., 2000), while DiD has similar diffusion properties as DiI ($DiIC_{18}(3)$) (Agmon et al., 1995). This difference in lateral diffusion between DiD and DiO is interpreted as accounting for the different results obtained dependent on which of the co-culture partners is labelled with DiD, as DiD will diffuse further into the membrane of DiO labelled cells, than DiO would be able to diffuse into DiD labelled cells.

Although it seems appropriate to exercise similar caution in interpretation of observations using DDAO-SE, CFSE and GFP as cytoplasmic markers, in light of the binding of DDAO-SE and CFSE to a wide variety of proteins via free amine residues on peptides such as Lys and Arg across proteins of all sizes, as well as the fact that GFP is a small but nonetheless substantial protein label, the impact of differential diffusion rates between adjacent cells would seem less a source of potentially confounding data, compared with studies using the membrane markers DiD and DiO. Nonetheless, there was a clear predominance of cells with dual labelling of cytoplasm and having nuclei of SAOS-GFP origin, and it is tempting to conclude that there is preferential bulk transport of cytoplasm from HGF to the smaller SAOS-2. This would seem consistent with the greater size of cytoplasmic dual labelled cells compared with SAOS-2 cultured alone (Figure 4.12). However, it is nonetheless acknowledged that amongst all proteins labelled with DDAO-SE in HGF, some will be considerably smaller and perhaps have a greater diffusion rate compared with GFP, so that it may not be warranted to make strong conclusion regarding the bulk-transport of cytoplasm between HGF and SAOS-2 in these experiments. Irrespective of this, it does seem reasonable to conclude that, similar to findings with plasma membrane, co-culture of HGF with SAOS-2 establishes physical continuity between the cytoplasm of the two cell types sufficient to permit the exchange of proteins.

The uptake of membrane and cytoplasmic components from stromal by neoplastic cells may have significant functional impact, and for lack of an established better term, this thesis proposes ‘cellular sipping’ as a descriptor for the harvesting of stromal cellular elements by neoplastic cells.

Actual cell surface area is determined not only by the apparent surface area profile as seen in the light microscope, but also by the height and surface roughness of cells, as well as by the length of cell processes, and none of these additional details could be reliably measured in the photomicrographs used for morphometric analysis. It would not have been possible to collect all of the necessary data from the many thousands of cells counted in the current study, to determine true cell surface area measurements. Nonetheless, of interest for this study was not the absolute value of true surface area of each cell, but rather the relative shifts in size for populations of cells cultured in different conditions, while the approach used was both practical and effective at collecting data that could be subjected to statistical analysis to make such comparisons.

While TNF-α did increase binding of SAOS-2, to HGF and also increased the proportion of cells expressing both membrane and cytoplasmic dual labelling (Figures 2.3 and 4.5), it is interesting that this cytokine did not appear to significantly affect either the size or circularity of any of the cell populations identified in co-cultures (Figures 4.10 and 4.11). This is interpreted as suggesting that the effect of TNF-α was purely with regard to increasing binding between cells which then had opportunity for more exchange of membrane or cytoplasmic contents, and that TNF−α did not directly stimulate the mechanisms responsible for exchange between cells.

It was interesting that the circularity of HGF in co-culture with SAOS-2 was greater than that of HGF cultured alone, as this suggests a degree of specificity amongst HGF with regard to which cells exchange membrane and cytoplasmic markers with SAOS-2, with more elongated cells being favoured for such exchange. Such micro-environmental permissive activity echoes the theme repeatedly emerging from work in

this thesis. An alternative and perhaps more likely interpretation of the data, however, is that SAOS-2 co-culture induced morphological rounding of HGF, despite the absence of any clear change in apparent HGF surface area profile.

Transfer of cytoplasmic components between cells without the transfer of the nucleus is observed between cardiomyocytes with fibroblasts (Driesen et al., 2005) and bone marrow derived cells (Alvarez-Dolado et al., 2003). Some precedent for exchange of plasma membrane components relevant to signalling between adjacent cells is established in work demonstrating transfer of the glycosylphosphatidylinositol attached co-receptor Cripto-1 across different tumour cell lines (Watanabe and Salomon, 2010), and it seems necessary to note that one of the membrane markers studied in this thesis, alkaline phosphatase, is also attached via glycosylphosphatidylinositol (Noda et al., 1987). In addition, there is growing interest in the role of intercellular transfer of both membrane and cytoplasmic proteins across cells, particularly with regard to the immune system (Ahmed and Xiang, 2011, Davis, 2007, Caumartin et al., 2006, Ahmed et al., 2008, LeMaoult et al., 2007, Rechavi et al., 2009, Waschbisch et al., 2009). Of particular interest to the current thesis, is a report demonstrating bi-directional movement of both lipid membrane and cytoplasmic components across a wide variety of cells (Niu et al., 2009). The work in the current thesis appears, however, to be the only study investigating the relationship between such phenomena and cytokine stimulation of stromal cells.

No evidence for fusion or sharing of nuclei was found in any of the experiments performed in this thesis, so that exchange of material between HGF and SAOS-2 appears to fall short of true cell fusion. Nonetheless, the membrane and cytoplasmic

exchange phenomena seen between SAOS-2 and HGF in the current chapter, could be interpreted as a highly limited form of tumour parenchymal- stromal cell fusion, a process that has been suggested as facilitating: transition into a malignant phenotype; expression of phenotypic and genotypic diversity among the tumour cells; increased migratory potential; and as a possible mechanism for immune evasion and chromosomal repair (Pawelek, 2000, Parris, 2006, Pawelek and Chakraborty, 2008a, Rachkovsky et al., 1998). The fusion process entails a series of events that encompass cell adhesion, membrane fusion, cellular material transfer and nuclear fusion. The presence of inflammatory cytokines in the tumour microenvironment contributes towards tumour progression and has been associated with increased tumour cell adhesion (Harimaya et al., 2000, Zhu et al., 2002, Bereta et al., 1991), invasion (De Wever and Mareel, 2003, Hagemann et al., 2004, Yanase et al., 1995, Zhu et al., 2002) and spread (Negus and Balkwill, 1996, Panozzo et al., 1996).

In context of the current findings, it is tempting to speculate that exchange of plasma membrane markers between neoplastic and host stromal cells may play a role in immune evasion. It is also possible, that 'cellular sipping' by neoplastic cells might comprise an indirect mechanism whereby cells with severely damaged genomes may be able to harvest the products of normal and more fully functional housekeeping genes.

It seems inherent in the data presented that exchange of membrane and cytoplasmic material between neoplastic and stromal cells would contribute the overall phenotypic diversity amongst neoplastic cells, so that 'the third population of cells' bearing both neoplastic and stromal cell markers may play a role in driving tumour progression.

Study earlier in this thesis showed increased SAOS-2 binding by HGF stimulated with TNF-α, while work in the current chapter indicated this had the effect of increasing exchange of membrane and cytoplasmic markers between SAOS-2 and HGF. There is some precedent of a role of cytokines in driving cell fusion, which although not complete in the current study does seem to have some features in common with exchange of material between SAOS-2 and HGF. Multinucleated giant cells (MNGCs) are characteristic of granulomatous inflammation first described by Langhans in 1868, and result from the fusion of macrophages. Cytokines such as IFN-γ, IL-13, IL-4 and GM-CSF play a role in inducing macrophage fusion for giant cell formation (Enelow et al., 1992, Most et al., 1990, Nagasawa et al., 1987, DeFife et al., 1997). Separately, RANK-L is known to induce osteoclast formation from isolated macrophages (Lau et al., 2007, Haynes et al., 2001). There is additional literature suggesting that inflammation increases cell fusion between other cell types, with increased intestinal epithelial cell fusion in the presence of inflammation and epithelial proliferation contributing towards tumour progression (Davies et al., 2009). In the presence of cytokines such as TNF-α, and or IFN-γ, augmentation in fusion between bone marrow derived mesenchymal cells and cerebellar neurons is observed (Kemp et al., 2011). Similarly, increased incidence of fusion between haematopoietic cells and purkinje neurons is noted (Johansson et al., 2008). A considerable increase in fusion between lymphocytes and skeletal muscle cells, cardiomyocytes and hepatocytes in vivo is also reported (Nygren et al., 2008). Variation in the extent of cellular material transfer between HGF and SAOS-2 across experiments is consistent with findings by other researchers (Busund et al., 2003, Chakraborty et al., 2001, Mortensen et al., 2004, Rachkovsky et al., 1998, Pawelek, 1993, Wakeling et al., 1994).

The morphology of cells in co-culture was studied in an attempt to identify any possible phenotypic change accompanying not only co-culture, but also exchange of membrane and cytoplasmic markers. Supporting this idea is that cell contact is reported to affect cell shape (Lecuit and Lenne, 2007). We interpreted the change in surface area and circularity as reflecting intimate exchange of cellular material between SAOS-2 and HGF, while the intermediate morphological properties of dual labelled cells supports the idea of phenotypic effects. Morphological changes in tumour cells is associated with an increase in migratory and invasive phenotype (Friedl and Wolf, 2003). Shape determined directed cell migration is observed with normal fibroblasts, endothelial cells as well as with transformed cells (Jiang et al., 2005). Similar to the current observations, squamous carcinoma cells (SCC) have increased surface area in the presence of fibroblasts, and this is associated with augmentation of migratory behaviour and the presence of hepatocyte growth factors (Xylas et al., 2010).

Observations of membrane and cytoplasmic marker transfer in this thesis, together with altered morphology of dual labelled cells intermediate to HGF and SAOS-2, lead to the suggestion that SAOS-2 may enjoy the benefits of at least partial normalization by cellular sipping of HGF components. This may confer survival advantages for affected neoplastic cells in tumours, thus contributing to tumour progression. Cellular sipping could thus provide a mechanism for overcoming the effects of extensive genetic injury inherent to neoplasia, and inevitable where there is failed segregation of chromosomes with increasing aneuploidy.

Chapter 5: Contact of HGF with SAOS-2 Decreases HGF Synthesis of GM-CSF, G-CSF and IL-6 in Response to TNF-α

5.1 INTRODUCTION

Work described in Chapters 2 and 3 revealed a high level of complexity in TNF-α directed interactions between SAOS-2 and HGF, such that the neoplastic and stromal cells studied had strong but opposing responses to the cytokine with regard to their ability to bind to each other. This raised the concept of there being 'permissive histories' for individual cells migrating throughout tumour microenvironments, as well as there being 'permissive microenvironments', and a role for such permissive effects was suggested as potentially contributing to internal tumour diversity and tumour progression.

However, it should be noted that aspects of the idea of permissive effects have been foreshadowed by earlier workers, as it has long been accepted that cytokines produced throughout tumours may play important roles in determining biological outcomes by creating local microenvironments promoting tumour growth and progression (Negus and Balkwill, 1996, Panozzo et al., 1996). It is plausible that direct neoplastic and stromal cell interactions evoke altered stromal cell cytokine production.

While preceding chapters demonstrated effects of TNF-α in increasing adhesion of SAOS-2 to HGF, as well as in encouraging exchange of markers between the two cell types, the current chapter examines interactions between SAOS-2 and HGF with regard to the synthesis of the cytokines IL-6, GM-CSF, G-CSF and FGF.

Enzyme linked immunosorbent assay (ELISA) is a convenient, reliable and widely used method for quantitative evaluation of cytokines (Martin et al., 2006, Segundo et al., 2009, Jensen and Gad, 2010, Swartzman et al., 1999), while FACS analysis is also widely used to

evaluate expression of cell associated cytokines (Vignali, 2000, Foster et al., 2007, Jung et al., 1993, Prussin and Metcalfe, 1995), and a more recently developed approach is electrochemiluminescence using magnetic beads linked with antibody directed against cytokines (Sennikov et al., 2003, Nold et al., 2008). Simultaneous detection of multiple cytokines can be carried out by enzyme linked immunosorbent spot assays (Jansson et al., 2003, Fujihashi et al., 1993, Meierhoff et al., 2002). Cytokine mRNA quantification has traditionally been by Northern blot (Hein et al., 2001, Powell et al., 1999), but is increasingly by reverse transcriptase real-time polymerase chain reaction (RT-RTPCR) (Stordeur et al., 2002, Rama Iniguez et al., 2006, Huang et al., 1999). Given the range of methods currently available to study cytokine expression, it was decided that the current study would use an ELISA approach, for which the laboratory within which this work was performed was equipped, and which continues to be a widely used, accurate and sensitive method for detection and quantification of cytokines.

As mentioned previously, a number of cytokines and growth factors produced by neoplastic and stromal cells can directly or indirectly contribute towards tumour progression by modulating cell survival, growth, motility and attachment (Balkwill and Mantovani, 2001, Coussens and Werb, 2001, Negus and Balkwill, 1996, Panozzo et al., 1996). Elevated levels of several cytokines in tumours underscores the potential of these signalling molecules, while examples of cytokines increased in some tumours include: IL-6 (Berek et al., 1991, Blay et al., 1992, Seymour et al., 1995), FGF (Nguyen et al., 1994, Nguyen et al., 1993, O'Brien et al., 1995), G-CSF (Tachibana et al., 1995, Braun et al., 2004), TNF (Naylor et al., 1993, Balkwill et al., 1987, Nakashima et al., 1995, Szlosarek et al., 2006), TGF (Nishimura et al., 1986), GM-CSF (Chopra et al., 1997) and IL-1 (Uefuji et al., 2005).

Directed migration of neoplastic cells may be driven by some cytokines or cell surface molecules (Moore, 2001). An interesting example of stromal-neoplastic cell interaction in metastasis is seen in a form of rat mammary carcinoma, where gradients of epidermal growth factor (EGF) and CSF-1 drive migration required for metastasis, but where the EGF and CSF-1 receptors are confined to carcinoma cells and macrophages respectively, such that cooperation between the two cell types is necessary for metastasis to occur (Wyckoff et al., 2004). Cytokines such as FGF, TGF-β, VEGF-A, EGF and PDGF are expressed by tumour associated stromal cells (al-Sarireh and Eremin, 2000, Bhowmick et al., 2004), and appear important for recruitment of secondary inflammatory cells such as neutrophils and mast cells which themselves secrete cytokines sustaining further inflammation, angiogenesis and expression of pro-tumourigenic proteases that support tumour progression (Lin and Pollard, 2004, Meininger and Zetter, 1992, Schaider et al., 2003). Altered cytokine production by tumour associated stromal cells is seen relative to healthy tissue (Silzle et al., 2004, al-Sarireh and Eremin, 2000, Silzle et al., 2003), and roles for these cytokines in tumour development are suggested via adhesion (Harimaya et al., 2000, Zhu et al., 2002, Bereta et al., 1991), epithelial-mesenchymal transformation (Yamauchi et al., 2010, Bates and Mercurio, 2003) and neovascularisation (Ribatti et al., 2003). Macrophages comprise a likely rich source of tumour associated cytokines, and express IL-6, IL-1, IL-8, GM-CSF, IL-10 and TNF-α, to affect tumour growth, angiogenesis, invasion and dissemination, while IL-10 has been suggested as specifically immunosuppressive (Yanagawa et al., 1999, al-Sarireh and Eremin, 2000). Countering tumour promoting activities of cytokines, are some tumour negating effects, with IL-1 and TNF-α stimulating macrophage induced apoptosis for example (al-Sarireh and Eremin, 2000). The presence of an inflammatory response in and around the tumour as evidenced by a high density of tumour infiltrating lymphocytes as well as tumour associated

macrophages is associated with improved prognostic outcome as seen in colon, rectal and breast cancer (Fridman et al., 2011, Hsu et al., 2010, Roxburgh and McMillan, 2011). Higher counts of stromal as well as intratumoural CD3+ correlated with better survival (Roxburgh and McMillan, 2011). Importantly, the shared association by an inflammatory response with tumour progression as well as improved prognosis supports a compelling role for pro-inflammatory factors in influencing and determining the tumourigenic process.

In light of the response to TNF-α seen in Chapters 2, 3 and 4 and the overall question of microenvironmental permissive signals, it became of interest in this thesis to investigate potential changes in the cytokine synthetic profile of SAOS-2 and HGF during co-culture. It is stressed that despite the literature's reasonable focus on macrophages as an important source for tumour inflammatory cytokines (Dranoff, 2004, Le Bitoux and Stamenkovic, 2008, al-Sarireh and Eremin, 2000, Allavena et al., 2008), that it is fibroblasts which form the great bulk of extravascular stromal cells, so that cytokines expressed by fibroblasts may have significant impact on determining microenvironmental conditions in tumours.

Normal fibroblasts produce basal levels of cytokines such as IL-6, IL-1, IL-8, GM-CSF and G-CSF (Silzle et al., 2004, Rockman et al., 2001, Kotb and Calandra, 2003). An up regulation of IL-1β, IL-8, IL-6 and CCL2/MCP-1 occurs in fibroblasts when co-cultured with BLM and MV3 melanoma cell lines with increased production of IL-1β specifically contributing towards melanoma cell invasiveness (Li et al., 2009). Similarly, myofibroblasts express high levels of IL-1, G-CSF, GM-CSF, M-CSF and IL-10 (Silzle et al., 2004, Powell et al., 1999).

IL-6 is produced by a variety of cells including fibroblasts, macrophages, endothelial T-cells, and B-cells (Dranoff, 2004). This cytokine facilitates spread and proliferation of myeloma tumour cells (Bataille et al., 1995, Asschert et al., 1999), while enhanced expression of IL-6 occurs in association with ovarian (Watson et al., 1990, Berek et al., 1991), colon (Galizia et al., 2002), renal (Blay et al., 1992), breast (Yamashita et al., 1996) and lung cancer (Yanagawa et al., 1995). Spontaneous secretion of IL-6 is seen by fibroblasts isolated from breast carcinomas as opposed to fibroblasts from healthy tissue (Silzle et al., 2003). Also, IL-6 has an inhibitory effect on dendritic cell differentiation and activation in pancreatic cancers (Bharadwaj et al., 2007), and a growth stimulatory effect in some solid tumours (Okamoto et al., 1997, Voorzanger et al., 1996).

Colony stimulating factors in general are primarily involved in regulating haematopoietic cell function, proliferation and differentiation with regard to the generation of leukocytes (Koeffler et al., 1988, Gordon and Gabrilove, 1990, Hamilton, 2008) Separate to haematopoietic activity, however, CSFs have a wide range of separate activities including activation and differentiation of inflammatory leukocytes (Ho et al., 1990, Markowicz and Engleman, 1990), lipid metabolism (Irie et al., 2001, Le Meur et al., 2004), neurotrophism (Kannan et al., 2000, Pitzer et al., 2008), and migration of endothelial cells (Sunderkotter et al., 1994).

GM-CSF is expressed by many cells including fibroblasts, endothelial cells, macrophages, leukocytes and mast cells (Dranoff, 2004, Kotb and Calandra, 2003). Mesenchymal stem cells after prolonged culture in the presence of GM-CSF undergo malignant transformation (Zhou et al., 2011) and increased GM-CSF expression is noted in colon carcinoma cells

with an enhanced desmoplastic reaction and aggressive malignant behaviour (Bretscher et al., 2000).

G-CSF is expressed by fibroblasts, endothelial cells, macrophages and lymphocytes (Koyama et al., 1998, Kotb and Calandra, 2003). It is interesting that G-CSF expression by bladder carcinoma cells is associated with aggressive tumour growth and poor prognosis (Tachibana et al., 1995), and G-CSF appears to facilitate proliferation of bladder carcinoma cells via enhanced fibroblast growth factor production (Ohmi et al., 2003). G-CSF also increases colon cancer growth, seemingly by mediating pro-angiogenic effects (Natori et al., 2002). However, an inhibitory effect on the differentiation and activation of dendritic cells in pancreatic carcinoma by G-CSF has also been described (Bharadwaj et al., 2007).

FGF plays an important role in cell function, proliferation, migration, differentiation (Yun et al., 2010) and angiogenesis (Presta et al., 2005). FGF is unusual in that it is produced without a leader sequence, and so accumulates in the cytoplasm of cells, seemingly pre-loaded in the event of cell damage and lysis when it is released into the environment to drive cell proliferation and angiogenesis (Lawrence et al., 2006, Renko et al., 1990, Yun et al., 2010). Aberrant FGF production could conceivably alter cell turn-over in tissues and thus contribute to a variety of tumour associated processes including cell proliferation, invasion, tissue replacement, angiogenesis and desmoplasia. In light of the multiple effects of FGF with regard to neoplasia, it is perhaps not surprising that both tumour inhibitory and promoting activities have been proposed for FGF (Turner and Grose, 2010, Presta et al., 2005, Nomura et al., 2008). Increased levels of FGF are reported in lung (Ruotsalainen et al., 2002) and prostrate cancers (Kwabi-Addo et al., 2004). FGF

facilitates prostate cancer growth (Russell et al., 1998), and expression of FGF receptors by prostrate cancer cells enhances invasiveness via an increase in expression of membrane-type 1 matrix metalloproteinases (Sugiyama et al., 2010). Similarly, expression of FGF receptors in multiple myeloma is thought important for development of the disease (Chesi et al., 2001). FGF released by melanoma and other neoplastic cells drives proliferation and angiogenesis (Wang and Becker, 1997, Kerbel, 2000, Ribatti et al., 2003, Bikfalvi, 1995, Figg et al., 2008). FGF can also activate anti-apoptotic pathways promoting survival of tumour cells, and may provide resistance to some anti-cancer drugs (Pardo et al., 2002, Pardo et al., 2003, Pardo et al., 2006).

5.2 MATERIALS

5.2.1 Materials for Cell Culture

Most materials for culture of HGF, SAOS-2 were the same as those used and described in section 2.2.1. In addition, transwells with a pore size of 0.4 μm were purchased from Corning Costar (USA).

5.2.2 Materials for ELISA

Duoset® ELISA Development Systems, used for the detection of IL-6, GM-CSF, G-CSF, IL-1α, TNF-α and FGF, were obtained from R & D Systems (Minneapolis, USA) while streptavidin-HRP substrate reagents and sulphuric acid (H_2SO_4) were purchased from APS Chemicals (NSW, Australia). PBS tablets were acquired from Oxoid (Hampshire, England). Bovine serum albumin (BSA) and Tween-20 were from Sigma-Aldrich (St. Louis, USA). ELISA plates were bought from Becton Dickinson Labware (NJ, USA).

5.3 METHODS

5.3.1 Isolation, Culture and Storage of HGF

HGF cells were isolated, cultured and stored as described earlier in sections 2.3.1, 2.3.2 and 2.3.5 respectively.

5.3.2 Culture and Storage of SAOS-2

SAOS-2 cells were cultured and stored as described earlier in section 2.3.7.

5.3.3 Cell Culture Conditions for Direct Co-Culture and Preparation of Samples for ELISA

Confluent T75 cm^2 flasks of HGF were harvested using trypsin/EDTA as outlined in section 2.3.2, and seeded in pre-gelatinised 12 well tissue culture plates for incubation in CM at $37^{o}C$, under CO_2 (5%) and at 95% relative humidity. Note that the antibiotics penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (0.25 µg/ml) were present throughout all cell culture procedures, identical to use described in previous chapters. HGF reached confluence in 1 to 2 days as determined by light microscopy, and monolayers were washed once with M199 before pre-incubation with or without TNF-α (3 nM) for a period of 24 hrs in M199 with antibiotics. Confluent SAOS-2 were released with trypsin/EDTA and pelleted by centrifugation as outlined in section 2.3.7, before resuspension in the appropriate amount of M199 with antibiotics and BSA (4%) and application of 3.6×10^5 cells per well to HGF. Prior to this, the pre-incubated HGF monolayers were washed with M199 and the media replaced with M199 and antibiotics with BSA (4%). HGF and SAOS-2 cultured alone in M199 with antibiotics and BSA (4%)

served as controls. Supernatants were collected from 0 to 24 hrs and centrifuged to remove remnants of dead or floating cells, while monolayers were also collected by gently washing twice with M199 before preparing cell lysates by scraping of attached cells into PBS with triton-X 100 (0.5%). Supernatants and lysates were stored at -80^{o}C until analysis by ELISA.

5.3.4 Procedures Used in Transwell Experiments

Confluent HGF were harvested as described in section 2.3.2, and cells seeded above transwell inserts in 12 well transwell culture plates (Figure 5.1) for incubation in CM. Once HGF monolayers were established, they were washed once with M199 and pre-incubated with TNF-α (3 nM) for 24 hrs. Immediately prior to co-culture with SAOS-2, HGF monolayers in transwell inserts were gently washed with M199 and the media replaced with M199 containing antibiotics and BSA (4%). SAOS-2 were harvested from confluent T75 cm^{2} flasks using trypsin/EDTA as outlined in section 2.3.7, and seeded in M199 with antibiotics and BSA (4%) in lower transwell chambers so that HGF were suspended above SAOS-2 on the porous transwell membrane.

After 24 hrs incubation, supernatants were collected separately from above and below inserts and centrifuged to remove the remnants of dead or floating cells. Similarly, lysates were also collected as outlined in section 5.3.3, but separately from HGF above and SAOS-2 below the inserts. Both the supernatants and lysates were stored at -80^{o}C until analysis by ELISA.

5.3.5 Enzyme Linked Immunosorbent Assays (ELISA)

Assays were performed according to the manufacturer's protocol (R & D systems) and the amount of cytokine present in samples calculated according to standard curves using material provided by the manufacturer. The wells of 96-well ELISA plates were coated overnight at room temperature (RT) using 100 µl / well of cytokine specific capture antibody diluted to a prescribed working concentration in PBS (Table 5.1). The following day, wells were washed three times with wash buffer comprising tween 20 (0.05% in PBS) and then blocked for non-specific binding using 300 µl / well of reagent diluent comprising PBS with BSA (1%) for 1 hr. Wells were then washed three times with wash buffer and 100 µl / well of either experimental supernatant or lysate applied, while serial dilutions of standards diluted in reagent diluent were also loaded as appropriate before incubation for 2 hrs at RT (Table 5.1). Following this, the wells were washed three times with wash buffer before application of 100 µl / well of biotinylated detection antibody diluted in reagent diluent and further incubation for 2 hrs at RT (Table 5.1). After washing plates three times with wash buffer, wells were incubated for 20 mins with 100 µl / well of streptavidin-HRP conjugate, diluted 1:200 in reagent diluent, while avoiding direct light. Plates were then washed three times with wash buffer, before being incubated with 100 µl / well of substrate solution comprising 1:1 mixture of H_20_2 and tetramethybenzidine for 20 mins at RT avoiding direct light. The colour developing reaction was stopped by addition of 50 µl / well of stop solution (2N H_2SO_4). Absorbance was measured at 450 nm using a microplate reader (Bio-Rad Model 3550 plate reader). Statistical analysis of data and inter group comparisons was by Student t-test, and $p<0.05$ was regarded as being statistically significant.

Table 5.1: Working concentrations of capture antibody, standard, and detection antibody used for different cytokines measured by ELISA.

Cytokine	Capture Antibody (µg/ml)	S_1^* Standard Concentration (pg/ml)	Detection Antibody
IL-6	2	500	200 ng / ml
GM-CSF	2	500	0.5 µg / ml
G-CSF	2	500	200 ng / ml**
IL-1α	2	200	12.5 ng / ml
TNF-α	4	1000	250 ng / ml
FGF-b	2	1000	0.25 µg / ml

***** S_1 indicates the highest concentration of serial standard dilutions.

** Reagent diluent including 2% heat-activated goat serum was used to prepare detection antibody.

5.4 RESULTS

5.4.1 TNF-α Stimulated HGF Expression of IL-6, GM-CSF, G-CSF and FGF

Table 5.2 shows the average result of triplicate samples from one experiment. Unstimulated HGF in isolated culture had very low levels of GM-CSF and G-CSF which were often below detection limits for both supernatants and lysates, whereas IL-6 and FGF were present at appreciable levels such that IL-6 was primarily in supernatants and FGF in lysates. Neither IL-1α or TNF-α were detected in any samples tested. TNF-α (3nM) increased HGF expression of IL-6 ($p<0.001$), GM-CSF ($p<0.0001$), G –CSF ($p<0.0001$) and FGF ($p<0.0001$) over a 24 hr incubation period (Table 5.2), with levels rising mostly in supernatants with the exception of FGF which although elevated in the supernatant was reduced in cell lysates ($p<0.0001$) (Table 5.2)

Table 5.2. Cytokine concentrations in supernatants and lysates of HGF in isolated culture after 24 hrs of stimulation with TNF-α (3nM) compared with unstimulated controls

	Without TNF-α		**With TNF-α**	
Cytokine Type	**Supernatant (pg/ml)**	**Lysate (pg/ml)**	**Supernatant (pg/ml)**	**Lysate (pg/ml)**
IL-6	672 ± 47.9	144 ± 8.9	971.17 ± 31.45	516.29 ± 87.35
GM-CSF	18.17 ± 4.9	12.21 ± 7.81	221.15 ± 2.64	86.90 ± 9.21
G-CSF	15.16 ± 9.14	16.60 ± 5.98	1178.45 ± 38.93	148.85 ± 13.08
FGF	8.35 ± 6.17	1808.04 ± 37.69	276.81 ± 13.42	699.13 ± 38.88

Data are expressed as Mean ± SD of triplicate samples.

5.4.2 SAOS-2 had Negligible Levels of IL-6, GM-CSF, G-CSF and FGF, while SAOS-2 Reduced Cytokine Levels in HGF Pre-treated with TNF-α

Figures 5.2 and 5.3 show the results of representative experiments comparing the levels of cytokines studied in supernatants and lysates respectively of TNF-α pre-treated HGF alone, SAOS-2 alone and co-cultures of TNF-α stimulated HGF with SAOS-2, while similar results were obtained in three experiments using TNF-α pre-treated HGF isolated from three donors.

Comprising a convenient control for the fidelity of the measurement system used, no appreciable levels of cytokines were present in supernatants of any cultures studied at the 0 hr time point, although FGF was detected at significant levels in lysates of both HGF pre-treated with TNF-α and co-cultures with SAOS-2 as expected for this cell associated cytokine. Very low or undetectable levels of all cytokines studied were also present in the supernatants and lysates of SAOS-2 cultured alone (Figures 5.2 and 5.3).

In the supernatants collected from both TNF-α pre-treated HGF cultured alone and co-cultured with SAOS-2, levels of IL-6 ($p<0.0001$), G-CSF ($p<0.0001$) and GM-CSF ($p<0.001$) were consistently higher in supernatants than in cell lysates , while in the case of FGF, TNF-α pre-treated FGF cell lysates had higher levels compared with supernatants levels in co-cultures ($p<0.0001$) (Figure 5.2 and 5.3). At the 4 hr time point, only IL-6 was detected in the supernatants alone and in co-culture with SAOS-2, and there was no clear difference between these two culture conditions (Figure 5.2). All cytokines studied were found at high levels by 24 hrs in the supernatants of TNF-α pre-treated HGF cultured alone and co-cultured with SAOS-2, while levels were consistently lower in co-cultures

compared with TNF-α pre-treated HGF alone for IL-6 ($p<0.05$), GM-CSF ($p<0.001$), G-CSF ($p<0.0001$) and FGF ($p<0.0001$) (Figure 5.2).

A broadly similar pattern was seen in lysates, in that at the 24 hr time point, there were lower levels of IL-6 ($p<0.01$), GM-CSF ($p<0.02$) and G-CSF ($p<0.01$) in lysates of TNF-α pre-treated HGF in co-culture with SAOS-2, than in TNF-α pre-treated HGF cultured alone (Figure 5.3). A difference, however, was in the case of FGF, where co-cultures of TNF-α pre-treated HGF with SAOS-2 had higher levels of cytokine relative to TNF-α pre-treated HGF cultured alone ($p<0.001$). Another difference seen in results from lysates as opposed to supernatants, was that IL-6 appeared to be at a higher level at the 4 hr time point in co-cultures of TNF-α pre-treated HGF with SAOS-2 compared with TNF-α pre-treated HGF cultured alone ($p<0.001$), although this relationship was reversed by the 24 hr time point (Figure 5.3).

5.4.3 SAOS-2 Derived Soluble Factors Increased Cytokine Expression in Co-Culture

To determine if direct cell contact was required for the effects of co-culture with SAOS-2 on cytokine production by HGF seen in Figures 5.2 and 5.3, further experiments were performed using transwell membranes. The results of representative experiments with transwell inserts are shown in Figures 5.4 and 5.5, comparing levels of cytokines in supernatants and lysates respectively of HGF alone, SAOS-2 alone and co-cultures of HGF with SAOS-2, while similar results were obtained in three experiments using HGF isolated from three donors.

Cytokine levels were consistently higher in both supernatants and lysates of the upper well compartments containing HGF, than in the lower well compartments containing SAOS-2, consistent with findings in Figures 5.2 and 5.3 that HGF are the principal source of all cytokines studied in this model system (Figures 5.4 and 5.5).

Contrasting with results of experiments with direct co-culture (Figures 5.2 and 5.3), however, was that when the two cell types were separated across transwell membranes, HGF had increased rather than decreased production of IL-6 ($p<0.03$), GM-CSF ($p<0.001$), and G-CSF ($p<0.01$) in transwell co-culture with SAOS-2 as compared with HGF cultured alone. No difference, however, was seen in FGF levels between HGF cultured alone or in transwell co-culture with SAOS-2 (Figure 5.4).

Regarding cell lysates in transwell experiments, there was a similar increase in IL-6 ($p<0.02$), GM-CSF ($p<0.02$) and G-CSF ($p<0.01$) when HGF were subjected to transwell co-culture with SAOS-2 as compared with HGF cultured alone, while again there was no difference in FGF lysate levels in these two conditions (Figure 5.5).

Figure 5.1 Diagramatic representation of the transwell culture system. Fibroblasts were grown on the microporous membrane in the upper chamber, whereas SAOS-2 were seeded into the lower chamber. This system allows for bi-directional transfer of soluble factors across the membrane whilst avoiding cell contact. Membrane pore size = 0.4 μM.

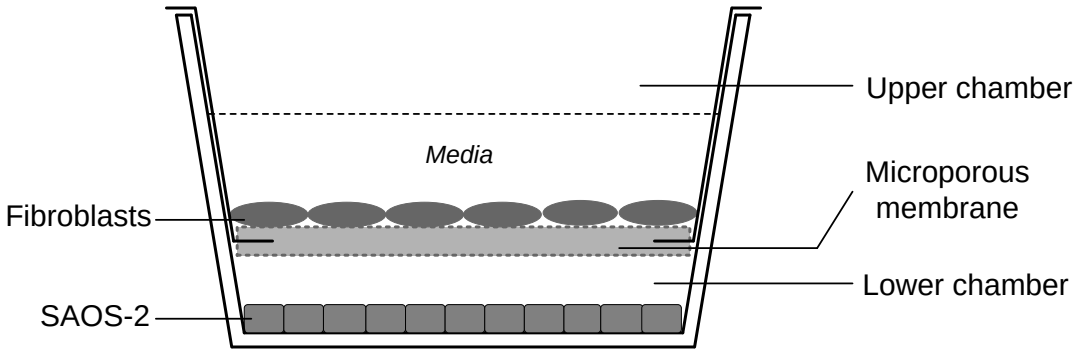

Upper chamber
Media
Microporous membrane
Fibroblasts
Lower chamber
SAOS-2

Figure 5.2 Histograms showing the result of a representative experiment in which protein levels of IL-6, GM-CSF, G-CSF and FGF were determined by ELISA in supernatants of: HGF pre-treated with TNF-α for 24 hr; SAOS-2 cultured alone; or both cell populations co-cultured together. None of the cytokines studied were detected at appreciable levels in SAOS-2 cultures alone, while all cytokines studied accumulated in supernatants of HGF pre-treated with TNF-α by 24 hrs. Co-culture of TNF-α stimulated HGF with SAOS-2 significantly reduced levels of IL-6 ($p<0.05$), GM-CSF ($p<0.001$), G-CSF ($p<0.0001$) and FGF ($p<0.0001$) Similar results were obtained in three separate experiments with HGF from three different donors. Data is expressed as Mean $\pm$ SD, (n=3).

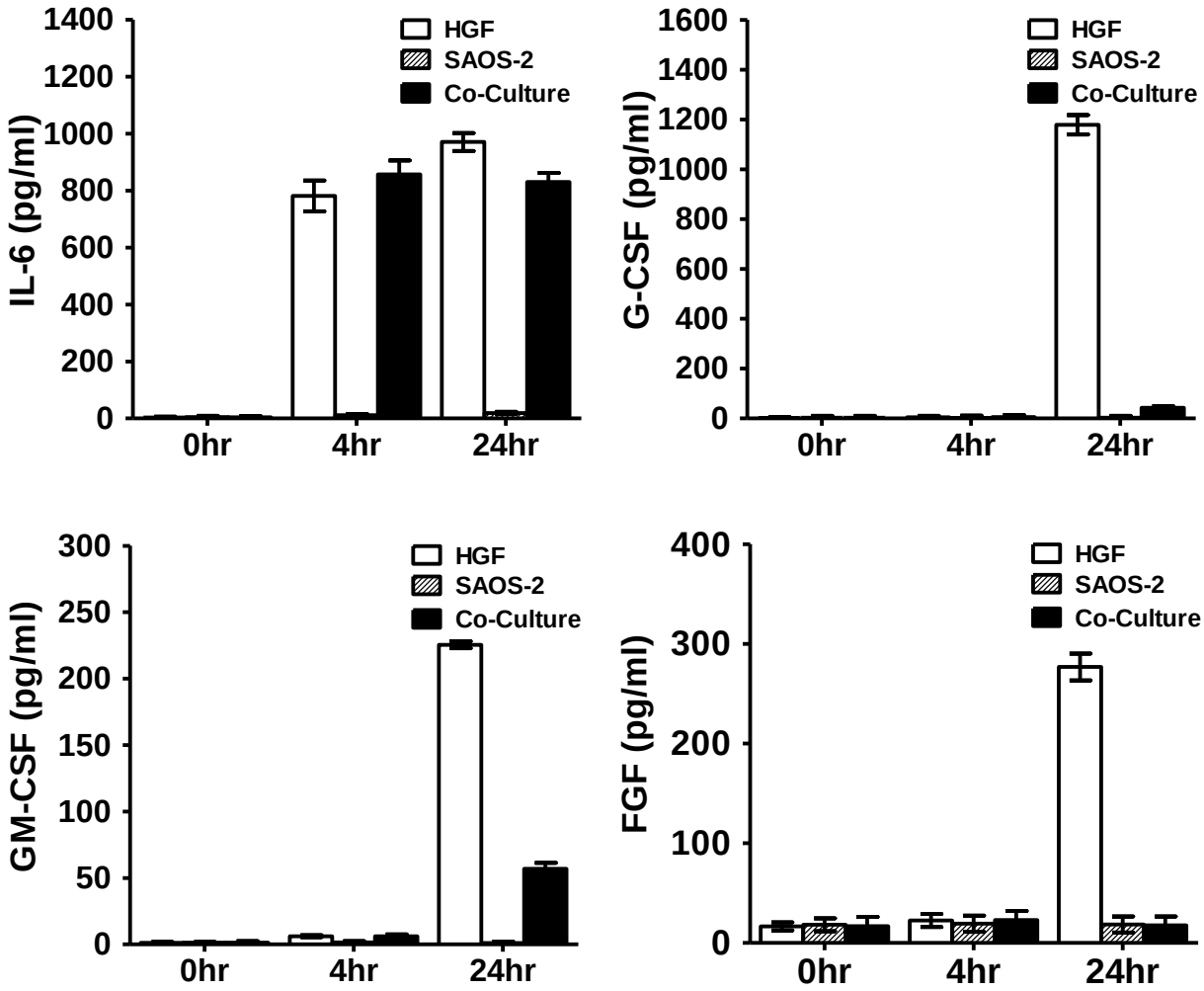

IL-6 (pg/ml)
1400
1200
1000
800
600
400
200
0
0hr
4hr
24hr
HGF
SAOS-2
Co-Culture
G-CSF (pg/ml)
1600
1400
1200
1000
800
600
400
200
0
0hr
4hr
24hr
HGF
SAOS-2
Co-Culture
GM-CSF (pg/ml)
300
250
200
150
100
50
0
0hr
4hr
24hr
HGF
SAOS-2
Co-Culture
FGF (pg/ml)
400
300
200
100
0
0hr
4hr
24hr
HGF
SAOS-2
Co-Culture

Figure 5.3 Histograms showing the result of a representative experiment in which protein levels of IL-6, GM-CSF, G-CSF and FGF were determined by ELISA in lysates of: HGF pre-treated with TNF-α for 24 hr; SAOS-2 cultured alone; or both cell populations co-cultured together. None of the cytokines under study were detected in lysates of SAOS-2. HGF pre-treated with TNF-α, however, did express appreciable levels of all cytokines studied in cell lysates by the 24 hr time point. Co-culture of these TNF-α stimulated HGF with SAOS-2 did, however, result in decreased levels of IL-6 ($p<0.01$), GM-CSF ($p<0.02$), G-CSF ($p<0.01$), whereas there was an increase in FGF levels ($p<0.001$) Similar results were obtained in three separate experiments with HGF from three separate donors. Data is expressed as Mean $\pm$ SD, (n=3).

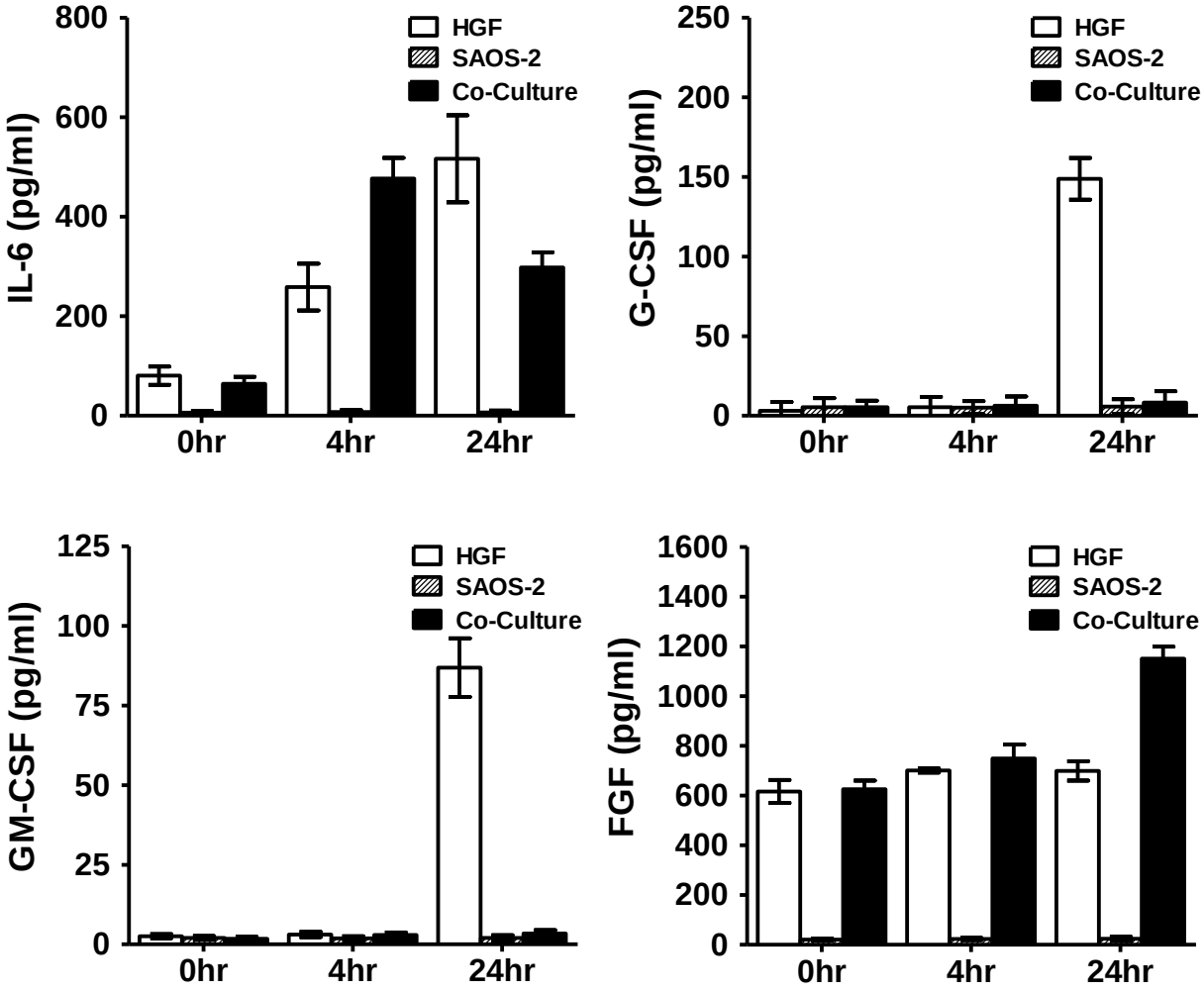

IL-6 (pg/ml)
800
600
400
200
0
HGF
SAOS-2
Co-Culture
0hr
4hr
24hr
G-CSF (pg/ml)
250
200
150
100
50
0
HGF
SAOS-2
Co-Culture
0hr
4hr
24hr
GM-CSF (pg/ml)
125
100
75
50
25
0
HGF
SAOS-2
Co-Culture
0hr
4hr
24hr
FGF (pg/ml)
1600
1400
1200
1000
800
600
400
200
0
HGF
SAOS-2
Co-Culture
0hr
4hr
24hr

Figure 5.4 Histograms showing the result of a representative experiment in which protein levels of IL-6, GM-CSF, G-CSF and FGF were determined by ELISA in supernatants from the upper (A) and lower (B) compartments of transwell cultures of: HGF pre-treated with TNF-α for 24 hr; SAOS-2 cultured alone; or both cell populations co-culture together. An increase in HGF expression levels of IL-6 ($p<0.03$), GM-CSF ($p<0.001$), G-CSF ($p<0.01$) was noted at 24 hrs in co-culture relative to HGF cultured alone; however no difference in FGF levels were noted. The graphs show one representative experiment for each cytokine out of three experiments with three different donors. Data is expressed as Mean $\pm$ SD, (n=3).

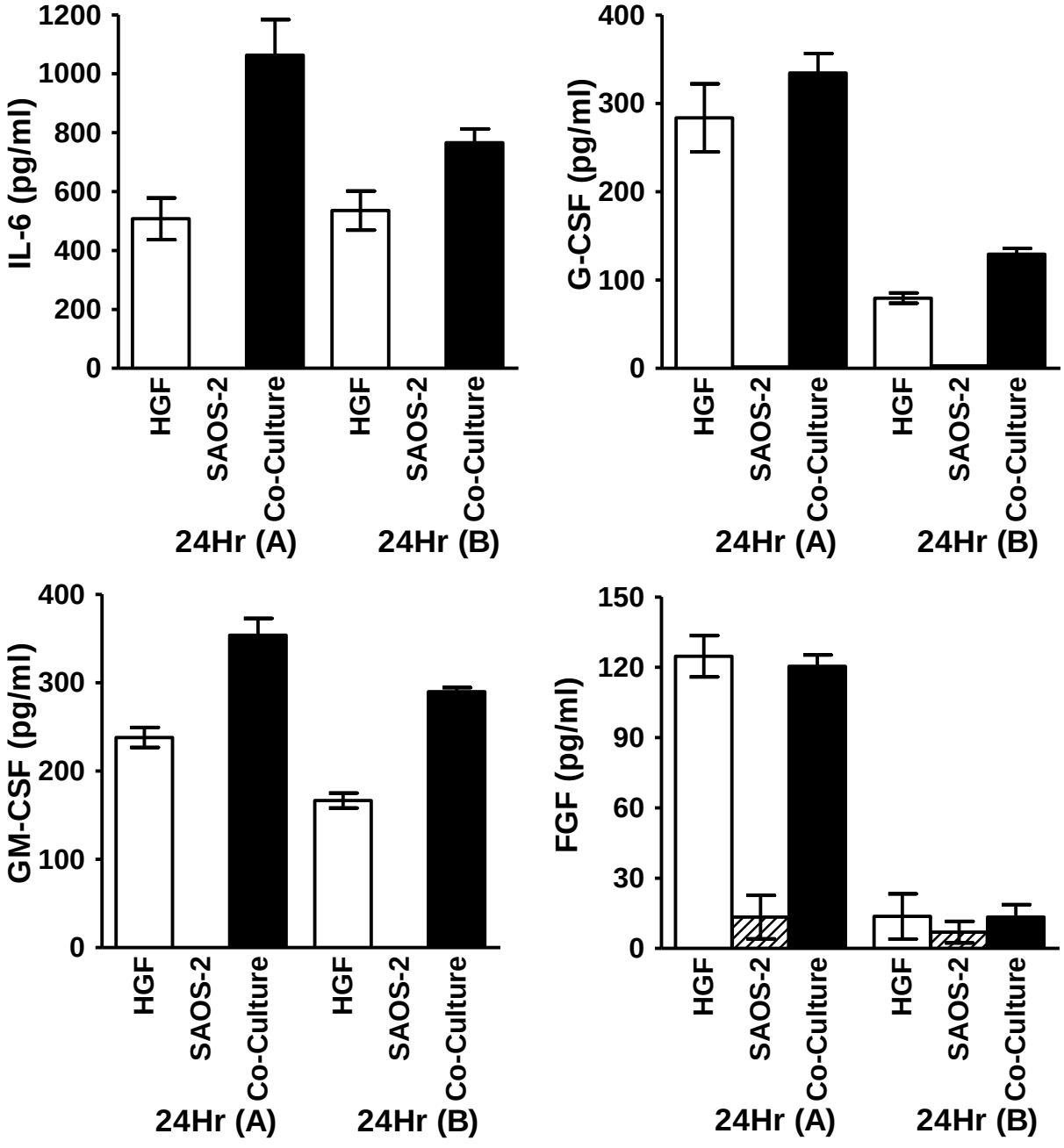
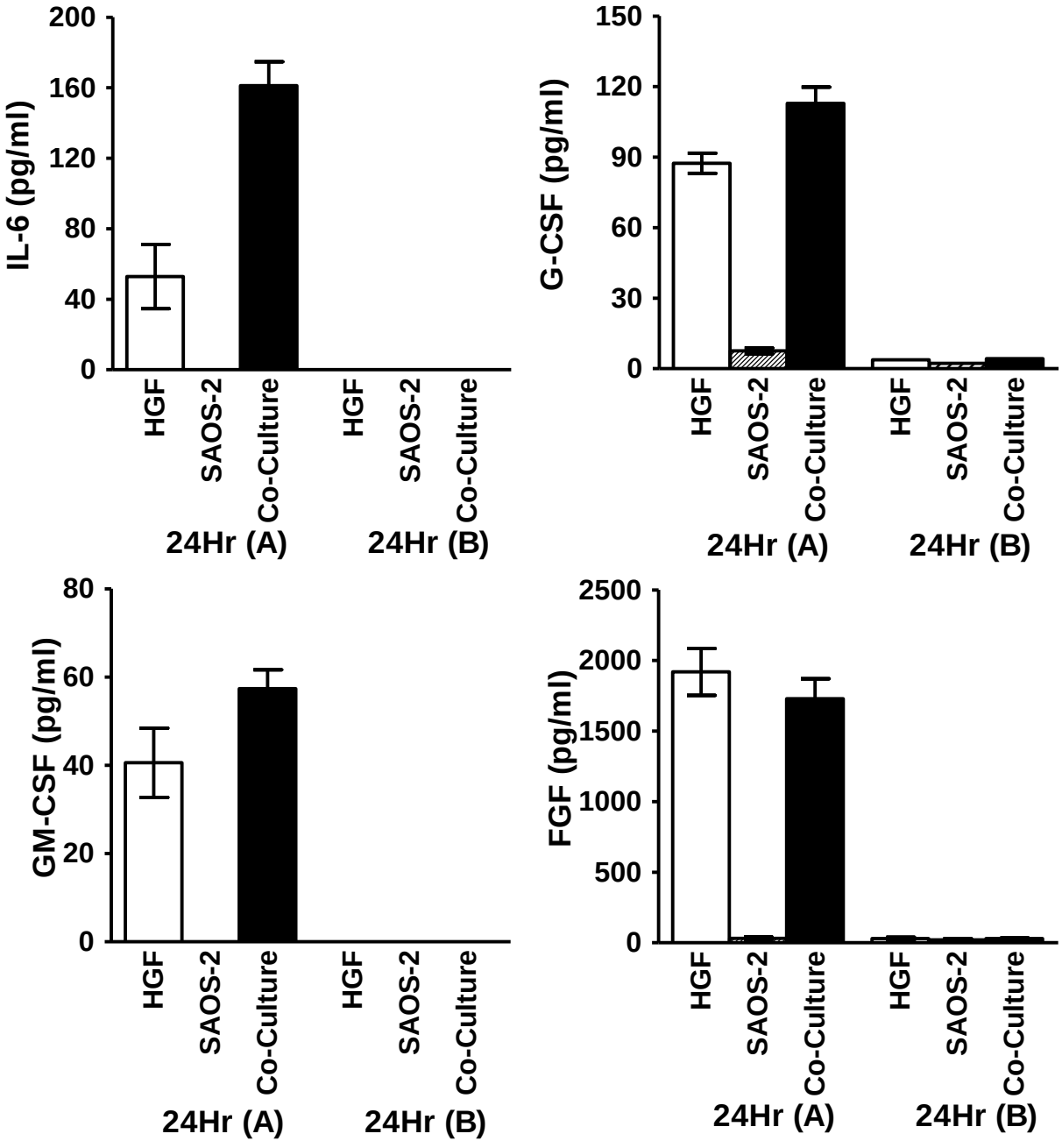

IL-6 (pg/ml)
1200
1000
800
600
400
200
0
HGF
SAOS-2
Co-Culture
HGF
SAOS-2
Co-Culture
24Hr (A)
24Hr (B)
G-CSF (pg/ml)
400
300
200
100
0
HGF
SAOS-2
Co-Culture
HGF
SAOS-2
Co-Culture
24Hr (A)
24Hr (B)
GM-CSF (pg/ml)
400
300
200
100
0
HGF
SAOS-2
Co-Culture
HGF
SAOS-2
Co-Culture
24Hr (A)
24Hr (B)
FGF (pg/ml)
150
120
90
60
30
0
HGF
SAOS-2
Co-Culture
HGF
SAOS-2
Co-Culture
24Hr (A)
24Hr (B)

Figure 5.5 Histograms showing the result of a representative experiment in which protein levels of IL-6, GM-CSF, G-CSF and FGF were determined by ELISA in lysates from the upper (A) and lower (B) compartments of transwell cultures of: HGF pre-treated with TNF-α for 24 hr; SAOS-2 cultured alone; or both cell populations co-culture together. Negligible levels of cytokine were detected in cultures of SAOS-2 alone, while lysates of the upper chambers containing HGF in cultures of TNF-α pre-treated HGF did have detectable levels of all cytokines studied. Transwell co-culture with SAOS-2 increased levels of IL-6 ($p<0.02$), GM-CSF ($p<0.02$), and G-CSF ($p<0.01$) in pre-activated HGF, while there was no clear effect of co-culture on levels of FGF in these cells. Similar results were obtained in three experiments with HGF from three separate donors. Data is expressed as Mean $\pm$ SD, (n=3).


IL-6 (pg/ml)
200
160
120
80
40
0
HGF
SAOS-2
Co-Culture
HGF
SAOS-2
Co-Culture
24Hr (A)
24Hr (B)
G-CSF (pg/ml)
150
120
90
60
30
0
HGF
SAOS-2
Co-Culture
HGF
SAOS-2
Co-Culture
24Hr (A)
24Hr (B)
GM-CSF (pg/ml)
80
60
40
20
0
HGF
SAOS-2
Co-Culture
HGF
SAOS-2
Co-Culture
24Hr (A)
24Hr (B)
FGF (pg/ml)
2500
2000
1500
1000
500
0
HGF
SAOS-2
Co-Culture
HGF
SAOS-2
Co-Culture
24Hr (A)
24Hr (B)

5.5 DISCUSSION

Work in this chapter was to explore the effect of HGF co-culture with SAOS-2 on the production of cytokines potentially relevant to driving inflammatory or proliferative events. IL-6, G-CSF and GM-CSF were selected as a cytokines of interest because of their well characterized role in driving systemic as well as local aspects of inflammation (Xing et al., 1998, Fonseca et al., 2009, Eyles et al., 2008, Hamilton, 2008, Martins et al., 2010), while FGF was of interest in context of its roles in angiogenesis and cell proliferation (Parsons-Wingerter et al., 2000, Montesano et al., 1986, Hu et al., 1999, Schweigerer et al., 1987). Although IL-1 and TNF-α are of inherent interest as key inflammatory signals, preliminary experiments failed to detect these cytokines in detectable quantity.

The heterogeneity in expression of cytokines by fibroblasts from different donors seen in the current study, both in isolated HGF cultures as well in co-culture with SAOS-2, reflects an innate biological variability amongst individuals that has been observed by other investigators (Watari et al., 1989, Danis et al., 1995, Thomson and Lotze, 2003). Nonetheless, similar patterns of response were seen in multiple experiments with cells from different donors, so that despite the heterogeneity in magnitude of response across donors of HGF, meaningful conclusions can be drawn from our data.

Preliminary experiments revealed that SAOS-2 have negligible levels of the cytokines studied, and this is convenient for interpretation of data in co-culture experiments, in that changes in cytokine levels in co-cultures can reasonably be thought of as representing changes in HGF cytokine production due to the influence of SAOS-2.

Work in the current chapter focused on HGF pre-treated with TNF-α as opposed to unstimulated HGF. One reason for this is that, without pre-stimulation of HGF, levels of several of the cytokines under study would have been so low as to be often undetectable, and hence render any co-culture experiments uninterpretable. Beyond this, however, it was found earlier in this thesis that interaction between SAOS-2 and HGF was enhanced by HGF pre-treatment with TNF-α, so that it seems reasonable to focus on TNF-α stimulated as opposed to naive HGF in the current work.

Fibroblasts are reported to express basal levels of cytokines such as IL-6, IL-8, IL-1, GM-CSF and G-CSF, thought to play a role in maintaining tissue function (Silzle et al., 2004, Rockman et al., 2001, Kotb and Calandra, 2003). Nonetheless, in the present study HGF expressed low levels of IL-6 and FGF whereas GM-CSF and G-CSF were often below detection levels. Stimulation with TNF-α, however, significantly increased expression of these cytokines, and this is consistent with enhanced production of cytokines such as IL-1, IL-8, GM-CSF, G-CSF by fibroblasts upon exposure to inflammatory cytokines and other stimuli such as hypoxia reported by others (Fitzgerald et al., 2003, Larsen et al., 1989, Galindo et al., 2001, Nakao et al., 1995, Okada et al., 1997, Pang et al., 1994, Sakuta et al., 1998, Watari et al., 1994, Tamm et al., 1998). Further, TNF-α is specifically reported to affect production of multiple cytokines including IL-8, GM-CSF, IL-6, G-CSF, M-CSF and FGF (Fitzgerald et al., 2003, Sakuta et al., 1998, Larsen et al., 1989, Nakao et al., 1995, Okada et al., 1997, Zoellner et al., 1992, Leizer et al., 1990, Filonzi et al., 1993, Hamilton et al., 1993).

Direct co-culture experiments of TNF-α pre-treated HGF with SAOS-2 revealed a strongly inhibitory effect of SAOS-2 upon HGF production of the secreted cytokines IL-6, G-CSF

and GM-CSF, although the cell associated cytokine FGF was slightly elevated in lysates of HGF co-cultured with SAOS-2. This general reduction in secreted cytokine levels was in strong contrast to findings in trans-well co-cultures, where the reverse occurred and SAOS-2 stimulated further secretion of IL-6, GM-CSF and G-CSF. Two major conclusions can be drawn from this contrast. Firstly, that there are soluble factors released by SAOS-2 that increase production of these secreted cytokines by HGF. And secondly, that direct inter-cellular contact between HGF and SAOS-2 strongly inhibits production of the secreted cytokines studied, dampening the effect of prior HGF stimulation by TNF-α, and overcoming the stimulatory effect of soluble factors released by SAOS-2.

Other possible interpretations of these data include, however, HGF contact dependent inhibition of SAOS-2 release of the soluble factors that stimulate HGF cytokine production; or that SAOS-2 mediate inhibition of HGF cytokine production by soluble factors too labile to survive diffusion across the transwell chamber membrane, as opposed to other SAOS-2 derived factors which are sufficiently stable to survive the journey across transwell chambers.

While it is not possible from the available data to definitively exclude these two further possibilities, they do seem less likely than the preferred interpretation made. Irrespective of the specific mechanisms involved, however, the data presented do illustrate yet further complexity in the interactions between stromal and parenchymal neoplastic cells, that could have significant impact on events in-vivo. At the very least, a direct transcription of the current cell culture data to the invading tumour front, would suggest that the production of some cytokines by fibroblasts may be greatly increased by soluble factors released by neoplastic cells and that these cytokines may promote local structural changes associated

with inflammation in advance of the invading neoplastic front, but that once neoplastic cells come into actual contact with these fibroblasts, there would be abrupt change in cytokine synthetic profile potentially inhibiting inflammation and favouring retention of remnant ECM to support the invasive neoplastic cells. It is admitted that this is a highly speculative interpretation from the limited data presented, but nonetheless seems a reasonable interpretation of the possible significance of these findings.

Other studies support the general idea that complex stromal-parenchymal interactions contribute significantly to tumour development. For example, there is an increase in IL-6 production by bone marrow stromal cells upon adhesion of myeloma tumour cells (Uchiyama et al., 1993, Lokhorst et al., 1994). Also, IL-6, IL-8, GRO/GRO-α (CXCL1) and macrophage stimulating protein-α (MSP-α) are up regulated when human mammary fibroblasts are co-cultured with breast cancer cells, and this has been linked with tumour progression (Olsen et al., 2010). Further, there is an increase in IL-10 and a decrease in TNF-α and IL-12 levels in monocytes in the presence of tumour cells (Baj-Krzyworzeka et al., 2004).

In light of the demonstration of exchange of membrane and cytoplasmic markers between SAOS-2 and HGF in Chapter 4, it is interesting to consider the extent to which the new mixed populations of cells may contribute to changes in cytokine production seen in the current work. It is possible for example, that reduced cytokine production reflects perverted signalling mechanisms required for maintained cytokine production by HGF, as a result of acquisition of SAOS-2 membrane and or cytoplasmic components. It does seem possible that, following direct contact with SAOS-2, HGF become unresponsive, as demonstrated by a decreased production of IL-6, GM-CSF and G-CSF. In support of this

interpretation, monocytes become selectively unresponsive following contact with tumour cells as demonstrated by decreased TNF-α and IL-12 expression and increased IL-10 production (Baj-Krzyworzeka et al., 2004, Mytar et al., 2003).

Down regulation in cytokine production by HGF seems selective, as reflected by the suppression of IL-6, GM-CSF and G-CSF production, without affecting FGF expression. This is suggestive of a mechanism by which SAOS-2 subvert the fibroblasts to a tumour promoting phenotype (Silzle et al., 2003).

In contact independent (transwell) cultures, an increase in IL-6, GM-CSF and G-CSF expression was observed; indicating that cytokine induction in co-culture occurs through SAOS-2 released soluble factors. Similar observations are reported in other studies where tumour cell derived soluble factors increase cytokine or chemokine production by stromal cells (Nakamura et al., 1997, Toulza et al., 2005). That cancer related inflammation provides a favourable environment for tumour growth and metastasis is supported by findings in other studies (Macarthur et al., 2004, Schwartsburd, 2003, Schottenfeld and Beebe-Dimmer, 2006, Balkwill and Mantovani, 2001, Rakoff-Nahoum, 2006) and is further substantiated by studies showing reduced incidence of colorectal, breast, lung and prostrate tumours with the administration of non-steroidal anti-inflammatory drugs (Harris, 2009, Cuzick et al., 2009, Thun et al., 1991, Schreinemachers and Everson, 1994, Harris et al., 2002, Harris et al., 2003).

A number of studies have verified the importance of cytokines in regulating tumour cell adhesion (Harimaya et al., 2000, Zhu et al., 2002, Bereta et al., 1991), growth (Negus and Balkwill, 1996, Ohmi et al., 2003, Panozzo et al., 1996), invasion (De Wever and Mareel,

2003, Hagemann et al., 2004, Yanase et al., 1995, Zhu et al., 2002) and spread (Negus and Balkwill, 1996, Panozzo et al., 1996). These findings are reminiscent of the association between increased cytokine expression and poor prognosis of tumours (Chopra et al., 1997, Berek et al., 1991, Balkwill, 2006, Balkwill, 2009, Balkwill and Mantovani, 2001).

Our results suggest that both direct contact between neoplastic and stromal tumour cells, as well as soluble tumour cell derived factors play important roles in modifying the tumour microenvironment. Further investigation is needed to identify the cell surface molecules involved in mediating the SAOS-2 induced down regulation of fibroblast cytokine expression and soluble SAOS-2 derived factors responsible for their up regulation.

Chapter 6: General Discussion

6.1 MAJOR FINDINGS AND SIGNIFICANCE OF WORK CARRIED OUT IN THIS THESIS

Tumour parenchymal-stromal cell interactions significantly contribute towards tumour growth and progression (Mahadevan and Von Hoff, 2007, Stetler-Stevenson et al., 1993, Wernert, 1997, Tlsty and Hein, 2001, Proia and Kuperwasser, 2005).

Work in this thesis commenced with a view to better understand the dynamic interactions between SAOS-2 tumour cells and fibroblasts, particularly to seek explanation for the apparent reduction in HGF culture density seen in co-cultures with SAOS-2 in the absence of HGF apoptosis (Huynh, 2007), in contrast with the earlier report from this laboratory that SAOS-2 induce apoptosis in HUVEC in a contact dependent manner (McEwen et al., 2003). While the work by Huynh (2007) had demonstrated exchange of fluorescent labels between HGF and SAOS-2, experiments had been performed using the membrane marker DiD in SAOS-2, and the cytoplasmic marker CFSE in HGF, so that there was uncertainty regarding the differential movement of membrane as opposed to cytoplasmic labels, while no nuclear labelling had been performed. In this way, although it seemed reasonable to conclude that the reduction in apparent HGF culture density induced by SAOS-2 was likely due to transfer of alkaline phosphatase from SAOS-2 to HGF, the data supporting this conclusion was not strong, and required the additional work described in this thesis to make a convincing argument.

Early work in this thesis included confirmation that SAOS-2 induced contact dependent reduction in apparent HGF cell culture density, as a necessary starting point for investigation. The approach then taken, was to first investigate what was deduced as a

necessary first step in SAOS-2 – HGF interaction, that of SAOS-2 binding to HGF. The methods used in doing this work were comparable to those long applied in studying the binding of leukocytes to endothelium (Sano et al., 1999, Gamble et al., 1985, Yu et al., 1985, Miossec et al., 1988, Masuyama et al., 1986), and since there is an extensive literature demonstrating modulation of leukocyte adhesion to endothelium by inflammatory cytokines (Gamble et al., 1985, Hashimoto, 1994, Weill et al., 1995), as well as a comparable literature describing cytokine effects upon neoplastic cells (Bertomeu et al., 1993, Dejana et al., 1988, Gangopadhyay et al., 1998, Kim et al., 1993, Lauri et al., 1991, Rice et al., 1988, Steinbach et al., 1996, ten Kate et al., 2004), the current study was extended to investigate the effect of the powerful tumour-associated cytokine TNF-α.

TNF-α was found to increase SAOS-2 binding to HGF, and this was largely mediated by increased ICAM-1 expression by HGF. Differences in ICAM-1 expression were noted between HGF donors, and this possibly reflects the innate biological variability between the donors which was also observed with the adhesion assays. Cytokines are reported to up regulate tumour cell attachment to stromal cells by the modulation of adhesion molecule expression in a number of other model systems (Kim et al., 1993, Mackay et al., 1993, Steinbach et al., 1996, Yanase et al., 1995). The findings of this thesis, together with those of other workers (Kim et al., 1993, Mackay et al., 1993, Steinbach et al., 1996, Yanase et al., 1995), support the idea that differences in capacity for adhesion molecule expression throughout the population may contribute to differences between individuals with regard to the progression of individual tumours.

A role for heat sensitive serum derived factors in mediating adhesion was also determined, although the identity of these factors remains unknown. Nonetheless, as outlined in

Chapter 3 as well as below in 6.2, there is a possible role for fibrinogen as a bridge between HGF-ICAM-1 and SAOS-2. It was also interesting that fixation of HGF by a variety of means abrogated TNF-α induced binding, but not 'background binding' of SAOS-2 to HGF, and that SAOS-2 subjected to fixation lost all ability to bind to HGF (Chapter 2).

Since adherence of neoplastic cells to the stroma is a necessary step in tumour invasion, observations made in Chapters 2 and 3 may have relevance to malignant invasive processes. Expression of specific adhesion molecules and cytokines not only by the neoplastic cells but also the tumour stromal cells has capacity to encourage tumour cell attachment, growth, invasion and metastasis (Honn and Tang, 1992, LaBiche et al., 1993, Nicolson, 1988, Chirivi et al., 1994). Various cytokines including TNF-α augment tumour cell adhesion to endothelial cells, fibroblasts and mesothelial cells (Bereta et al., 1991, Bertomeu et al., 1993, Bendall et al., 1997, Lauri et al., 1991, Rice et al., 1988, Steinbach et al., 1996, Stolze et al., 1998, van Grevenstein et al., 2007). Experiments aimed at identifying SAOS-2 binding to HGF in the presence of TNF-α demonstrated a significant increase in SAOS-2 cell attachment. It should be noted that treatment of SAOS-2 with TNF-α failed to modify binding to otherwise unstimulated HGF, indicating that the observed effect is reflective of the changes in fibroblasts brought about by TNF-α treatment and not that of the tumour cells. A similar augmentation of SAOS-2 binding after TNF-α stimulation was also seen with HUVEC and HUASMC. Although HGF and HUVEC demonstrate similar effects with regard to SAOS-2 adhesion, it is interesting to note the differences in the outcome of HUVEC and HGF after direct interaction with SAOS-2, where HUVEC undergo apoptosis whereas this was not the case with HGF.

Many tumour and stromal cells have detectable TNF-α mRNA, and this is often associated with poor prognosis (Balkwill, 2006, Balkwill, 2009, Balkwill and Mantovani, 2001). Data in Chapters 2 and 3 revealed that although TNF-α was not essential for the baseline attachment of SAOS-2 to HGF, that this cytokine does contribute towards interactions between these cells.

The effect of TNF-α increasing the capacity of SAOS-2 to reduce the apparent culture density of HGF as defined by the absence of alkaline phosphatase (Chapter 3), supported the idea that TNF-α increases exchange between HGF and SAOS-2 developed in Chapter 4, through either or both increasing initial adhesion and or activating mechanisms for inter-cellular exchange (Chapter 3).

Emerging from the data, were the twin ideas that: microenvironmental signals such as inflammatory cytokines may determine the specific interactions between stromal and neoplastic cells; and that the specific history of individual cells could affect the outcome in such interactions. Particularly striking, was the effect of TNF-α pre-treatment of SAOS-2 of inhibiting consequent SAOS-2 binding to similarly treated HGF (Chapter 2). In this way, the phenomenon of cytokine induced binding of SAOS-2 to HGF, a 'microenvrionmental permissive event', is itself modulated by what seems reasonable to call a 'permissive cellular history' of the SAOS-2 involved, as only SAOS-2 which have not been previously stimulated by the cytokine are bound by TNF-α stimulated HGF. Related to this is also the effect of decaying cellular response following initial cytokine stimulation, examined in Chapter 2 with regard to the binding of SAOS-2 by HGF stimulated with TNF-α.

Recognizing that malignant tumours in-vivo are not static, but that there is simultaneously invasion of neoplastic cells into the surrounding normal tissue stroma, as well as incorportation and migration of stromal elements into the tumour mass as part of the desmoplastic and angiogenic response (De Wever and Mareel, 2003, Folkman et al., 1989, Hanahan and Weinberg, 2000, Kiaris et al., 2004, Mueller and Fusenig, 2004), the dual concepts of 'permissive microenvironmental responses', and 'permissive cellular histories' for specific responses, may help to account for the phenotypic diversity seen within individual neoplasms. Notably, architectural and cytological pleomorphism in neoplasms is usually attributed to the outgrowth of divergent clones of cells bearing discrete genetic injuries, whereas findings in the current thesis support a likely additional contribution of permissive microenvironmental and permissive cellular historical effects.

Work in Chapter 4 demonstrated that HGF and SAOS-2 in co-culture establish continuity between their plasma membranes and cytoplasm, such that there is significant exchange of membrane and cytoplasmic labels between the two cell types. These interactions, however, fell well short of actual cell fusion, such that there was no evidence for the exchange or sharing of nuclear material, and earlier time-lapse photography studies suggested at most only transient glancing interaction between individual SAOS-2 and HGF (Huynh, 2007). TNF-α increased the extent of exchange between the two cell types, although this was a comparatively modest effect.

While it was tempting to conclude significant bulk-transport of membrane and cytoplasm between the two cell types in co-culture, it was realized from the contradictory results of experiments performed using different configurations of fluorescent labels, that the specific diffusion rates of the labels strongly affected results. An appropriately cautious

interpretation of data thus excludes conclusion of significant bulk-transport of membrane and or cytoplasm between HGF and SAOS-2, but does nonetheless permit confident statement of significant physical continuity between HGF and SAOS-2 plasma membranes and cytoplasm, with exchange of membrane and cytoplasmic contents between the cells.

Cell shape and size are convenient, although indirect, indicators of phenotypic state (De Paiva et al., 2006, Goessl et al., 2001, Folkman and Moscona, 1978, Chen et al., 1997), and the current thesis exploited this to evaluate phenotypic change amongst co-cultured cells. Objective numerical comparison of cell surface area profile and circularity was possible using appropriate image analysis software, and revealed an increase in the size of cytoplasmic dual labelled cells, bearing the nuclear label for SAOS-2. While this does support the idea of possible bulk transport of cytoplasm from HGF to SAOS-2, it is recognized that actual cell volume could not be determined by the methods used in the current study, and that cell surface area profile may be increased by the stretching of cells as much as by increased true cell volume. Also, there were some, although fewer, cytoplasmic dual labelled cells with nuclei labelled as of HGF origin, indicative of significant diffusion of GFP from SAOS-GFP to HGF (Chapter 4).

Irrespective of whether or not bulk transport of cytoplasm occurred between SAOS-2 and HGF, it is clear that proteins were transferred between HGF and SAOS-2, and it is here suggested that this would have functional significance. The progressive accumulation of genetic injuries is inherent in the process of malignant neoplasia, while loss of capacity for normal chromosome segregation accelerates generation of progressively poorly controlled cells (Beckman and Loeb, 2005, Albertson et al., 2003, Lengauer et al., 1998, Jallepalli and Lengauer, 2001). The problem of an excessively scrambled genome seems an inevitable

challenge to neoplastic cells, and the extensive death by both apoptosis and necrosis of many neoplastic cells in-vivo (Kumar et al., 2005, Arends et al., 1994, Mooney et al., 1995), supports the general assumption that clones of cells unable to survive are simply lost but eventually outgrown by those neoplastic cell clones with the traits needed for survival (Kumar et al., 2005). Findings in this thesis, however, suggest an alternative strategy for some neoplastic cells, of harvesting functional cytoplasmic and membrane proteins from non-neoplastic stromal cells. For lack of a better term, it is here suggested that such harvesting of cellular components by neoplastic cells from stromal cells, be called 'cellular sipping'.

Apart from the direct collection of stromal cell proteins by cellular sipping, there may also be collection of mRNA from stromal cells, so that there may be capacity for neoplastic cells to reconstitute an otherwise defective protein complement using essentially foreign mRNA. Cellular sipping by neoplastic cells from stromal cells may thus play an important role in facilitating survival of those clones of neoplastic cells which have suffered the greatest number of genetic injuries. Importantly, it is such highly genetically damaged clones of cells, which seem most important for tumour progression, and it would seem that it is particularly these cells which would most benefit from cellular sipping.

A similar argument can be made regarding the collection of plasma membrane components from stromal cells by neoplastic cells, while the further possibility that reverse transfer of potentially immunogenic components from the surface of neoplastic cells to stromal cells could facilitate immune evasion in tumours. Further, the increased diversity amongst neoplastic cells conferred by both membrane and cytoplasm uptake from stromal cells, would likely contribute to morphological diversity throughout tumours.

Supporting the idea that exchange of cellular elements has phenotypic effects, is the observation that dual labelled cells had a surface area profile intermediate to that of HGF and SAOS-2, and circularity comparable to that of SAOS-2. A similar increase in area of squamous carcinoma cells is reported when co-cultured with fibroblasts (Xylas et al., 2010).

While the observations in the current thesis are novel with respect to osteosarcoma cells, fibroblasts and TNF-α, exchange of cellular material between neoplastic cells and stromal cells is reported for macrophages, endothelial, epithelial and bone marrow derived cells (Mortensen et al., 2004, Busund et al., 2003, Pawelek and Chakraborty, 2008, Bhatia et al., 2008). A role for inflammatory cytokines in facilitating exchange phenomena such as those described in the current thesis, is supported by increased heterotypic cell fusion in response to chronic inflammation (Johansson et al., 2008, Kemp et al., 2011, Nygren et al., 2008).

In light of the powerful modulating effects of cytokines upon cellular behaviour, Chapter 5 investigated the possible effect upon cytokine synthesis of HGF co-culture with SAOS-2. While SAOS-2 did not appear to contribute significantly themselves to production of any of the cytokines studied, TNF-α stimulated HGF release of IL-6, GM-CSF and G-CSF was significantly reduced in co-cultures with SAOS-2. This effect was contact dependent, while separate experiments using transwell culture systems, indicated a reverse effect of SAOS-2 soluble factors upon release of these cytokines by HGF. These data suggest a high degree of selectivity in response of HGF to SAOS-2, dependent on whether there is direct cell contact or alternatively, if the cells act upon each other at a distance, and this reinforces the importance of microenvironmental factors in determining the precise nature

of interactions between neoplastic and stromal cells. Comparable observations have been made of tumour derived soluble factors increasing cytokine production (Nakamura et al., 1997, Toulza et al., 2005). Similarly monocytes become selectively deactivated in the presence of tumour cells (Baj-Krzyworzeka et al., 2004, Mytar et al., 2003).

The augmentation of cytokine production in fibroblasts by tumour derived soluble factors, possibly favours a mechanism for the establishment of a pro-metastatic inflammatory environment. Interestingly, cancer related inflammation correlating with an increase in metastatic potential has been reported in other studies (Mantovani et al., 2008, Germano et al., 2008, Schottenfeld and Beebe-Dimmer, 2006, Schwartsburd, 2003, Lorusso and Ruegg, 2008).

In summary, results in this thesis demonstrate that SAOS-2 and HGF interact intimately, through establishment of continuity between plasma membranes and cytoplasm, and that the resulting transfer of membrane and cytoplasmic material produces four ultimate populations of cells in co-culture being: HGF, SAOS-2, HGF with SAOS-2 membrane and cytoplasmic elements; and SAOS-2 with HGF cytoplasmic and membrane elements. Furthermore, a role for TNF-α in facilitating HGF and SAOS-2 binding via ICAM-1, as well as in mediating exchange of material between cells is demonstrated. The complexity of these interactions is underscored by the opposing effect upon SAOS-2 binding to HGF of pre-treatment of either cell type with TNF-α, as well as by the opposite cytokine synthetic profile of HGF co-cultured in direct contact with SAOS-2, as opposed to separated by a transwell membrane. These observations support the idea of there being ‘permissive microenvironments’ for a range of interactions between stromal and neoplastic cells, as well as the idea of a ‘permissive cellular history’ for some responses. The

paracrine and contact dependent interactions identified support subversion of stromal cell function in support of neoplastic cells. Also, cellular sipping seems to provide a novel mechanism through which malignant neoplastic cells with irreparably damaged genomes may be able to gain survival advantage by capturing normal cytoplasmic and membrane elements from surrounding stromal cells. In addition, findings relating to permissive microenvironments, permissive cellular histories, and the generation of complex cell populations through cellular sipping, provide further explanation of architectural and cytological pleomorphism in malignant neoplasms, till now attributed primarily to the emergence of divergent sub-clones of neoplastic cells through the accumulation of genetic lesions.

6.2 FUTURE WORK

Although significant progress was made in this thesis in developing an understanding of interactions between SAOS-2 and HGF, it is clear that a number of important questions remain unanswered, and that further work is required.

One outstanding question is the molecular mechanism mediating and regulating exchange of membrane and cytoplasm between HGF and SAOS-2. Although ICAM-1 was identified as an important initial adhesion signal for TNF-α induced SAOS-2 binding to HGF, as well as a factor increasing the extent of exchange between the two cell types studied, there is no direct evidence confirming ICAM-1 involvement in actual trans-cellular exchange.

Related to this, the SAOS-2 binding partner for ICAM-1 mediated binding to HGF has not been identified, and it seems reasonable to assume this would likely be an integrin, further

careful investigation of this possibility is required. A comparatively simple experimental approach would be to determine the effect of a panel of appropriate blocking antibodies for integrins known to bind ICAM-1, although it is also possible that a novel and as yet uncharacterized SAOS-2 surface molecule is responsible, in which case a combination of protein chemical and molecular biology strategies would be required to identify the SAOS-2 – ICAM-1 binding partner. Separately, ICAM-1 does bind fibrinogen (Pluskota and D'Souza, 2000, Gardiner and D'Souza, 1997, Gardiner and D'Souza, 1999, Tsakadze et al., 2002), and it is possible that this is the serum factor contributing to binding events characterized in Chapter 2. One difficulty with this interpretation, is that serum is greatly depleted of fibrinogen by the formation of fibrin from plasma. Nonetheless, it is possible that trace fibrinogen surviving conversion of plasma to serum, or perhaps alternatively proteolytic fragments of fibrinogen, acts as a bridge between HGF-ICAM-1 and SAOS-2 fibrinogen binding molecules.

Identification of the SAOS-2 ICAM-1 binding partner would be particularly interesting as a starting point for further characterization of the inhibitory effect of TNF-α treatment of SAOS-2, upon subsequent binding to TNF-α treated HGF. Even without the advantage of knowledge of the SAOS-2 – ICAM-1 binding receptor, further study of the effect of pre-treatment of SAOS-2 with TNF-α is warranted, including dose response studies and time course experiments determining the rate at which decreased binding occurs following stimulation, as well as the time during which decreased binding is retained after removal of the cytokine stimulus. Knowing from earlier time-lapse microscopy studies that SAOS-2 are highly active and mobile, it would also be interesting to investigate the effect of cytokine stimulation on SAOS-2 migration, and to interpret the findings in context of the known cytokine dependent binding phenomena to HGF.

It seems likely that proteins other than ICAM-1 and its SAOS-2 partner, play an important role in mediating HGF binding of SAOS-2, so that further identification of these other binding molecules would be of interest in future work. Some molecules of potential interest include: CD44, syncitin, CD47 and ADAM12 (Bjerregaard et al., 2006, Galliano et al., 2000, Parris, 2006, Tian et al., 2002, Vignery, 2000).

Further characterization of data obtained at the protein level, by investigation of expression of relevant mRNA levels would seem warranted, including for ICAM-1, G-CSF, GM-CSF, IL-6 and FGF by northern blotting and or RT-PCR amplification (Iademarco et al., 1995, Marlor et al., 1992). The analysis of the functional impact of the gene knockdown of ICAM-1 or other involved proteins could be determined in vivo by analysis of loss-of-function mutants and performing RNAi experiments (Pluvinet et al., 2004, Rosette et al., 2005). Further on, it would be possible to determine if reconstitution of ICAM-1 or other proteins of interest in these mutants by transfection with protein specific cDNA, would completely restore their ability to adhere.

While the focus in this thesis has been on TNF-α, and this was justified on the basis of this being an important cytokine across inflammation, immunity and tumour biology (Aggarwal et al., 2002, Apostolaki et al., 2010, Balkwill, 2006, Mannel et al., 1993, van der Merwe, 1988), it would nonetheless be interesting to determine if similar or perhaps opposite effects are seen if other cytokines such as IL-1, IL-6, TGF-β, IFN-γ or IL-4 were studied. Similarly, measurement of a wider range of HGF derived cytokines, as well as of any thus far unmeasured SAOS-2 derived cytokines in co-cultures, would seem a worthwhile extension of the current study.

A further insight into TNF-α activity can be obtained by analysing the expression levels of receptors TNF-R55 and TNF-R75 in unstimulated HGF by flow cytometry using antibodies directed against the specific receptors. A further confirmation of receptor activity in mediating SAOS-2 adhesion can be determined by blocking the activity of the receptors separately.

FACS sorting could be used to separate the dual labelled cells from HGF and SAOS-2 in mixed cultures, and this would provide a valuable tool for further characterization of these cells, particularly with regard to their increased or perhaps decreased capacity to contribute to important tumour related processes. For example, it would be interesting to determine the rate of cell proliferation amongst dual labelled cells with SAOS-2 nuclei, relative to SAOS-2 alone. Other activities of interest would be: apoptosis; production of proteinases and proteinase inhibitors; migratory behaviour; and the binding capacity to differential substrates as well as to endothelium and HGF. mRNA / cDNA microarray and proteomic comparisons of these populations may be particularly informative. Similar studies of dual labelled cells with the nuclei of HGF would also be interesting, and would shed light on the functional significance of cellular sipping between stromal and neoplastic cells.

Although the focus in the current thesis has been upon HGF, it would also be interesting to confirm similar in HUASMC that the same process is responsible for reduction in apparent HUASMC culture density in co-culture with SAOS-2. Further, while SAOS-2 have provided a valuable model tumour cell line for these studies, wider applicability of these findings could only be established with confidence were these studies repeated with a wide range of other tumour cell lines, preferably including lines of both carcinoma and sarcoma origins.

Establishment of in-vivo significance requires that animal experiments be performed. Nude mice with congenital thymus deficiency provide a convenient animal model able to accept tumour cell lines of human origin without immune rejection (Sharkey and Fogh, 1984, Fogh et al., 1977), so that these mice would seem appropriate for in-vivo studies. Before commencing experiments with nude mice, however, it does seem important to first confirm similar interactions between SAOS-2 and fibroblasts isolated from the mice, as inability of mouse fibroblasts to interact with human SAOS-2 in the same way that HGF do, would render this model system useless for the purposes of this particular study. Subcutaneous implantation of sponge material has been used in this laboratory as part of a wound healing model in which there is a clear invading front of reparative granulation tissue, with progressive wound maturation at the periphery eventually extending to the centres of implants (Bolitho et al., 2010). It would be interesting to modify this animal model system to include SAOS-2 impregnated into the sponge material, so that collision between SAOS-2 and invading granulation tissue would be orientated in a way that definition of defined microenvironments would be possible histologically. If nude mice expressing GFP were used, as opposed to SAOS-2 pre-labelled with DDAO-SE, then dual-labelled cells would be readily identified by fluorescence microscopy, while SAOS-2 labelled with DiD would also provide means of identifying migration of tumour membrane marker to host stromal cells. Cytokine could be readily introduced by either: pre-impregnation of sponges before implantation; delivery via an osmotic pump to one side of the tumour cell containing implant, such that the opposing side could be used as one form of control; or microinjection of cytokine into the sponge or surrounding tissues as desired. Colloidal carbon could be used to track injection sites histologically, while analysis of cellular interactions including the emergence or otherwise of tumour pleomorphism would

provide support for, or alternatively refute, in-vivo significance of the observations made in cell culture in this thesis.

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