

**A NOVEL, ANGIOGENESIS-INDEPENDENT ROLE
FOR PERICYTES IN OVARIAN CANCER
PROGRESSION AND METASTASIS**

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Dedicated to my parents, for everything.

Declaration

This thesis work was performed under the supervision of Professor Christopher Parish and Associate Professor Pritinder Kaur. The majority of experimental work described in this thesis was performed by the author, unless otherwise indicated in the text. Pericyte scoring and *in silico* analyses on patient gene expression data was performed by Dr. Joshy George (Figures 1.7, 1.8, 5.11 and Appendix A); Pilot *in vivo* and *in vitro* data on the OVCAR-5 model were generated by Dr. Lynn Chong (Appendix B); Immunohistochemical staining on patient TMAs was performed by Dr. Holger Schlüter (Figures 5.7, 5.9 and 5.10); Gene set enrichment analysis was performed in collaboration with Ms. Susan Mönchgesang (Table 5.2 and Appendix D); Transmission electron microscope images were acquired with the assistance of Dr. Jill Danne and Dr. Sarah Ellis; FFPE sections for histology were prepared by members of The Peter MacCallum Cancer Centre Histology and Microscopy Core; Flow cytometry was performed with the assistance of Mr. Ralph Rossi, Ms. Viki Milovac and Ms. Sophie Curcio.

This thesis conforms to The Australian National University guidelines and regulations. The work presented in this thesis has not been submitted for the purpose of obtaining a degree at this or any other university.

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"You gain strength, courage, and confidence by every experience in which you really stop to look fear in the face. You must do the thing which you think you cannot do."

-Eleanor Roosevelt

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Publications

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- Pericytes act independently of angiogenesis to promote ovarian cancer progression
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Abstract

Cancer-associated fibroblasts (CAFs) are a heterogeneous population of cells known for their pro-tumourigenic effect in the microenvironment of solid tumours. One of their major sources is bone marrow derived mesenchymal stem cells (BM MSCs), a population with a demonstrated ability to infiltrate the site of the tumour and engage in paracrine cross-talk with the tumour cells. However, the role of a locally resident perivascular MSC population, i.e. pericytes, has not been explored in this context. Pericytes are contractile cells located around microvessels and are known for their prominent role in maintenance and stabilisation of the blood vasculature in homeostasis, and during tumour angiogenesis. However, previous data from our laboratory has shown that pericytes have the ability to promote the growth of normal epithelial cells, independent of angiogenesis. In addition, interrogation of a gene expression dataset of 215 serous ovarian cancer patients revealed that patients carrying a pericyte-specific gene expression signature bore a significantly higher risk of relapse and a lower overall chance of survival. This indicated that pericyte activity is a strong predictor of cancer recurrence and mortality. Further, this high-risk patient group was distinct from that identified by an angiogenic signature.

Therefore, this thesis work was undertaken with the hypothesis that pericytes have a direct, pro-proliferative role in promoting tumour growth and metastasis, independent of their known roles in regulating angiogenesis. In the work presented here, co-injection of exogenous human pericytes with tumour cells resulted in increased tumour growth and metastasis in a xenograft model of ovarian cancer. Importantly, this effect was not associated with a concomitant increase in the extent and distribution of blood vessels, or alterations in other crucial angiogenic processes. Further, histological analyses of the xenograft tumours revealed increased tumour invasion leading to epithelial mesenchymal transition (EMT) in the presence of pericytes.

Interestingly, co-injection of pericytes led to an increased infiltration of alpha smooth muscle actin (SMA) positive cells to non-blood vessel-associated sites within xenograft tumours – a notable feature in tumor tissue from patients with relatively earlier recurrence of disease. In consistence with this observation, alpha SMA expression was predictive for relapse in ovarian cancer patients. Further *in silico* analysis revealed that extracellular matrix (ECM) remodelling and focal adhesion were the primary pathways overexpressed in ovarian cancer patients with early recurrence of disease. Together, findings from this study led to the conclusion that the MSC-like pericytes, when dissociated from blood vessels or mislocalised at non-vascular sites, can directly engage in paracrine crosstalk with tumour cells, resulting in increased proliferation, invasion and metastasis, without affecting angiogenesis. Thus, this study is the first clinical and experimental evidence for a novel, angiogenesis-independent, CAF-like role for pericytes in the ovarian tumour microenvironment and presents the possibility of pericyte-specific genes being used at the protein level as prognostic markers. Further studies focussing on deciphering the molecular mechanisms through which pericytes exert their pro-proliferative effects in the tumour microenvironment are required for better understanding of their biology and precise role in cancer pathogenesis.

Abbreviations

µg	Microgram
µl	Microlitre
µm	Micrometre
Ab	Antibody
AEC	3-Amino-9-ethylcarbazole
ALDH	Aldehyde dehydrogenase
Ang	Angiopoetin
ANOVA	Analysis of variance
AOCS	Australian Ovarian Cancer Study
APC	Allophycocyanin
αSMA	Alpha smooth muscle actin
AX	Aptamer
BLI	Bioluminescent imaging
BM MSC	Bone-marrow mesenchymal stem cell
BMP	Bone morphogenetic protein
bri	Bright
BSA	Bovine serum albumin
BV	Blood vessel
CA MSC	Cancer-associated mesenchymal stem cell
CA125	Cancer antigen 125
CAF	Cancer-associated fibroblast
CC3	Cleaved caspase 3
CD	Cluster of differentiation
CSC	Cancer stem cell
CSF	Colony stimulating factor
CSPG	Chondroitin sulphate proteoglycan
CTL	Cytotoxic T lymphocyte
CXCL12	C-X-C motif chemokine 12
DAB	Diamino-benzidine
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
EC	Endothelial cell
E-cad	E-cadherin

ECM	Extracellular matrix
EDTA	Ethylenediamine tetra-acetic acid
EGM-2	Endothelial Growth Media-2
EMT	Epithelial mesenchymal transition
EndMT	Endothelial mesenchymal transition
EOC	Epithelial ovarian cancer
EpCam	Epithelial cell adhesion molecule
expts	Experiments
FACS	Fluorescence activated cell sorting
FAP α	Fibroblast activation protein alpha
FC	Flow cytometry
FCS	Fetal calf serum
FDR	False discovery rate
FFPE	Formalin fixed paraffin embedded
FGF	Fibroblast growth factor
FIGO	Fédération internationale des gynaecologues et obstétristes
FITC	Fluorescent isothiocyanate
FSC	Forward scatter
FSP	Fibroblast specific protein
GEO	Gene expression omnibus
GFP	Green fluorescent protein
GI	Gastrointestinal tract
GO	Gene ontology
GPCR	G-protein coupled receptor
GSEA	Gene-set enrichment analysis
H&E	Haematoxylin and eosin
HD	Human dermis
HGSC	High-grade serous carcinoma
HIF-1 α	Hypoxia inducible Factor-1 alpha
HSC	Haematopoietic stem cell
i.p.	Intraperitoneal
IFC	Immunofluorescence
IFC-Fr	Immunofluorescence-frozen
IFC-P	Immunofluorescence-paraffin
IHC	Immunohistochemistry
IL	Interleukin
Inc.	Incorporated
kDA	Kilo Dalton

KEGG	Kyoto Encyclopedia of Genes and Genomes
LGSC	Low-grade serous carcinoma
LMP	Low malignant potential
luc	Luciferase
MCAM	Melanoma cell adhesion molecule
mg	Milligram
ml	Millilitres
mm ²	Millimetre square
mm ³	Millimetre cube
MMP	Matrix metalloproteinase
MPI	Microvessel pericyte index
MQ	Milli-Q
MSC	Mesenchymal stem cell
MVD	Microvessel density
NBF	Neutral buffered formalin
NES	Normalised enrichment score
NG2	Neural glial 2
NIH	National Institute of Health
NK	Natural killer
NLRD	Notifiable low risk dealings
NOD	Non-obese diabetic
ns	Not significant
nu/nu	Nude/nude
O.C.T.	Optimal cutting temperature
OSD	Overall survival
OSE	Ovarian surface epithelium
OVCAR	Ovarian carcinoma
OVCAR-5+F	OVCAR-5 + fibroblasts
OVCAR-5+P	OVCAR-5 + pericytes
OVCAR-8+P	OVCAR-8 + pericytes
p1	Passage 1
p2	Passage 2
p4	Passage 4
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with Tween 20
PC	Principal component
PCA	Principal component analysis
PDGF	Platelet derived growth factor

PDGFR β	Platelet derived growth factor receptor beta
PE	Phycoerythrin
PECAM-1	Platelet endothelial cell adhesion molecule-1
pen-gent	Penicillin-gentamycin
PFA	Paraformaldehyde
PFS	Progression free survival
PI	Propidium iodide
PVDF	Polyvinylidene fluoride
RGS5	Regulator of G-protein signalling 5
RIP	Rat insulin promoter
RT	Room temperature
Sca-1	Stem cell antigen-1
SCID	Severe combined immunodeficiency
SD	Standard deviation
SDF-1	Stromal derived factor-1
SEM	Standard error of mean
SSC	Side scatter
ST-HRP	Streptavidin horseradish peroxidase
TAMs	Tumour associated macrophages
TEM	Transmission electron microscopy
TGF β	Transforming growth factor beta
TMA	Tissue microarray
TNB	Tris and sodium chloride blocking buffer
TNT	Tris and tween
TSA	Tyramide signal amplification
v/v	Volume by volume
VEGF	Vascular endothelial growth factor
VLA	Vascular leukocyte antigen
VSMC	Vascular smooth muscle cell
vWF	von Willebrand factor
w/v	Weight by volume

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Chapter 1
Literature Review

1.1. Introduction to the tumour microenvironment

In spite of the impressive scientific advances in the knowledge and research into the molecular and cellular biology of cancer, it still remains one of the most complex of human diseases affecting thousands of lives every day. Once a normal cell transforms into a neoplastic state, several cell-intrinsic and extrinsic factors come into play in determining the course of tumour progression – from initiation to invasive growth and finally dissemination to secondary sites or the process of cancer ‘metastasis’. In fact, it has become increasingly evident in the last few years of our understanding of cancer biology that tumours are not just comprised of an isolated collection of proliferating cancer cells but constitute an evolving niche of various other non-cancer cell types and a connective tissue framework - together known as the tumour stroma (Mueller and Fusenig, 2004). A range of autocrine and paracrine interactions within these cell types and the tumour cells together results in the proliferation of the tumour (Hanahan and Weinberg, 2011; Mueller and Fusenig, 2004). The tumour microenvironment continuously evolves throughout the multistep process of tumour progression. The interactions within these cells create a succession of tumour microenvironments thereby allowing tumour progression through primary, invasive and metastatic stages (Hanahan and Weinberg, 2011) via a complex signalling circuitry (*Figure 1.1*).

Apart from the cancer cells, the tumour microenvironment is comprised of the extracellular matrix (ECM), inflammatory cells, fibroblasts, endothelial cells of the vasculature and pericytes. Tumour cells initiate the stromal activation process by the secretion of proteases and growth factors that act in autocrine and paracrine manners – ‘resting’ fibroblasts undergo morphological and functional changes to become ‘activated’ fibroblasts (Schauer et al., 2011), the epithelium becomes hyperplastic and a number of macrophages and other inflammatory cells are recruited to the site (Mueller and Fusenig, 2004). Sustained angiogenesis is maintained resulting in a multitude of ‘leaky’ tumour vessels and finally tumour cells degrade and invade the basement membrane to infiltrate the blood vessels and metastasise to distant organs (Gerhardt and Semb, 2008; Hanahan and Weinberg, 2011; Kalluri and Zeisberg, 2006).

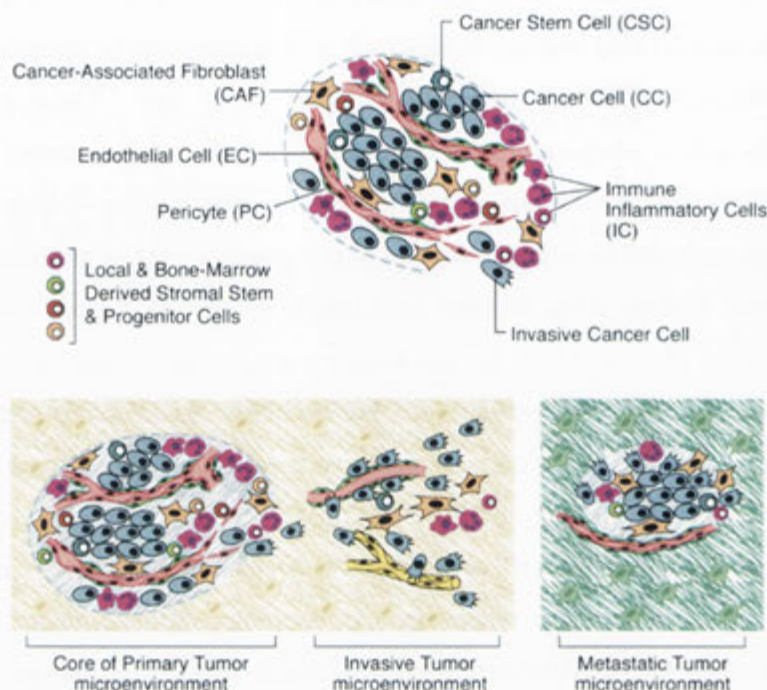


Figure 1.1. The tumour microenvironment. The tumour microenvironment comprises of various stromal cell types and concertedly evolves in histological complexity and paracrine crosstalk through primary, invasive and metastatic stages (From Hanahan and Weinberg, 2011).

It is thus important that each of the cell types within the tumour microenvironment, their functions, evolution through the course of tumour progression and crosstalk with other cells, be studied individually and in conjunction with each other, to better understand the biology of cancer pathogenesis. To this end, brief reviews of the scientific literature on important components of the tumour microenvironment have been summarised in the following sections.

Tumour cells form the basis of the tumour microenvironment. The tumour cell population is thought to be initially homogeneous, but with increased genomic instability and rapid proliferation turns into a niche of distinct clonal subpopulations, often in different stages of proliferation (Hanahan and Weinberg, 2011). In the past decade, there has been increasing evidence to establish the existence of a progenitor subpopulation within tumour cells – the ‘cancer stem cells’ (CSCs). CSCs are

defined by their ability to seed new tumours upon inoculation into recipient host mice (Cho and Clarke, 2008; Lobo et al., 2007) and expressing markers which are co-expressed by normal stem cells in their tissue of origin (Al-Hajj et al., 2003). Although still perceived to be a subject of scientific debate, several recent studies have reported that up to 25% of tumour cells in certain cancers bear CSC properties (Kelly et al., 2007; Quintana et al., 2009). The CSC theory further states that these cells are a 'reservoir of self-sustaining cells with the exclusive ability to self-renew and maintain the tumour' (Clarke et al., 2006). There is also evidence that the CSC model is plastic – enabling the conversion of non-CSCs to CSCs under appropriate conditions (Lobo et al., 2007; Mani et al., 2008).

The CSC theory bears significant implications from a therapeutic perspective. Not only does it explain why certain cancers and even the same cancers at different stages of progression show striking differences in resistance to therapeutic targeting, but also provides a probable explanation for disease recurrence after surgical/therapeutic removal of the primary tumour (Hanahan and Weinberg, 2011; Mueller and Fusenig, 2004). This highlights the need for looking into therapies targeting both CSC and non-CSCs specifically and in combination, bearing in mind their phenotypic plasticity, in order to achieve better treatment and cure.

Bearing in mind the scope of the work carried out in this thesis, the non-inflammatory cellular components of the tumour microenvironment, i.e. endothelial cells, fibroblasts, mesenchymal stem cells (MSCs) and pericytes, have been reviewed in comprehensive detail in the following sections.

1.1.1. Endothelial cells

Endothelial cells are a particularly important cell type within the tumour stroma for their primary role in functionally supporting tumour growth by forming the all-important tumour associated vasculature (Carmeliet and Jain, 2000). Early evidence on the role of endothelial cells in sustaining and maintaining solid tumours came from the seminal experiments conducted by Folkman and colleagues, who showed that the lack of blood supply severely restricted the growth of a tumour, while contrarily transplanting a tumour fragment in a highly vascular tissue resulted in aggressive tumour growth (Folkman et al., 1971). Further studies using electron microscopy showed that tumour vasculature is abnormal and dysfunctional and that they do not form the hierarchical artery-arteriole-capillary branching (Konerding et al., 1999; Warren et al., 1978). High vascular endothelial growth factor A (VEGF-A) expression and interstitial fluid pressure in most solid tumours have been put forth as a possible cause for hyper-dilated vessels with abnormal structure (Nagy et al., 2007). Increased production of other pro-angiogenic factors like platelet derived growth factor beta (PDGF β), fibroblasts growth factors (FGFs) and transforming growth factors (TGFs), from either stromal or tumour origin, also lead to hyper-proliferative vessels with aberrant structures, resulting in more nutrient supply to the tumour and contributing to increased tumour size (Carmeliet and Jain, 2000). In addition, angiogenesis and metastasis have shown to be co-dependent processes, given that blood vessel intravasation is the most potent route of metastasis in solid tumours. Thus, high microvessel density (MVD) has been correlated to increased metastasis in gastric, breast, ovarian, prostate and squamous cell carcinomas, among many others. Therefore, anti-angiogenic therapy was put forth as an attractive therapeutic means of achieving tumour regression (Carmeliet and Jain, 2000; Gerhardt and Semb, 2008).

However, most anti-angiogenic therapies targeting VEGF-A have not shown much promise, possibly because pericytes, fibroblasts, MSCs (Kalluri and Zeisberg, 2006) and cells of hematopoietic lineage (Murdoch et al., 2008) all engage in cross-talk with endothelial cells to stabilise and maintain tumours blood vessels. In addition, tumour blood vessels are postulated to have heterogeneous cells of origin (Dudley,

2012). Also emerging are unique gene expression profiles of cell surface markers of normal and tumour-associated endothelial cells (Chi et al., 2003; Nagy A et al., 2010), which show substantial variability of gene expression within vessels from the same tumour. Investigating these would be highly instrumental in deciphering signalling changes that occur at the molecular level during tumour angiogenesis. To add to the complexity, recent studies have revealed that tumour cells themselves can pose as CD31⁺ endothelial cells and channel the flow of blood for their sustenance – a process termed ‘vasculogenic mimicry’, which has opened up a new direction of research in tumour angiogenesis (Kirschmann et al., 2012).

In summary, it is undeniable that endothelial cells are crucial for the growth and sustenance of tumours and are hence vital components of the tumour stroma. Further research into their origins and interactions with other stromal cells is required to unravel key functional aspects through which they operate.

1.1.2. Cancer-Associated Fibroblasts (CAFs)

1.1.2.1. Introduction

Fibroblasts are essentially non-epithelial, non-vascular, non-haematopoietic cells of the connective tissue (Parsonage et al., 2005). First described in the 19th century based on their association with the extracellular matrix and elongated morphology (Duvall, 1879; Virchow, 1858), fibroblasts remain a rather heterogeneous population of cells with varied expression of molecular markers, mostly identified by their negativity for markers of other lineages (Cirri and Chiarugi, 2011). A study undertaken with the aim of understanding the biology and heterogeneity of human fibroblasts, performed genome-wide analysis on cells from 50 human fibroblast cultures isolated from 16 different anatomical sites. Results showed a wide divergence of gene expression among samples - mainly stemming from variance in expression of ECM proteins like collagen types I, III and V and fibronectin, alluding to tissue-specific functions of fibroblasts in adhesion and matrix remodelling (Chang et al., 2002).

The most widely used markers for fibroblasts *in vivo* are the filament associated proteins vimentin, desmin and fibroblast specific protein 1 (FSP-1) (Cirri and Chiarugi, 2011). However, none of these markers are exclusive to fibroblasts.

Specifically, vimentin is also observed in certain epithelial cells, neurons and myoepithelial cells (Franke et al., 1979; Mørk et al., 1990; Schmid et al., 1982); desmin is expressed in perivascular smooth muscle cells (Schmid et al., 1982) while FSP-1 has been detected in invasive carcinoma cells that have undergone epithelial mesenchymal transition (EMT) (Strutz et al., 1995). In addition, myofibroblasts and activated fibroblasts (present in reactive stroma of inflamed tissues e.g. fibrosis, arthritis and skin wounds) also express alpha smooth muscle actin (α SMA) and the serine protease fibroblast activation protein alpha (FAP α) (Tomasek et al., 2002). Again, neither are specific to fibroblasts - α SMA is widely expressed in smooth muscle cells and pericytes (Cirri and Chiarugi, 2011) while there is evidence of FAP α being detected in activated melanocytes (Ramirez-Montagut et al., 2004; Rettig et al., 1993).

Functionally, fibroblasts have been implicated in regulating ECM plasticity, remodelling and organisation and as cytokine sources regulating epithelial differentiation and inflammation (Tomasek et al., 2002). Hence, it is not surprising that fibroblasts are a widely studied cell type in the contexts of wound healing and cancer. The role of tumour stromal fibroblasts or cancer associated fibroblasts (CAFs) in accentuating the rate of tumour formation and invasiveness over normal fibroblasts has been reported in several cancer types - most notably, in breast and prostate carcinomas (Kalluri and Zeisberg, 2006; Pietras and Ostman, 2010). The following section aims to describe the current literature on the origin of CAFs and their reported role in cancer progression.

1.1.2.2. Postulated origins and role in cancer development

Historically, the first evidence of the involvement of stroma-derived fibroblasts in tumours came from a seminal study by Dawe et al., showing that cells from polyoma virus-induced tumours in mouse salivary glands could be expanded in culture only in the presence of an embryonic salivary gland mesenchyme (Dawe et al., 1966). A subsequent study prompted the coining of the term 'CAF' – alluding to the fact that fibroblasts isolated from cancers (rhabdomyosarcoma) showed a different chemotactic response to fibronectin compared to skin fibroblasts in an *in vitro* Boyden chamber assay (Mensing et al., 1983). Subsequently, Picard et al. demonstrated that subcutaneous co-injection of fibroblasts with cancer cells from rat

or human origin substantially increased both tumourigenicity and tumour growth rates in nude mice (Picard et al., 1986). Interestingly, fibroblasts also increased the lung colonisation potential of the weakly metastatic rat rhabdomyosarcoma cell line 9-4/8 (Picard et al., 1986). This study also showed that co-injection of fibroblast-conditioned media had similar effects, but not to the extent observed with the injection of fibroblasts themselves, indicating that tumour cell – fibroblast paracrine cross-talk is important for the growth and sustenance of solid tumours (Picard et al., 1986). Gleave et al. showed the importance of fibroblasts from metastatic sites. Specifically, human bone and lung fibroblasts promoted growth of the prostate cancer cell line LnCAP tumours in nude mice (Gleave et al., 1991), as did fibroblasts from normal human prostate tissue (Tuxhorn et al., 2002). Further, CSML100, a highly metastatic murine mammary carcinoma cell line failed to metastasise in FSP-1^{-/-} mice, a phenotype that was partially rescued by the co-injection of wild-type fibroblasts (Grum-Schwensen et al., 2005).

The cell-intrinsic differences between normal fibroblasts and those derived from malignant tissues were further highlighted in two seminal studies. The first showed that *in vivo* co-grafting of SV-40 transformed prostate epithelial cells together with prostate carcinoma-derived CAFs in collagen gels led to neoplastic lesions, while similar co-grafting of fibroblasts from normal prostate tissue did not (Olumi et al., 1999). Another study showed the capacity of CAFs to promote increased tumour growth in a human MCF-7 breast cancer xenograft model, in comparison to normal fibroblasts. Importantly, this study also alluded to a possible mechanism of action of CAFs – demonstrating that secretion of the stromal derived factor 1 (SDF-1), also known as CXCL12, specifically by CAFs, resulted in the recruitment of bone-marrow derived endothelial cells. This led to increased angiogenesis which prompted accelerated growth of the MCF7 mammary tumours (Orimo et al., 2005).

The TGF β pathway components have been implicated as important molecular mediators through which CAFs exert their pro-tumourigenic effect, particularly in the initiation of malignancy in normal epithelial tissues. Specifically, Kuperwasser et al. showed that while human mammary epithelial cells could form normal ducts within the mouse mammary fat pad, overexpression of TGF β and the hepatocyte growth factor (HGF) in the mouse stromal fibroblasts led to spontaneous mammary

tumour formation (Kuperwasser et al., 2004). A concurrently published study further demonstrated concrete evidence that TGF β activation in normal fibroblasts leads to cancer initiation in adjacent epithelia cells (Bhowmick et al., 2004). This study showed that obliterating the TGF β receptor type II specifically in mouse fibroblasts using a FSP-1 promoter, resulted in epithelial neoplasia in the prostate gland and the formation of squamous cell carcinomas in the forestomach. Interestingly, this led to increased expression of HGF and loss of cell cycle inhibitors p21 and p27, a probable cause of almost four-fold increase in proliferation rates of both transformed epithelial cells as well as fibroblasts (Bhowmick et al., 2004).

CAF-derived MMPs have also been implicated as potent enhancers of tumour growth. Given that the major function of MMPs is the proteolytic degradation of the ECM, their role in directly affecting the invasive and migratory capacities of cancer cells have been investigated (Stetler-Stevenson et al., 1993). Specifically, protease activated receptor 1 (PAR-1), a receptor for MMP-1 was shown to promote invasion of breast cancer cells by regulating PAR1-dependent Ca²⁺ signals (Boire et al., 2005). MMP-3 or stromelysin-1 was also demonstrated as an important mediator of epithelial mesenchymal transition or EMT, a process tumour cells employ to invade adjacent tissue and intravasate (Sternlicht et al., 1999). Quite recently, it was also shown that MMP-9 is a CAF-derived pro-tumourigenic protease which can be inhibited by omega-3 polyunsaturated fatty acids (Taguchi et al., 2014).

Put together, the evidence from these and other studies concertedly point towards a bimodal effect of CAFs in the environment of solid tumours. The first role involves the secretion of pro-proliferative factors that set the tone for increased neoplasia resulting in accentuated tumour growth; the second function involves remodelling the ECM, mostly by proteolytic degradation thereby setting up a paracrine dialogue with cancer cells, leading to EMT, increased invasion and migration. Additionally, there are also suggestions that CAFs can regulate mechanical factors like matrix stiffness, thus indirectly affecting the migratory behaviour of cells (Xing et al., 2010). More evidence on this front has been shown recently in an elegantly conducted study by Sahai and colleagues (Tozluoglu et al., 2013). They isolated CAFs from a variety of primary human cancer tissues and demonstrated that an autocrine self-reinforcing loop of tissue remodelling and actomyosin contractility is

essential for maintenance of the pro-tumourigenic phenotype of CAFs. This loop is regulated by the transcription factor YAP and Src kinases, and ROCK inhibition targeted at disrupting this loop led to reversal of CAF phenotype (Tozluoğlu et al., 2013). This study highlighted the possibility of therapeutic use of ROCK inhibitors directed at stroma specific pro-tumourigenic components of the microenvironment.

Although an increasing number of studies are now focussing on deciphering the role of CAFs in the microenvironment of various cancer types, the precise cells of origin of CAFs remain a key question. There is increased recognition of the fact that the origins of CAFs are diverse – they are a heterogeneous population of cells that vary with specific environmental cues, tumour histotypes as well as within different areas of the same tumour (Kalluri and Zeisberg, 2006). It has also been recently proposed that CAFs be considered as a cell ‘state’ rather than a cell ‘population’, given its wide heterogeneity (Madar et al., 2013). The following sections focus on the current literature on the various origins of CAFs.

1.1.2.2.1. Resident stromal fibroblasts

Function of CAFs within a tumour is often reminiscent of myofibroblasts in a wound – reinforcing the idea of tumours being ‘a wound that never heals’ (Dvorak, 1986). During wound healing, PDGF and TGF β secretion by platelets are the first cues for resident tissue fibroblasts to be recruited at the wound site, where they transdifferentiate into an ‘activated’ myofibroblast state. Myofibroblasts then secrete a cohort of matrix proteins to create a fibrotic stroma, the primary ones being collagens type I and III, fibronectin, proteoglycans and MMPs – all directed at repairing the damaged epithelia, while simultaneously providing pro-proliferative signals to the adjacent epithelial cells (Kalluri and Zeisberg, 2006).

In tumourigenesis, PDGF and TGF β are primarily secreted by the transformed malignant cells, which have been postulated to recruit resident fibroblasts in a similar manner (Schäfer and Werner, 2008). This was validated *in vivo* in a human breast cancer xenograft model, where resident fibroblasts were shown to transdifferentiate into CAFs due to an activating signal cascade regulated concertedly by TGF β and CXCL12 (Kojima et al., 2010). Another study also reported that CXCL12 was a crucial cytokine that mediated the conversion of

resident fibroblasts to CAFs (Toullec et al., 2010). Recruitment of resident stromal fibroblasts has been reported at metastatic sites as well. Mueller et al showed that CAFs isolated from colorectal cancer liver metastasis tissue originated from resident portal-located liver fibroblasts; these CAFs were also shown to secrete inflammatory cytokines TNF α and IL-8, generating a pro-inflammatory stromal environment in the liver (Mueller et al., 2007). Elevated expression of α SMA, assumed to represent CAFs, was also shown in lymph node metastases of oral squamous cell carcinoma (Vered et al., 2010).

1.1.2.2.2. EMT and endothelial-mesenchymal transition (EndMT)

Epithelial mesenchymal transition (EMT) is a key process in malignant transformation. Cells that undergo EMT lose adhesion to basement membrane and cell-cell contact and gain mesenchymal properties (Kalluri and Weinberg, 2009). Carcinoma cells that have undergone EMT have been postulated as a potential source of CAFs. Genomic studies showing non-random genetic alterations that were common between cancer cells and stromal CAFs indicated a common origin (Petersen et al., 2003). Induction of oxidative stress driven by MMPs also resulted in mutations that allowed cancer cells to undergo a 'specialised' EMT, thus trans differentiating into CAFs (Radisky et al., 2005).

Kalluri and colleagues have shown that transition of non-malignant epithelial cells into myofibroblasts via EMT occurs during fibrosis, leading them to postulate that such EMT of cancer cells could similarly be a putative source of CAFs in solid cancer types (Zeisberg et al., 2003). In addition, Kim et al. showed that ~30% of vimentin positive activated fibroblasts in lung fibrosis were derived from β -galactosidase marked lung epithelial cells (Kim et al., 2006). Another study also established that the majority of FSP-1⁺ fibroblasts in a mouse renal fibrosis model were epithelial-derived (Iwano et al., 2002).

A seminal study by Kalluri and colleagues also showed the transition of endothelial cells into a mesenchymal shaped pro-tumourigenic cell type, leading them to postulate that endothelial mesenchymal transition or EndMT can be a potential source of CAFs (Zeisberg et al., 2007). This process was validated in three independent transgenic mouse models in this study. B16F10 melanomas were

generated in mice expressing endothelial-cell specific β -gal; β -gal⁺ endothelial cells were shown to express FSP1 and α SMA. Similar results were obtained in the RIP1Tag2 spontaneous pancreatic carcinoma model. In a third model, using a Tie-2 Cre-lox mouse model expressing lacZ exclusively in endothelial cells, ~30% of FSP1⁺ cells were shown to be lacZ⁺. In addition, endothelial cells were shown to gain FSP1 and lose CD31 expression with *in vitro* TGF β treatment, indicating a transition to a CAF-like state (Zeisberg et al., 2007).

1.1.2.2.3. Adipose stem cells

Several studies have also indicated that tissue resident adipose stem cells are a potential source of CAFs. Yu et al. demonstrated that *in vitro* co-culture and *in vivo* co-injection of lung cancer cells together with adipose stem cells resulted in increased tumour cell proliferation and decreased apoptosis (Yu et al., 2008). Similarly adipose stem cells injected in close proximity to the site of PC3 prostate carcinoma subcutaneous tumours led to their recruitment into the tumour and increased tumour growth (Lin et al., 2010). Injection of the melanoma cell line A375 along with adipose stem cells also led to decreased latency, increased tumour take rates and larger tumour volumes; this study also alluded to adipose stem cells as a locally resident population of MSCs (Kucerova et al., 2010).

1.1.2.2.4. Bone marrow derived mesenchymal stem cells (BM MSCs)

A multitude of studies have established bone marrow mesenchymal stem cells (BM MSCs) as one of the major sources of CAFs. MSCs are multipotent progenitor cells, and are best known for responding to tissue injury as a regenerative reservoir with potential to differentiate into bone, fat, cartilage and muscle tissues. Despite their regenerative role, MSCs remain a poorly characterised population in comparison to haematopoietic stem cells (Studený et al., 2004). A landmark study (Karnoub et al., 2007) showed that human BM MSCs migrated rapidly in a transwell assay towards media derived from MCF7/Ras or MDA-MB-231 cultures in comparison to control cultures. In addition, GFP labelled human MSCs injected into MCF7/Ras or MDA-MB-231 human breast cancer tumour bearing mice localised specifically to the developing xenograft tumours, with no observable accumulation in other tissues. This indicated that BM MSCs could migrate from the bone marrow to sites of

cancerous growth and promote tumour growth (Karnoub et al., 2007). In a similar study, Ishii et al. transplanted bone marrow from β -galactosidase mice into irradiated NOD/SCID mice, and demonstrated the accumulation of β -gal⁺ myofibroblasts within and around the edges of xenograft tumours generated from the pancreatic cell line Capan-1 (Ishii et al., 2003).

Similarly, when allogenic bone marrow transplantation was carried out from male donors to female patients of gastrointestinal cancer, a large proportion of α SMA⁺ cells observed in the primary tumours were of donor (male) origin (Worthley et al., 2009). Direkze et al. showed that ~25% of myofibroblasts in the spontaneously generated pancreatic tumours in the RIP1Tag2 mice were of BM MSC origin (Direkze et al., 2004), while another study similarly established that ~20% of CAFs in gastric cancer were bone-marrow derived (Quante et al., 2011). Prostate cancer derived CAFs were shown to express CD90, an MSC marker; although in this model, these cells failed to express any other MSC marker except CD24, this was attributed to the fact that MSCs possibly lost their 'stem cell' markers on differentiating into CAFs/myofibroblasts (Zhao and Peehl, 2009). Subcutaneous co-injection of MSCs with the ovarian cancer cell line SKOV3 also resulted in significantly increased tumour cell proliferation (Spaeth et al., 2009). Mishra et al. showed that MSCs co-cultured with conditioned media from breast cancer cells expressed α SMA and CXCL12, with a simultaneous gain in tumour promoting properties, indicating that MSCs are possibly recruited to the tumour microenvironment in response to signals from malignant cells, where they differentiate into CAFs and provide growth-promoting signals to aid the sustenance and progression of the tumour (Mishra et al., 2008).

Figure 1.2 highlights the various origins of CAFs and illustrates the bimodal effect of CAFs in the epithelial tumour microenvironment.

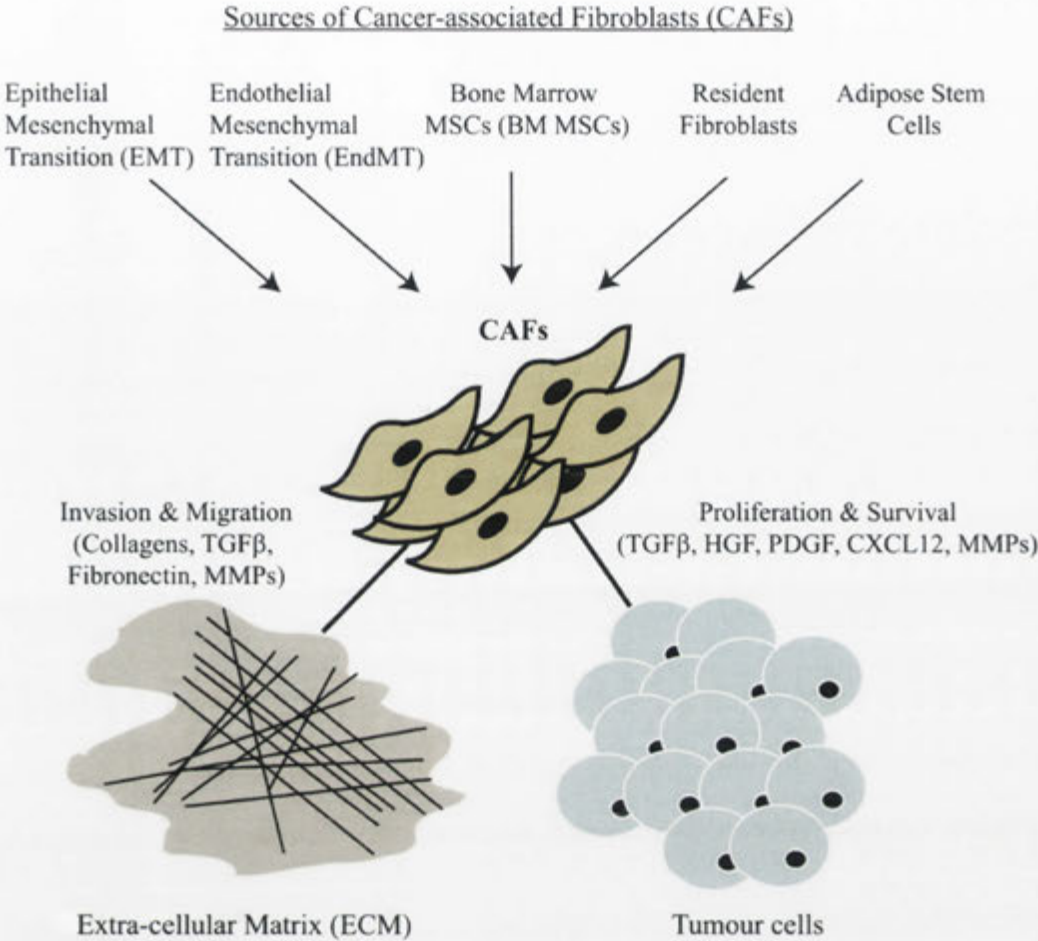


Figure 1.2. The postulated origins of CAFs and their chief roles in promoting tumour growth and modulating the ECM (Figure modified from Brennen et al., 2012)

Interestingly, pericytes, a progenitor population that reside close to microvessels in all vascularised organs of the body, are multipotent MSCs (Crisan et al., 2008a; Paquet-Fifield et al., 2009). However, because of their close association with blood vessels and homeostatic function of angiogenesis regulation (Bergers and Song, 2005), their MSC aspect has never been explored in the context of cancer. The following sections take a detailed look at this elusive cell type, with a comprehensive review of the literature on their physiological roles in homeostasis and cancer.

1.1.3. Pericytes

1.1.3.1. Introduction

Pericytes are classically defined as contractile mesenchymal cells that share a common basement membrane with endothelial cells of the microvasculature. They are best known for supporting endothelial cell function - particularly regulation of proliferation, differentiation and microvessel permeability through paracrine crosstalk with endothelial cells. Specific vascular sites from where pericytes have been reported include postcapillary and collecting venules, capillaries and precapillary arterioles (Armulik et al., 2005; Shepro and Morel, 1993).

Pericytes were first described by Rouget (1873) who referred to them as 'adventitious cells'. The term 'pericyte' was later coined by Zimmermann (1923), to denote their perivascular location. Subsequently, they have also been called Rouget cells, mural cells and pericapillary cells (reviewed in Sims, 1986).

The most common molecular markers of pericytes are α SMA, platelet derived growth factor receptor beta (PDGFR β), desmin, neural glial 2 (NG2, also known as chondroitin sulphate proteoglycan 4) and regulator of G-protein signalling 5 (RGS5). However, none of these markers are exclusive to pericytes. In addition, pericytes are often confused with vascular smooth muscle cells (VSMCs), which are present on larger arteries or veins, given their overlapping markers and periendothelial location. However, a key difference lies in their respective ultrastructures – while pericytes share the basement membrane with the adjacent endothelial cells, VSMCs are subadjacent, creating their own separate matrix along the vascular wall. However, these differences are not absolute, especially given that there are no molecular markers that distinguish a pericyte from a VSMC and even certain fibroblasts, resulting in any mesenchymal cell in the periendothelium being termed as 'pericytes' in the literature. Pericyte markers show some variation across species and anatomical location. For example, brain pericytes, which have a different ontogeny to other pericytes, do not express α SMA; while desmin is not expressed in skeletal and cardiac muscles. This can be attributed to the fact that further to their key function of vessel maintenance, pericytes possibly have additional tissue-specific roles,

especially in specialised organs like the heart and brain (Bergers and Song, 2005; Gerhardt and Semb, 2008; Sims, 1986).

Key pericyte markers, other cells that they have been described in, and their functional relevance have been enlisted in *Table 1.1*.

Table 1.1. Key Pericyte markers (modified from Armulik et al., 2011)

Pericyte Marker	Other Cell Types Expressing the Marker	Functional Relevance	References
α SMA	Smooth muscle; myofibroblasts; CAFs; MSCs	Structural protein; up-regulated in tumours and in inflammation	(Nehls and Drenckhahn, 1993)
PDGFR β	Interstitial mesenchymal cells during development; smooth muscle; neurons and neuronal progenitors; myofibroblasts; MSCs	Receptor tyrosine kinase; functionally involved in pericyte recruitment during angiogenesis	(Lindahl, 1997; Winkler et al., 2010)
NG2	Developing cartilage, bone, muscle; early postnatal skin; adult skin stem cells; adipocytes; VSMCs; neuronal progenitors;	Integral membrane chondroitin sulphate proteoglycan;	(Ozerdem et al., 2001)
Desmin	Skeletal, cardiac, smooth muscle; myofibroblasts; CAFs	Structural protein; useful pericyte marker outside skeletal muscle and heart	(Nehls et al., 1992)
RGS5	VSMCs	Regulate heterotrimeric G proteins by activating GTPases; marker for pericytes on angiogenic vessels	(Bondjers et al., 2003; Cho et al., 2003)
Endosialin	VSMCs, myofibroblasts; fibroblasts; T cells	Transmembrane cell surface glycoprotein; expression on pericytes is dynamic; down-regulated during development	(Christian et al., 2008)

1.1.3.2. Ultrastructure & Ontogeny

Pericytes appear as elongated polymorphic cells attached to the abluminal surface of endothelial cells. A key feature of pericyte ultrastructure is the presence of long cytoplasmic processes that allow one cell to cover the surface of two to three endothelial cells in general, albeit incompletely. These cytoplasmic extensions

change with the type of vessels they are associated with – e.g. pericytes on postcapillary venules have cells with more radial processes encircling the vessel wall whereas those on micro capillaries have longitudinal processes extending along the vascular axis (reviewed in Sims, 1986).

Both endothelial cells and pericytes secrete components of the basement membrane that separates the two cells, although the contribution of pericytes in secreting matrix proteins is higher (Stratman et al., 2010). Direct endothelial cell: pericyte contact is frequently made through disruptions in the basement membrane, allowing pericytes to exert contractile pressure on the vessels to regulate blood flow, a key function they are associated with (Díaz-Flores et al., 2009). *Figure 1.3* illustrates the ultrastructure of pericytes and their intimate association with endothelial cells.

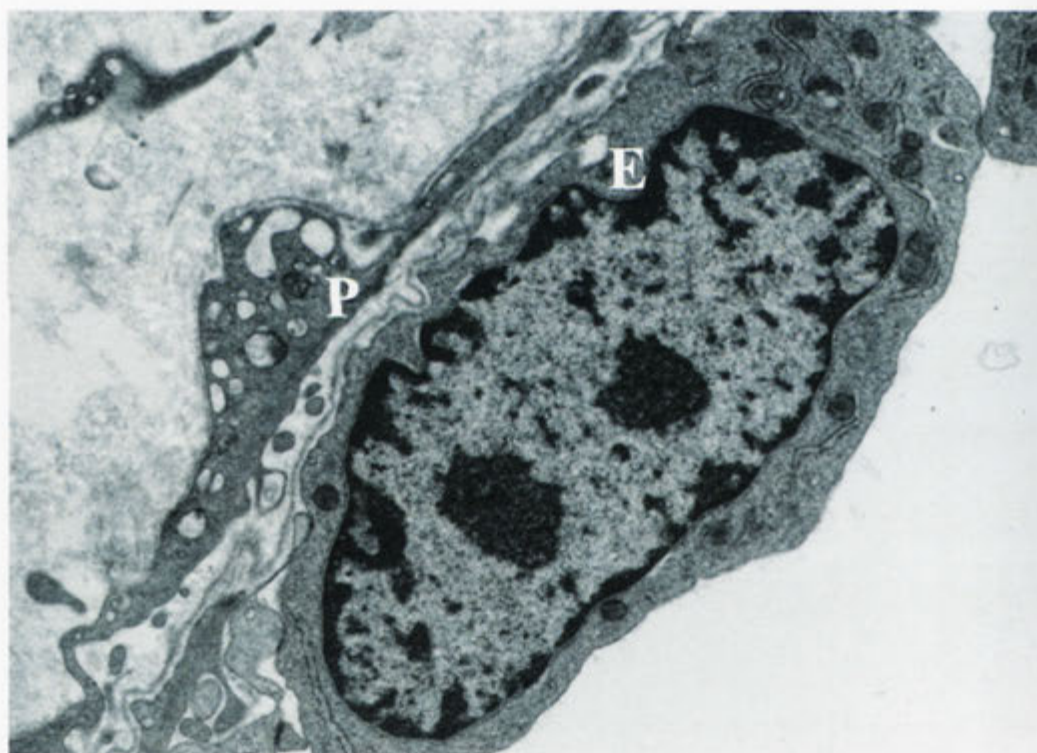


Figure 1.3. Transmission electron micrograph of a pericyte (P) and endothelial cell (E) from murine dermal tissue taken during this study. Note the long cytoplasmic extensions and inclusion bodies of pericytes and a common basement membrane between the two cells.

Pericyte distribution varies largely across vessel types and anatomy; however, in general pericyte: endothelial cell distribution ranges from 1:1 to 1:10 under homeostasis (Sims, 1986).

Interestingly, the ontogeny of pericytes shows a divergence in their developmental origin (Armulik et al., 2011). While pericytes in the liver (Asahina et al., 2011), gastrointestinal tract (Wilm et al., 2005), lung (Que et al., 2008) and cardiac tissue (Wessels and Pérez-Pomares, 2004; Zhou et al., 2008) are mesoderm-derived, pericytes in the brain, the central nervous system (CNS) and thymus are derived from the neural crest (Etchevers et al., 2001; Korn et al., 2002). Even within neuroectoderm derived pericytes, glial pericytes respond to different molecular signals than pericytes isolated from the thymus (Foster et al., 2008). This possibly alludes to their specific functions in vascular regulation within each tissue type. In contrast, mature VSMCs have a diverse ontology, arising from a mosaic of different developmental origins even within the same tissue (Majesky et al., 2011). However, ontogenic and lineage relationship of pericytes and VSMCs is still not fully understood (Armulik et al., 2011).

1.1.3.3. Function of pericytes

1.1.3.3.1. Pericytes and angiogenesis

The major functions of pericytes are regulation of endothelial cell proliferation and differentiation (by paracrine signalling with endothelial cells), and maintenance of vascular structure (mainly by secreting ECM proteins).

Three main molecular pathways have been implicated in pericyte: endothelial cell crosstalk. The first is the interaction of angiopoietin (Ang), expressed by pericytes (Sundberg et al., 2002) with its receptor Tie-2 on the endothelial cells (Dumont et al., 1993). This crosstalk has been shown to be functional in co-ordinating intra/extravasation by alteration of vascular permeability and regulating processes that ensure maturation and stability of endothelial cells (Gaengel et al., 2009).

The second pathway central to pericyte function is the PDGF axis – specifically the interaction of PDGF secreted by endothelial cells and its interaction with its receptor PDGFR β on pericytes. The pronounced role of PDGF β interactions are in recruiting

pericytes at new vessel sites during angiogenesis. Thus, endothelial tip cells are shown to express more PDGF β than stalk cells (Lindblom et al., 2003). Interestingly, PDGF β , although important, can be compensated for by other mechanisms in an organ-specific manner. Thus, knockout models of both PDGF β and PDGFR β resulted in partial obliteration of pericytes from the brain, kidney and skin, but liver pericytes, i.e. the hepatic stellate cells, were unaffected (Hellström et al., 1999; Lindahl, 1997). Moreover, endothelial cell secreted PDGF β binds to the ECM through heparin sulphate proteoglycans. However, specific deletion of the retention motif of PDGF β (PDGF $\beta^{\text{ret}/\text{ret}}$ mice) results in only a partial reduction in pericyte numbers (Lindblom et al., 2003), while ablation of heparin sulphate does not affect pericyte recruitment or function (Stenzel et al., 2009).

Pericytes have also been demonstrated to employ the TGF β signalling pathway to regulate endothelial cell proliferation and differentiation. While particular roles of TGF β pathway members in pericyte : endothelial cell crosstalk are not properly understood, knocking out crucial TGF β genes like TGF β 1 (Dickson et al., 1995), SMAD4 (Lan et al., 2007), SMAD5 (Chang et al., 1999), Alk-1 (Urness et al., 2000) and Alk-5 (Larsson et al., 2001) have resulted in embryonic lethal phenotypes with abnormal yolk sac vasculature and defective pericytes. Interestingly, two specific TGF β 1 receptors, Alk-1 and Alk-5, have been shown to play synergistic roles in maintaining endothelial cell function. Specifically, during angiogenesis, Alk-1 signalling promotes proliferation and migration of endothelial cells; Alk-5 signalling then regulates differentiation and ECM production (Goumans et al., 2002, 2003; Oh et al., 2000) to stabilise the newly formed vessel.

Moreover, Notch (Joutel et al., 1996; Ruchoux et al., 1995), sonic hedgehog (Vokes et al., 2004) and ephrin-Eph receptor (Foo et al., 2006) signalling have also been implicated in the biology of pericyte: endothelial cell crosstalk. Furthermore, pericytes have been shown to play a crucial role in maintaining the blood-brain barrier (Armulik et al., 2010). From a mechanotransduction perspective, an important function of pericyte contractility is the maintenance and regulation of capillary constriction indirectly affecting vascular traffic, the biology of which is gradually unfolding with the help of advanced imaging techniques (Dai et al., 2009;

Vates et al., 2010). Figure 1.4 illustrates the various functions of pericytes in regulation and maintenance of angiogenesis.

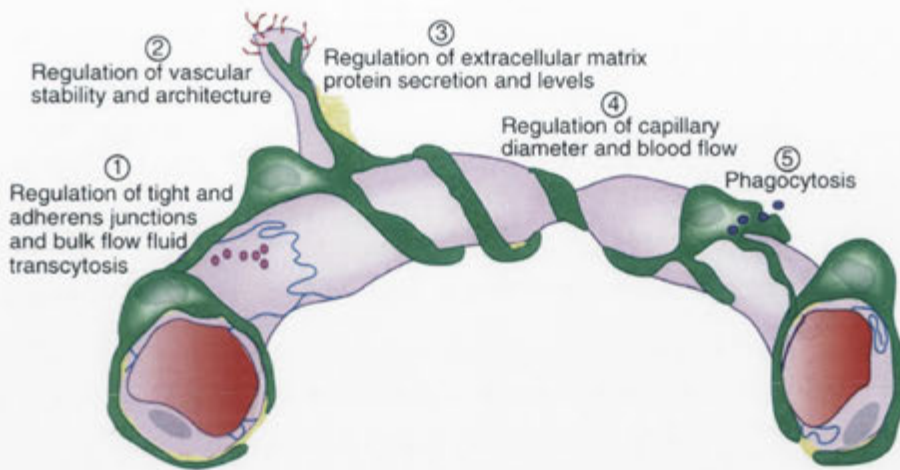


Figure 1.4. Key functions of pericytes in regulating angiogenesis. (From Winkler et al., 2011)

1.1.3.3.2. Pericytes as Mesenchymal Stem Cells (MSCs)

In addition to their roles in supporting angiogenesis, multiple studies have shown that pericytes are MSCs, a local reservoir of progenitor cells harbouring a capacity to differentiate into bone, fat, cartilage and muscle cells. Pericytes from multiple vascularised tissues including skeletal muscle, pancreas, adipose tissue and placenta have been shown to have multi-lineage potential (bone and muscle) with expression of key MSC markers CD73, CD44, CD105, CD146 and CD90 and negativity for endothelial, haematopoietic and epithelial markers (Crisan et al., 2008a). Similarly, our laboratory has shown that dermal pericytes are multipotent MSCs, with osteogenic, adipogenic and chondrogenic potential (Paquet-Fifield et al., 2009). Pericytes have also been demonstrated as a local reservoir for odontoblasts (Feng et al., 2011), muscle stem cells (Dellavalle et al., 2007) and progenitors for white adipocytes (Olson and Soriano, 2011; Tang et al., 2008). *In vitro* multi-lineage potential of pericytes as MSCs has also been demonstrated in a number of other studies on prospectively isolated cells (Collett et al., 2003; Crisan et al., 2008b; Dellavalle et al., 2007; Doherty et al., 1998; Farrington-Rock et al., 2004).

Although the non-exclusivity of pericyte markers poses certain limitations in isolating pericytes, there is an increasing body of evidence that suggests pericytes might be ‘more MSC-like’ than classical BM MSCs, in terms of plasticity, regenerative capacity and specific functional effects. For example, pericytes, not BM MSCs, have been shown to bear a potential to regenerate testicular Leydig cells (Davidoff et al., 2004), when they are experimentally obliterated. The more specialised pericytes in the CNS can differentiate into neural stem cells (Dore-Duffy et al., 2006). However, a fair criticism of this study is that CNS pericytes are isolated based on expression of NG2 and nestin (Belachew et al., 2003; Dore-Duffy et al., 2006). Neither marker is specific, especially considering that the perivascular niche has also been implicated as a reservoir of neural stem cells (Tavazoie et al., 2008). Nevertheless, these studies indicate the degree of plasticity inherent in these multipotent cells is possibly more than that of BM MSCs.

Collectively, these data show that pericytes prospectively isolated from various tissue types have a certain degree of uniformity of MSC marker expression and multi-lineage potential. However, further research into defining more markers specific to these elusive cells might unravel interesting new roles for these cells that can extend beyond those defined for classical MSCs.

1.1.3.3.3. Pericytes in cancer

The presence of pericytes has been detected in almost all solid cancer types, given that tumours operate like ‘vascularised organs’. However, not much is understood about how they function within tumours, their recruitment and their specific molecular interactions beyond the endothelium.

Decrease in pericyte coverage of blood vessels results in dysfunctional and hyper-permeable vessels (Gerhardt and Semb, 2008; Raza et al., 2010). The accepted notion in the field is that pericytes maintain their homeostatic functions of blood vessel stabilisation in malignant tumours; hence, increased pericyte coverage leads to better vascularisation and aggressive tumours, while ablation of pericytes coincides with hypoxia-driven haematogenous metastasis. Although there is general agreement in the field that increased pericyte coverage does render a survival advantage to

tumour due to better vascularisation, the specific roles of pericytes in metastasis remain unclear (reviewed in Gerhardt and Semb, 2008).

Pericyte biology in tumours have been primarily studied using two mouse models – the PDGF $\beta^{ret/ret}$ mouse that harbours a mutation in the C-terminal retention motif of PDGF (Abramsson et al., 2003; Lindblom et al., 2003; Nisancioglu et al., 2010; Sennino et al., 2007; Xian and Håkansson, 2006) and the RIP1Tag2 mouse model that generates spontaneous pancreatic tumours (Bergers et al., 2003; Sennino et al., 2009).

Experiments on various tumour models on the PDGF $\beta^{ret/ret}$ mouse have led to conclusions like the absence of pericytes does not affect tumour vasculature (Nisancioglu et al., 2010) or that the presence of pericytes limit tumour metastasis (Xian et al., 2006). However, careful review of this model reveals that while pericyte numbers within tumours in PDGF $\beta^{ret/ret}$ mice were fewer, there was also a substantial increase in their dissociation from vessels (Abramsson et al., 2003; Nisancioglu et al., 2010). Moreover, tumour-driven PDGF expression could partially increase pericyte numbers, but they remained vessel-dissociated (Abramsson et al., 2003). Further, reduction in pericyte numbers reported in these studies varied with tumour types, ranging from 35% to 65% (Lindblom et al., 2003; Nisancioglu et al., 2010).

Similarly, inhibition of PDGF β signalling using an aptamer resulted in only a partial decrease in tumour vasculature and pericyte numbers in the RIP1Tag2 model (Sennino et al., 2007). Although Bergers et al. was able to show decreased tumour growth with dual targeting of pericyte PDGF β signalling and anti-angiogenic VEGF-A treatment (Bergers et al., 2003), neither model evaluated metastasis (Bergers et al., 2003; Sennino et al., 2007). Interestingly, a positive correlation between metastasis and dissociation of pericytes from blood vessels was observed in human neuroendocrine pancreatic tumours (cited as unpublished observation by McCarthy et al. in Gerhardt and Semb, 2008).

Surprisingly, specific molecular pathways through which pericytes have been postulated to ‘limit’ metastasis have not been identified thus far. The only evidence of pericytes limiting metastasis comes from them being a mechanical obstacle - a ‘gatekeeper of metastasis’, as termed by Gerhardt and Semb, 2008. Thus while most

of these studies show increased metastasis with pericyte dissociation from blood vessels, majority of them do not address or acknowledge the fact that pericyte ‘ablation’ and pericyte ‘dissociation to non-vascular sites’ are inherently different events. Therefore, results from these studies collectively pose the question whether the increased metastasis observed is due to the removal of a mechanical barricade from the vessels, or the dissociation of pericytes to other sites from where they exert a direct pro-metastatic signal. Given that pericytes are a potent MSC population that can secrete ECM components and are known for paracrine crosstalk, it is rather likely that they will engage in paracrine interactions with adjacent cells even at vessel-dissociated sites.

Indirect evidence in favour of the latter hypothesis comes from further studies showing that tumour pericytes exhibit extended processes towards the tumour parenchyma away from blood vessels (Hall, 2006; Morikawa et al., 2002; Ozawa et al., 2005). This was illustrated elegantly by advanced electron microscopy techniques capturing the ultrastructure of tumour pericytes (Baluk et al., 2005). Moreover, in a melanoma model, Furuhashi et al. observed increased numbers of pericytes with PDGF over-expression. However, this increase did not correlate with increased microvessel density, but rather with increased proliferation of tumour cells. More importantly, a substantial number of pericytes in this model were vessel-dissociated (Furuhashi, 2004). Co-culture of fetal pericytes increased the invasiveness of MDA-231 cells *in vitro*; co-injection of pericytes and MDA-231 cells also led to an increase in tumour volume (Bagley et al., 2005). Therefore, these data collectively pose the argument that independent of their ‘gatekeeper’ function, pericytes have other roles within the microenvironment that are exerted once they dissociate from vessels.

Data proposing pericytes as regulators of immune cell passage have also been reported. *In vivo* imaging has demonstrated that pericytes regulate T-cell extravasation in the thymus (Zachariah and Cyster, 2010), leukocyte transmigration (Alon and Nourshargh, 2013; Daneman et al., 2010), and that inflammatory cells often tend to extravasate at sites without pericyte coverage (Voisin et al., 2010; Wang et al., 2006). In addition, in RGS5 knock-out mice, tumours exhibited an

increased infiltration of CD4⁺/CD8⁺ T cells on adoptive transfer (Hamzah et al., 2008).

1.1.4. Summary

Put together, a review of the current literature on CAFs, MSCs and pericytes highlight a major caveat in the field i.e. no single definitive marker unequivocally distinguishes pericytes from perivascular VSMCs, perivascular MSCs, α SMA⁺ myofibroblasts or even α SMA⁺ MSC-like CAFs. As a consequence, “pericyte” is often used in the literature to describe any microvascular peri-endothelial cell of mesenchymal lineage and morphology (Armulik et al., 2005; Gaengel et al., 2009).

In fact, it is now clear that myofibroblasts, activated stromal fibroblasts and CAFs can all be induced to express α SMA under TGF β stimulation (Hall, 2006; Kalluri and Zeisberg, 2006). Interestingly, a prospective function of pericytes as myofibroblasts has been brought to light through data obtained from studies in kidney (Schrimpf and Duffield, 2011), fibrogenesis in liver (Fabris and Strazzabosco, 2011) and systemic sclerosis (Scholten et al., 2010). Some of these and other novel fate mapping studies (Duffield and Humphreys, 2011; Scholten et al., 2010) traced the origin of fibroblasts to PDGFR β ⁺CD73⁺ interstitial MSCs, which, although not conclusive, points towards a pericyte-like origin of myofibroblasts. In addition, two recent studies have demonstrated that pericytes are progenitors of stromal cells that contribute to fibrotic tissue at injury sites (Dulauroy et al., 2012; Göritz et al., 2011).

Together, data from these and other studies led to the hypothesis that MSC-like pericytes, when dissociated from blood vessels, can engage in paracrine cross talk with cancer cells in a CAF-like manner, increasing tumour cell proliferation and creating a permissive environment for metastasis. Thus, in addition to BM MSCs, resident perivascular MSCs, i.e. pericytes could be a putative source of CAFs in solid tumours.

In the work undertaken in the thesis, this hypothesis has been tested in *ovarian cancer* as a disease model. Therefore, the second part of this Chapter attempts to review the literature on ovarian cancer - specifically its disease biology, pathogenesis and role of the microenvironment.

1.2. Ovarian Cancer

1.2.1. Introduction

Ovarian cancer is the fourth leading cause of cancer death in women from developed countries, with a ten year survival probability of less than 35% and the highest case-to-fatality ratio among gynaecological malignancies (Baldwin et al., 2012; Coleman et al., 2011). In Australia, ovarian cancer is the second most commonly diagnosed gynaecological cancer and the 7th most common cause of cancer death in women (data from Australian Institute of Health and welfare, www.aihw.gov.au and Coleman et al., 2011).

Unfortunately, early detection of ovarian cancer is rare with more than 75% of cases being reported only at stages III or IV. Hence, the disease presents with one of the highest mortality rates among gynaecological cancers (Coleman et al., 2011). This is mostly due to the clinical non-specificity of early symptoms, and the lack of convenient screening tests like those available for cervical cancer (pap smears), prostate cancer (prostate examination and blood test for prostate specific antigen) and breast cancer (mammograms).

Ovarian tumours present wide heterogeneity in histology and pathogenesis across patients. According to the World Health Organisation (WHO) classification of malignant ovarian disease, most ovarian tumours can be normally placed into three main categories based on their anatomical tissue of origin - ovarian surface epithelial tumours, germ cell tumours and tumours of the ovarian mesenchyme (stroma and sex chord) (Kaku et al., 2003).

1.2.1.1. Histotypes and pathogenesis of epithelial ovarian cancer (EOC)

The vast majority of clinically reported cases of ovarian cancer are of ovarian surface epithelial (OSE) in origin. These can be further grouped into several histological subtypes, the most common ones being serous, endometrioid, mucinous, clear cell and transitional cell (Brennan cell) tumours (Auersperg et al., 1998; Bast et al., 2009).

Serous ovarian cancer, the major focus of the work undertaken in this thesis, is clinically classified into four stages based on the progression of the primary tumour; stage I is when cancer growth is limited to one or both ovaries; at stage II the growth of the cancer involves extension into the pelvic region; stage III comprises of malignant progression beyond the pelvis into the abdominal lining and organs within the peritoneal cavity and/or to the lymph nodes; and stage IV represents the most advanced stage of serous ovarian cancer, where extensive metastasis to local and visceral organs are observed (Bast et al., 2009; Morrison, 2005; Vang et al., 2009).

An alternate classification of serous ovarian cancer is based on its pathology, genetic alterations and progression. Low-grade serous carcinomas (LGSCs), mostly arising from adenofibromas or borderline tumours of low malignant potential (LMP), present low-grade nuclei with infrequent mitotic figures. In contrast, high-grade serous carcinomas (HGSCs) have high-grade nuclei and numerous mitotic figures, progress rapidly into metastatic disease and have a relatively higher mortality rate (Auersperg et al., 1998; Vang et al., 2009). LGSCs are mostly non-invasive and have better outcome than high-grade tumours. HGSCs, comprise of approximately one-third of all reported cases of serous ovarian cancers, with an incidence of approximately 50% of all ovarian neoplasms (Coleman et al., 2011; Kaku et al., 2003).

Genetically, LGSCs present a gradual accumulation of somatic mutations in their slow transition from benign to malignant state. These mutations are most commonly observed in K-Ras, BRAF, and PPP2R1A, pathways that are implicated in growth control and regulation of cell division (Shih et al., 2011). HGSCs, on the other hand are more genetically diverse aggressive tumours with relatively smaller numbers of *de novo* somatic mutations, mostly in the tumour suppressor p53 (Tp53) or the breast cancer susceptibility proteins type 1 and II (BRCA1/BRCA2) DNA repair pathways (Crum et al., 2007; Geisler et al., 2002; Tone et al., 2008). Interestingly, 10% of all HGSC cases diagnosed result from hereditary BRCA1/BRCA2 germline mutations (Risch et al., 2001, 2006). *Figure 1.5* summarises the histopathological and genetic hallmarks of low and high-grade serous ovarian cancers.

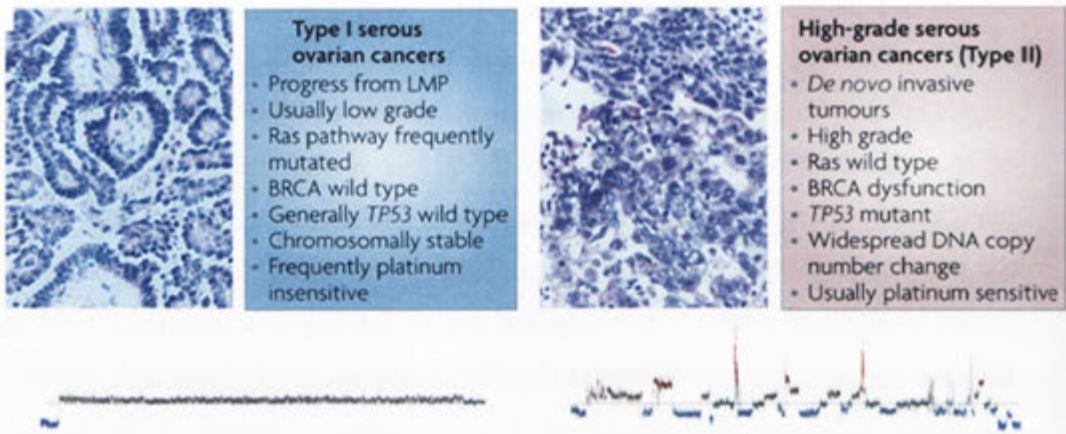


Figure 1.5. A comparative view of Type I (low-grade) and type II (high-grade) serous ovarian tumours. (From Bowtell, 2010)

1.2.2. Epithelial ovarian cancer - Disease Biology

1.2.2.1. Initiation

Three proximate but anatomically distinct sites have been implicated in the origin and initiation of serous ovarian cancer – the OSE, the fallopian tube epithelium and the layer of mesothelium covering the surface of the ovary. The OSE is a mucinous monolayer of simple squamous to cuboidal epithelial cells and the most common site of origin of serous ovarian cancer. This is possibly due to the recurrent cycles of growth and repair the OSE undergoes during ovulation, thus resulting in benign cyst formation which leads to malignant transformation (Auersperg et al., 1998; Fathalla, 1972). In contrast, the fallopian epithelium is a monolayer of fully differentiated columnar, secretory and ciliated epithelial cells. The OSE is developmentally derived from the coelomic epithelium, while the adjacent uterine, cervical and fallopian tissues are derived from müllerian (paramesonephric) ducts, which arise from the mesoderm. Intriguingly, the histology of OSE derived ovarian cancer resembles that of the normal fallopian tube and endometrial cells, raising the question how simple epithelial cells could be malignantly transformed to resemble tissues of the müllerian duct (Dubeau, 2008). Also, contrary to the theory that the hallmark of neoplastic progression is the ‘dedifferentiation’ of malignant cells (Hanahan and Weinberg, 2000), EOC seemingly presents the transition of simple epithelial cells into a more complex, organised and differentiated epithelia.

One possible explanation is that the junction of the ovary and fallopian tubes represent a site of epithelial transition that is commonly susceptible to malignant changes, similar to the cervical transition zone (Auersperg et al., 2008) or Barrett's epithelium (Gibson et al., 2013). The other theory is that the dynamic process of ovulation leads to the deposition of fallopian-derived cells on the OSE, which then proceed to form benign inclusion cysts subsequently leading to malignancy (Kurman and Shih, 2010).

1.2.2.2. Progression

Progression of EOC through the clinical stages described earlier (*Section 1.2.1.1*) generally displays a biology that is distinct from the classical haematogenous and lymphatic metastasis routes commonly observed in breast, prostate and colon carcinomas. Specifically, the organised multistep process of intravasation and extravasation (reviewed in Gupta and Massagué, 2006), is less crucial in EOC, given that cancer cells directly disseminate into the peritoneal cavity as single cells or malignant ascites (Cannistra, 2004; Dubeau, 2008). These are then carried to adjacent tissues via the physiological pressure of the peritoneal fluid, a feature unique to the biology of ovarian cancer. Thus, while ovarian cancer disseminates and grows efficiently within tissues and organs of the peritoneal cavity, progression to visceral organs are a relatively rare occurrence, mostly presented in very advanced stage IV patients (Bast et al., 2009; Rosen et al., 2009). In light of Paget's 'seed and soil' hypothesis (Paget, 1989), the layer of mesothelium is the 'soil' for EOC, given that it covers all organs within the peritoneal cavity, including the omentum.

Although haematogenous metastasis is not the first or more preferred route of EOC metastasis, EMT is still crucial to metastatic progression. EMT is a complex, transformative process central to development and cancer. It is comprised of a cascade of molecular and cellular events resulting in normal or transformed epithelial cells losing cell-cell adhesion and apical-basal polarity, and gaining cell motility, invasiveness and increased resistance to apoptosis (Kalluri and Weinberg, 2009). The transformed cells bear a characteristic 'fibroblastic' or 'mesenchymal' shape which is representative of the molecular changes it has undergone. These specifically include the loss of key epithelial features like the expression of E-cadherin and concomitant gain of mesenchymal markers like N-cadherin, vimentin and fibronectin, among several others (Christiansen and Rajasekaran, 2006; Guarino

et al., 2007). The concerted role of the microenvironment in triggering the EMT process in various forms of solid epithelial cancers has been demonstrated (Lee et al., 2006; Vergara et al., 2010). The TGF β signalling pathway, in particular, and its associated cytokines have been the most extensively studied as inducers of EMT in cancer pathogenesis (Massagué, 2008; Xu et al., 2009). Moreover, the role of hypoxia as a process that can trigger EMT at the cores of growing solid tumours has also been demonstrated in several cancer types (Kalluri and Weinberg, 2009). Specifically, hypoxic conditions at tumour cores either due to angiogenic stress or therapeutic obliteration of the tumour vasculature, signals the up-regulation of pro-angiogenic molecules like the hypoxia inducible growth factor 1 alpha (HIF-1 α), VEGF and PDGF, which can bring about EMT of tumour cells and prompt dissemination (Tsai and Wu, 2012).

Progression from stage I/II to stage III of EOC involves the key process of disruption of the solid tumour encapsulation and dissemination of single malignant cells, cellular aggregates or malignant ascitic spheroids. Thus, EMT in the context of EOC is crucial since it allows loosening of desmosomes regulating cell-cell and cell-matrix adhesion, allowing the cells to be motile and invasive. In fact, it has been shown that E-cadherin expression in cancer cells disseminated in the ascitic fluid is either lost or significantly lower than cells within the primary tumour; EOC cells with lower E-cadherin expression are more invasive as well (Veatch et al., 1994). In addition, loss of E-cadherin in EOC patients predicts poor survival (Daraï et al., 1997).

The presence of malignant cells as ascites in the peritoneum, another unique clinical feature of ovarian cancer, is still a confounding factor; it is unclear if ascites are a sign of more advanced disease that has progressed into secondary organs or is indeed a biomarker for initial dissemination from the primary tumour. It is also not certain if single disseminated cells aggregate to form spheroids or cells detach as aggregated clumps, the leading edges having undergone EMT, although current studies possibly indicate the latter (Cannistra, 2004). Proteolytic activity is also rather crucial in the detachment of cancer cells from the basement membrane in the OSE. MMPs, specifically MMP-2 and MMP-14, have been implicated as effectors of this proteolytic degradation process. While MMP-2 is preferentially expressed in

spheroids than monolayer cultures of EOC cells (Davidson et al., 2001), activated MMP-14 has been shown to cleave $\alpha 3$ -integrin, contributing to degradation of matrix (Moss et al., 2009).

The mesothelium is the preferred target of EOC metastasis. It is primarily a mesothelial layer of cells attached to the basement membrane of organs of the peritoneal cavity like the peritoneum, bowel serosa, omentum and the lining of the diaphragm, which, as a result are the most common sites of ovarian cancer metastasis observed in stage III/IV patients (Cannistra, 2004). The mesothelium is rich in collagen types I and IV, laminin and fibronectin, possibly secreted by embedded fibroblasts and macrophages. Free flowing single tumour cells or spheroids employ these factors to adhere and invade the mesothelial layer (Kenny et al., 2007, 2009). $\beta 1$ integrin is a key adhesion molecule that has been implicated in this process, given that antibodies against $\beta 1$ integrin can inhibit adhesion (Strobel and Cannistra, 1999). Vascular cell adhesion molecule (VCAM-1) from mesothelial cells has also been shown to bind to $\alpha 4\beta 1$ integrin on tumour cells (Lössner et al., 2008), while CD44, a cell surface receptor for hyaluronic acid expressed on most ovarian cancer cells, has also been implicated to aid adhesion (Cannistra et al., 1993). MMPs have been implicated at this step of ovarian cancer progression as well; xenograft ovarian tumours in MMP-9 knock-out nude mice displayed impaired angiogenesis and tumour growth, while injection of MMP-9^{+/+} macrophages rescued this phenotype (Huang et al., 2002).

While adhesion and proteolytic degradation are the predominant mechanisms that dictate early metastasis into the mesothelial layer and adjacent organs, processes that govern progression of ovarian cancer metastasis into advanced stages is poorly understood. This is primarily due to the unavailability of experimental models that correlate well with clinical progression of the disease. Some insight into advanced metastatic disease was provided by several genomic studies. One of the first studies that analysed global genomic data of ovarian cancers reported that a substantial proportion of EOCs do not undergo much genomic alterations as they progress, and were largely clonal in nature (Jacobs et al., 1992). Subsequent studies provided further evidence of this observation, showing that comparative gene expression (mRNA) of tumours from primary versus metastatic sites differed by only 64 (Hibbs

et al., 2004), and 35 (Adib et al., 2004) genes. These studies were further validated by comparative genomic hybridisation (Israeli et al., 2004) and SNP analysis (Haverty et al., 2009). This is biologically quite different to observations in breast cancer (Ding et al., 2010; Popławski et al., 2010) and melanoma (Riker et al., 2008; Turajlic et al., 2012) for example, where tumours show substantial genomic heterogeneity and evolve as they progress into metastatic disease. However, this also conforms to the basic biology of EOC, since the mechanism of dissemination in EOC is rather passive, since tumour cells often do not undergo classical transformative steps required for haematogenous metastasis.

1.2.2.3. Role of the microenvironment

As highlighted in *Section 1.1*, like other cancer types, the stromal environment around ovarian tumours play a major role in pathogenesis and disease progression. It has been shown that infiltration of CTLs and CD3⁺ T cells improve prognosis (Milne et al., 2009; Sato et al., 2005; Zhang et al., 2003) whereas tumour associated macrophages (TAMs) of the M2 phenotype facilitate metastasis (Geller et al., 2010; Wang et al., 2010; Zhang et al., 2010) in ovarian cancer.

MSCs have been studied both as BM-derived progenitor cells as well as cells that can differentiate into CAFs. Importantly, an interesting study showed that resident MSCs (cancer-associated, referred to as CA-MSCs) showed a more pronounced effect in aggravating tumour growth than BM MSCs (McLean et al., 2011).

Key components of the ECM, particularly hyaluronan (HA) (Gardner et al., 1996; Ween et al., 2011) and $\alpha 5 \beta 1$ integrin (Casey et al., 2001; Strobel and Cannistra, 1999) have been shown to play important roles as well.

Pericytes in ovarian cancer have been studied with respect to their angiogenesis-supportive functions. Specifically, it has been shown that dual targeting of pericytes as well as endothelial cells could increase the efficacy of anti-angiogenic therapies (Lu et al., 2007, 2008, 2010).

A comprehensive review of key studies focussing on deciphering the roles of various stromal components in ovarian cancer has been listed in *Table 1.2* below.

Collectively, these data show that the microenvironment has a substantial bearing on the pathogenesis of ovarian cancer, and therapeutic strategies particularly aimed at components of the microenvironment, when combined with conventional chemotherapy, might result in increased patient survival. However, lessons from anti-angiogenic therapies caution against the risk of taking a simplistic view on combined treatments. Thus further research into the interrelationship among various stromal components, their cells of origin and their concerted evolution through tumour progression is required to understand the complexity of ovarian cancer pathogenesis.

Table 1.2. List of key studies targeting components of the tumour microenvironment in ovarian cancer.

Study#	Stromal population	Cell line/Disease subtype/Model	Details of study	Reference
1	T lymphocytes	Advanced EOC patients	- The presence of CD3⁺ intratumoural T cells improved prognosis in advanced stage ovarian carcinoma - immunohistochemical analysis of 186 frozen specimens	(Zhang et al., 2003)
2		EOC patients	- Intraepithelial CD8⁺ tumour infiltrating lymphocytes (TILs) s and a high CD8⁺/Treg cell ratio improved prognosis - immunohistochemical analysis of 117 patient samples	(Sato et al., 2005)
3		EOC patients	- Foxp3, CD20 and T cell intracellular antigen (TIA-1) are prognostic predictors - immunohistochemistry & survival analysis	(Milne et al., 2009)
4	Tumour associated macrophages (TAMs)	Stage III/IV EOC patients	- Stromal macrophage derived CSF-1 expression correlated with histological malignancy, skewing TAMs to a M2 phenotype	(Chambers et al., 1997; Kawamura et al., 2009)
5		<i>In vivo</i> , IGROV (EOC cell line)	- Targeting ovarian TAMs using folate-conjugated liposomes improved survival in a xenograft model of advanced EOC	(Turk et al., 2004)
6		<i>In vivo</i> , MA148 (EOC cell line)	- Chemotherapy-induced up-regulation of MCP-1 led to infiltration of TAMs in the ovarian tumour microenvironment	(Geller et al., 2010)
7		Advanced EOC patients	- FXII and thrombin stimulation facilitated ovarian cancer metastasis by activating macrophages to an M2 stage	(Wang et al., 2010; Zhang et al., 2010)

Study#	Stromal population	Cell line/Disease subtype/Model	Details of study	Reference
8	Mesenchymal Stem Cells (MSCs)	<i>In vivo</i> , SKOV3 (EOC cell line)	- Human MSCs injected subcutaneously with SKOV3 cells acquired a CAF phenotype (FAP, FSP and Tenascin C expression) - MSC/CAF secreted IL-6 led to increased tumour cell proliferation and overall increase in tumour size	(Spaeth et al., 2009)
9		<i>In vitro</i> + Mouse model, SKOV3 (EOC cell line) & MSCs derived of patient EOC tissue	- Cancer-associated MSCs (CA-MSCs), when co-cultured <i>in vitro</i> or co-injected <i>in vivo</i>, aggravated tumour cell growth via alterations in the BMP signalling pathway	(McLean et al., 2011)
10		<i>In vitro</i> , OVCAR-3 and SKOV3(EOC cell line)	- MSCs treated with ovarian cancer cell derived exosomes showed increased αSMA expression, indicative of differentiation to myofibroblasts - Associated increase in tumour-promoting cytokines CXCL12 and TGFβwere also observed	(Cho et al., 2011)
11	Cancer-associated fibroblasts (CAFs)	<i>In vivo</i> , MLS (EOC cell line)	- Pre-labelled fibroblasts injected i.p. were recruited to primary subcutaneous EOC tumours	(Granot et al., 2007)
12		<i>In vitro</i> , SKOV3 (EOC cell line)	- Conditioned media from SKOV3 cells or TGFβ1 stimulation led to activation of fibroblasts to myofibroblasts - Higher expression of CLIC4 and αSMA	(Yao et al., 2009)

Study#	Stromal population	Cell line/Disease subtype/Model	Details of study	Reference
13		<i>In vitro</i> , HO-8910PM (EOC cell line)	- TGFβ stimulation increased FAPα expression - Increased FAPα expression in stromal fibroblasts accentuates proliferation, invasion and migration of HO-8910PM cells <i>in vitro</i>	(Chen et al., 2009)
14		<i>In vitro</i> , ES2 (clear cell ovarian carcinoma cell line)	- Conditioned media from ES2 cells induced urokinase type plasminogen activator (uPA) in fibroblasts - uPA is a protease implicated in cancer invasion and migration	(Noskova et al., 2009)
15		EOC patient derived cancer cells and CAFs; normal fibroblasts from ovaries of patients undergoing non-malignant surgery	- An increased number of CAFs (identified by αSMA and FAPα expression) were observed in EOC than borderline and benign tumour tissue - Increase in CAF numbers in EOC tissue correlated with increased microvessel density (MVD) and lymph node, omental and peritoneal metastasis	(Zhang et al., 2011)
16		EOC patient derived cells and normal fibroblasts from omental tissue of patients undergoing non-malignant ovarian surgery	- TGFβ1 from cancer cells stimulated activation and proliferation of normal fibroblasts into CAFs which creates a premetastatic niche in the omentum, increasing number of omental metastatic nodules - <i>In vitro</i> co-culture of EOC cells and normal fibroblasts also led to the up-regulation of HGF and MMP-2	(Cai et al., 2012)
17	Pericytes	<i>In vivo</i> , HeyA8, SKOV3ip1, HeyA8-MDR (EOC cell lines)	- Anti-angiogenic therapeutic targeting of both pericytes (STI571 or PDGF-aptamer, AX102) and endothelial cells (VEGFR inhibitor AEE788 or bevacizumab) led to decreased growth of orthotopic xenograft EOC tumours	(Lu et al., 2007, 2008, 2010)

Study#	Stromal population	Cell line/Disease subtype/Model	Details of study	Reference
18	Adipocytes	<i>In vivo</i> , SKOV3 (EOC cell line)	- Ovarian cancer cells preferentially migrated to the omentum, an adipocyte- enriched organ - Mediating cytokines identified were adipokines like IL-6, IL-8, CCL-2 and adiponectin	(Nieman et al., 2011)
19		<i>In vitro</i> , OVCAR-3 and A2780 (EOC cell lines)	- ER transcriptional activation via STAT-3 signalling led to ovarian cancer cell stimulation via the adipose tissue derived hormone, leptin	(Choi et al., 2011)
20	Extra cellular Matrix (ECM)	<i>In vitro</i> , OVCAR-5, OVCAR-3, SKOV-3 (EOC cell lines)	- Versican induced a pro-metastatic migratory behaviour in EOC cells <i>in vitro</i> - This metastasis inducing activity of versican could be blocked by small hyaluronan (HA) oligosaccharides - Human ovarian cells bind to HA via membrane CD44	(Gardner et al., 1996; Ween et al., 2011)
21		<i>In vitro</i> , OVCAR-5 36M2, CAOV-3, SKOV3 (EOC cell lines)	- $\alpha 5 \beta 1$ integrin increased the multicellular spheroid formation capacity of ovarian cancer cell lines <i>in vitro</i> - $\beta 1$ integrin also found to mediate the capability of EOC cells to bind to the peritoneal mesothelium	(Casey et al., 2001; Strobel and Cannistra, 1999)
22		<i>In vitro</i> , OV90, IGROV-1, SKOV3 (EOC cell lines)	- Inhibition of tissue transglutaminase (TG2) resulted in decreased cell adhesion of ovarian cancer cell <i>in vitro</i> by inhibiting cross-linking of integrin and fibronectin networks	(Khanna et al., 2011)

1.2.3. Epithelial ovarian cancer –Treatment & Prognosis

1.2.3.1. *Diagnosis, Treatment and Resistance*

As discussed previously, early detection of EOC is rare, with most patients being presented clinically at stage II/III, with locally metastasised disease into the peritoneum, omentum, abdominal and reproductive organs (Cannistra, 2004). Mild to major discomfort due to obstruction of the gastrointestinal tract by peritoneal or omental tumours are often diagnosed to be the initial symptom at presentation. Unfortunately, over the past couple of decades, ‘first treatment’ of EOC has remained surgical debulking of the primary (or metastatic) tumour, or *en bloc* resection of the reproductive organs and parts of the colon, where affected. Such surgical debulking is effective only in cases where the disseminated cells in the peritoneal fluid remain contained within the abdominal/peritoneal cavity, and invasion into secondary organs remain superficial. In addition, complete resection is often impossible, and the residual primary tumour triggers further invasive disease (Ledermann and Kristeleit, 2010). In fact, clinically, ‘optimal debulking’ refers to cases where the residual primary tumour <1cm (Goodman and Schorge, 2010; Schorge et al., 2010). However, in spite of these limitations, studies have also shown that cytoreduction of primary disease by surgery significantly improves patient survival (Bristow et al., 2002; Winter et al., 2007).

Post-operative treatment consists of platinum-based chemotherapy agents (carboplatin or cisplatin) and taxanes (paclitaxel/Taxol or docetaxel) (Ledermann and Kristeleit, 2010). Although intraperitoneal (i.p.) administration of chemotherapy can lead to a high degree of abdominal toxicity, a study of i.p. versus intravenous (i.v.) routes of administration in 415 stage III EOC patients showed a moderate increase in progression-free survival (PFS) with the former route (Armstrong et al., 2006). The initial response rates of the traditional carboplatin/paclitaxel administration are about 70-80%; however a vast majority of patients subsequently relapse. Thus, while such treatment rarely brings about a significant difference to PFS, overall survival rates are gradually on the rise (Kyrgiou et al., 2006), possibly due to better standards of care.

Platinum resistance, one of the chief causes of disease recurrence after first-line treatment, is a major downfall of traditional chemotherapy-based treatments regimens (Cree et al., 2007). However, fairly recent studies have shown that a dose-fractionated therapy of carboplatin and weekly paclitaxel can overcome platinum resistance to a certain degree (Cadron et al., 2007; Sharma et al., 2009). Hormone therapy is another alternative for patients with platinum resistance and those with low-tolerance for chemotherapy (Makar, 2000). However, clinical trials have not been able to demonstrate a significant survival advantage with hormone therapy using tamoxifen or leuporelin, in comparison to traditional chemotherapy in EOC patients (du Bois et al., 2002; Kristensen et al., 2008).

1.2.3.2. Prognosis – key markers and molecular subtypes

Epidemiological phenotypes that significantly correlate with patient survival are called ‘prognostic’ factors. Assessment of key prognostic factors at the time of diagnosis often impact the therapeutic routes physicians take; while epidemiological research on these factors allows biologists to better understand disease pathogenesis. Age, parity status, histological subtype, tumour grade, disease stage at presentation and/or surgery and residual tumour volume after surgical debulking are independent prognostic factors in EOC (Berman, 2003; Clark et al., 2001; Winter et al., 2007).

Cancer antigen 125 (CA125), a high molecular weight glycoprotein identified in 1981, is one of the most extensively studied molecular prognostic markers of EOC (Bast et al., 1981). High serum expression of CA125 is observed in ~90% of advanced EOC patients and in ~50% patients with stage I disease (Nustad et al., 1996). Thus, assessment of serum CA125 after each bout of chemotherapy is accepted as standard treatment of care (Guppy and Rustin, 2002; Markman, 2003).

Cytokines and immune cells characteristic of a reactive, inflamed tumour stroma have also been implicated as prognostic markers of EOC. Specifically, increased serum IL-6 and elevated IL-6 expression in ascites correlated with poor prognosis and decreased PFS (Lane et al., 2011; Scambia et al., 1995). Elevated CSF-1 (Chambers et al., 1997) and FGF18 (Wei et al., 2013) levels also led to significantly shorter disease-free survival. Conversely, an anti-inflammatory immune signature of CD20, FoxP3 and TIA-1 corresponded with improved mortality rates (Milne et al., 2009).

Rapid advances in global genomic analyses techniques have also facilitated research into defining novel molecular signature-based subtypes of EOC (reviewed in Bowtell, 2010). A seminal paper from The Australian Ovarian Cancer Study (AOCS) led to the identification of six novel molecular subtypes of serous and endometrioid ovarian cancer. Specifically, the study involved optimal *in silico* clustering of gene expression data of 285 patient-derived tumours to reveal novel signatures with significant differences in mortality rates. Importantly, a reactive stromal gene expression signature had the worst prognosis, which correlated with high desmoplasia in the samples. Another group represented a more 'mesenchymal' subtype, with overexpression of N-cadherin and low expression of CA125 (Tothill et al., 2008).

A larger dataset of 415 serous ovarian adenocarcinoma samples was analysed by the consortium 'The Cancer Genome Atlas' (TCGA), which aimed to create a library of genomic signatures of various cancer types to better understand EOC biology and pathogenesis. The TCGA analysis reiterated the importance of DNA repair mechanisms in the initiation of malignant transformation, showing that TP53 mutations were present in 96% of the tumours; BRCA1/BRCA2 mutations were present in high frequencies as well (The Cancer Genome Atlas Research Network, 2011).

Another recent study on 1538 patient derived samples identified further novel subtypes by grouping molecular signatures by the expression of EMT and stem cell markers. Thus, the subtype of patients with a more mesenchymal signature showed poorer survival, as did patients with increased expression of stem cell markers (Tan et al., 2013).

1.2.4. Epithelial ovarian cancer –Role of Cancer Stem Cells (CSCs)

The biology and clinical implications of the CSC model has been briefly reviewed in *Section 1.1*. A growing number of studies in ovarian cancer have focussed on identifying and characterising ovarian CSCs and exploring their impact on pathogenesis. CD133 (prominin-1), implicated as a CSC marker in glioblastoma (Liu et al., 2006; Singh et al., 2004), prostate and endometrial cancer (Friel et al., 2008), was first explored as an ovarian CSC marker by Baba et al. This study demonstrated the ability of CD133⁺ ovarian A2780 and PE01 cells to generate both CD133⁺ and

CD133⁻ populations; CD133⁺ cells also exhibited increased chemoresistance *in vitro* (Baba et al., 2009). Another study showed that CD133⁺ cells purified from primary patient ovarian tumours showed increased tumourigenicity in NOD/SCID mice compared to CD133⁻ cells. More importantly, resulting xenograft tumours recapitulated the CD133 heterogeneity similar to the parent tumours, a hallmark of CSCs (Ferrandina et al., 2008). Enzymatic activity of aldehyde dehydrogenase isoform 1 (ALDH) has also been used to isolate prospective CSC populations in breast, colon and liver carcinomas (Deng et al., 2010; Ma et al., 2008). ALDH⁺ cells from a platinum resistant ovarian cell line showed a 50-fold increase in tumourigenic potential than ALDH⁻ cells from the same line (Landen et al., 2010). More recently, both markers have been utilised together to further stratify 'true CSCs'; specifically Silva et al. showed that ALDH⁺CD133⁺ primary human EOC cells had the highest tumourigenic and colony forming potential (Silva et al., 2011), the results of which were subsequently replicated in another study (Kryczek et al., 2012).

Notably, CD44⁺ (Alvero et al., 2009; Zhang et al., 2008), CD117⁺ (Luo et al., 2011) and side population cells (Dou et al., 2011; Moserle et al., 2008) from primary ovarian tumours have also been implicated as putative CSCs in ovarian cancer. The concept of side population being the CSC subset also stems from the fact that effluxing ability of cellular toxins, the characteristic ability of ABCG2⁺ side population cells, is a possible mechanism through which CSCs exert increased resistance to chemotherapeutic agents (Dou et al., 2011; Hu et al., 2010).

Ovarian CSC biology research, although in its infancy compared to CSC research in breast cancer (Iqbal et al., 2013) and glioblastoma (Stopschinski et al., 2013), presents novel possibilities of developing therapeutic interventions specifically targeted at this potent, self-renewing population of cancer-initiating cells.

1.3. Previous work leading up to this study

1.3.1. Characterisation of human dermal pericytes

In a seminal study from our laboratory, a novel monoclonal antibody, HD-1 (human dermis – 1) which recognises the vascular leukocyte antigen -1 (VLA-1) also known as $\alpha 1\beta 1$ integrin, was identified while attempting to detect specific stem-like populations within human dermal cells that might affect the tissue reconstitution capability of epithelial cells (Paquet-Fifield et al., 2009). Importantly, this antibody specifically exhibited a perivascular pattern of staining in the human dermis (*Figure 1.6A*). In flow cytometry analysis on an enzyme-digested pool of freshly isolated human dermal cells, HD-1 fractionated two distinct positive populations – the HD-1^{dim} and the HD-1^{bri} (*Figure 1.6B*).

Closer examination revealed that HD-1 stained for perivascular cells surrounding skin microvessels (stained with the endothelial cell marker von Willebrand factor or vWF) and co-expressed α SMA (*Figure 1.6C-D*). Further careful morphological and histological characterisation in this study led to the identification of the HD-1^{bri} population as ‘pericytes’ and the dim population as ‘fibroblasts’. Furthermore, the inclusion of HD-1^{bri} pericytes in the dermal equivalent of organotypic cultures (comprising of fibroblasts), exhibited significant increase in the intrinsic capacity of $\alpha 6^{\text{dim}}$ keratinocytes to generate a stratified, multilayered epithelium (*Figure 1.6E-F*). Given that the 3D organotypic system employed in this study was devoid of vasculature, this demonstrated a novel, pro-proliferative role for pericytes in the epithelial microenvironment. In addition, this study also demonstrated that human HD-1^{bri} pericytes were multi-potent MSCs with an ability to differentiate in to bone, fat and cartilage lineages *in vitro* under appropriate media conditions; and also expressed the characteristic MSC markers CD73, CD90 and CD146 (*Figure 1.6G-J*) (Paquet-Fifield et al., 2009).

The results from this study therefore provided a potent tool for isolating pericytes and fibroblasts and prospectively studying their specific effects independent from each other.

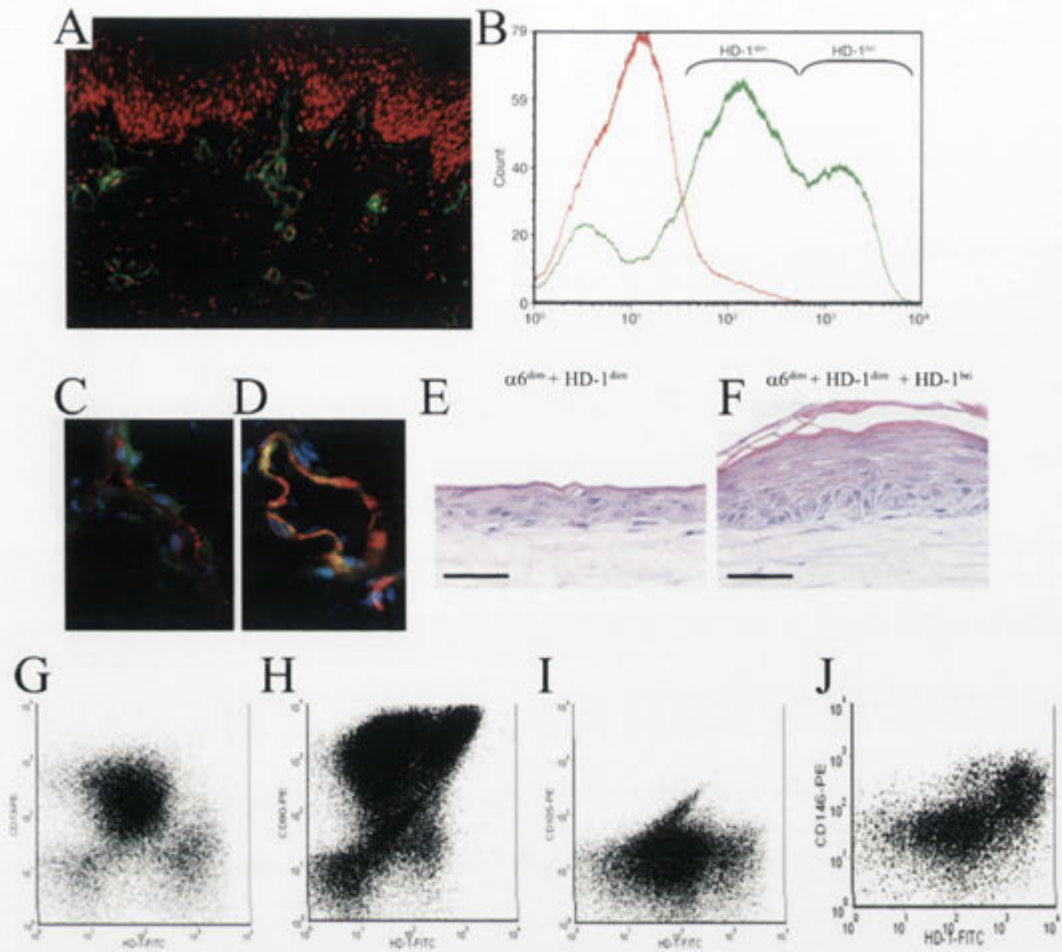


Figure 1.6. Summary of key data from Paquet-Fifield et al., 2009. (A) *In situ* localisation of dermal pericytes revealed by staining for HD-1 (green). Nuclei counterstained with propidium iodide (red). (B) Representative flow cytometry analysis for HD-1 expression on dermal cells indicating the presence of the HD-1 negative, dim and bright populations. (C) HD-1⁺ cells (green) were observed adjacent to vWF⁺ (red) vessels indicating their perivascular location. (D) HD-1⁺ cells (green) show co-expression of αSMA (red). (E-F) Co-culture with (F) HD-1^{bri} cells enhance the *in vitro* proliferation and of α6^{dim} keratinocytes observed with HD-1^{dim} cells. (G-H) Flow cytometry analysis for expression of key MSC markers on HD1⁺ cells. HD-1^{bri} cells were weakly positive for (G) CD73 and highly positive for (H) CD90 and (J) CD146, compared to HD-1^{dim} cells. Neither population expressed CD105 (I).

Global gene expression analysis of the two populations re-confirmed the up-regulation of pericyte specific markers like α SMA, NG2, PDGFR β and CD146 in the HD-1^{bri} cells, with more characteristic fibroblast markers like FAP α being overexpressed in the dim population. *Table 1.3*, adapted from Paquet-Fifield et al., 2009, lists some selected candidates of interest that were up-regulated in the HD-1^{bri}/VLA-1^{bri} pericytes compared to the HD-1^{dim}/VLA-1^{dim} fibroblasts.

Table 1.3. List of selected pericyte markers overexpressed in HD-1^{bri} pericytes. (*data from Paquet-Fifield et al., 2009*)

Gene symbol	Gene name	GenBank accession no.	log2 fold	Adjusted P value
Cell-surface markers				
AOC3	Adhesion protein 1	NM_003734	4.43	2.85×10^{-8}
CDH6	Cadherin 6, type 2, K-cadherin	AU151483	4.39	2.12×10^{-9}
		BC000019	4.39	2.12×10^{-9}
		BF344237	2.54	1.46×10^{-7}
GJA4	Gap junction protein, alpha 4, 37kDa (connexin 37)	M96789	3.5	6.36×10^{-8}
		NM_00206	2.9	1.15×10^{-7}
ITGA7	Integrin, alpha 7	AF072132	4.17	3.07×10^{-8}
		AK022548	3.92	1.12×10^{-8}
JAG1	Jagged 1	U61276	2.76	6.76×10^{-8}
		U77914	2.14	1.11×10^{-7}
		AI457817	2.36	2.03×10^{-6}
		U73936	2.04	1.12×10^{-7}
MCAM (CD146)	Melanoma cell adhesion molecule	M29277	4.12	8.73×10^{-9}
		AF089868	3.69	904×10^{-9}
		BE964361	3.21	1.98×10^{-8}
		M28882	3.28	4.60×10^{-9}
NOTCH3	Notch homolog 3	NM_000435	3.54	1.36×10^{-8}
ITGA1	Integrin alpha 1, Pelota homolog	BG619261	3.4	1.48×10^{-7}
		X68742	2.63	6.91×10^{-5}
		NM_015946	1.61	8.96×10^{-7}
		AA156783	3.41	3.19×10^{-7}
Secreted factors				
A2M	Alpha-2-macroglobulin	NM_000014	1.82	1.97×10^{-6}
ANGPT2	Angiopoietin 2	NM_001147	3.03	1.11×10^{-5}

Gene symbol	Gene name	GenBank accession no.	log2 fold	Adjusted P value
		AA083514	2.72	9.29×10^{-6}
		AF187858	2.46	8.50×10^{-6}
BMP2	Bone morphogenetic protein 2	NM_001200	1.26	1.19×10^{-5}
		AA583044	1.35	1.52×10^{-6}
CCL8	Chemokine (C-C motif) ligand 8	AI984980	3.82	2.56×10^{-7}
ECRG4	Esophageal cancer related gene 4 protein	AF325503	5.04	2.78×10^{-7}
FRZB	Frizzled-related protein	U91903	2.83	1.13×10^{-7}
		NM_001463	2.76	2.32×10^{-7}
PDGFA	Platelet-derived growth factor alpha polypeptide	NM_002607	3.59	2.18×10^{-7}
		AW205919	3.3	7.45×10^{-8}
		X03795	2.97	1.31×10^{-7}
ECM proteins				
ADAMTS5	ADAM metallopeptidase (aggrecanase-2)	AI123555	1.4	1.70×10^{-6}
		BF060767	1.4	3.01×10^{-6}
		NM_007038	1.28	5.07×10^{-6}
COL4A1	Collagen, type IV, alpha 1	NM_001845	2.35	3.41×10^{-6}
		AI922605	1.62	1.51×10^{-6}
COL4A2	Collagen, type IV, alpha 2	AA909035	2.13	1.61×10^{-6}
		X05610	1.66	1.67×10^{-6}
LAMA3	Laminin, alpha 3	NM_000227	3.03	4.58×10^{-8}
LAMA5	Laminin, alpha 5	BC003355	2.39	2.69×10^{-7}
NTN4	Netrin 4	AF278532	1.51	4.62×10^{-5}
TNC	Tenascin C	AI266750	2.2	2.58×10^{-6}
Pericyte markers				
ACTA2	Actin, smooth muscle aorta	AI917901	2.94	5.03×10^{-6}
		NM_001613	2.96	2.03×10^{-8}
MYH9	Myosin, heavy polypeptide 9, non-muscle	AI827941	1.23	3.69×10^{-6}
TPM1	Tropomyosin 1	NM_000366	2.73	1.11×10^{-7}
		NM_000366	1.7	4.75×10^{-6}
		Z24727	2.15	3.22×10^{-7}
TPM2	Tropomyosin 2	M19267	2.33	5.39×10^{-8}
		NM_003289		
RGS5	Regulator of G-protein signalling 5	AF493929	1.29	9.68×10^{-7}
		AI183997	5.83	1.52×10^{-8}
		AF159570	5.23	4.24×10^{-8}
		NM_025226	5.73	1.52×10^{-8}

Gene symbol	Gene name	GenBank accession no.	log2 fold	Adjusted P value
CSPG4/NG2	Chondroitin sulfate proteoglycan 4	NM_001897	1.03	2.07×10^{-6}
		BE857703	1.75	3.50×10^{-6}
PDGFRB (CD140B)	Platelet-derived growth factor receptor	NM_002609	1.28	1.22×10^{-6}
ENPEP	Glutamyl aminopeptidase (aminopeptidase A)	L12468	2.77	9.17×10^{-6}
		NM_001977	2.83	2.39×10^{-6}
CNN1	Calponin 1, basic smooth muscle	NM_001299	2.34	3.41×10^{-7}
CALD1	Caldesmon 1	AI685060	1.89	1.44×10^{-5}
		AL577531	1.83	2.95×10^{-7}
		NM_004342	2.2	3.44×10^{-7}
		AL583520	1.04	7.15×10^{-6}

1.3.2. Preliminary patient prognosis data (AOCS)

The mRNA signature of pericytes obtained in Paquet-Fifield et al., 2009 provided us with a potent tool for evaluating if pericytes, akin to their effect on normal keratinocytes, indeed had a direct, pro-proliferative role as MSC-derived CAFs on malignantly transformed epithelial cells. Gene expression data of 215 high-grade stage III/IV serous ovarian cancer patients available from the AOCS, was deemed to be an ideal dataset for testing this hypothesis. Notably, this dataset was previously used to demonstrate that patients with a reactive ‘stromal’ signature had significantly poorer clinical outcome (Tothill et al., 2008),

Accordingly, the previously described mRNA gene expression signatures (Table 1.3) of VLA-1^{bri} pericytes and VLA-1^{dim} fibroblasts (Paquet-Fifield et al., 2009) were compared *in silico* with the ‘stromal signature’ deduced from the mRNA expression of laser-capture micro-dissected ovarian CAFs (Tothill et al., 2008). Viewed as a proportion of the total ovarian stromal set of genes, a greater overlap of the stromal signature was observed with fibroblasts, compared to pericytes (~24% compared to ~10%) (George, Bowtell and Kaur, unpublished data) (Figure 1.7).

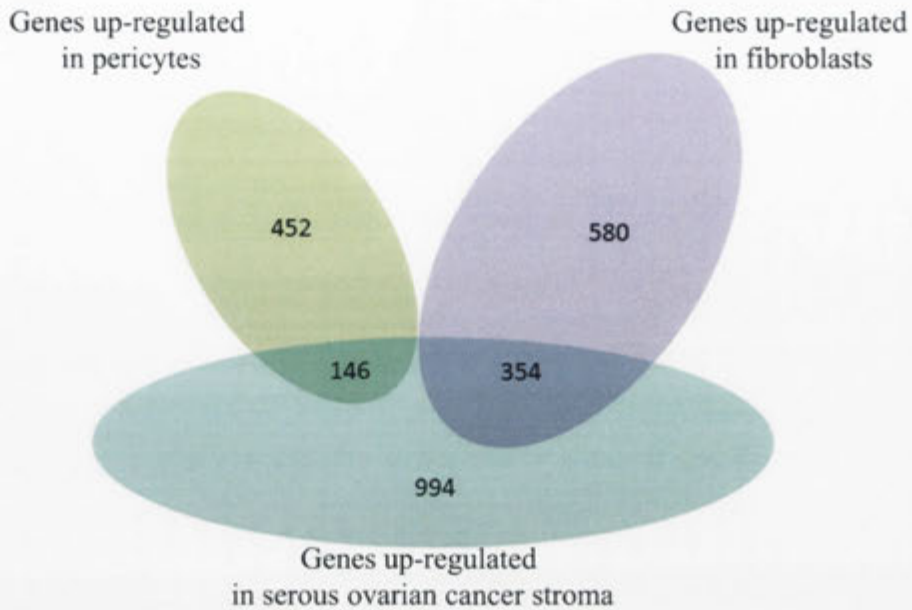


Figure 1.7. Relationship between genes up-regulated in the ovarian cancer stroma and those up-regulated in VLA-1^{bri} pericytes and VLA-1^{dim} fibroblasts. 354/934 normal human neonatal fibroblast genes were co-expressed by serous ovarian cancer stroma, while only 146/598 normal human neonatal pericyte genes were co-expressed by serous ovarian cancer stroma. (George, Bowtell and Kaur, unpublished data)

Despite this, the pericyte signature was the more potent predictor of poor prognosis, early relapse and mortality over that of the VLA-1^{dim} fibroblasts. Specifically, those ovarian cancer patients with a high VLA-1^{bri} pericyte-specific signature (i.e. a positive 'pericyte score' – a statistically determined median value derived from the log₂fold change of all the probes relating to the genes up-regulated in the VLA-1^{bri} pericytes) were at a much greater risk of earlier recurrence of their disease and poorer disease-free survival post-treatment ($p=0.00067$; Figure 1.8A), compared to those that bore a high VLA-1^{dim} fibroblast signature ($p=0.001$; Figure 1.8B). Importantly, this showed a significant improvement in stratifying a gene-set to extricate patients with poor prognosis over the published ovarian tumour stromal signature ($p=0.0011$, Tothill et al., 2008).

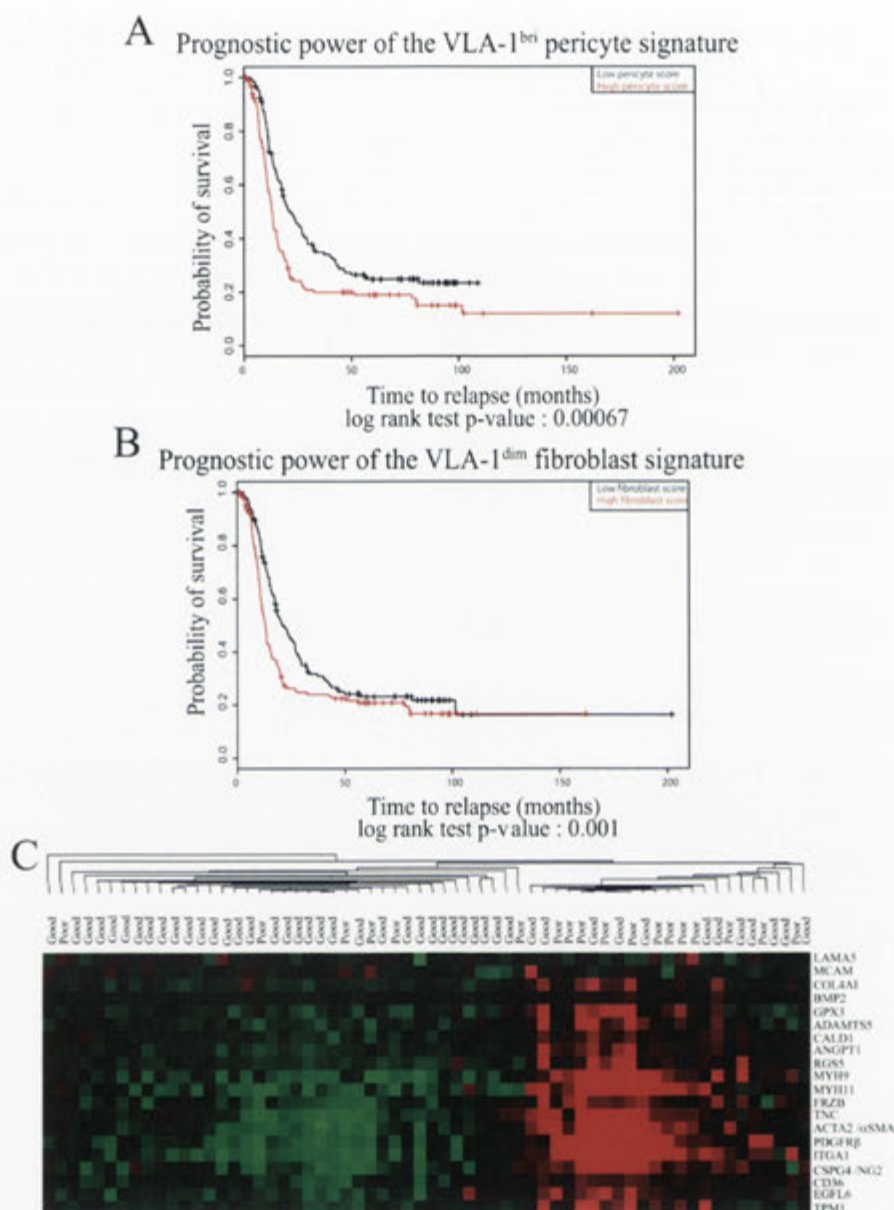


Figure 1.8. The pericyte-specific gene signature predicts poor prognosis in ovarian cancer patients. (A-B) Kaplan-Meier curves showing significantly poorer progression-free survival rate among 215 high grade serous ovarian cancer patients with a (A) high score of pericyte-specific genes compared to those with a (B) high score of fibroblast-specific genes. (C) Heatmap showing expression of 20 representative pericyte genes from 62 patients annotated for ‘Poor’ i.e. those that relapsed at ≤ 6 months or ‘Good’ prognosis i.e. relapsed at ≥ 9 months from primary surgery and initiation of treatment. (George, Bowtell and Kaur, unpublished data)

Notably, although there was less overlap of the ovarian stromal genes with the VLA-1^{bri} gene-set compared to the VLA-1^{dim}, the net effect on patient mortality and relapse was higher in the former, suggesting that some of these overlapping genes may be mechanistically more potent participants in the active ovarian stroma, rendering the cancer more aggressive and fatal (*Appendix A, list of 146 overlapping genes*). The correlation between pericyte gene expression and poor prognosis was also evident in *Figure 1.8C*, showing a heat map of 20 representative ‘pericyte’ genes selected based on reported pericyte markers in the literature, where ‘poor’ prognosis was defined as mean PFS of <6 months whereas ‘good’ prognosis was a mean PFS >9 months (*George, Bowtell and Kaur, unpublished data*).

1.4. Research Question

The significant prognostic capability of the pericyte signature in serous ovarian cancer progression and mortality and our previous data on their direct pro-proliferative effect on normal epithelial cells (Paquet-Fifield et al., 2009), led us to further investigate the precise role of pericytes in the proliferation of transformed malignant epithelial cells through the work undertaken in this thesis. *The underlying hypothesis was that tissue resident pericytes with MSC properties, on dissociation from vessels, could directly engage in paracrine crosstalk with tumour cells in a CAF-like manner. This could result in increased tumour cell proliferation and further pro-metastatic signals from pericytes could lead to increased metastasis. Therefore, like BM MSCs, resident perivascular MSCs or pericytes could be a putative source of CAFs.*

The first of these is the fact that the world is not a uniform whole, but a collection of many different parts, each with its own characteristics and laws. This is the principle of diversity, which is the basis of all knowledge and science. The second is the fact that the world is not a static whole, but a dynamic whole, constantly changing and evolving. This is the principle of change, which is the basis of all history and progress. The third is the fact that the world is not a chaotic whole, but a ordered whole, governed by certain laws and principles. This is the principle of order, which is the basis of all morality and justice.

The fourth is the fact that the world is not a separate whole, but a connected whole, where all parts are interrelated and interdependent. This is the principle of unity, which is the basis of all love and compassion. The fifth is the fact that the world is not a material whole, but a spiritual whole, where the mind and soul are the true reality. This is the principle of spirituality, which is the basis of all wisdom and enlightenment. These five principles are the foundation of all knowledge and science, and they are the keys to understanding the world and ourselves.

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Chapter 2

Materials and Methods

2.1. Materials

2.1.1. Chemical Reagents and kits

All reagents and media were made up in water purified by the Milli-Q (MQ) reverse osmosis (RiOS) filtration system (Millipore, North Ryde, Australia) and filter-sterilised with Millipore syringe-driven Millex-GV 13 mm ethylene oxide-sterilised filter units. For small volumes, low protein binding PVDF membranes (0.22 μ m pore size, Millipore, Cat. SLGV 013 SL) were used for filtration. For larger volumes, vacuum-driven Steritop-GP polyethersulfone 500 ml 33mm γ -irradiation-sterilised filter units with low protein binding PES membrane (0.22 μ m pore size, Millipore, Cat. SCGPS05RE) were used unless otherwise indicated. Reagents that required 'pre-warming' before use were brought to 37°C by incubating in a water bath.

Chemicals and reagents were obtained from laboratory stocks or standard commercial suppliers and were of analytical grade or of the highest purity available. A comprehensive table of general reagents is listed in Table 2.1.

Table 2.1. List of reagents.

Reagent	Source	Catalogue Details
4',6-diamidino-2-phenylindole (DAPI)	Sigma Aldrich	D9542-5MG
AEC Kit	Vector Labs	SK-4200
AldeFluor™ kit	Stem Cell Technologies	1700
Aquatex mounting medium	Merck	1085620050
Bovine serum albumin (BSA)	Sigma Aldrich	A9418-500G
Citric acid	Sigma Aldrich	251275-500G
Collagenase Type 1 (Collagenase A)	Worthington	LS004197
Collagenase Type 4	Worthington	LS004189
Dimethylsulphoxide	Bio Scientific	RES2166D-A102X
Dispase I	Roche	4942086001
Dispase II	Roche	165859
D-luciferin	Gold Biotechnology	115144-35-9
DNase	Sigma Aldrich	D4527
EDTA (ethylenediaminetetraacetic acid)	Astral Scientific	AM0105

Reagent	Source	Catalogue Details
EGTA (ethylene glycol bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid)	Merck	324626.25
Ethanol	Merck	1009836010
FITC-dextran	Sigma Aldrich	52471-1G
Fluorogold antifade mounting medium	Life Technologies	P36930
Formalin (10% neutral buffered)	Sigma Aldrich	HT501128
Glutamax	Life Technologies	35050079
Hank's Balanced Salt Solution (HBSS)	Life Technologies	14175095
Hepes pH 7.4	Sigma Aldrich	H0887-100ML
Hydrogen peroxide, 30%	Merck	1072091000
Isopropanol	Merck	1096342500
Matrigel™	BD Biosciences	Standard
Methanol	Merck	1060182500
O.C.T. compound	TissueTek	4583
Polybrene	Sigma Aldrich	107689
Polyvinyl alcohol	Sigma Aldrich	P8136-250G
Sodium chloride	Astral Scientific	AMX190
Sodium citrate	Sigma Aldrich	W302600-1KG-K
Sodium pyruvate	Life Technologies	11360070
Tissue culture plasticware	BD Biosciences & Sarstedt	Various
Tris base	Astral Scientific	AM0497
Trypan Blue	Life Technologies	15250061
Trypsin 0.05%	Life Technologies	25300120
Trypsin 0.25%	Sigma Aldrich	T4549-100ML
Tween-20	Merck	US1655205-250ML
Tyramide signal Amplification (TSA) kit	Perkin Elmer	NEL700001KT
Xylene	Sigma Aldrich	534056-500ML

2.1.1.1. Eosin

A working solution of 0.1% (w/v) eosin was made by diluting 10 ml of a 10 mg/ml stock solution of alcohol soluble Fluka eosin (Sigma Aldrich, #45230) into 90 ml MQ H₂O and adding 2 g calcium chloride. The solution was filtered through Whatman No. 1 paper prior to use.

2.1.1.2. Mayer's haematoxylin

To make a 1 L working solution the following were added: Haematoxylin (Sigma, Cat. H3136; 1g), MQ H₂O (1 L), potassium sulphate (50 g), sodium iodate (0.2 g), citric acid monohydrate (1 g) and chloral hydrate (50 g).

The haematoxylin, potassium sulphate and sodium iodate were dissolved in the MQ H₂O overnight at room temperature. The chloral hydrate and citric acid were then added and the mixture boiled for 5 minutes, cooled and filtered through Whatman No. 1 prior to use.

2.1.1.3. Scott's tap water

Scott's tap water was prepared by dissolving 7 g sodium bicarbonate (Sigma Aldrich) and 40 g magnesium sulphate in 2 L MQ H₂O.

2.1.2. Buffers and media

2.1.2.1. Flow cytometry buffers

To enable specific labelling of freshly isolated cells for flow cytometry, samples were resuspended at $2 - 4 \times 10^6$ cells/ml in freshly filter-sterilised blocking buffer consisting of 2% (v/v) fetal calf serum (FCS) and 2% (w/v) bovine serum albumin (BSA) in PBS⁺. Labelling buffer consisted of 2% (w/v) BSA in PBS⁺ and was used to dilute antibodies and wash cells after each step.

2.1.2.2. Immunohistochemistry buffers

2.1.2.2.1. Citrate retrieval buffer

9 ml of 0.1 M citric acid pH 6.0 stock solution and 41 ml of 0.1 M sodium citrate pH 6.0 stock solution was made up to 500 ml with MQ H₂O. pH was adjusted to 6.

2.1.2.2.2. Tris retrieval buffer

10 mM TRIS base, 1 mM EDTA solution and 0.05% Tween 20 was made up in MQH₂O. pH was adjusted to 9.

2.1.2.2.3. Trypsin retrieval buffer

260 mg calcium chloride and 50 mg Trypsin was made up to 200 mL with 0.05 M TRIS (pH 8.0).

2.1.2.2.4. Peroxidase blocking buffer

Hydrogen peroxide was added to a final concentration of 3% in methanol or MQ H₂O.

2.1.2.2.5. Tris and sodium chloride (TNB) blocking buffer

0.1 M TRIS-HCl, pH 7.5 and 0.15 M sodium chloride was made up to 500 ml MQ H₂O. 0.5% Blocking Reagent (from TSA kit) was added slowly in small increments while stirring. Buffer was then heated gradually to 60°C with continuous stirring to dissolve the Blocking Reagent and aliquoted and stored at -20°C for long term use. TNB buffer was only used while staining for CD31 (BD; #553370).

2.1.2.2.6. Tris and Tween (TNT) washing buffer

100 mL 1 M TRIS, pH 7.5 and 30 mL 5 M sodium chloride was made up to 1000 mL with distilled water and mixed by vigorous shaking. 500 µL of Tween20 was added to this buffer and mixed well by shaking further. TNT washing buffer was only used while staining for CD31 (BD; #553370).

2.1.2.2.7. Blocking buffer

1% (w/v) BSA in PBS with 0.05% (v/v) Tween-20 (PBS-T) was used as a blocking buffer to prevent non-specific binding of antibodies and to dilute primary, isotype and secondary antibodies unless otherwise indicated.

2.1.2.2.8. Washing buffer

PBS with 0.05% (v/v) Tween-20 (PBS-T) was used as a washing buffer between staining steps unless otherwise indicated.

2.1.2.2.9. Sorensen's buffer (for electron microscopy)

1 M stock solution of Sorensen's buffer was made up with 1 M disodium phosphate and 1 M potassium dihydrogen phosphate and pH adjusted to 7.2.

2.1.2.3. Cell culture buffers and media

2.1.2.3.1. Trypsin-EGTA (TEG) buffer

40 mg ethylene glycol bis (2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) and 10 mg polyvinyl alcohol were added to in 90 mL warmed PBS. 10 ml of 2.5% Trypsin was added further upon cooling. pH was adjusted to 7.4.

2.1.2.3.2. Antibiotics for cell culture reagents

For 100mL of 500X penicillin-gentamycin (pen-gent), 60mg penicillin (Commonwealth Serum Laboratories, Parkville, Australia) and 800mg gentamicin (Life Technologies, Gaithersburg, MD, U.S.A.) were dissolved in a final volume of 100ml MQ H₂O.

The 500X pen-gent solution was filter-sterilised and stored at -20°C in 1ml aliquots. Tissue culture media used for the cultivation of primary cells were supplemented with 2X pen-gent (2.4µg/ml and 32µg/ml, respectively). All other reagents utilised in the isolation and preparation of live cells for culture were supplemented with 5X pen-gent (6µg/ml and 80µg/ml, respectively).

2.1.2.3.3. Dulbecco's phosphate buffered saline, without MgCl₂ + CaCl₂ (PBS)

To make up a 5 L solution, the following were combined: KCl (1 g), KH₂PO₄ (1 g), Na₂HPO₄ (5.75 g) and NaCl (40g). Approximately 2 L of MQ H₂O was poured into a 5 L flask. The above powdered ingredients were added and the volume brought up to 5 L with MQ H₂O and stirred well with a metal spoon until dissolved. The solution was checked to ensure pH 7.0-7.5 and osmolarity of 270±13.5mM and filter-sterilised using a 20 L pressure tank and Sartobran filter. Sterilised PBS was stored at 4°C. PBS supplemented with 1X pen-gent (1.2 µg/ml, 16 µg/ml) and 10X pen-gent (12 µg/ml, 160 µg/ml) was referred to as PBS⁺ and PBS⁺⁺⁺ respectively.

2.1.2.3.4. Dispase solution for separation of the epithelium

Neutral dispase II powder was made up to 6 mg/ml in PBS⁺⁺, filter-sterilised and chilled to 4°C for immediate use.

2.1.2.3.5. Collagenase-dispase solution for enzymatic digestion of dermal cells

Collagenase type IV and dispase were added at 3 mg/ml and 4 mg/ml concentrations respectively in a total volume of 10 ml PBS⁺. Once dissolved, the solution was filter-sterilised and used immediately.

2.1.2.3.6. Cell Culture media

All tissue cultures reagents utilised to harvest and manipulate live primary cells were supplemented with 5X pen-gent. All media used for freshly isolated primary cultures was also supplemented with Diflucan® anti-fungal solution. All media were stored

in dark at 4°C and used within 30 days. Required volumes were aliquoted and pre-warmed for immediate use.

2.1.2.3.6.1. DMEM-10

DMEM (Life technologies; #A14430-01) powder with 4.5 g/L D-glucose, 4.5 g/L L-glutamine and 110 mg/L sodium pyruvate was made up in MQ H₂O as per manufacturer's instructions. Sodium bicarbonate was added to 74 g/L and the solution filter-sterilised using a 20 L pressure tank and Sartobran filter and stored in darkness at 4°C. For immediate culture, heat-inactivated FCS (45 minutes at 56°C) was added to 10% (v/v). DMEM-10 was used in this study for culturing VLA-1^{dim} fibroblasts and HEK293T cells.

2.1.2.3.6.2. RPMI-1640

RPMI 1640 (Life technologies; #11875) was supplemented with 25mM HEPES buffer and filter-sterilised using a 20L pressure tank and Sartobran filter and stored in darkness at 4°C. For immediate culture, heat-inactivated FCS (45 minutes at 56°C) was added to 10% (v/v). RPMI-1640 was used in this study for culturing the ovarian cancer cell lines OVCAR-5 and OVCAR-8.

2.1.2.3.6.3. EGM-2

Endothelial Basal media (EBM-2) (Lonza; #CC-3156) was supplemented with EGM-2 Singlequots™ (Lonza; #CC-4147) which comprised of hEGF, hydrocortisone, GA-1000 (Gentamicin, Amphotericin-B), VEGF, h-FGF B, IGF-1, ascorbic acid, heparin and 25ml FCS to make up EGM-2. EGM-2 was used in this study for culturing VLA-1^{bri} pericytes.

2.1.3. Mice

Female athymic nude Balb/c and NOD/SCID mice were obtained from Walter and Eliza Hall Medical Research Institute, Melbourne. Mice were housed in a pathogen-free 12 hour light-dark environment and fed *ad libitum*. 6-8 week old mice were used for tumourigenicity assays. All experiments were performed with Peter MacCallum Animal Research Ethics Committee approval (# E394 and #504).

2.1.4. Patient tissue microarrays (TMAs)

Stage III/IV high grade serous ovarian cancer patient TMAs were obtained through the Australian Ovarian Cancer Study Group (AOCS), courtesy of Professor David Bowtell, after approval by the AOCS review board. Patients underwent laparotomy for diagnosis, staging and tumour debulking and subsequently received first-line platinum based chemotherapy. Tumour material for the study was excised at the time of primary surgery, prior to the administration of chemotherapy. Surgical staging was assessed in accordance with FIGO (Fédération Internationale des Gynaecologues et Obstétristes) classification. Optimal debulking was defined as ≤ 1 cm (diameter) residual disease, and sub-optimal debulking was more than 1 cm (diameter) residual disease.

The patients included in this study specifically had Stage III or IV serous invasive disease and received treatment including a platinum-based agent (carboplatin or cisplatin) administered within 6 weeks of surgery and completed (at least 4 cycles of platinum treatment) unless disease progression was evident while on first line of treatment. TMAs constituted 4 μ m cores of formalin-fixed, paraffin-embedded, high-grade serous ovarian cancer biopsy tissues. TMA versions V2.0, V4.0 and V5.0 were used for this study.

2.1.5. Human neonatal samples

Neonatal (1-2 weeks old) foreskin tissues were collected from routine clinical circumcision surgeries from Circumwise, Vermont South, Victoria, after informed consent was obtained in writing from parents or legal guardians. All experimentation with human neonatal tissue was approved by the Peter MacCallum Cancer Centre Human Research Ethics Committee (project 03/44). For the purposes of this project, neonatal foreskin tissue was used to generate primary human neonatal VLA-1^{bri} pericytes and VLA-1^{dim} fibroblasts.

2.1.6. Cell lines

VLA-1^{dim} and VLA-1^{bri} cells were generated as previously described (Paquet-Fifield et al., 2009) and described in *Section 2.2.2.3*. OVCAR-5 is an aggressive serous ovarian cancer cell line derived from a stage III biopsy sample of a 57-year old patient before cisplatin treatment (Hamilton et al., 1984a, 1984b). OVCAR-8

(Schilder et al., 1990) is a relatively non-aggressive serous ovarian cancer cell line derived from a patient who underwent cisplatin treatment. OVCAR-8 and OVCAR-5 cell lines were kindly provided by the Bowtell Laboratory, Peter MacCallum Cancer Centre, Melbourne. HEK293T cells were kindly provided by Dr. Camerone Johnstone, Anderson Laboratory, Peter MacCallum Cancer Centre, Melbourne, and were used in this study for lentiviral production. Cells were maintained in their appropriate culture medium and incubated at 37 °C with 5% CO₂. Cells were split at about 70 - 90% confluency, using 0.05% trypsin after washing with PBS^{-/-} supplemented with 0.5% 0.5M EDTA.

2.1.7. Antibodies

Tables 2.2 and 2.3 contain a list of antibodies employed in this study.

2.1.8. Lentiviral Vector

The replication defective 3rd generation lentiviral vector employed in this study was promoted by co-transfection of pFUGW, HIV-1 packaging vector and VSV-G envelope glycoprotein expressing green fluorescent protein (GFP) from jellyfish *Aequorea victoria* in pKs-CMV-GFP derived from pEGFP-C1 (Clontech Inc.) and luciferase from firefly.

2.1.9. Software

SPOT advanced software 5.1 (Spot Imaging Solutions, Inc.) was used to capture light microscopic images used in this study using the Olympus BX-61 light microscope. The Leica TCS SP5 microscope was used for confocal microscopy. Transmission electron microscopy (TEM) was carried out on a JEOL 1011 and TEM images were acquired using the iTEM software. Leica SCN400 slide scanner (Leica camera) was employed to scan patient TMAs. Photoshop CS 6.0 (Adobe Inc.), Metamorph (Molecular devices) and Image J 1.47 (NIH software) were used for morphometric analyses of photomicrographs. For statistical analyses of patient gene expression data, the biostatistics software R (version 3.2) and several bioconductor packages were used (Crispin J Miller; Gentleman et al., 2004; R Core Team, 2013). All other statistical analyses were performed using the Prism v5.02 (GraphPad Inc.). Flowlogic (Inivai Technology Inc.) was used for analysing flow cytometry data.

Principal Component Analysis (PCA) was carried out using PRISMTC PCA 3D Visualiser (<http://www.prismtc.co.uk/pca-3d-visualiser/>).

2.1.10. Ethics

Experimental use of human neonatal tissue was approved by the Peter MacCallum Human Research Ethics Committee (#03/44) after obtaining appropriate consents from parents/legal guardians. All animal experiments employed in this study were approved by the Peter MacCallum Animal Research Ethics Committee (E394 and E504). Use of lentiviral vectors were approved under the NLRD dealing #09/2007. Use of ovarian cancer patient primary biopsy samples in the form of tissue microarrays (TMAs) were approved by the Australian Ovarian Cancer Study (AOCS) review board.

Table 2.2. List of primary antibodies employed in this study.

Antigen	Type	Product description	Source/Catalogue	Dilution	Retrieval method	Application
GFP	unconjugated	rabbit IgG polyclonal	Abcam; #ab290	1:1000	Heat mediated; Tris buffer pH 9.0	IHC-P, IFC-P
Ki67	unconjugated	mouse IgG1 clone MIB-1	Dako; #M7240	1:100	Heat mediated; Tris buffer pH 9.0	IHC-P, IFC-P
α SMA	unconjugated	mouse IgG2a clone 1A4	Dako; #M085129	1:200	Heat mediated; Tris buffer pH 9.0	IHC-P, IFC-P, IFC-Fixed cells
Human mitochondrial protein	unconjugated	mouse IgG1 clone 113-1	Millipore; #MAB1273	1:500	Heat mediated; citrate buffer pH 6.0	IHC-P
CD31	unconjugated	rat IgG2a clone MEC 13.3	BD; #553370	1:50	Enzyme retrieval; trypsin, 37°C, 20'	IHC-P
PDGFR β	unconjugated	rabbit IgG polyclonal	Abcam; #ab32570	1:100	Heat mediated; citrate buffer pH 6.0	IHC-P, IFC-Fixed cells
CD34	unconjugated	mouse IgG1 clone QBEnd-10	Dako; #15632	neat	Heat mediated; citrate buffer pH 6.0	IHC-P
CD34	unconjugated	rat IgG2a clone MEC 14.7	Abcam; #ab8158	1:50	Heat mediated; Tris buffer pH 9.0	IHC-P, IFC-P
CD73	unconjugated	rabbit IgG polyclonal	Abcam; #ab71822	1:50	Heat mediated; Tris buffer pH 9.0	IFC-P, IFC-Fr
Sca-1	unconjugated	rat IgG2a clone LEW	BD; #557403	1:200	Heat mediated; Tris buffer pH 9.0	IFC-P, IFC-Fr
CD105	unconjugated	goat IgG polyclonal	R&D systems; #AF1320	1:20	Enzyme retrieval; trypsin, 37°C, 45'	IFC-P, IFC-Fr
CXCL12/SDF-1	unconjugated	mouse IgG1 clone 79018	R&D systems; #MAB350	1:100	Heat mediated; Tris buffer pH 9.0	IFC-P, IFC-Fr

Antigen	Type	Product description	Source/Catalogue	Dilution	Retrieval method	Application
Ki67	unconjugated	rabbit IgG polyclonal	Novocastra; #301119	1:2000	Heat mediated; Tris buffer pH 9.0	IFC-P, IFC-Fr
E-cadherin	unconjugated	mouse IgG2a clone 36/E-Cadherin	BD; #610182	1:100	Heat mediated; citrate buffer pH 6.0	IFC-P, IFC-Fr
EpCam	unconjugated	mouse IgG1 clone VU1D9	Cell Signalling; #2929	1:500	Heat mediated; citrate buffer pH 6.0	IFC-P, IFC-Fr
HIF1 α	unconjugated	rabbit IgG polyclonal	Novus; #NB100-449	1:75	Heat mediated; Tris buffer pH 9.0	IHC-P
NG2	unconjugated	mouse IgG1 clone LHM	R&D systems; #MAB2585	1:50	NA	IFC-Fixed cells
FAP α	unconjugated	sheep IgG polyclonal	R&D systems; #AF3715	1:20	NA	IFC-Fixed cells, FC-analysis
VLA-1	FITC-conjugated	mouse IgG1 clone TS 2/7	Serotec; #MCA1133FT	1:10	NA	FC - cell sorting
CD45	PE-conjugated	mouse IgG1 κ clone HI30	BD; #555483	1:100	NA	FC - cell sorting, FC-analysis
CD34	APC-conjugated	mouse IgG2a κ clone 561	Biolegend; #343607	1:800	NA	FC-analysis
CD73	PE-conjugated	at IgG2a κ clone TY/23	BD; #561014	1:100	NA	FC-analysis
CD90	unconjugated	mouse IgG1 κ clone 5E10	BD; #555592	1:100	NA	FC-analysis
CD105	PE-conjugated	mouse IgG1 κ clone 266	BD; #560839	1:100	NA	FC-analysis
CD146	unconjugated	goat IgG polyclonal	R&D; #AF932	1:100	NA	FC-analysis

FC=Flow cytometry; IFC=Immunofluorescence; IHC = Immunohistochemistry, Fr = Frozen; P= Paraffin

Table 2.3. List of secondary antibodies employed in this study.

Reagents/Kits	Corresponding primary	Application	Source & catalogue details	Dilution
Rabbit Impress™ kit	GFP, HIF-1α, PDGFRβ	IHC-P, IFC-fixed cells	Vector Laboratories; #MP-7401	neat (pre-diluted)
Rat Impress™ kit	CD34	IHC-P	Vector Laboratories; #MP-7444	neat (pre-diluted)
Mouse Impress™ kit	CD34, αSMA	IHC-P	Vector Laboratories; #MP-7402	neat (pre-diluted)
Mouse Vectastain™ ABC kit	Human mitochondrial protein	IHC-P	Vector Laboratories; #PK-6102	neat (pre-diluted)
Goat anti-rabbit Alexa-Fluor™ 488	GFP, Ki67	IFC-P, IFC-Fr	Life Technologies; #A-11008	1:200
Goat anti-rabbit Alexa-Fluor™ 555	GFP, Ki67	IFC-P, IFC-Fr	Life Technologies; #A-21428	1:200
Goat anti-rat Alexa-Fluor™ 488	CD34	IFC-P, IFC-Fr	Life Technologies; #A-11006	1:200
Goat anti-rat Alexa-Fluor™ 555	CD34	IFC-P, IFC-Fr	Life Technologies; #A-21434	1:200
Goat anti-mouse IgG1 Alexa-Fluor™ 488	Ki67, EpCam, CXCL12/SDF-1	IFC-P, IFC-Fr	Life Technologies; #A-21121	1:200
Goat anti-mouse IgG2a Alexa-Fluor™ 568	αSMA, E-cadherin	IFC-P, IFC-Fr	Life Technologies; #A-21134	1:200

Reagents/Kits	Corresponding primary	Application	Source & catalogue details	Dilution
Goat anti-mouse IgG2a Alexa-Fluor™ 647	αSMA	IFC-P, IFC-Fr	Life Technologies; #A-21235	1:200
Donkey anti-goat Alexa-Fluor™ 488	CD105	IFC-P, IFC-Fr	Life Technologies; #A-11055	1:200
Goat anti-mouse IgG1 Alexa-Fluor™ 647	CD73, CD90, CD105	FC-analysis	Life Technologies; #A-21240	1:200
Sheep/goat biotin	CD146, FAPα	FC-analysis, IFC-fixed cells	Sigma Aldrich; #B3148	1:300
Streptavidin-APC	sheep/goat biotin	FC-analysis	BD; #349024	1:100
Streptavidin-Texas Red	sheep/goat biotin	IFC-fixed cells	BD; #551487	1:100

FC=Flow cytometry; IFC=Immunofluorescence; IHC = Immunohistochemistry, Fr = Frozen; P= Paraffin

2.2. Methods

2.2.1. Cell isolation, culture and cryopreservation

2.2.1.1. Trypsinisation and passage

Culture media was aspirated using a sterile Pasteur pipet. Adherent cell monolayers were washed once or twice with a small volume of pre-warmed PBS without Ca^{2+} and Mg^{2+} to remove any residual FCS that may inhibit the action of trypsin. An appropriate volume of 37°C trypsin/EDTA solution was added to cultures to cover adhering cell layers and placed in a 37°C incubator for 1-2 minutes. Plates were then gently tapped on the countertop to dislodge cells. Trypsin activity was quenched by adding an equivalent amount of pre-warmed medium containing FCS. Cell suspensions were centrifuged (5 minutes at 400g, 4°C), resuspended in appropriate media and equal volumes split (passaged) into new sterile cell culture flasks.

2.2.1.2. Cell counting

The hemacytometer slide and coverslip were cleaned with 70% alcohol. Cells to be counted were detached from the surface of culture flasks using trypsin/EDTA and diluted as needed to obtain a uniform homogeneous suspension. 40 µl of cell suspension was mixed well with 40 µl of trypan blue stain and a sterile Pasteur pipet was used to transfer 10 µl of this suspension to edge of the hemacytometer counting chamber. A hand-held cell counter was used to count cells in each of the four grids and cell number per ml of suspension determined by the following calculations:

$$\text{Cells/ml} = \text{average count per square} \times 2 \text{ (dilution factor)} \times 10^4$$

Total cell number = cells/ml $\times$ total original volume of cell suspension from which sample was taken

For determining cell viability, total numbers of dead (blue) cells were counted.

Percentage of viable cells was calculated as follows:

$$(\text{Total \# cells} - \text{Total \# dead cells}) / \text{Total \# cells} \times 100$$

2.2.1.3. Dermal cell isolation

Dermal tissue digestion was carried out as described previously (Paquet-Fifield et al., 2009). Briefly, freshly removed neonatal human foreskin tissue was washed in 70% ethanol and rinsed twice in fresh PBS⁺⁺⁺, trimmed of subcutaneous adipose tissue, cut into pieces of no more than 2 mm², and immersed in 4 mg/ml dispase II solution overnight at 4°C. After removal of the epidermis following overnight incubation, dermal tissue was gently scraped to ensure complete removal of any residual epidermal tissue, washed in two changes of fresh PBS⁺⁺⁺ and minced (~5 mm² pieces) with a fresh #22 scalpel blade on inverted lid of a sterile petri dish. The minced tissue was added to 10 ml pre-warmed 3 mg/ml collagenase and 4 mg/ml dispase solution and incubated for 2 hours at 37°C, with gentle agitation every ~15 minutes to promote digestion. Collagenase-dispase digestion was quenched by adding an equal volume of DMEM with 2% FCS. The digested dermis fragments were then filtered through a 70 µm cell strainer followed by a 40 µm filter to remove undigested tissue and debris. The resulting cell suspensions were centrifuged (5 minutes at 400g, 4°C) and resuspended in FACS blocking buffer at approximately 10⁶ cells/ml for counting. Cell suspensions were kept on ice throughout. All tissue and cell samples were handled with autoclaved instruments using aseptic techniques in a Class II Biological Safety Cabinet.

2.2.1.4. Cryopreservation

For cryopreservation, cells were at 80-90% confluency and resuspended in 90% FCS/10% dimethylsulfoxide (DMSO) and aliquoted into cryovials. Cryovials were placed in a Nalgene “Mr Frosty” freezing container with 100% isopropyl alcohol and transferred immediately to a -80°C freezer. After 24 hours, cryovials were removed from the container and stored at -80°C or transferred to liquid nitrogen for prolonged storage. To thaw, frozen cryovials were immersed rapidly in a 37°C water bath. The appropriate pre-warmed growth medium was carefully added to the suspension to dilute the DMSO. Cells were then resuspended in fresh medium and centrifuged at 1400 g for 5 mins. The supernatant was decanted, the pellet resuspended in fresh medium and transferred to an appropriate tissue culture flask for immediate culture.

2.2.1.5. Lentivirus production

HEK293T cells were plated at 2×10^5 cells per 60 mm plate in 3 ml DMEM containing 10% FCS, and allowed to adhere and grow overnight at 37°C in a humidified incubator containing 5% (v/v) CO₂. HEK293T cells were transfected using FuGENE 6 (Roche Diagnostics, Mannheim, Germany) at a FuGENE : DNA ratio of 3:1. The appropriate amount of FuGENE 6 was added to serum free media and incubated for 5 minutes at room temperature. The FuGENE/media mix was then added to the DNA mix (equalised for concentration with empty vector) and incubated for 15 minutes at room temperature. 100 µl of the FuGENE/DNA/media mix was then added drop-wise per 60 mm plate and incubated at 37°C for 24-48 hours.

2.2.1.6. Lentiviral transduction

One day prior to the procedure, target cells (pericytes, OVCAR-5 or OVCAR-8 cells) were plated into 6-well plates at a concentration of approximately 1×10^5 cells per well. Virus was concentrated by placing viral supernatant into each sterile tube and loading into a SW28 rotor. The harvested viral supernatants were centrifuged at 1000 g at 25°C for 90 minutes in an ultracentrifuge. After spinning, the media was aspirated and the viral pellet resuspended in 1mL of EGM-2 or RPMI-10 media. For transduction, 2.5 µl (10 mg/ml) of polybrene was diluted in 2 ml of media and added to 500 µl of concentrated viral supernatant. This infection cocktail was applied to each well containing the target cells and incubated for 12 hours at 37°C with 5% CO₂. The next day, virus-containing medium was aspirated off and replaced with fresh media. Cells were checked on a fluorescence microscope for GFP transduction to assess transduction efficiency and grown in culture for up to two passages before sorting.

2.2.1.7. In vitro assays

For the *in-vitro* migration assays, p4 pericytes (1.5×10^5 in 200µl serum-free epidermalization medium) were added to the lower chamber of transwell culture inserts (12 mm polycarbonate inserts; BD Biosciences). The upper chambers of transwells were seeded with OVCAR-5-GFP cells until they were 80% confluent.

Inserts were lowered into chambers containing 600 µl of serum-free epidermalization media. After 5 hours incubation at 37 °C in 5% CO₂, the inserts were removed and fixed in 4% paraformaldehyde (w/v). The membrane was stained with DAPI (1 µg/ml in PBS), mounted onto a glass slide to count cells that had traversed to the basal side of the insert. Five fields, selected at random, were scored at x 200 magnification. Total number of OVCAR-5-GFP cells were counted and represented as fold migration.

The invasion assay was performed in a 96-well format consisting of a Boyden chamber-like set up made up of two chambers separated by a filter membrane (8 µm polyethylene, Cultrex). p4 pericytes (2×10^4 cells in 100µl in serum-free media) were seeded into the lower well to act as a chemoattractant while GFP⁺OVCAR-5 cells (5×10^4 in 50µl serum-free media) were mixed well with growth factor reduced Matrigel (BD Biosciences) and pipetted into the upper chamber. The media in the lower chamber of the transwell was replaced with fresh epidermalization media at the start of the assay and incubated at 37 °C in 5% CO₂ for 24 hours. Invasion was quantified by placing cell dissociation/Calcein AM solution in the bottom chamber, detaching the migrated cells from the filter. Calcein AM is internalized by the dissociated cells and intracellular esterases cleave the acetomethylester (AM) moiety. Fluorescent free calcein was then quantified to determine the number of cells that had invaded and plotted on a standard curve. Fluorescence was detected at excitation 540 nm and emission 610 nm using a BMG Labtech (Offenburg, Germany) POLARstar Optima fluorescent plate reader.

2.2.2. Flow cytometry

2.2.2.1. General considerations and machine set up

Fluorescence activated cell sorting (FACS) was conducted on a FACS Diva™ (BD Biosciences) flow cytometer equipped with a Spectraphysics I90 UV laser (355nm @ 50mW), a Coherent Sapphire laser (488nm @ 200mW) and a Spectraphysics I70 Spectrum laser (647nm @ 200mW).

The BD FACSCanto™ II system consisting of an excitation source with up to three lasers - blue (488-nm, air-cooled, 20-mW solid state), red (633-nm, 17-mW HeNe), and violet (405-nm, 30-mW solid state) was used for flow cytometric analysis.

The flow cytometers were operated with the aid of Cellquest of BD FACS Diva™ software version 4.1 (BD Biosciences).

Photodiode (light scatter) and photomultiplier tube (fluorescence) voltage settings were adjusted using control samples stained for viability only; compensation adjustments for overlapping emission signals were made using single-colour positive controls. Forward scatter (FSC), side scatter (SSC) and diamidino-phenyl-indole (DAPI) emission signals were displayed in linear mode; all other fluorochromes were displayed in logarithmic mode. Cells were analysed and sorted with a sheath fluid pressure of 20 psi with a throughput of $\sim 1 \times 10^4$ cells per second. For each sample, a minimum of 1×10^4 events were collected in list mode and saved for subsequent analysis using Flowlogic® (Inivai software).

2.2.2.2. Sample preparation

Cells were suspended in PBS with 2% FCS (FACS staining buffer) at a concentration of 1×10^7 /ml for cell sorting, and 5×10^5 /ml for analysis. Controls for each cell sorting/analysis group comprised of an unstained negative control, a DAPI control for assessing cell viability, isotype controls, and single colour controls for each antibody. Antibodies were added to each tube at the appropriate dilutions outlined in Table 2.2. Primary antibodies and corresponding isotype controls were incubated at 4°C for at least 45-60 minutes with gentle agitation every 15 minutes. After two washes with antibody labelling buffer, the secondary antibody (where applicable) was added and incubated at 4°C for at least 30-45 minutes with gentle agitation every 15 minutes. After two washes with FACS buffer, cells were re-suspended in antibody labelling buffer and DAPI was added (1:100 dilution to 2 µg/ml final concentration) 15 minutes prior to flow cytometry to all sample tubes barring unstained controls. Cells to be sorted were then passed through a 40 µm cell strainer on a filter-capped 5 ml polystyrene FACS tube (BD Biosciences, Cat. 352235), collected in fresh FACS tubes and sorted on the FACS Diva™ cell sorter (BD Biosciences) or analysed on a Canto™ II (BD Biosciences). Sorted fractions were routinely re-analysed for purity.

2.2.2.3. Dermal cell sorting using VLA-1

Sorting for VLA-1^{bri} pericytes and VLA-1^{dim} fibroblasts was carried out as described previously (Paquet-Fifield et al., 2009). Briefly, following tissue digestion and separation of the dermal and epidermal fractions, the dermal fraction was stained with VLA-1 and CD45. CD45 was used to detect and gate out haematopoietic cells, prior to selecting the VLA-1^{bri} and VLA-1^{dim} populations. Isotype controls used for the experiment were mouse IgG1 (Dako; #X0931) and mouse IgG2a (BD; #551074). Sorting and analysis was performed in the background of the fluorescent viability dye DAPI or propidium iodide (PI). The collected fractions were reanalysed to confirm sort purity.

2.2.3. Immunohistochemistry and immunofluorescence

2.2.3.1. Histology: haematoxylin and eosin (H&E) staining

Paraffin was removed from formalin-fixed, paraffin embedded (FFPE) sections with xylene (2 × 5 minutes) and tissue sections rehydrated by immersion in a graded series of alcohol (absolute 3 × 1 minute, 90% 1minute, 70% 1minute), followed by a wash in running tap water (1minute). Slides were immersed in Mayer's haematoxylin for approximately 4 minutes and then washed in running tap water for 2 minutes. Excess non-specific stain was removed by 3 dips in 70% ethanol followed by a wash in running tap water (1minute) and the nuclear stain blued in Scott's tap water (1 minute), followed by another wash in running tap water (1 minute). Sections were counterstained with 0.1% (w/v) eosin solution for roughly 3 minutes and rinsed with 2 dips in running tap water. Stained tissue was then dehydrated through a graded series of alcohol (70% 2 dips, 90% 4 dips, absolute 4 × 1minute), cleared in xylene (3 × 4 minutes) and mounted in Aquatex™.

2.2.3.2. Immunohistochemistry

For immunohistochemical staining of xenograft tumour sections and patient TMAs harvested tissue sections were fixed in 10% neutral buffered formalin, dehydrated, and embedded in paraffin (FFPE). Sections were then de-paraffinised (as described

in the previous section) and processed for antigen retrieval by incubation in a pressure cooker at 125°C for 3 minutes in 0.1 M Tris/HCL pH 9.0 or 10 mM citrate buffer pH 6. Individual sections were outlined with a PAP pen (Dako) to prevent loss of antibody solution placed on the tissue specimen during staining procedures. Endogenous peroxidase were inactivated by immersing slides in a solution of 30% (v/v) H₂O₂ diluted 1:10 in 100% methanol (3.0% v/v final) for 15 minutes at room temperature, followed by blocking in 1% BSA in PBS for 1 hour at room temperature. Primary antibodies were also diluted in 1% BSA in PBS at the dilutions indicated in Tables 2.2 and incubated for 1 hour at room temperature or overnight at 4°C, followed by incubation with the corresponding secondary antibody (Impress or Vectastain kit, VectorLabs; Table 2.3) for 1 hour at room temperature. The sections were then developed by DAB staining according to the manufacturer's instructions, followed by counterstaining with haematoxylin and mounted with Aquatex (Merck). Washes between steps were carried out using PBS-T.

For CD31 staining, antigen retrieval was carried out by incubating sections with the CD31 antibody for 20 minutes in trypsin at 37°C - CaCl₂ buffer (260 mg CaCl₂ and 50 mg trypsin made up to 200 mL with 0.05M pH 8.0 Tris buffer), blocked in TNB blocking buffer, stained with appropriate antibodies using the Tyramide Signal Amplification kit (Perkin Elmer) according to manufacturer's instructions and visualised with AEC substrate. Briefly, post primary incubation at 4°C overnight, sections were incubated in diluted (1:100) streptavidin horseradish peroxidase (ST-HRP) for 30 minutes at room temperature and then incubated in diluted (1:50) biotinylated tyramide signal amplification (TSA) solution for 5 minutes, rinsed and incubated again in ST-HRP for 30 minutes at room temperature. Post TSA amplification, 2 drops of buffer stock solution, AEC stock solution and H₂O₂ solution (from the AEC kit) were added sequentially while mixing thoroughly. Sections were then incubated in the freshly made up AEC solution until desired stain intensity was observed, rinsed in MQ H₂O, mounted in Aquatex (Merck) and left to dry overnight. For CD31 staining, washes between steps were carried out with TNT wash buffer.

2.2.3.3. Immunofluorescence

For immunofluorescent staining of xenograft tumours, formalin-fixed sections were de-paraffinised and processed for antigen retrieval by incubation in a pressure cooker at 125°C for 3 minutes in 0.1 M Tris/HCL pH 9.0 or 10 mM citrate buffer pH 6. Alternatively, tissue snap-frozen in liquid nitrogen and stored as blocks in O.C.T. (TissueTek) were brought to -20°C and cryosections of 5 µm thickness cut in a Leica CM3050 cryostat (Leica Microsystems). Cryosections were brought to room temperature prior to staining, air-dried and then fixed by immersion in acetone at -20°C for 10 minutes. Sections were then blocked in 10% BSA and incubated with the appropriate primary antibody overnight at 4°C followed by incubation with the corresponding fluorescent secondary antibody for 1 hour at room temperature together with DAPI. Washes between steps were carried out using PBS-T. Sections were mounted in fluorescent anti-fade mounting medium (Life technologies) and stored in the dark at 4°C. All fluorescent reagents were handled with minimal exposure to light.

2.2.3.4. Transmission electron microscopy (TEM)

Edges of tumour tissue (~1-2 mm³) were carefully cut on ice post-harvest. Tissues were washed in PBS three times and incubated in 2% paraformaldehyde in 0.08 M Sorensen's buffer overnight at 4°C. Tissue pieces were then rinsed in 0.08 M Sorensen's buffer and post-fixed in 2% osmium tetroxide in 0.15 M Sorensen's buffer. Post-fixed tissue pieces were processed for embedding into Spurr's resin using a Lynx II automatic tissue processor (Electron Microscopy Sciences, Inc.) through the steps described in Table 2.4.

Table 2.4. Protocol for sample processing in a Lynx II automatic tissue processor for transmission electron microscopy.

Treatment	Time	Temperature	Agitation	Vacuum
Distilled water wash	20 minutes	RT	Yes	No
Distilled water wash	20 minutes	RT	Yes	No
Distilled water wash	20 minutes	RT	Yes	No
50% ethanol	1 hour	RT	Yes	No
70% ethanol	1 hour	RT	Yes	No

Treatment	Time	Temperature	Agitation	Vacuum
90% ethanol	1 hour	RT	Yes	No
95% ethanol	1 hour	RT	Yes	No
100% ethanol	1 hour	RT	Yes	No
100% ethanol	1 hour	RT	Yes	No
100% acetone	1 hour	RT	Yes	No
100% acetone	1 hour	RT	Yes	No
1:1 Acetone/Spur's resin	2 hours	RT	Yes	No
1:1 Acetone/Spur's resin	2 hours	RT	Yes	No
100% Spur's resin	3 hours	RT	Yes	Yes
100% Spur's resin	3 hours	RT	No	Yes
100% Spur's resin	3 hours	RT	No	Yes

2.2.4. *In vivo* assays

2.2.4.1. *Tumourigenicity assays*

Cells from 80-90 % confluent flasks were trypsinised and re-suspended in a 1:1 ratio of PBS to Martrigel™ (5 µg/ml) on ice (i.e. 8×10^6 OVCAR-5 cells and 8×10^5 pericytes/fibroblasts per 100 µl). A Hamilton syringe with a 26G needle was used to inject 100 µl of the cell suspension under the flank of Balb/c nude mice subcutaneously. Once tumours were palpable, their growth was measured with electronic callipers every alternate day, until the primary tumour size reached the ethical limit of 1500 mm³ or experimental end-point. The site of injection was the flank midway between the spine and the haunch.

2.2.4.2. *Tumour Measurement and monitoring*

Mice were kept under observation after injection of cells to ensure full recovery. Mice were observed daily for signs of stress and measurement of tumour volume used as a metric to monitor the growth of the tumour. The volume was calculated in mm³ using the following standard formula:

$$\text{Tumour volume} = (\text{width})^2 \times \text{length} \times 0.5$$

2.2.4.3. Bioluminescent imaging (BLI)

Cells injected into mice were labelled with GFP and firefly luciferase to enable *in vivo* tracking (NLRD #09/2007) using a lentiviral transduction method. Metastatic spread (and pericyte survival) was monitored by BLI using the Xenogen Real-Time Imaging System (IVIS) by tracking luciferase⁺ cells *in vivo*. The substrate for the enzyme luciferase, sodium luciferin (Gold Biotechnology Inc.) [(S)-2-(6'-hydroxy-2'-benzothiazolyl) thiazoline-4-carboxylic acid], was injected subcutaneously (on the non-tumour bearing flank) at a concentration of 150 µg per gram of mouse body weight in 100 µl sterile PBS. Mice were allowed free movement for 6-8 minutes and then anaesthetised in 2.5% Forthane® (commercial isoflurane anaesthetic; Abbott) in oxygen. Anaesthetised mice were then transferred to the inhalation nose cones on the induction chamber, mounted on heated platforms and imaged within 10-12 minutes of luciferin injection at the same exposure time for all groups of mice. For tracking metastatic spread, imaging was repeated every 7 days till experimental/ethical end-point starting Day 14 when primary tumours were palpable. Care was taken to ensure that the total time mice were under anaesthesia did not exceed three minutes. At end-point, since the luminescent signal from the primary tumours became saturated and hence risked blocking signals from smaller metastatic nodules, mice were allowed free movement for 6-8 minutes post injection, euthanised, primary tumours carefully excised, the peritoneal cavity surgically opened in order to visualise organs with metastases, and imaged with a longer exposure time.

2.2.4.4. Vascular permeability assay

Mice were injected via the tail vein with 100 µl of 10 mg/ml FITC-dextran (2,000,000 MW, Sigma) in sterile PBS one hour before sacrifice. Tumours were collected upon sacrifice, snapfrozen in liquid nitrogen, embedded in O.C.T. and stored at -80°C. Cryosections from O.C.T.-embedded tissue were cut with minimal light exposure, co-stained with the blood vessel marker CD31 and analysed directly by fluorescent microscopy. The ratio of intra:extra-vascular FITC-dextran was used as a readout of vascular permeability (Cook et al., 2012). This procedure was only conducted once per animal at experimental/ethical end-point.

2.2.5. Morphometric analyses

2.2.5.1. Staining intensity

Tumour sections derived from control and pericyte co-injected mice were analysed with the same threshold and results plotted as percent staining intensity per visual field ($\times 10$ or $\times 20$) or per unit area and analysed by Metamorph (Molecular Devices, Inc.). For relapse correlation analyses, individual biopsy sections from patient TMAs were plotted as percent staining intensity per visual field ($\times 4$) and also analysed by Metamorph.

2.2.5.2. Microvessel density (MVD) & microvessel pericyte coverage index (MPI)

For calculating microvessel density (MVD) in the xenograft tumours, the number of microvessels per visual field ($\times 20$) was counted using a modification of the Cell Counter plug-in of ImageJ. Microvessel pericyte coverage index (MPI) was calculated as the percentage of vessels per visual field ($\times 60$) that were covered by αSMA^+ cells using the Cell Counter plug-in of ImageJ.

2.2.5.3. Vessel association

For analysing vessel association of αSMA^+ cells in the xenograft tumours, the number of CD34^+ vessel-associated and stroma-associated αSMA^+ cells was counted per visual field ($\times 60$) using the Cell Counter plug-in of ImageJ and plotted as a percentage of the total number of αSMA^+ cells per unit area. For analysing vessel association of αSMA^+ cells in the patient biopsy tissue microarrays, the number of vessel-associated and stroma-associated αSMA^+ cells was counted per visual field ($\times 40$) using the Cell Counter plug-in of ImageJ and plotted as a percentage of the total number of αSMA^+ cells per unit area.

2.2.6. *In silico* analyses

For prognostic analyses using gene expression signatures of particular cell types, the biostatistics software R (version 3.2) and several bioconductor packages were used (Crispin J Miller; Gentleman et al., 2004; R Core Team, 2013). Genes differentially expressed by fibroblasts or pericytes (GEO accession number: GSE17014) (Paquet-Fifield et al., 2009) were assessed in the ovarian cancer patient data set (GEO accession number: GSE9899) (Tothill et al., 2008). A false discovery rate of 5% was defined as the signature gene set and used to compute a score in each of the tumour samples using a previously described method (Helland et al., 2011). Since the score is an objective measure of the quantity of the particular cell type, samples with high and low levels of pericytes were identified based on the median pericyte score. The clinical significance of this pericyte signature was then computed by testing the statistical significance of survival difference in the form of a Kaplan-Meier plot using Cox proportional hazards model. The same procedure was used to identify the prognostic significance of the fibroblast-specific gene set (Paquet-Fifield et al., 2009).

For analysing patient biopsy TMA samples, the morphometric ratio of the stained area to that of the total area was calculated using MetaMorph (Molecular Devices, Inc.) and was used to denote the measure of protein expression. The clinical significance of protein expression was assessed by stratifying the samples into two groups based on the median expression level and visualised using Kaplan-Meier curves. Statistical significance was assessed using Cox proportional hazards model. For prognostic correlation with the probability of relapse, the relationship between a particular protein expression and pericyte score was visualised using scatter plots. The statistical significance of the relationship was assessed using a linear regression model.

For enrichment analyses of related genes differentially overexpressed in the early versus late relapse patients, Gene Ontology terms enriched were tested among the overexpressed genes (70 genes, >2-fold, $p < 0.05$) in the early relapse group. A Fisher's exact test was applied in combination with the classic algorithm from the topGO package using query terms from Ensembl (Durinck et al., 2005, 2009). Results were corrected for multiple testing according to Benjamini-Hochberg

($p \leq 0.05$, $FDR \leq 5\%$) (Benjamini, Yoav; Hochberg, 1995), revealing 70 genes as overexpressed (2-fold, $p < 0.05$) in patients who relapsed early. Gene Set Enrichment Analysis was run in its java desktop application with data sets for GO biological processes and KEGG pathways, provided by MSigDB (v4.entrez.gmt). Default parameter settings were applied and results with $FDR > 0.25$ were dismissed (Subramanian et al., 2005).

2.2.7. Statistical analyses

Statistical analyses to compare between two groups were performed using paired or unpaired (as applicable) t-test. Statistical analyses between more than two groups were conducted using one-way ANOVA. Two-way ANOVA was used for repeated measures tests and multiple comparisons. Error bars were used to represent the standard error of the mean (SEM) or standard deviation (SD) as applicable. A non-linear regression line was fitted to the growth curve of each tumour growth experiment so that a second order polynomial quadratic equation could be formulated to determine mean tumour volume for each time-point. All statistical analyses were performed using the software Prism version 6.0 (GraphPad Inc.). A p-value < 0.05 was considered statistically significant.

Chapter 3

**Pericytes promote tumour growth in a
xenograft model of ovarian cancer
without affecting angiogenic processes**

3.1. Introduction and Rationale

Recent scientific literature has demonstrated a crucial role of CAFs in promoting tumour growth and invasiveness over normal fibroblasts in several carcinomas (Cirri and Chiarugi, 2011; Kalluri and Zeisberg, 2006). Although CAFs are a widely heterogeneous population, several seminal studies have established BM MSCs as a one of their major sources (Ishii et al., 2003; Karnoub et al., 2007; Mishra et al., 2008; Quante et al., 2011) (discussed in *Chapter 1, Section 1.1.2.2.4*).

Despite a large body of evidence of BM MSCs infiltrating the tumour site and differentiating into CAFs, the role of a locally resident MSC population, i.e. the pericytes, have remained rather understudied. This is possibly due to the absence of good markers capable of distinguishing pericytes from MSCs and myofibroblasts. Pericytes and BM MSCs are particularly indistinguishable, given that both populations express α SMA and are CD73⁺CD90⁺CD146⁺ (classic human MSC markers) (Crisan et al., 2008a, 2009; Paquet-Fifield et al., 2009). Moreover, α SMA is widely expressed in myofibroblasts and smooth muscle cells (Bergers and Song, 2005). As a consequence, “pericyte” identity is predominantly determined by morphological evidence, mostly identifiable by perivascular α SMA expression (Armulik et al., 2005; Gaengel et al., 2009).

In addition, due to their intimate association with blood vessels, pericytes have not been studied in the cancer context independent of their vasculature-supporting roles. Interestingly, previous data from our laboratory showed that VLA-1^{br} pericytes were able to enhance the proliferation of untransformed epithelial cells *in vitro*, in a 3D co-culture system devoid of vasculature (*Figure 1.6*; Paquet-Fifield et al., 2009). Moreover, the gene expression signature of pericytes was prognostic for progression-free survival in serous ovarian cancer patients (*Figure 1.8*; George et al., unpublished data). Therefore, in this study it was postulated that ***pericytes, a perivascular reservoir of MSCs, can exert CAF-like pro-tumourigenic effects without affecting the tumour vasculature.***

An *in vivo* co-injection xenograft model, previously developed in our laboratory to study the tumour-promoting effects of pericytes (*Lynn Chong Ph.D. thesis, 2013*;

Appendix B), was employed in this thesis work to investigate the role of VLA-1^{bri} pericytes in a xenograft ovarian tumour microenvironment, particularly with respect to key angiogenic processes.

Hence, the specific aims of this Chapter were to (i) validate the maintenance of key expression markers in cultured VLA-1^{dim} fibroblasts and VLA-1^{bri} pericytes (ii) dissect the specific effects of pericytes versus fibroblasts in an *in vivo* xenograft co-injection model of ovarian cancer; (iii) explore changes in angiogenic processes with the introduction of exogenous pericytes in the tumour microenvironment and; (iv) investigate the intratumoural persistence of exogenous pericytes *in vivo*, with respect to their localisation and survival.

3.2. Results

3.2.1. Cultured p4 VLA-1^{bri} cells retain stable expression of MSC and pericyte markers

The numbers of VLA-1^{bri} pericytes and VLA-1^{dim} fibroblasts required for *in vivo* assays were determined to be in the order of 10^5 from previously conducted experiments in the laboratory. These stromal cells were isolated from neonatal tissue by previously described methods (*Chapter 2, Section 2.2.2.3*; Paquet-Fifield et al., 2009) and expanded *in vitro* until sufficient numbers were obtained (passage 4).

However, before *in vivo* studies were undertaken, it was important to determine that at passage 4 (p4), pericytes in particular, retained the expression of classical 'pericyte' and MSC markers. Previous studies have demonstrated that freshly isolated pericytes from a diverse range of vascularised organs expressed classical pericyte markers i.e. α SMA, PDGFR β and NG2; and were also positive for a range of MSC markers including CD73 (5' nucleotidase), CD146 (MCAM), CD90 (Thy-1) and CD105 (endoglin) (Crisan et al., 2008a; Paquet-Fifield et al., 2009). In fact, Crisan et al. were also able to show that these markers continued to be expressed up to passage 7 *in vitro* (Crisan et al., 2008a). Hence, prior to proceeding with *in vivo* assays using passage 4 pericytes, experiments were carried out to determine if the expression of standard pericyte and MSC markers observed in freshly isolated VLA-1^{bri} pericytes (Paquet-Fifield et al., 2009, summarised in *Figure 3.1A*) were retained in culture.

3.2.1.1. Cultured (p4) VLA-1^{bri} pericytes maintain expression of MSC markers

VLA-1^{bri} pericytes and VLA-1^{dim} fibroblasts from healthy human neonatal tissue were isolated by flow cytometry using established procedures (Paquet-Fifield et al., 2009). After FACS separation, pericytes and fibroblasts were grown in EGM-2 and DMEM-10 respectively for four passages. P4 cells were analysed for the expression of known MSC markers CD73, CD105, CD90 and CD146. While p4 pericytes retained the expression of all the MSC markers examined (*Figure 3.1B-E*), p4

fibroblasts also showed positive staining for these markers (Figure 3.1F-I). CD45 and CD34 were included in the analyses as negative controls (data not shown).

However, it was interesting to note that while the fibroblasts gained the expression of MSC markers in culture, they still differed from the pericytes in the expression of the key fibroblast specific marker FAP α (Figure 3.1J-K).

3.2.1.2. Cultured (p4) VLA-1^{bri} pericytes maintain expression of known pericyte markers

Next, p4 pericytes were examined for the retention of known pericyte markers α SMA (ACTA2), PDGFR β and NG2 (CSPG4). Since the antibodies employed to detect these markers were either unsuitable or yielded suboptimal results in flow cytometry analyses, their expression was examined via immunofluorescence on fixed p4 cells. While p4 pericytes were shown to retain their characteristic positive expression of these markers (Figure 3.2A,C,E), p4 fibroblast showed gain in expression of all three ‘pericyte’ markers (Figure 3.2B,D,F) suggesting possible activation to a ‘myofibroblast-like’ state *in vitro*. Consistent with the previous flow cytometry analysis in Figure 3.1, p4 fibroblasts were also positive for the activation marker FAP α by immunofluorescent analysis as well, while p4 pericytes were not (Figure 3.2G,H). This demonstrated that the two populations were inherently different, and that they were distinguishable using at least one marker.

A

Expression of key MSC markers in primary pericytes and fibroblasts ^a		
Marker	Primary VLA-1 ^{bri} pericytes	Primary VLA-1 ^{dim} fibroblasts
CD105	negative	negative
CD90	bright	negative
CD146	bright	negative
CD73	dim	bright

^aAdapted from data in Paquet-Fifield et al. 2009, shown in Chapter 1, Figure 1.6

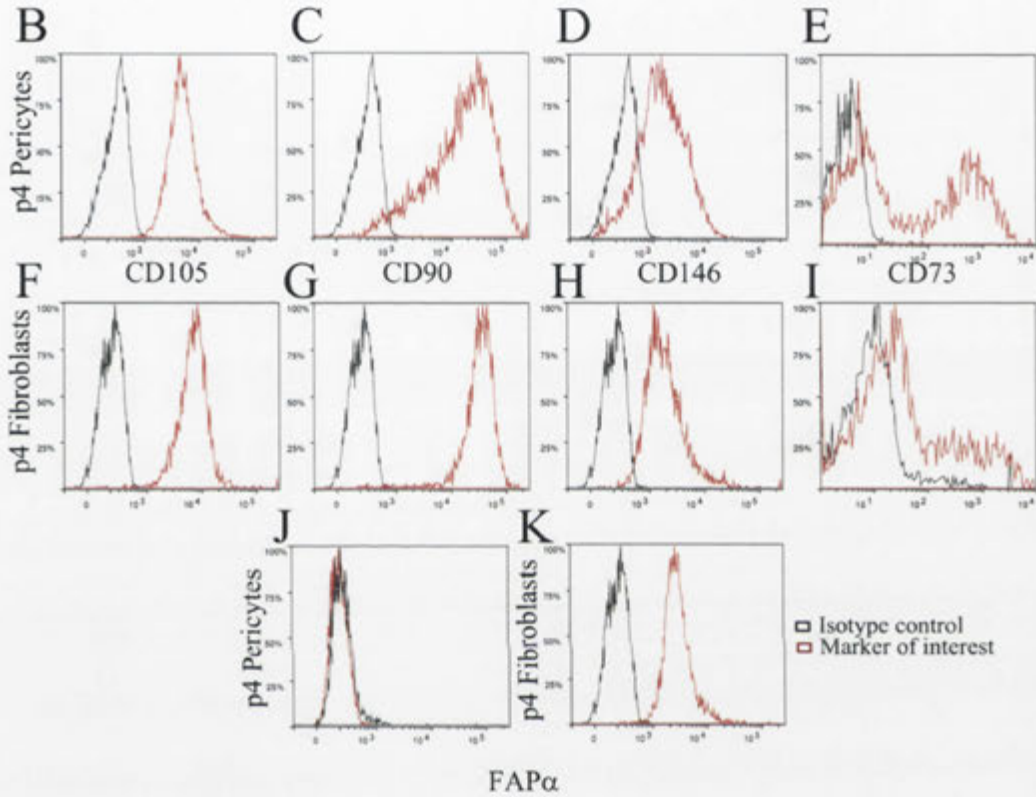
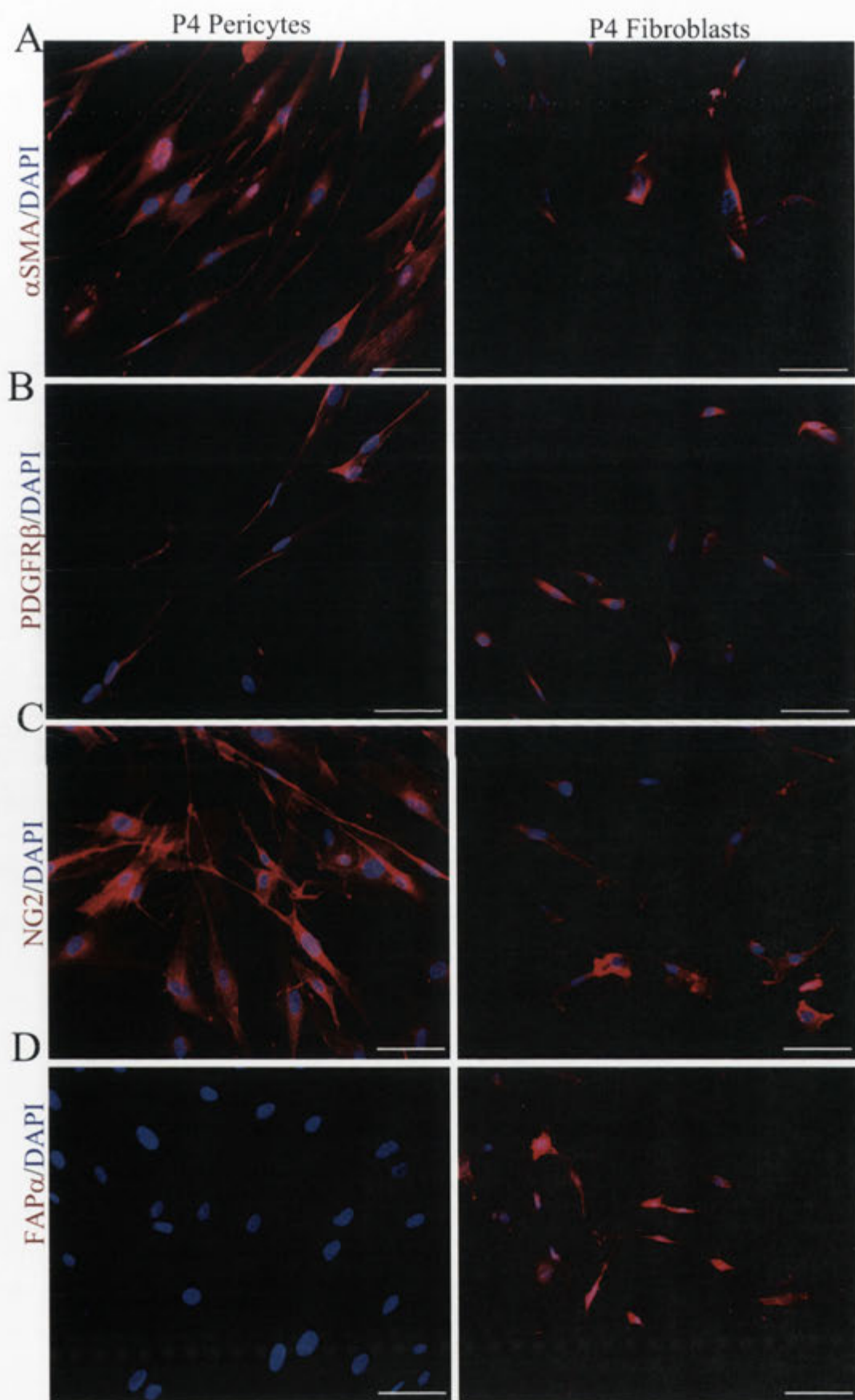


Figure 3.1. Expression profile of MSC markers in cultured (p4) pericytes and fibroblasts.(A) Summary of data from Paquet-Fifield et al., 2009 on the expression of MSC markers in freshly isolated primary pericytes and fibroblasts. (B-K) Flow cytometric analysis of cultured VLA-1^{bri} pericytes stained with monoclonal antibodies against surface markers (B,F) CD105, (C,G) CD90, (D,H) CD146, (E,I) CD73 and (J-K) FAPα for p4 pericytes and fibroblasts respectively (shown in red). Respective isotype controls are shown in black. Both pericytes and fibroblasts in culture were CD90⁺CD146⁺CD73⁺. Contrary to freshly isolated cells, both p4 populations were also positive for the MSC marker CD105. Notably, while p4 fibroblasts were FAPα⁺, p4 pericytes were not. 100,000 viable propidium iodide (PI) negative events were collected. Plots are representative of 2 independent experiments, performed in two technical replicates with VLA-1^{bri} pericytes and VLA-1^{dim} fibroblasts pooled from 6 and 8 healthy neonatal tissue samples respectively.

Figure 3.2. Expression profile of MSC markers in cultured (p4) pericytes and fibroblasts. Immunofluorescent staining of fixed p4 pericytes and p4 fibroblasts for antibodies against (A) α SMA (B) PDGFR β (C) NG2 and (D) FAP α . Both populations were α SMA⁺PDGFR β ⁺NG2⁺ while only p4 fibroblasts were FAP α ⁺. Notably, pericytes appear moderately larger than fibroblasts by histology (both in terms of nuclei size and long cytoplasmic processes), a recurrent observation in our laboratory. Nuclei counterstained with DAPI. Results representative of staining on cells derived from 3 independent experiments each performed in three technical replicates. Scale bar = 50 μ m.



3.2.2. Pericytes promote the growth of human ovarian cancer cells *in vivo*

3.2.2.1. Pericytes stimulate increased ovarian tumour growth evidenced by increased end-point tumour volume and mass

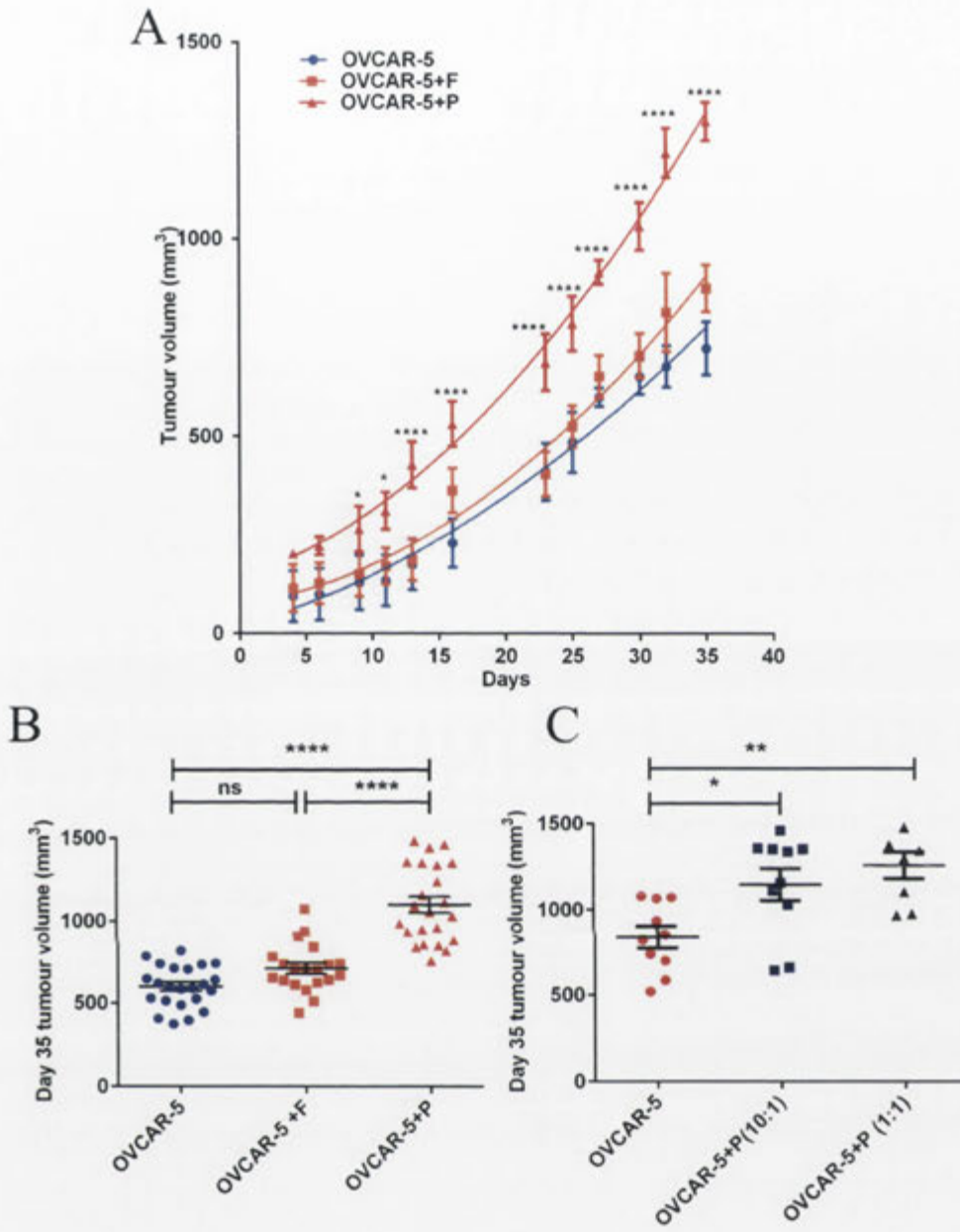
Having ascertained that pericytes maintain their inherent MSC properties and expression of known markers, further experiments were carried out to investigate whether pericytes could promote ovarian tumour growth *in vivo* in a xenograft model. The OVCAR (ovarian carcinoma) series of cell lines derived from primary biopsy samples of patients with high-grade serous ovarian cancer were employed for tumourigenicity experiments in this study. Specifically, OVCAR-5 – derived from an advanced stage untreated patient and known to form aggressive xenograft tumours (Godwin et al., 1992; Molpus et al., 1996) was chosen for the majority of experiments in this study.

To assess the effects of pericytes on ovarian xenograft tumour growth, a co-inoculation model previously developed in our laboratory was adopted. OVCAR-5 cells were injected with or without 10% VLA-1^{bri} pericytes or VLA-1^{dim} fibroblasts derived from the same neonatal tissue of origin. 8×10^6 OVCAR-5 cells were injected in diluted Matrigel™ (1:1 in PBS) subcutaneously into the flanks of 6-8 weeks old female nude mice either alone, or together with 8×10^5 p4 VLA-1^{dim} fibroblasts or p4 VLA-1^{bri} pericytes, in a ratio of 10:1 tumour: stromal cells. Tumour size was measured from the time they were palpable, usually between Days 6-11, for 35 days (experimental end-point) or until tumours reached the size of 1500mm³ (ethical end-point). Pericyte co-injection consistently led to significantly accelerated tumour growth compared to OVCAR-5 controls ($p < 0.0001$; Figure 3.3A). In contrast, fibroblast co-injected OVCAR-5 tumours did not exhibit a significantly altered growth rate (Figure 3.3A). Total tumour volume measured at the end-point (Day 35 post-injection) was significantly higher in the pericyte co-injected group ($p < 0.0001$; Figure 3.3B) whereas no significant differences were observed upon co-injection with fibroblasts, compared to OVCAR-5 control tumours.

3.2.2.2. Increasing pericyte: tumour cells ratio further increases tumour growth

Next, further experiments were carried out to investigate if there was a dose-dependent effect of pericytes on tumour growth by increasing the proportion of pericytes in the inoculum from 10:1 to 1:1 tumour cells: pericytes, while keeping the number of OVCAR-5 cells constant. Since this meant using an increased number of cells per injection and the ethical limit for the maximum number of cells per injection was 1×10^7 , 5×10^6 OVCAR-5 cells were injected alone or together with 5×10^5 (10:1) or 5×10^6 (1:1) p4 pericytes. End-point (Day 35) tumour volumes in both the pericyte co-injected groups were significantly greater than tumour only controls ($p < 0.05$ and $p < 0.01$ respectively) (*Figure 3.3C*). Further, the tumour volumes obtained in mice receiving 1:1 pericytes tended to be larger than those with 10:1 pericytes (*Figure 3.3C*) indicating a direct pro-proliferative effect of pericytes on tumour growth, although this difference was not statistically significant.

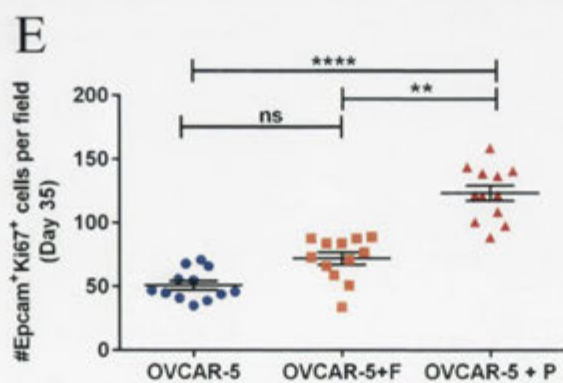
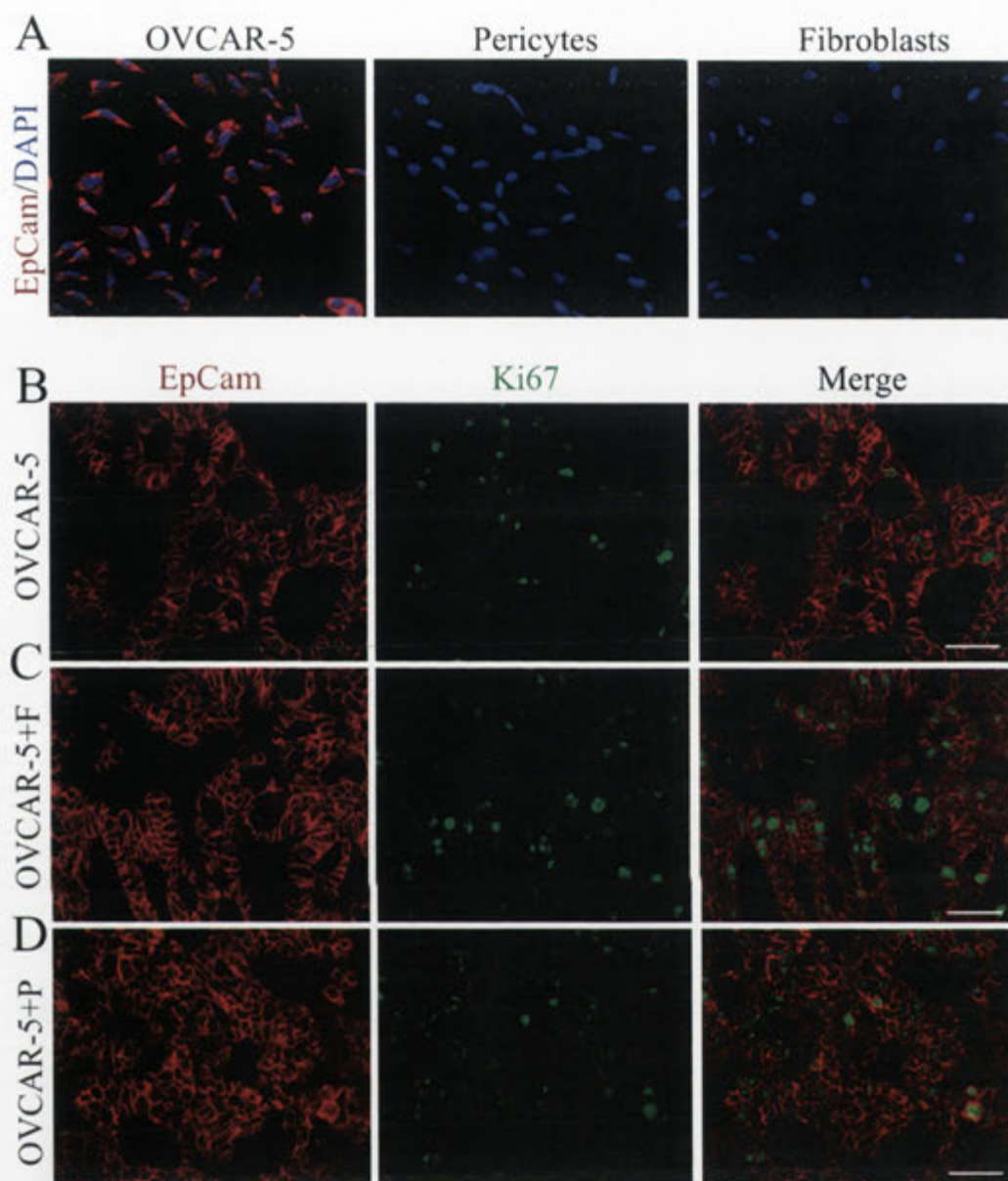
Figure 3.3. Pericytes promote ovarian tumour growth *in vivo*. (A) Non-linear regression fit of tumour volumes generated from the injection of OVCAR-5 cells alone or together with 10:1 fibroblasts or pericytes. Data represented as mean tumour volume $\pm$ SD of 26 mice per group from 5 independent experiments. Repeated measures data for each time-point was compared using two-way ANOVA (Tukey's multiple comparison test). (B) Quantification of end-point tumour volume at Day 35. Data represented as mean tumour volume $\pm$ SEM from 22-26 mice per group from 5 independent experiments injected with 8×10^6 OVCAR-5 cells with or without stromal cells in a 10:1 ratio. (C) Quantification of end-point tumour volume at Day 35 from tumours generated from the injection of 5×10^6 OVCAR-5 cells with or without pericytes in a ratio of 10:1 and 1:1. Data represents mean volume $\pm$ SEM from 8-10 mice per group from 2 independent experiments. Statistical analysis in (B) and (C) was performed using one-way ANOVA. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$, ns=not significant.



3.2.2.3. Increase tumour size results from increased proliferation of the injected tumour cells

To address if the increased tumour size was indeed a result of an increase in the number and proliferation of OVCAR-5 cells and not due to expansion of the co-injected pericyte themselves, tumours were co-stained with human EpCam – a marker for human epithelial cells and human Ki67 – a marker of cell proliferation. Prior to this, stable expression of EpCam in OVCAR-5 cells was confirmed by immunofluorescent staining (*Figure 3.4A*). In comparison, p4 fibroblasts and pericytes were predictably negative for EpCam (*Figure 3.4B-C*). Immunofluorescent staining on histological sections of tumours obtained at Day 35 demonstrated a significant increase in the overall number of EpCam⁺ OVCAR-5 cells in tumours co-injected with p4 pericytes. Moreover, a significantly higher proportion of these cells were Ki67⁺, compared to controls and fibroblast-coinjected tumours (*Figure 3.4C-D*), as indicated by the morphometric quantification in *Figure 3.4E* ($p < 0.0001$ and $p < 0.01$ respectively). This demonstrated an increase in the proliferative fraction of EpCam⁺ OVCAR-5 cells in the presence of pericytes.

Figure 3.4. Pericytes increase tumour growth by promoting the proliferative capacity of the tumour cells. (A) Immunofluorescent staining of fixed cells showing that the epithelial marker EpCam was expressed in 100% of the OVCAR-5 tumour cells, but not in p4 pericytes or fibroblasts. (B-D) Immunofluorescent co-staining of xenograft tumours at Day 35 resulting from the injection of (B) OVCAR-5 cells alone and co-injected with (C) fibroblasts and (D) pericytes with human-specific antibodies against the epithelial marker EpCam and the proliferation marker Ki67. Images indicate increased number of Ki67⁺ cells in the OVCAR-5+P group compared to the other two experimental groups. Nuclei counterstained with DAPI. (E) Morphometric quantification of the immunofluorescent staining data showing a significant increase in the number of Ki67⁺EpCam⁺ tumour cells in the OVCAR-5+P group. Error bars represent mean $\pm$ SEM. Results shown are representative of 2 random fields from 3 tumours per group from 2 independent experiments. Statistical analysis was performed using one-way ANOVA. ** $p < 0.01$, **** $p < 0.0001$, ns = not significant. Scale bar = 100 μ m.



3.2.3. Angiogenic processes remain unaltered despite the injection of exogenous pericytes

Given the pronounced role of pericytes in regulating endothelial cell proliferation and sustaining angiogenesis, it could be argued that the increased tumour growth observed was due to enhanced vascularisation promoted by the addition of exogenous human pericytes. Hence, changes in the extent of vascularisation and several other angiogenesis related processes were evaluated next to determine if the increased tumour growth observed in the presence of pericytes is an indirect result of increased angiogenesis.

3.2.3.1. Pericyte co-injection does not affect extent of vascularisation

First, to assess any changes in the tumour vasculature as a consequence of vascularisation in the xenograft tumours were examined. Staining with the endothelial marker CD31 did not exhibit any significant qualitative differences in extent and distribution between OVCAR-5 tumours and those co-injected with pericytes or fibroblasts, either at Day 11 or Day 35 (*Figure 3.5A-B*). Morphometric quantification of the staining area, shown in *Figure 3.5C-D*, confirmed this observation. Next, microvessel density (MVD) was calculated from these tumours by counting the number of microvessels observed per random photomicrograph field, which similarly did not show any significant differences between the three experimental groups, either at Day 11 and Day 35 (*Figure 3.5E-F*).

Similar analysis with a second endothelial marker, CD34, also demonstrated that there were indeed no qualitative (*Figure 3.6A-B*) or quantitative (*Figure 3.6C-D*) differences in tumour vascularisation. Blood vessel count (MVD) with CD34 did not reveal any significant differences between the experimental groups at Day 11 and Day 35 (*Figure 3.6E-F*).

Notably, for the quantitative analyses performed in *Figures 3.5 and 3.6*, photomicrograph images assessed were equally representative of tumour edges as well as centres, thus taking into account any variation in vessel distribution within each tumour.

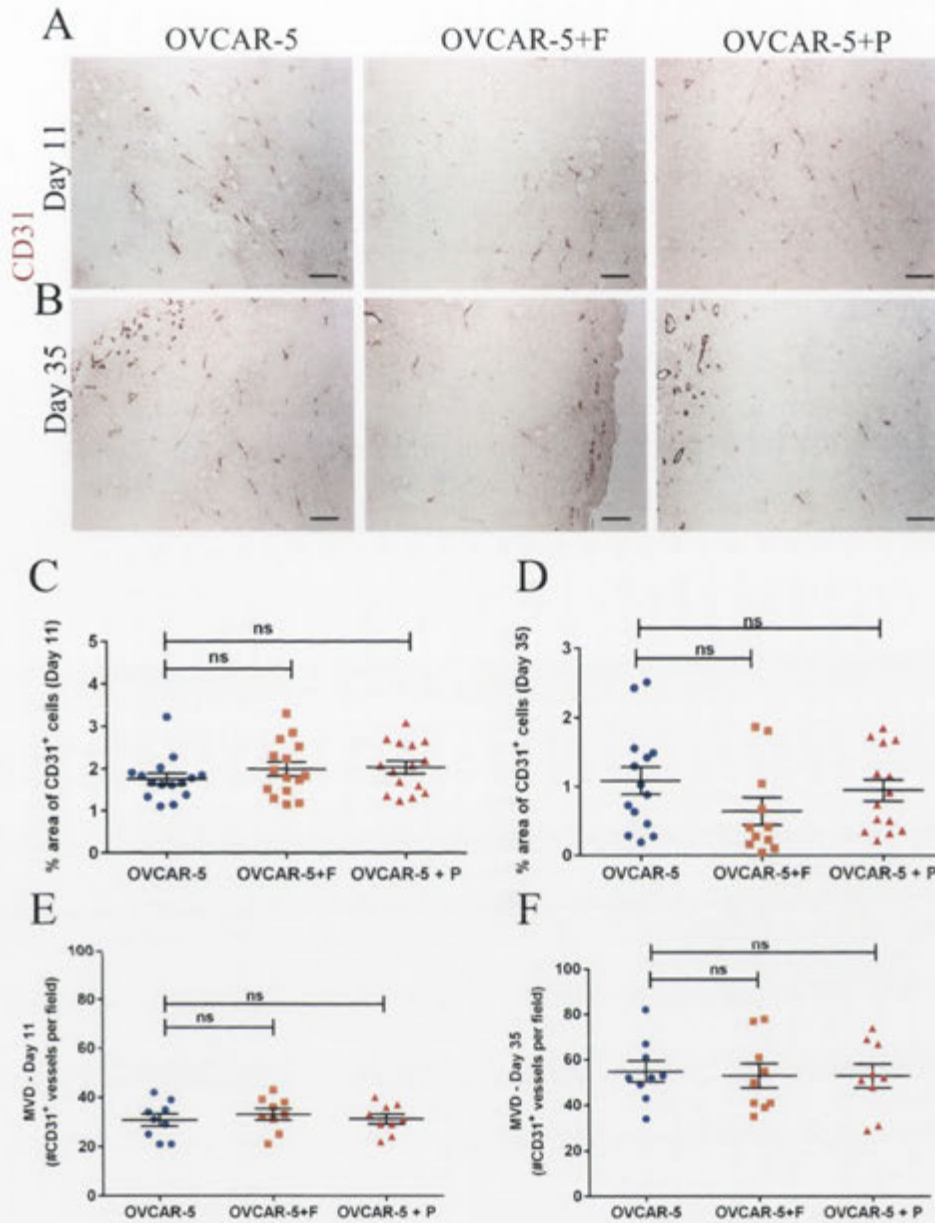


Figure 3.5. Addition of pericytes to the microenvironment does not affect the extent, distribution and density of vasculature (CD31). (A-B) Immunohistochemical staining for CD31, an endothelial marker, on xenograft tumour sections derived from the injection of OVCAR-5 cells alone or together with fibroblasts or pericytes at (A) day 11 and (B) day 35. (C-D) Morphometric quantification of CD31 staining showing no significant differences in the percentage of CD31 stained area at (C) Day 11 and (D) Day 35. (E-F) Quantification of CD31 vessel density (MVD) showing no significant differences at (E) Day 11 and (F) Day 35. Error bars represent mean $\pm$ SEM. Images are representative of 2-3 random fields from 5 tumours per experimental group from 2 independent experiments. Statistical analysis was performed using one-way ANOVA; ns = not significant. Scale bar = 100 μ m.

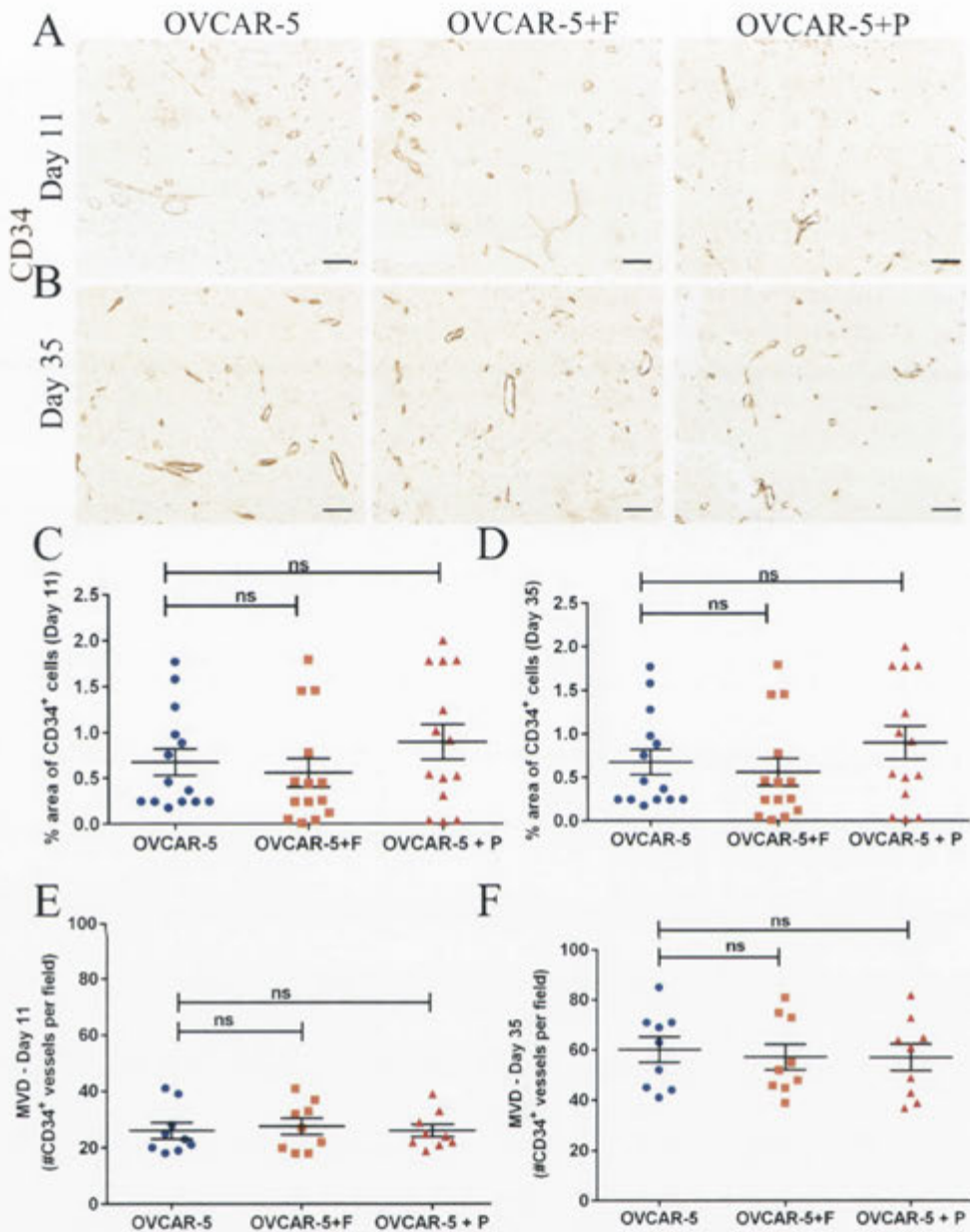


Figure 3.6. Addition of pericytes to the microenvironment does not affect the extent, distribution and density of vasculature (CD34). (A-B) Immunohistochemical staining for CD34, a second endothelial marker, on xenograft tumour sections derived from the injection of OVCAR-5 cells alone or together with fibroblasts or pericytes at (A) day 11 and (B) day 35. (C-D) Morphometric quantification of CD34 staining showing no significant differences in the percentage of CD34 stained area at (C) Day 11 and (D) Day 35. (E-F) Quantification of CD34 vessel density (MVD) showing no significant differences at (E) Day 11 and (F) Day 35. Error bars represent mean $\pm$ SEM. Images are representative of 2-3 random fields from 5 tumours per group from 2 independent experiments. Statistical analysis was performed using one-way ANOVA; ns = not significant. Scale bar = 100 μ m.

3.2.3.2. *Pericyte coverage of tumour blood vessels remain unaffected despite co-injection of exogenous pericytes*

Although the extent and distribution of tumour blood vessels was unaffected by the co-injection of pericytes or fibroblasts, their stability and permeability could be altered by increased pericyte coverage of individual vessels. Therefore, microvessel pericyte coverage index (MPI), defined as the percentage of CD31⁺ or CD34⁺ blood vessels that are enveloped by α SMA⁺ pericytes, was assessed. Xenograft tumour sections were stained for CD34 and α SMA (*Figure 3.7A-C*), photomicrographs taken and the resulting images analysed. The number of CD34⁺ vessels that were covered by α SMA⁺ pericytes were counted and plotted as a fraction of the total number of CD34⁺ vessels. Irrespective of the introduction of pericytes or fibroblasts, a steady MPI of ~ 90% (i.e. ~90% of the vessels in the tumours were enveloped by host pericytes in all the experimental groups) was observed with no significant differences in p-value (*Figure 3.7D*) between experimental groups. This indicated that the inclusion of pericytes did not alter the structural stability of the vascular architecture in the xenograft tumours.

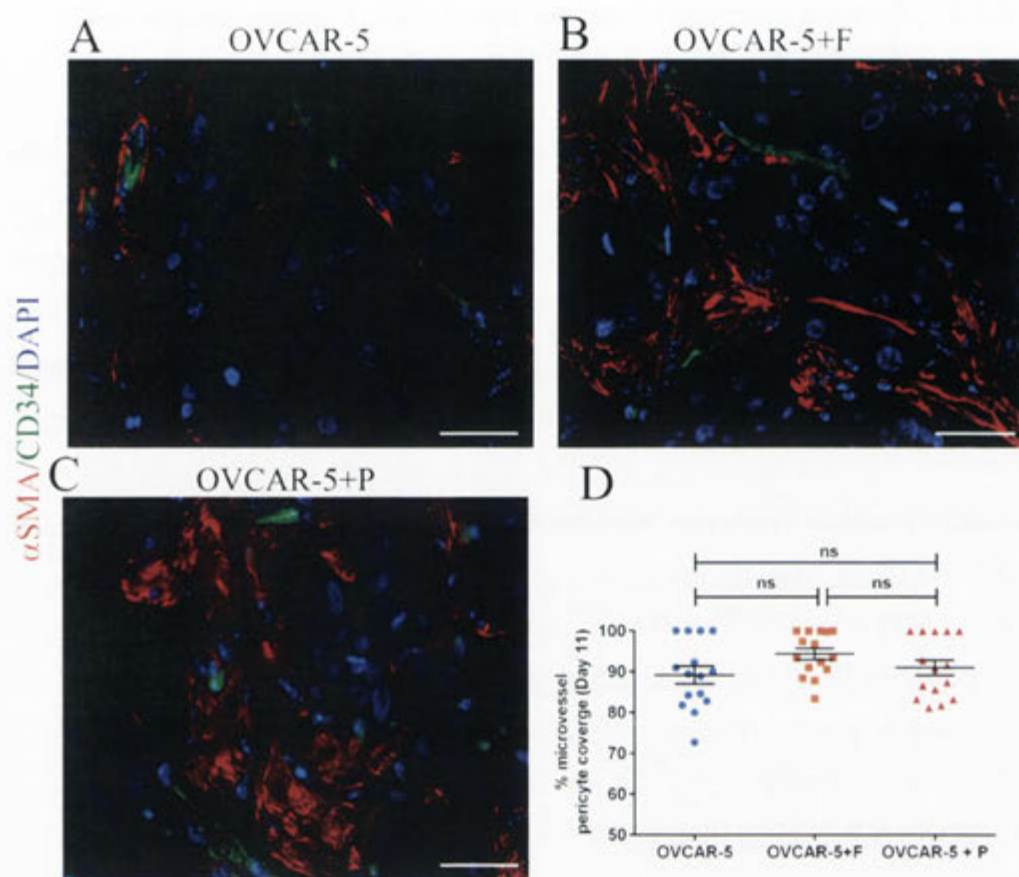


Figure 3.7. Introduction of exogenous human pericytes to the microenvironment does not affect tumour microvessel pericyte coverage index (MPI). (A-C) Immunofluorescent co-staining of xenograft tumours at Day 11 resulting from the injection of (A) control OVCAR-5 cells alone; and co-injected with (B) fibroblasts and (C) pericytes with antibodies against α SMA and the endothelial marker CD34. Images indicate no differences in the numbers of vessels that are completely enveloped by host pericytes. Nuclei counterstained with DAPI. (D) Morphometric quantification of the immunofluorescent staining data showing no significant differences in MPI, i.e. the percentage of vessels per field that are covered by pericytes. Error bars represent mean $\pm$ SEM. Results shown are representative of 3 random fields from 5 tumours per group from 2 independent experiments. Statistical analysis was performed using one-way ANOVA; ns = not significant. Scale bar = 50 μ m.

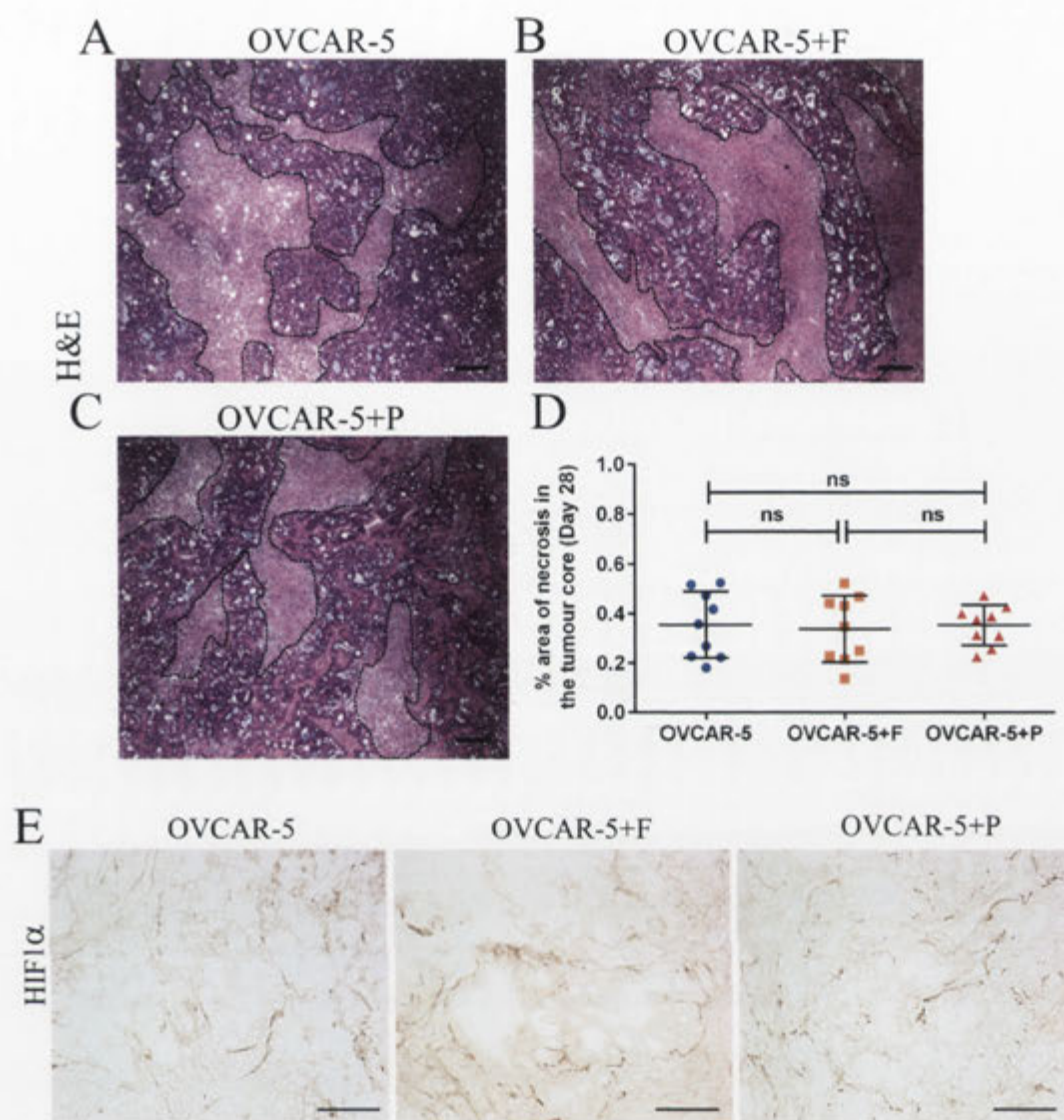
3.2.3.3. Necrosis and levels of hypoxia at tumour core remain similar despite co-injection of pericytes

Hypoxia resulting from necrosis at the tumour core has often been indicated as a major effector of cancer progression from primary to metastatic stage, as reviewed in *Chapter 1 Section 1.2.2.2*. As tumour mass increases, the core of the tumour starts becoming necrotic. This results in the increased expression of HIF1 α which can turn on a host of pro-angiogenic factors including VEGF and PDGF (De Craene and Berx, 2013; Wu et al., 2011). Hypoxia is also thought to be one of the major inducers of EMT, a process tumour cells employ to invade the surrounding tissue or enter the blood stream in order to disseminate into secondary sites (De Craene and Berx, 2013; Kalluri and Weinberg, 2009). Interestingly, pericytes have been implicated as a major functionary in this process. Lack of pericytes have been shown to cause blood vessels to become leaky and malfunctioned leading to hypoxia which in turn facilitates metastasis (Cooke et al., 2012). Hence, it was important to investigate whether introduction of exogenous pericytes resulted in differences in the levels of tumour necrosis and expression of HIF-1 α in this study. Since hypoxia arising from necrosis is a relatively mid/late stage event, it was crucial to examine these processes at a suitable time-point in the xenograft tumours. Day 11 was found to be too early i.e. tumour size not large enough for hypoxia to occur; whereas by Day 35, most of the tumour core in all the experimental groups were found to be necrotic. Therefore, Day 28 was the time-point investigated here.

In the analyses of patient pathology, H&E staining is regularly used as a standard means to identify necrotic areas with a characteristic increased eosin staining (eosinophilia) and cells with a glossy, homogeneous and rounded appearance (Kumar et al., 2007; Trump et al., 1997). Thus, Day 28 xenograft tumours were stained with H&E and images of the tumour cores were analysed. Necrotic area was delineated using ImageJ software and plotted as a percentage of total area (*Figure 3.8A-C*). Consistent with unaltered levels of angiogenesis, quantification of necrotic area did not reveal any significant differences in the extent of necrosis at tumour cores between the three experimental groups (*Figure 3.8D*), thereby excluding hypoxia arising from necrosis as a factor affecting these tumours. This was validated

by staining for the HIF-1 α , where no qualitative differences in the extent of staining were observed (*Figure 3.8E*) across experimental groups.

Figure 3. 8. Addition of pericytes to the microenvironment does not alter the status of necrosis and hypoxia at the tumour core. (A-C) Representative H&E stained xenograft tumour sections at Day 28 resulting from the injection of (A) OVCAR-5 cells alone and co-injected with (B) fibroblasts or (C) pericytes. The necrotic core was demarcated by a broken black line. (D) Morphometric quantification of the necrotic area showing no significant differences in the level of necrosis between the 3 experimental groups. Error bars represent mean $\pm$ SEM. Results shown are representative of 3 random fields from 3 tumours per group from 2 independent experiments. Statistical analysis was performed using one-way ANOVA; ns = not significant. (E) Immunohistochemical staining on xenograft tumour sections at Day 28 with an antibody against the hypoxia marker HIF1 α showing no qualitative differences in the extent of its expression across experimental groups. Scale bar = 200 μ m.



3.2.3.4. Vascular permeability is unaltered by the inclusion of exogenous human pericytes

It can be argued that blood vessels are strengthened in the presence of additional pericytes given their documented role in stabilising tumour vasculature thereby indirectly increasing tumour growth via an improved nutrient supply. To ascertain whether inclusion of pericytes resulted in change in vascular function specifically through alterations in blood vessel permeability (or leakiness), a small molecule dextran, conjugated to FITC, was employed in subsequent experiments. Dextran is a complex sugar of varying molecular weights that are routinely used in animal experiments as readout of vascular permeability in drug targeting and angiogenesis studies. Tumour-bearing mice were injected via the tail vein with 100 μ l of 10 mg/ml FITC-dextran in sterile PBS one hour before sacrifice. The resulting tumours were collected, snap-frozen in liquid nitrogen and sectioned with minimum photo exposure. They were then stained with the endothelial marker CD31 and visualised for intravascular: extravascular green (FITC) fluorescence. Both at Day 11 and Day 35, the overall intravascular pattern of FITC expression remained unaltered in xenografts generated in the presence and absence of exogenous pericytes (*Figure 3.9A-B*). Importantly, FITC signal was restricted to the lumen of blood vessels at both time-points, indicated by minimal extravascular leakage of green signal.

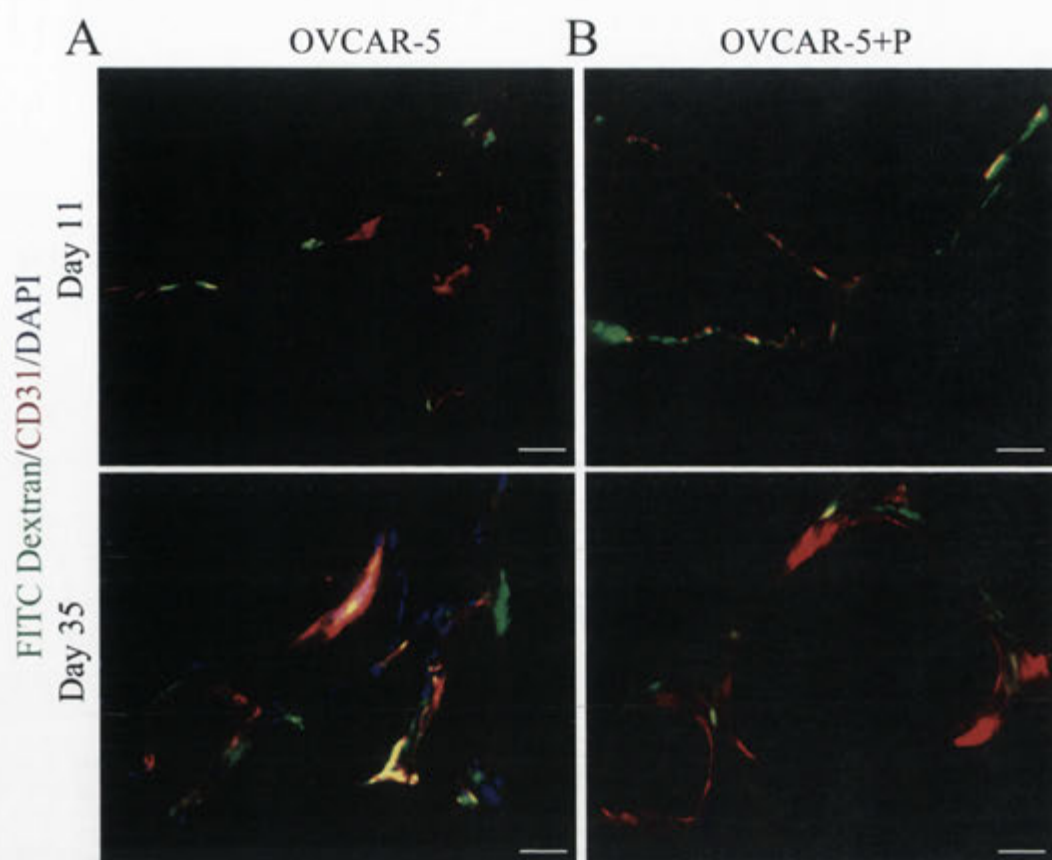


Figure 3.9. Addition of pericytes to the microenvironment does not alter the permeability of the tumour vasculature. (A-C) Immunofluorescent staining of xenograft tumour sections derived from mice injected intravenously with FITC-Dextran (green) and the endothelial marker CD31 (red) in (A) OVCAR-5 control and (B) pericyte co-injected tumours. Both at Day 11 and Day 35, no differences in the intravascular: extravascular ratio of FITC-Dextran was evident, indicating that introduction of exogenous pericytes in the tumour microenvironment did not alter the ‘leakiness’ of the tumour vasculature. Nuclei counterstained with DAPI. Results shown are representative of 6 tumours per group per time-point from 2 independent experiments. Scale bar = 20µm.

3.2.4. Injected human pericytes survive but do not proliferate in the xenograft tumours

Collectively, findings from *Section 3.2.3*, conclusively established that the introduction of pericytes, a population *only* known for its angiogenesis-supporting functions, resulted in significantly increased tumour size without *affecting the vasculature*, suggesting a new, undiscovered role for pericytes in the tumour microenvironment. Therefore, to further elucidate the role of injected human pericytes in the xenograft tumour microenvironment, experiments were carried out to determine the intratumoural persistence, localisation and characteristics of the injected human pericytes.

3.2.4.1. *p4 HD-1^{br1} GFP-luciferase⁺ pericytes maintain expression of known MSC and pericyte markers*

Firstly, in order to enable the detection of pericytes within the tumour post-injection in real-time *in vivo*, p1 pericytes were lentivirally transduced with a plasmid carrying a GFP-luciferase vector. Given that pericytes are primary human cells, they were incubated with the lentiviral supernatant for 1 hour only and expanded in EGM-2 for 2 passages. P3 cells were then subjected to flow cytometry to select GFP⁺ cells, the efficiency of transduction calculated, and positive cells expanded in culture to generate GFP-luciferase⁺ p4 pericytes ready for *in vivo* injection. To compensate for transduction efficiency, this process was repeated until cell numbers required for an appropriate number of p4 injections were obtained. A representative FACS sorting plot is illustrated in *Figure 3.10A*.

Next, it was important to ensure that GFP-luciferase labelling had not interfered with the p4 pericyte phenotype established earlier in *Section 3.2.1*. To verify this, p4 GFP-luciferase⁺ pericytes were similarly stained with antibodies for the MSC markers CD73, CD146, CD90 and CD105 and found to be positive for all of them (*Figure 3.10B-F*). Positive staining for α SMA, PDGFR β and NG2 and FAP α negativity (*Figure 3.11A-D*) also conclusively demonstrated the retention of their original pericyte markers after lentiviral transduction.

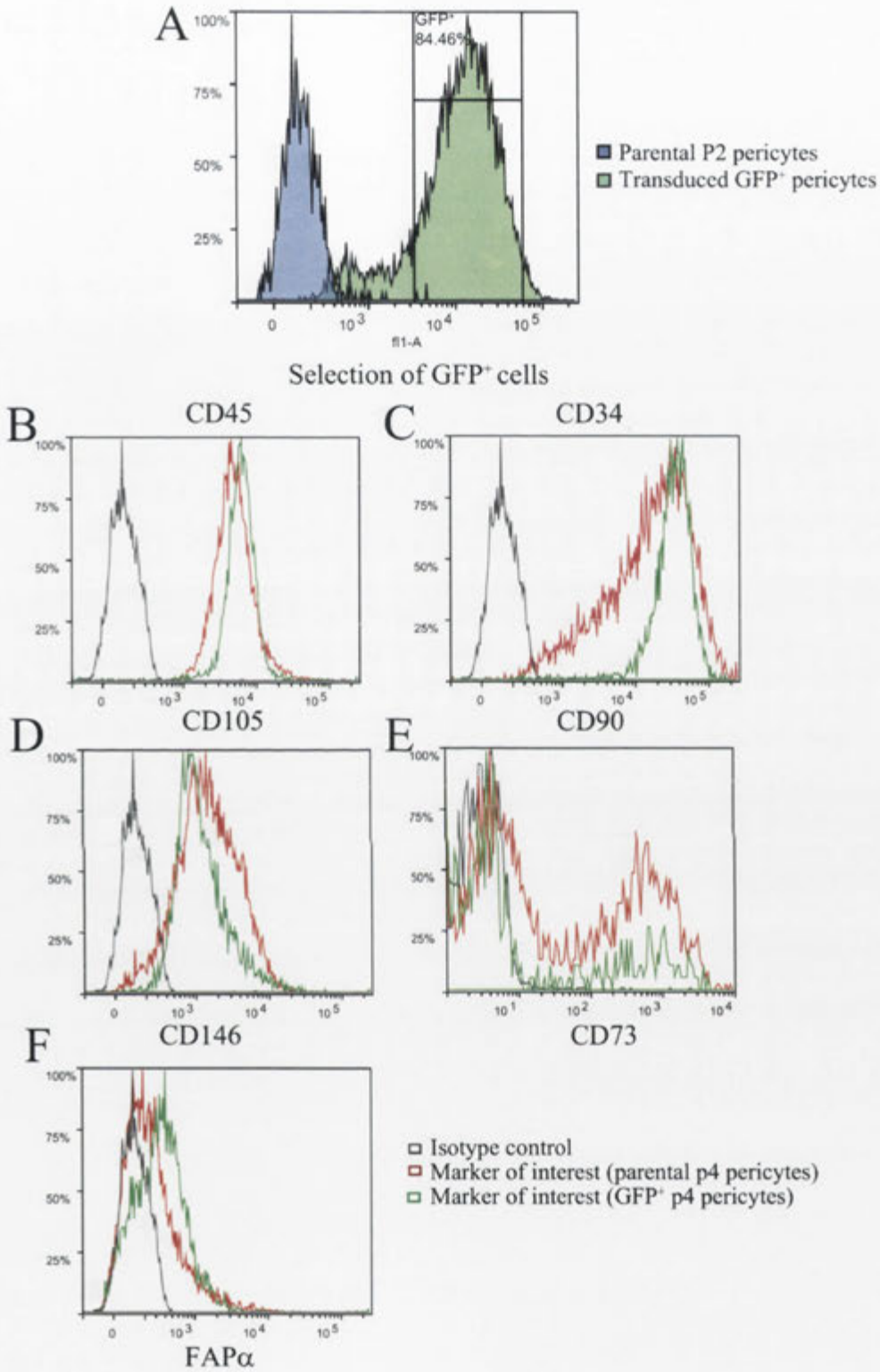


Figure 3.10. p4 GFP-luciferase⁺ pericytes maintain the expression of MSC markers after lentiviral transduction. (A) A representative flow cytometric analysis of untransduced parental p2 VLA-1^{bri} pericytes (purple) and GFP-luciferase transduced cells (green). Gate on FL-1 channel is indicative of GFP fluorescence and was used to sort for the GFP⁺ population. (B-H) Flow cytometric analysis of cultured p4 GFP-luciferase⁺ VLA-1^{bri} pericytes (green) stained with monoclonal antibodies against surface markers (B) CD105, (C) CD90, (D) CD146, (E) CD73 and (F) FAP α shown as overlay plots in comparison to their respective isotype controls (black) and parental cells (red). Pericytes maintained a CD105⁺CD90⁺CD146⁺CD73⁺ FAP α ⁻ profile after GFP transduction, similar to the parental p4 cells (*Figure 3.1*). All analyses were performed on viable propidium iodide negative cells and 50,000 viable events were collected per plot recorded. Plots are representative of 3 independent experiments, performed with two technical replicates each, with VLA-1^{bri} parental pericytes pooled from 6, 8 and 4 healthy neonatal tissue samples respectively.

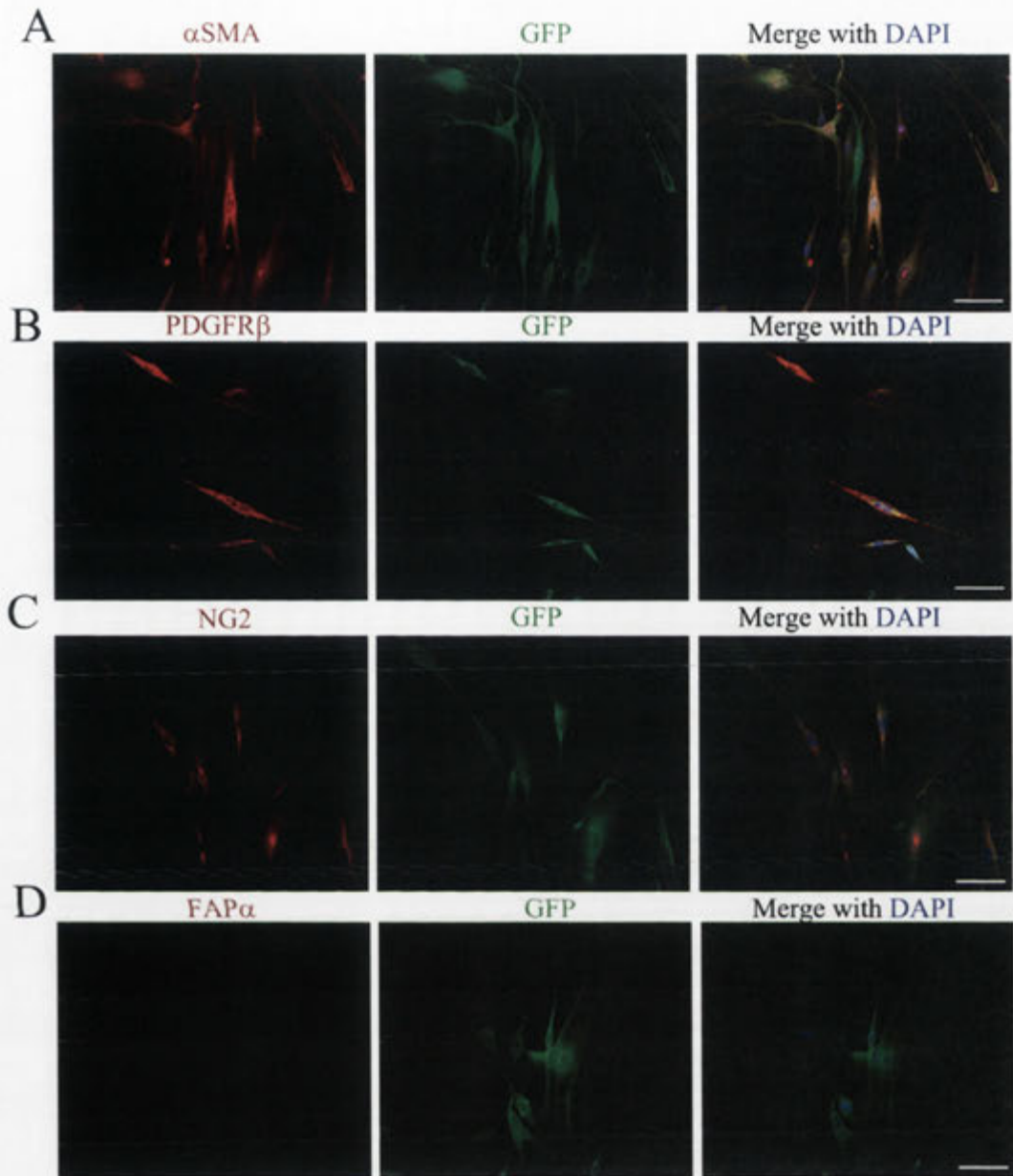


Figure 3.11. p4 GFP-luciferase⁺ pericytes maintain the expression of MSC markers after lentiviral transduction. Immunofluorescent staining of fixed p4 GFP-luciferase⁺ pericytes for antibodies against (A) αSMA (B) PDGFRβ (C) NG2 and (D) FAPα. p4 GFP-luciferase⁺ pericytes were also positive for αSMA, PDGFRβ and NG2 and negative for the fibroblast activation marker FAPα, similar to the profile of their parental cells shown in Figure 3.2. Nuclei counterstained with DAPI. Results representative of staining performed in triplicate on cells derived from 3 independent sort and transduction experiments. Scale bar = 50μm.

3.2.4.2. Tracking persistence of pericytes via *in vivo* bioluminescence imaging

To track the persistence of pericytes *in vivo*, OVCAR-5 cells were injected alone or with p4 GFP-luciferase⁺ pericytes as described previously (Section 3.2.2). The presence of pericytes in the developing tumours was tracked by *in vivo* bioluminescent imaging (BLI) up to the experimental end-point of day 35. Briefly, D-luciferin, a substrate for the enzyme luciferase was injected in both groups of mice (described in Chapter 2, Section 2.2.4.3). Mice were then anaesthetised and BLI images acquired between 10-12 minutes of luciferin injection, when the enzyme-substrate reaction reaches its kinetic peak. BLI at days 6, 9, 11, 18, 21, 28 and 35 post-injection revealed that the co-injected pericytes persist throughout the experiment and were detectable at all the time-points analysed (Figure 3.12A-G). At days 6, 11, 21 and 35, 2-3 mice per group were injected with luciferin, euthanised, their primary tumour excised and then imaged to determine if the injected pericytes had migrated to extra-tumoural locations (Figure 3.12A,C,E,G). The group injected with OVCAR-5 cells alone (i.e. without p4 GFP-luciferase⁺ pericytes) served as an appropriate negative control, since in the absence of luciferase in the system, no BLI signal was detected despite injection of luciferin (Figure 3.12A-G).

Figure 3.12. Exogenous pericytes survive but do not proliferate *in vivo*. (A-G) Representative BLI images of mice with unlabelled OVCAR-5 tumours and those co-injected with GFP-luciferase⁺ labelled pericytes imaged at days 6-35. Mice were harvested at alternate time-points and images taken after removing the primary tumour. BLI imaging was conducted in 3 mice per experimental group per time-point in 2 independent experiments.



3.2.4.3. Injected p4 pericytes do not proliferate in vivo and reside in vasculature-dissociated sites in the xenograft tumours

In order to confirm the BLI data in *Section 3.2.4.2* at a cellular level, histological sections of tumours harvested at Days 6, 11, 21 and 35 were stained for GFP. The presence of single GFP⁺ cells within the tumours confirmed that the injected pericytes persist in the ovarian xenograft tumour microenvironment; however, a steady decline in their number was evident over the course of the experiment (*Figure 3.13*). Notably, GFP⁺ pericytes were not Ki67⁺ at any of the time-points analysed indicating that they survive but do not proliferate during tumour progression (*Figure 3.13*). Further, immunofluorescent staining to visualise blood vessels with the endothelial marker CD34 revealed that the GFP⁺ pericytes were not associated with the CD34⁺ endothelial cells, but were instead localised at non-vascular sites in the tumour microenvironment (*Figure 3.14*).

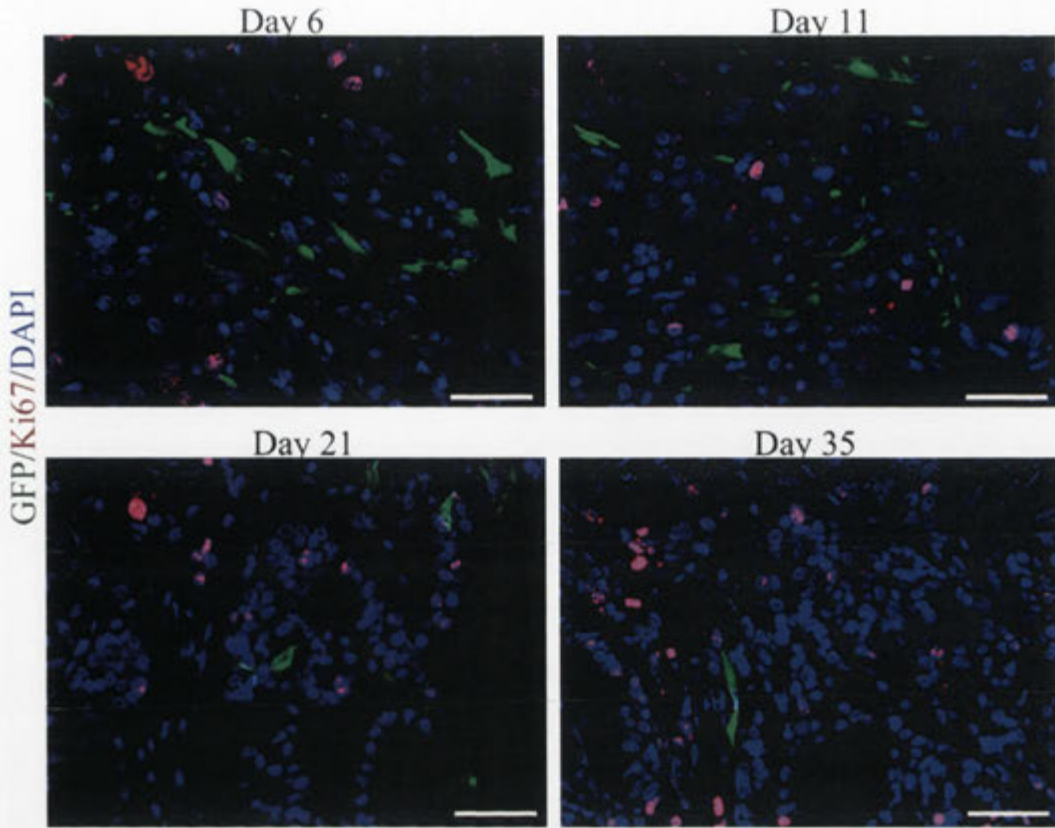


Figure 3.13. Co-injected pericytes decline in number over tumour progression *in vivo*. Immunofluorescent co-staining for GFP and the proliferation marker Ki67 at days 6, 11, 28 and 35 representing the course of tumour progression in the OVCAR-5 co-injection model. GFP⁺ pericytes show a steady decrease in number over time and do not co-express Ki67 at time-points analysed. GFP⁺Ki67⁺ cells detected at all time-points analysed were most likely proliferating OVCAR-5 tumour cells (based on data presented in *Figure 3.8*). Nuclei counterstained with DAPI. Images are representative of 3 random fields from 3 tumours per experimental group from 2 independent experiments. Scale bar = 50µm.

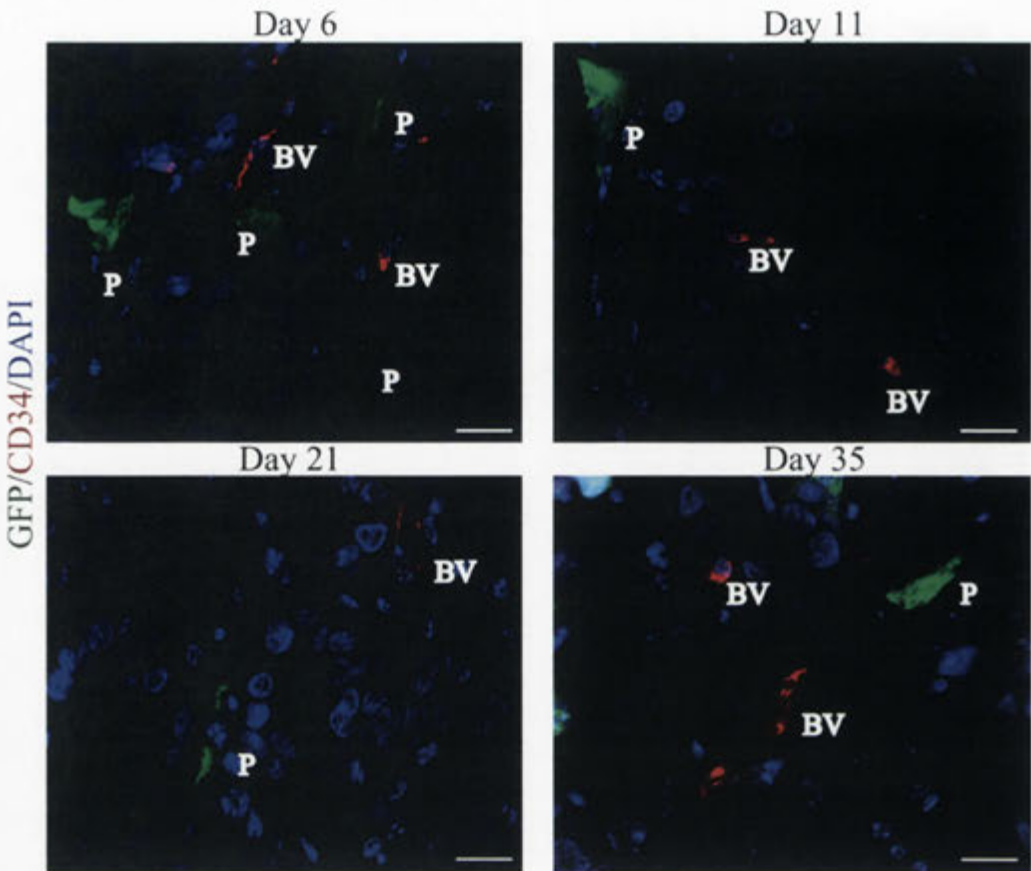


Figure 3.14. Co-injected pericytes do not associate with host blood vessels. Immunofluorescent staining for GFP and the endothelial marker CD34 illustrating that injected pericytes do not incorporate into host blood vessels at days 6, 11, 28 and 35, representing the course of OVCAR-5 tumour progression. Nuclei counterstained with DAPI. P=pericytes; BV=blood vessels. Images are representative of 3 random fields from 3 tumours per experimental group from 2 independent experiments. Scale bar = 20µm.

3.3. Discussion on key findings

The key findings of this Chapter are (i) human neonatal pericytes can be maintained in culture (p4) without affecting their phenotype; (ii) pericytes promote xenograft ovarian tumour growth while fibroblasts isolated from the same tissue of origin do not; (iii) increasing the ratio of pericytes brings about a further increase in tumour growth and; (iv) pericytes exert this effect by increased proliferation of the tumour cells without affecting angiogenesis, hypoxia, necrosis and vascular permeability; (v) injected exogenous pericytes do not associate with the tumour vasculature, suggesting non-vascular sites of action of pericytes within the tumour stroma.

In a seminal paper in the pericyte field, *Crisan et al.* successfully demonstrated that fetal and adult pericytes could be isolated from various organs including bone marrow, lung, skeletal muscle, pancreas, brain, placenta and skin based on the expression of the known 'pericyte' (and BM MSC) marker CD146 (melanoma cell adhesion molecule - MCAM) and negative selection of the hematopoietic lineages (CD45 and CD34) (Crisan et al., 2008a). They were able to successfully propagate these pericytes in culture in the endothelial growth media (EGM-2) and demonstrated that these cells retained the hallmark MSC markers and most accepted pericyte markers up to at least passage 7. Consistent with this study, our laboratory has extensively characterised VLA-1^{bri} pericytes and VLA-1^{dim} fibroblasts isolated from human neonatal tissue (discussed in *Chapter 1, Section 1.3.1*) and shown that freshly isolated VLA-1^{bri} dermal pericytes express all known pericyte markers including α SMA, PDGFR β , NG2, bore a CD45⁻CD34⁻CD146⁺CD73⁺CD90⁺ MSC phenotype and could differentiate into bone, fat and cartilage lineages under appropriate conditions (Paquet-Fifield et al., 2009). We have also been able to propagate VLA-1^{bri} pericytes for as long as 15 passages in EGM-2 (*unpublished data, Kaur Laboratory*).

Here, it was important to establish that these characteristics were still maintained in culture and upon lentiviral transduction (*Figures 3.1, 3.2, 3.10 and 3.11*). Interestingly, in our previous study, while the CD45⁻CD34⁻CD146⁺CD73⁺CD90⁺ phenotype of freshly isolated VLA-1^{bri} pericytes were in agreement with the *Crisan*

et al. signature, the only point of contention was the expression of CD105; while *Crisan et al.* cells were positive for CD105, p0 VLA-1^{bri} cells were not. Given that the *Crisan et al.* cells were isolated from skeletal muscle and VLA-1^{bri} cells from neonatal foreskin tissue, this was attributed to the fact that systemically present cells could also sometimes specially acquire (or lose) the expression of specialised markers depending on the organ they are associated with. However, interestingly, this singular disparity faded in culture when VLA-1^{bri} cells acquired expression of CD105, selected out possibly due to its role in promoting adhesion and inhibiting bacteria-induced cell detachment (Muenzner et al., 2005), conditions imperative for *in vitro* survival.

VLA-1^{dim} fibroblasts were previously shown to be a heterogeneous population with a CD146^{dim}CD73⁺CD90⁺CD105⁻ signature (Paquet-Fifield et al., 2009) and in this study, they were shown to be positive for these markers in culture (*Figure 3.1*). However they maintained steady expression of FAP α , a characteristic fibroblast marker, while pericytes maintained negativity for FAP α (*Figure 3.1G and 3.2D*). The inclusion of this key marker in these analyses was crucial since in the conundrum of overlapping markers, FAP α provided satisfying evidence that the VLA-1^{dim} cells were indeed of distinct fibroblast origin and conversely the VLA-1^{bri} FAP α ⁻ population were pericytes.

The acquisition of the ‘pericyte’ markers α SMA, PDGFR β and NG2 by FAP α ⁺ p4 fibroblasts probably indicate the activation of fibroblasts in culture to a ‘myofibroblast’ or ‘activated’ fibroblast-like state. Yet again, this points towards the ambiguity in the literature regarding the exclusiveness of classical ‘pericyte’ markers, highlighting how treacherous relying on a single marker can be in this context. Therefore, including the p4 fibroblasts in these studies (which are derived from the same tissue of origin, serving as the best possible internal control) further accentuates the influence of pericytes in the ovarian microenvironment, showing that any ‘stromal’ cell does not have this tumour-promoting and prognostic effect. Notably, growth conditions generally used to propagate “normal fibroblasts” do not permit propagation of pericytes which require more specialised media. In our laboratory, human neonatal pericytes adhere but do not proliferate in DMEM+10% FCS routinely used to propagate “fibroblasts” (Paquet-Fifield et al., 2009). Thus, any

experiments performed to date with “normal fibroblasts” passaged multiple times will have selected against pericytes – highlighting the importance of thoroughly characterising any ‘pericyte’ population for the expression of a range of markers for both their MSC and ‘pericyte-like’ characteristics, *before* performing genetic manipulations or *in vivo* experiments to draw conclusions about physiological significance.

The histological analyses of the xenograft tumours using EpCam and Ki67 established that the increase in tumour volume, mass and overall growth rate observed was due to the increased number and proliferation rate of the cancer cells and not the injected pericytes. In fact, it was previously shown in our laboratory that the VLA-1^{bri} pericytes injected alone in a Matrigel™ plug subcutaneously neither proliferate nor survive long term *in vivo*; additionally, the level of apoptosis in the OVCAR-5 tumours is unaltered despite the introduction of fibroblasts or pericytes (*Lynn Chong Ph.D. thesis, 2013; data shown in Appendix B, Figure 1*). This is consistent with the BLI (*Figure 3.12*) and immunofluorescent staining data (*Figure 3.13*) for GFP-luciferase⁺ pericytes, showing that the injected pericytes indeed do not proliferate within the tumour microenvironment and that their numbers clearly decrease over the course of tumour progression. This is profoundly indicative of how a small, non-proliferating or slow-cycling stem cell-like population could significantly affect the growth and progression of a tumour, highlighting the established but under-appreciated fact that the ability of tumour cells to attract specific subtypes of stromal cells to them (or migrate towards them) for various physiological reasons, can indeed alter the fate of the tumour and thus the patient.

Alterations in angiogenic processes in the presence of exogenous pericytes were investigated next. Endothelial cell markers CD31 and CD34 are commonly used in pathological analyses of tumour samples to determine the extent of tumour vasculature (Kutznier, 1993; Westra et al., 1994; De Young et al., 1998). CD31 or PECAM-1 (platelet endothelial cell adhesion molecule 1) is a 120-130 kDa transmembrane glycoprotein and a member of the immunoglobulin superfamily. It is expressed on the surface of platelets, monocytes, macrophages, and neutrophils and is a major constituent of the endothelial intercellular junction (Albelda et al., 1991). It plays a major role in the adhesion cascade between endothelial cell and other pro-inflammatory immune cells during inflammation in facilitating leucocyte migration

and between endothelial cells during angiogenesis. CD34, on the other hand, is a 110 kDa transmembrane glycoprotein present on the surface of endothelial cells and haematopoietic stem and progenitor cells (Westra et al., 1994). Interestingly, while CD31 is considered to be a stronger endothelial marker in normal tissues (Müller et al., 2002), CD34 has been deemed to be more reliable marker of angiogenesis in malignant tissue, as demonstrated in breast (da Silva et al., 2009), prostate (Trojan et al., 2004) and cervical cancer (Vieira et al., 2004). Thus, to account for variation in expression of markers, both CD31 and CD34 were employed in this study to characterise the extent of tumour vascularisation. Notably, since neither marker is exclusive to endothelial cells, it was also essential to validate the morphometric data by demonstrating that there was no difference in the actual counts of blood vessels, i.e. MVD, despite the introduction of exogenous pericytes or fibroblasts.

Another key process explored in this chapter was vascular permeability. Vascular permeability is defined as the capacity of a capillary or microvessel in particular to allow the influx or efflux of small molecules, drugs, immune cells like leukocytes during inflammation, and disseminating tumour cells during metastasis (Bates, 2010; Tozer et al., 2005). Fluorescent conjugated dextran and albumin tracers of various molecular weights have been systemically used since 1975 in the angiogenesis, diabetes and nephrology fields to histologically and quantitatively analyse vascular permeability (Olsson et al., 1975). Assessing whether adding pericytes affected the vascular permeability was relevant in the context of this study, since a number of studies have associated loss of pericytes with 'leaky' or hyper-permeable' blood vessels that are not only deemed dysfunctional, but also thought to allow relatively easier intravasation routes to escaping tumour cells, thereby allowing increased metastasis (Gerhardt and Semb, 2008; Raza et al., 2010; Xian and Håkansson, 2006). Although vascular permeability was not assessed in the tumours co-injected with fibroblasts, it is important to note that introducing exogenous fibroblasts to the environment did not alter extent of vascularisation, MVD, MPI, necrosis and hypoxia (*Figure 5.5 - 5.8*).

Collectively, systemic analysis of various structural and functional aspects of the tumour vasculature in our experimental model provided conclusive evidence for an angiogenesis-independent mechanism of action for pericytes that can cause increased

tumour cell proliferation. These conclusions are also supported by circumstantial evidence indicating that pericytes may promote tumour growth without affecting angiogenic processes. Pericytes have been reported to migrate away from the endothelium and extend into the tumour parenchyma in a mouse breast carcinoma model (Morikawa et al., 2002). *Furuhashi et al.* have shown that an increase in pericyte numbers did not correlate with increased blood vessel coverage but rather with a reduction in tumour cell apoptosis and increased tumour cell proliferation (Furuhashi et al., 2004). In addition to this, vessel-detached pericytes were found throughout the tumour stroma. Fetal pericytes have also been found to enhance the invasion of MDA-MB-231 breast cancer cells in a co-culture assay system while the subcutaneous co-injection of pericytes with MDA-MB-231 resulted in an increased tumour growth rate compared to cancer cells alone (Bagley et al., 2005).

Interestingly, it is critical to note that there is minimal overlap in the gene-set attributed to the angiogenesis signature in the Gene Ontology classification (*Table 3.1*) with the VLA-1^{bri} pericyte signature. This signature has only 1 gene (*ANGPT2*) common to pericytes – suggesting that the significantly earlier relapse observed in patients with overexpression of pericyte-specific genes, was not related to angiogenesis.

Table 3.1. List of genes in the gene-set attributed to the angiogenesis signature per the GO classification.

Symbol	GO Identifier	GO Term Name	Reference
ANGPT2	GO:0001525	angiogenesis	Ensembl:ENSMUSP00000033846
CAV1	GO:0001525	angiogenesis	Ensembl:ENSMUSP00000007799
HIF1A	GO:0001525	angiogenesis	Ensembl:ENSMUSP00000021530
NCL	GO:0001525	angiogenesis	Ensembl:ENSP00000318195
PHKG1a	GO:0001525	angiogenesis	ZFIN:ZDB-MRPHLNO-131121-1
ZC3H12a	GO:0001525	angiogenesis	UniProtKB-KW:KW-0037
CX3CL1	GO:0060055	angiogenesis involved in wound healing	Ensembl:ENSMUSP00000034230
VEGF-A	GO:0001525, GO:0002042	angiogenesis, cell migration involved in sprouting angiogenesis	Ensembl:ENSP00000361125

Symbol	GO Identifier	GO Term Name	Reference
BMP4	GO:0002043	blood vessel endothelial cell proliferation involved in sprouting angiogenesis	Ensembl:ENSP00000245451
BMPER	GO:0002043	blood vessel endothelial cell proliferation involved in sprouting angiogenesis	Ensembl:ENSP00000297161
EGR3	GO:0002042	cell migration involved in sprouting angiogenesis	Ensembl:ENSP00000318057
FGF2	GO:0002042	cell migration involved in sprouting angiogenesis	Ensembl:ENSP00000264498
GREM1	GO:0002042	cell migration involved in sprouting angiogenesis	Ensembl:ENSMUSP000000097170
SRF	GO:0002042	cell migration involved in sprouting angiogenesis	Ensembl:ENSMUSP000000015749
E2F8	GO:0002040	sprouting angiogenesis	Ensembl:ENSMUSP000000056778
LOXL2	GO:0002040	sprouting angiogenesis	UniProtKB:Q9Y4K0
LOXL2b	GO:0002040	sprouting angiogenesis	UniProtKB:Q9Y4K0
Notch1	GO:0002040	sprouting angiogenesis	MGI:MGI:1933835 MGI:MGI:245031
RNXN1a	GO:0002040	sprouting angiogenesis	ZFIN:ZDB-MRPHLNO-080516-7 ZFIN:ZDB-MRPHLNO-120719-2
PARVA	GO:0002040	sprouting angiogenesis	Ensembl:ENSMUSP000000033030
RAMP2	GO:0002040	sprouting angiogenesis	Ensembl:ENSMUSP000000122072

Finally, BLI data revealed the localisation of the exogenous pericytes at non-vascular sites within the tumour microenvironment (*Figure 3.14*). However it was not clear whether the exogenous pericytes were preferentially localised at non-vascular stromal sites or if this was due to species difference, given that the co-injected pericytes were of human origin while the vasculature was formed by murine endothelial cells. Nevertheless, the fact that the injected pericytes in this model were residing in the non-vascular areas of the stroma, presented a unique opportunity to investigate the role of vessel dissociation of pericytes and its effect on metastasis, which was carried out in the following Chapter.

Chapter 4

Pericytes promote ovarian cancer metastasis through invasion and epithelial mesenchymal transition (EMT)

4.1. Introduction and Rationale

As discussed in *Chapter 1, Section 1.1.3.3.3*, the evidence in the literature for the precise effects of pericytes on cancer metastasis remains ambiguous and divided. This is mainly because the identification of ‘true’ pericytes based on expression of key markers is rather challenging, given that no single marker is exclusively expressed by pericytes. In addition, most studies investigate the role of pericytes *only* with respect to their association to blood vessels, and not the precise molecular signals through which they might operate.

Specifically, in colorectal cancer, patient tumours with a low microvessel pericyte coverage index (MPI) exhibited significantly increased haematogenous metastasis and poorer prognosis compared to the high MPI group, using α smooth muscle actin (α SMA) as a pericyte marker (Yonenaga et al., 2005). Another study using h-caldesmon as a pericyte marker showed a similar inverse correlation of pericyte coverage and metastasis in malignant melanoma patients (Koganehira et al., 2003). A recent study has reported that pericyte depletion promotes metastasis via hypoxia and EMT in a mouse model of breast and renal cell carcinoma using NG2 as a pericyte marker (Cooke et al., 2012), which has been refuted recently by a study that showed high MPI correlates with increased metastasis in clear cell renal cell carcinoma patients using α SMA as a pericyte marker (Cao et al., 2013). Several others have drawn similar conflicting conclusions using the PDGFR^{ret/ret} mice as a model, the limitations of which have been discussed in *Chapter 1, Section 1.1.3.3.3*. The ambiguity of these results also stem from the fact that most studies do not distinguish between pericyte ‘ablation’ and ‘dissociation from vessels’ to other sites from where they might be exerting direct pro-metastatic effects.

Although it has been shown that dual targeting of vessels and pericytes leads to regression of orthotopic ovarian xenograft tumours (Lu et al., 2007, 2008, 2010), the role of pericytes specifically in ovarian cancer *metastasis* has not been studied thus far. As discussed previously (*Chapter 1, Section 1.2.2*), high-grade serous ovarian cancer, mostly arising from a single layer of simple epithelial cells on the surface of

the ovary, presents an excellent model for studying the epithelial tumour microenvironment. Epithelial ovarian cancer (EOC) is unique in that it is intimately associated with the surrounding tissue and mostly metastasises by direct dissemination into neighbouring organs, before metastasis to more distant visceral sites like the lung and the brain can take place via haematogenous and lymphatic routes. Early detection of ovarian cancer is rare, with the disease turning fatally aggressive by the time it is presented clinically in most cases (Auersperg et al., 1998; Morrison, 2005). The organs most frequently affected by ovarian cancer metastasis are the peritoneum, omentum, gastrointestinal (GI) tract and bladder. Late progression into the liver, spleen, kidney and lung are not uncommon (Cannistra, 2004).

In EOC, once the cancer cells are detached as single cells or clusters of spheroids, they disseminate passively, mostly carried by the dynamic pressure of the peritoneal fluid. However, this still requires the onset of epithelial mesenchymal transition (EMT), a key requisite for metastatic spread. EMT is crucial since it not only releases cells from adhesion to the basement membrane, but also from each other via loosening of cell-cell junctions, thereby signalling a cascade of molecular events within the cancer cell (Kalluri and Weinberg, 2009; Lee et al., 2006). E-cadherin has a pivotal role in the EMT process, since this membrane glycoprotein is responsible for maintaining cell-cell junctions (Huber et al., 2005; Vergara et al., 2010). A preferential loss of E-cadherin in floating, disseminated tumour cells compared to those within the primary tumours has been demonstrated in a wide range of cancers, including ovarian (Ahmed et al., 2007). Loss of E-cadherin sets up a 'cadherin-switch' – a series of molecular events leading to a motile, mesenchymal and 'fibroblastic' shaped cancer cell, ready to invade (Hudson et al., 2008; Patel et al., 2003).

In this study so far, a novel, angiogenesis-independent, pro-proliferative effect of pericytes in the ovarian tumour environment has been demonstrated (*Chapter 3*). Given that metastatic disease is key to cancer fatality, the next step for this study was to carefully dissect the effects of pericytes with respect to key processes like EMT, invasion and metastasis and their clinical consequences. Bearing in mind that the normal homeostatic role of pericytes revolves around remodelling the ECM and

regulating cell adhesion (Bergers and Song, 2005; Gerhardt and Semb, 2008), processes central to ovarian cancer metastasis, it was *hypothesised that the addition of exogenous pericytes would initiate metastasis of ovarian cancer cells to distant organs.*

The specific aims addressed in this Chapter were to (i) investigate whether co-injection of pericytes or fibroblasts with non-metastatic human ovarian cancer cell lines promote metastasis to secondary organs and; (ii) explore the role of invasion and epithelial mesenchymal transition (EMT) in xenograft tumours resulting from the co-injection of pericytes.

4.2. Results

4.2.1. Pericytes promote ovarian cancer metastasis in xenograft models of serous ovarian cancer

Luciferase labelling of cancer cells to monitor the progress of the primary tumour and metastatic spread by *in vivo* bioluminescent imaging is a powerful technique in studying metastasis in animal models of various cancer types. Although luciferase labelling of cells for *in vitro* cloning techniques has been in use since 1975, introducing luciferase⁺ cancer cells *in vivo* and monitoring its spread was first pioneered in an intraperitoneal model by Sweeney et al., 1999. This was subsequently followed by luciferase use in subcutaneous and intravascular models (Edinger et al., 1999), an orthotopic model of gliosarcoma (Rehemtulla et al., 2000) followed by several others (Caceres et al., 2003; Edinger et al., 2002; Nogawa et al., 2005). Luciferase imaging for monitoring primary tumour size and metastasis to secondary organs has since been a popular tool in cancer research, chiefly because of the technique's non-hazardous nature, reproducibility, and a very consistent correlation of bioluminescent signal with primary tumour volume calculated using caliper measurement. Moreover, BLI enables the monitoring of mice at selected intervals without the need to sacrifice animals at each time-point. In the experiments described in this Chapter, BLI using luciferase was used as a primary tool to investigate whether co-injection of pericytes facilitated metastasis of ovarian cancer cell lines in a subcutaneous xenograft model.

4.2.1.1. Lentiviral transduction to construct GFP-luciferase⁺ OVCAR-5 cells

In order to monitor the growth and dissemination of the tumour cells following subcutaneous injection, OVCAR-5 cells were lentivirally transduced with a plasmid carrying a GFP-luciferase vector. Cells were incubated with the viral supernatant for 6 hours and expanded in culture for 2 passages. Passaged cells were then subjected to flow cytometry to select GFP⁺ cells, efficiency of transduction calculated, and positive cells expanded for *in vivo* injection. A representative sort plot is shown in *Figure 4.1A*.

4.2.1.2. *In vivo* bioluminescence imaging (BLI) shows increased metastasis to distant organs in the presence of exogenous pericytes

GFP-luciferase⁺ OVCAR-5 cells were injected subcutaneously with or without cultured (p4) VLA-1^{brn} pericytes in a 10:1 cancer cell: pericyte ratio and progression of the tumour tracked weekly using *in vivo* bioluminescence imaging (Xenogen). BLI data revealed that tumours co-injected with pericytes were able to metastasise within the peritoneal cavity as early as Day 21 (*Figure 4.1B*). At Day 28, BLI images were acquired after the excision of the primary tumour from harvested mice, to eliminate the masking effect of strong bioluminescent signals from the primary tumour. By Day 28, metastases associated with the intestine, liver, and lung were detected in the group of mice co-injected with pericytes, whereas all mice in the control group were free of metastases (*Figure 4.1C*).

To address if the induction of metastasis was a specific effect of pericytes, it was crucial to determine if the co-injection of VLA-1^{dim} fibroblasts derived from the same tissue of origin as the pericytes, would have a similar metastasis-inducing effect. Nude mice bearing tumours resulting from the injection of either OVCAR-5 cells alone or together with fibroblasts were imaged at Day 21 (*Figure 4.2A*) where no metastatic signals were observed. At Day 28, BLI images acquired under identical experimental conditions after surgical excision of the primary tumour showed that mice co-injected with fibroblasts were completely free of metastasis (*Figure 4.2B*). This indicated that the metastasis-promoting property was specific to pericytes and that the introduction of any other mesenchymal stromal population will not have the same effect.

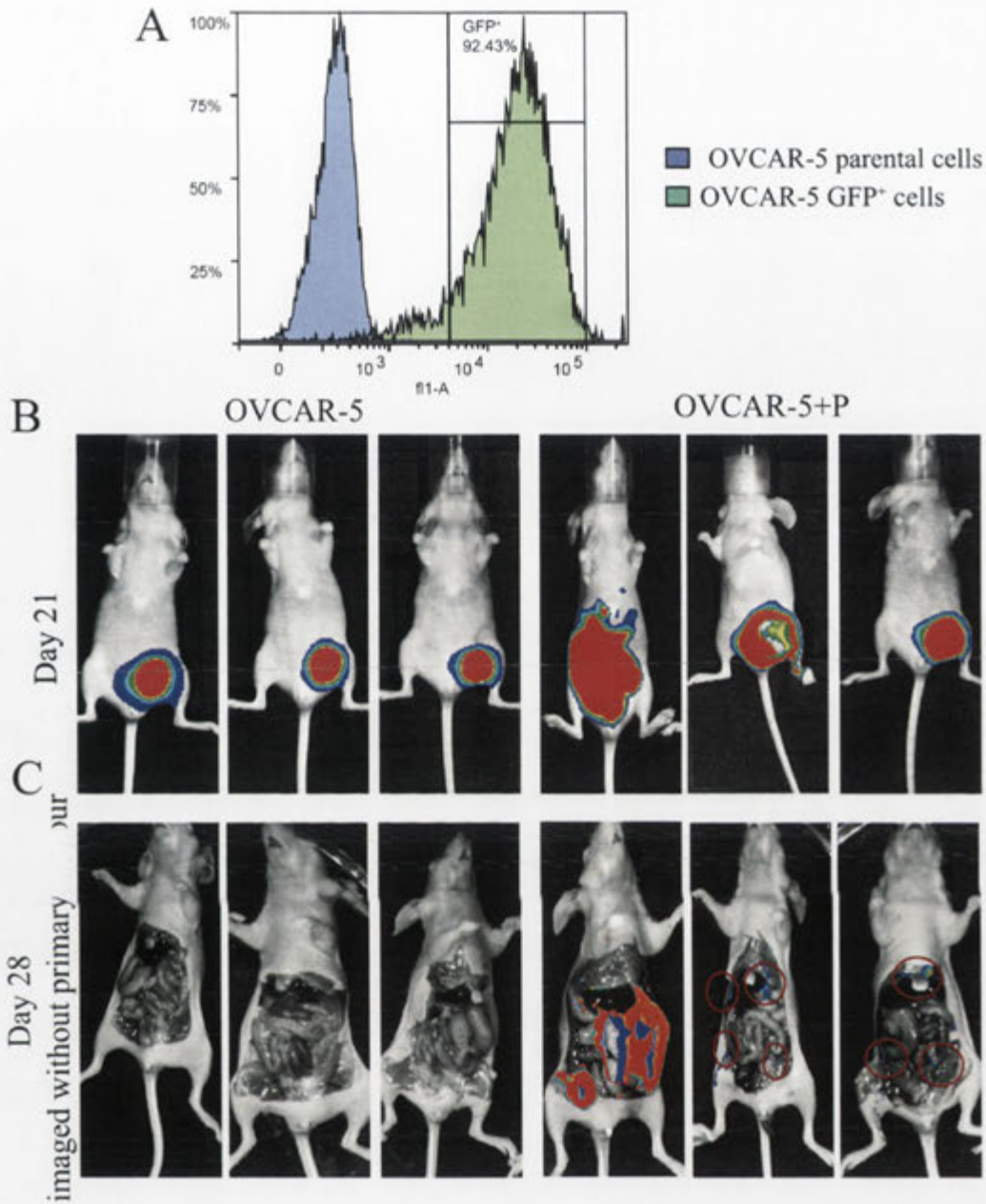


Figure 4.1. Pericytes promote metastasis in ovarian xenograft tumours in nude mice. (A) A Flow cytometric analysis of parental OVCAR-5 (purple) and GFP-luciferase transduced OVCAR-5 cells (green). Gate on FL-1 channel is indicative of GFP fluorescence and was used to sort for the GFP⁺ population. (B-C) Representative BLI images of nude mice carrying GFP-luciferase⁺ OVCAR-5 control tumours and those co-injected with pericytes at (B) Day 21, indicating position of primary tumour in anesthetised mice, and at (C) Day 28, after excision of primary tumour from euthanised mice. Red circles have been used to highlight signals from smaller metastatic nodules. Images are representative of 30-32 mice per experimental group from 5 independent experiments.

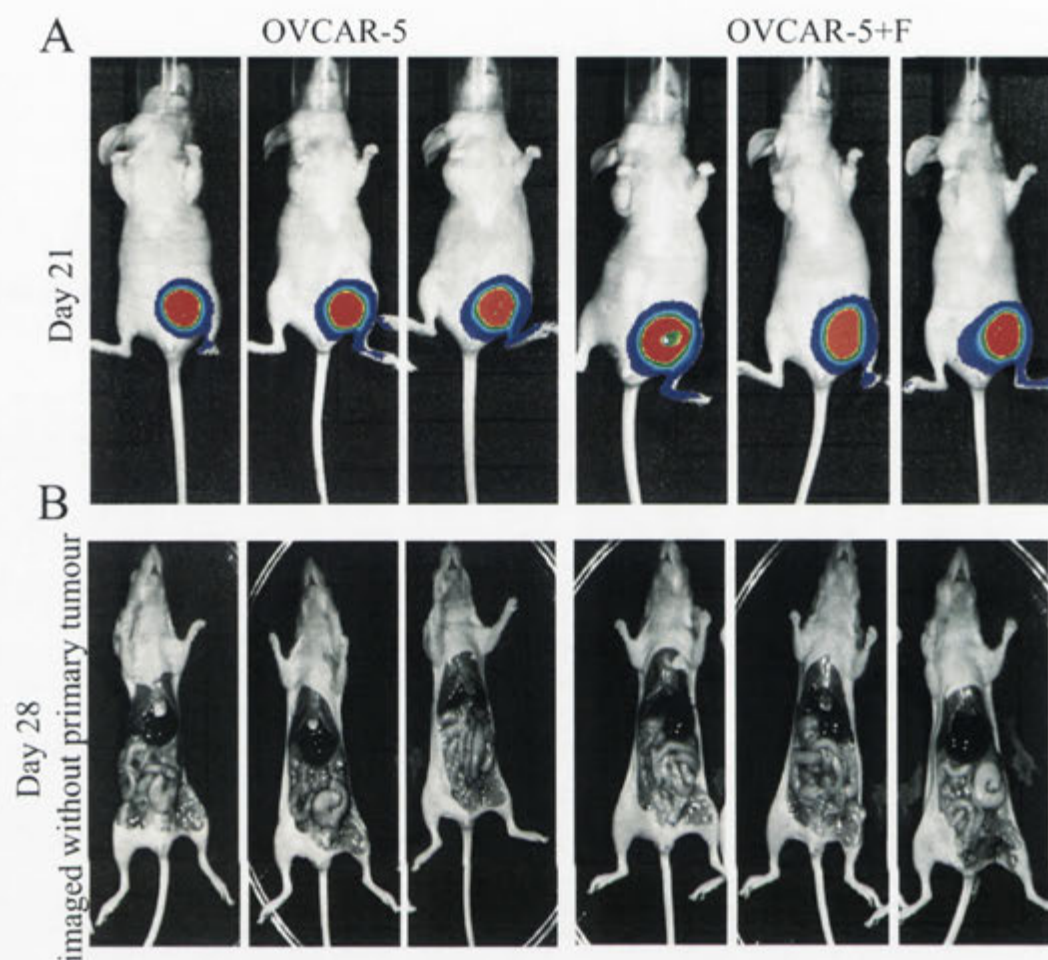


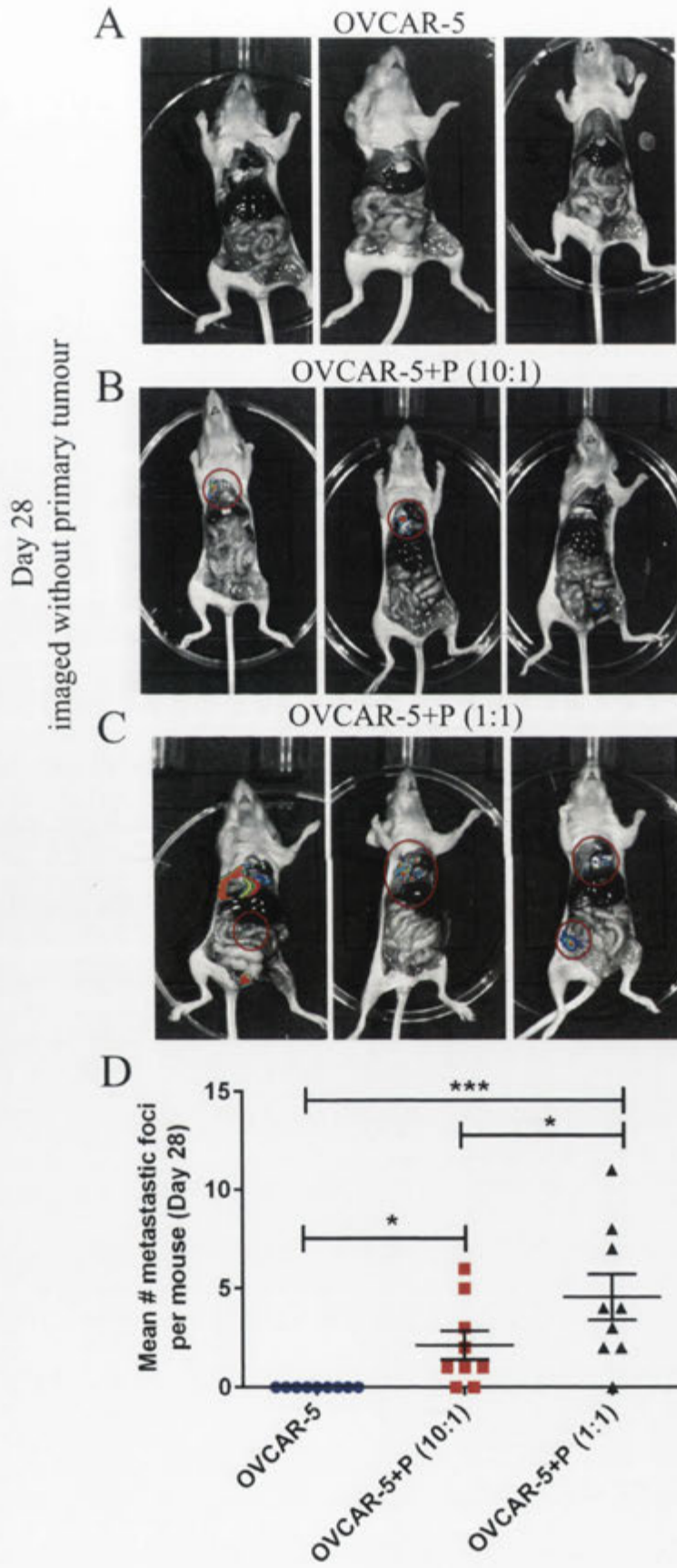
Figure 4.2. Addition of exogenous fibroblasts does not promote metastasis in ovarian xenograft tumours in nude mice. (A-B) Representative BLI images of nude mice carrying GFP-luciferase⁺ OVCAR-5 control tumours and those co-injected with fibroblasts at (A) Day 21, indicating position of primary tumour and at (B) Day 28, after excision of primary tumour from euthanised mice. There was no evidence of metastasis with the co-injection of fibroblasts. Images are representative of 10 mice per experimental group from 2 independent experiments.

Further experimentation with a higher proportion of exogenous pericytes revealed a pericyte dose-dependent effect on metastatic burden. Increasing the OVCAR-5 cell: pericyte ratio from 10:1 to 1:1 resulted in increased metastasis to local and visceral organs at Day 35. This increase was evident in terms of net metastatic burden, range of organs affected and the number of mice affected per group (*Figure 4.3A-C*). Quantification of the mean number of metastatic foci per mouse observed in secondary organs revealed a significant increase in the group injected with 1:1 OVCAR-5:pericyte ratio, compared to the group injected with a 10:1 ratio, as well as the OVCAR-5 controls, which were metastasis-free (*Figure 4.3D*).

Subsequently, similar experiments were conducted on the more immunocompromised NOD/SCID mice. Consistent with the data in nude mice (*Figure 4.1*), extensive metastasis in the abdominal organs were observed in the pericyte co-injected group of NOD/SCID mice (*Figure 4.4A-B*) at Day 28. Interestingly, some bioluminescent metastatic nodules were detected in control OVCAR-5 injected NOD/SCID mice as well. This was attributed to the increased susceptibility of NOD/SCID mice, given that their microenvironment was more permissive to tumour cell dissemination in the absence of tumour-suppressing immune cells like natural killer (NK) cells (Lapidot et al., 1997). Nevertheless, the net metastatic burden and the number of mice affected in the group co-injected with pericytes was undoubtedly higher (*Figure 4.4A-B*).

Figure 4.3. Pericytes exert a dose-dependent effect on metastatic burden in nude mice.

(A-C) Representative BLI images of nude mice carrying (A) GFP-luciferase⁺ OVCAR-5 control tumours and those co-injected with (B) 10:1 and (C) 1:1 pericytes. Red circles highlight the signals from smaller metastatic nodules. (D) Quantification of the number of metastatic nodules per mouse observed for each experimental group. Increased ratio of pericytes resulted in significantly increased metastatic burden. Results are representative of 10 mice per experimental group from 2 independent experiments. Error bars represent mean $\pm$ SEM; statistical analysis performed using one-way ANOVA; * $p < 0.05$, *** $p < 0.001$.



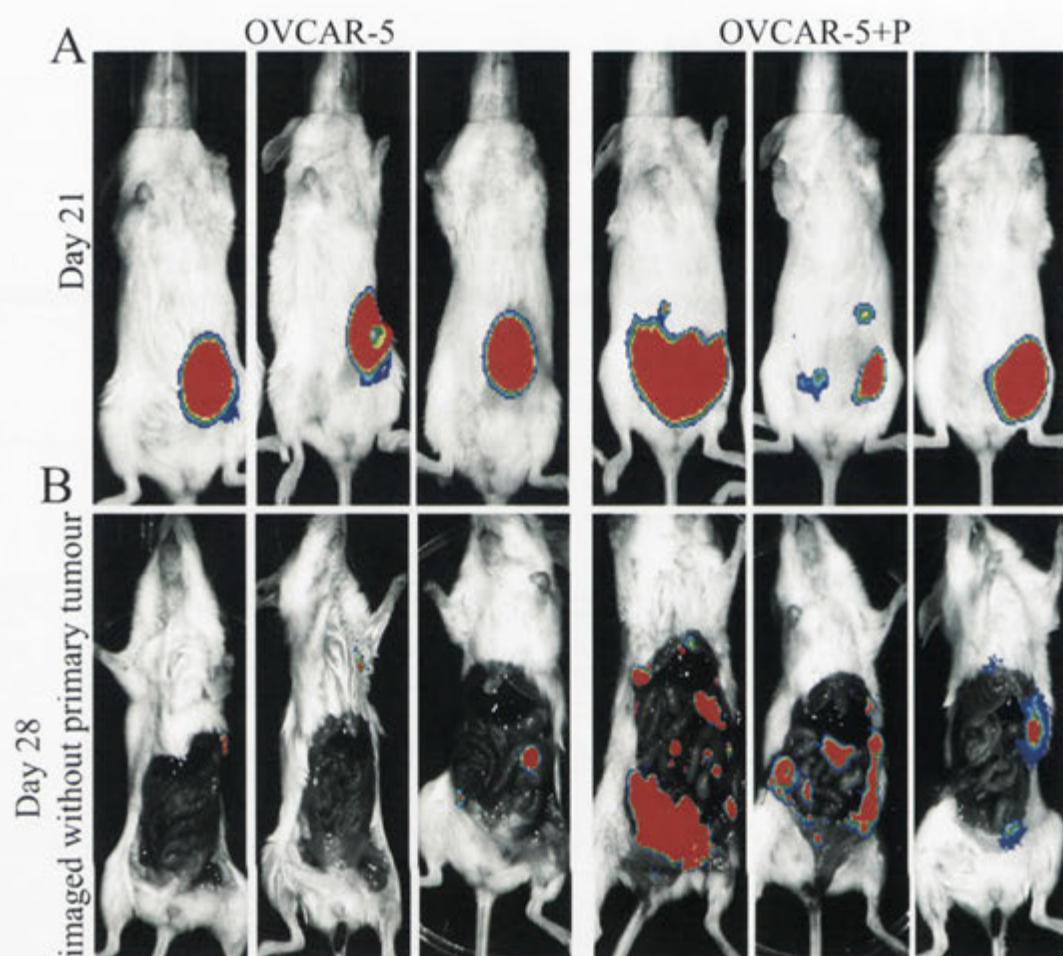


Figure 4.4. Pericytes promote more extensive ovarian cancer metastasis in NOD/SCID mice. (A-B) Representative BLI images of NOD/SCID mice carrying GFP-luciferase⁺ OVCAR-5 control tumours and those co-injected with pericytes at (A) Anesthetised mice were imaged at Day 21 indicating position of primary tumour, and (B) at Day 28, after primary tumour excision from euthanised mice. While baseline metastatic nodules were observed in the control group, more extensive metastases in local and visceral organs in the pericyte co-injected group were evident. Images are representative of 7 mice per experimental group from 2 independent experiments.

In a few instances where tumours in the nude mice were analysed beyond day 35, i.e. up to day 42, local metastases within the peritoneal cavity (especially the lower GI tract) were macroscopically visible upon harvesting, with tumour nodules in **both** control and pericyte co-injected mice (*Figure 4.5A-B*). However, metastatic spread to more distant organs such as the spleen, kidney and lung was **only** observed in the pericyte co-injected group (*Figure 4.5B*), demonstrating progression to more advanced disease in the presence of pericytes.

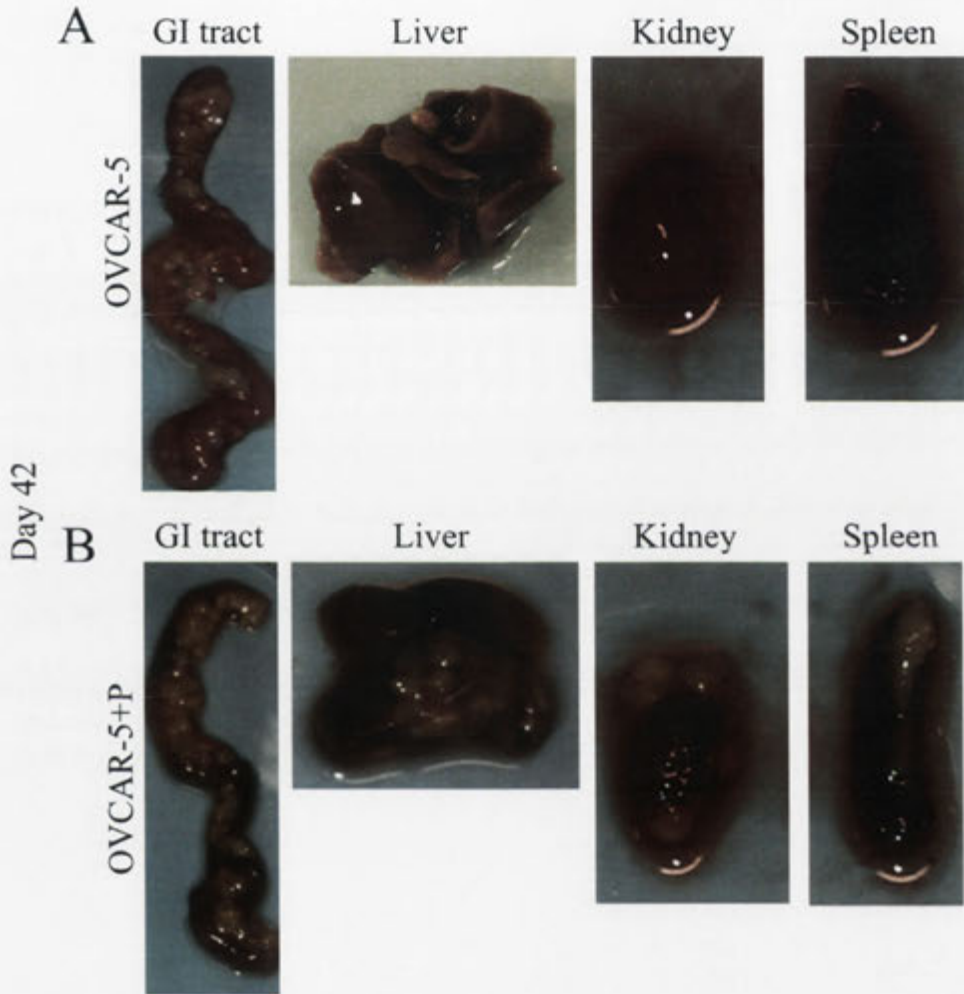


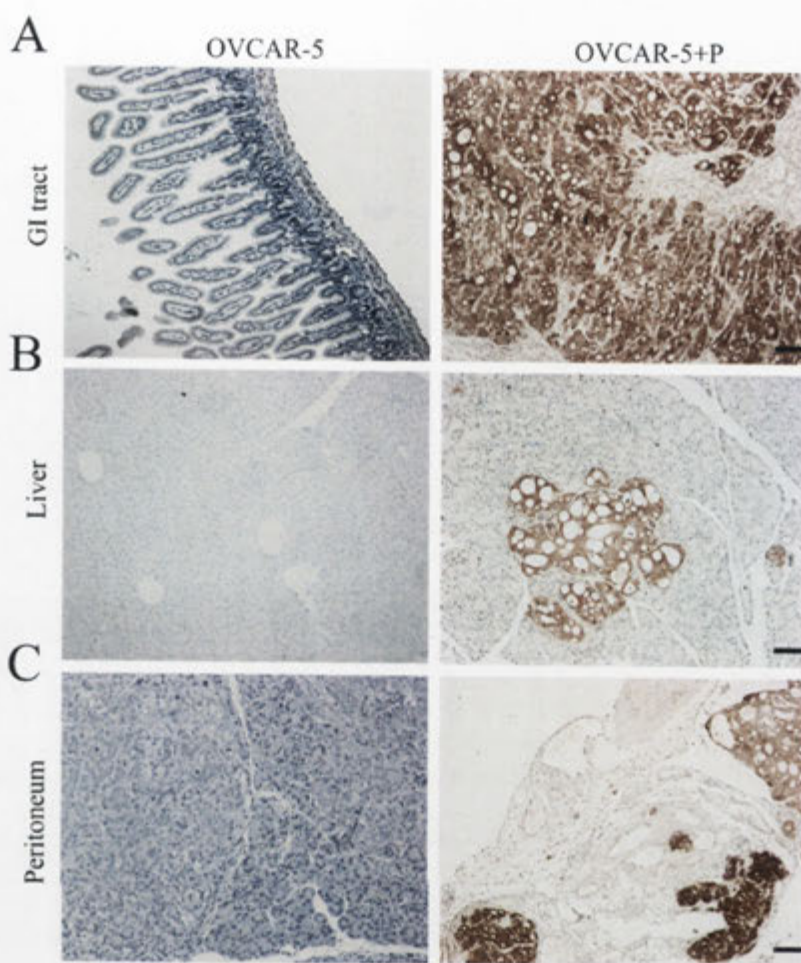
Figure 4.5. Evidence of increased macroscopic metastatic spread in pericyte co-injected nude mice at Day 42. (A-B) Organs harvested from nude mice carrying (A) GFP-luciferase⁺ OVCAR-5 control tumours and (B) those co-injected with pericytes at Day 42. Macroscopic metastatic nodules were observed along the GI tract of both control and pericyte-co-injected mice. However, at this stage, extensive metastases to the liver, kidney and spleen was observed only in the pericyte co-injected group of mice.

4.2.1.3. Histological validation of metastases detected by BLI confirmed their OVCAR-5 cellular origin

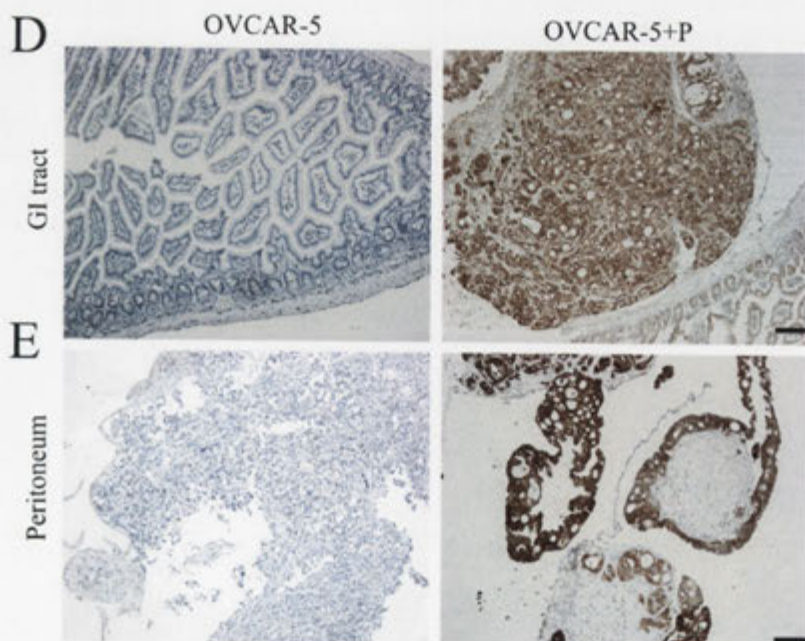
Although BLI is an excellent means of detecting the location and spread of metastasis, it is important to histologically confirm that BLI signals detected are indeed 'true' metastases originating from the injected tumour cells. Histological validation also gives a proper insight into the morphology of the metastatic nodule, histology of the metastatic environment, and the extent of metastasis. Here, it was advantageous to have a second marker, i.e. GFP, along with luciferase which was employed to validate observations from the BLI experiments.

In keeping with the BLI data (*Figure 4.1*) and macroscopic observations (*Figure 4.5*), immunohistochemical staining for GFP revealed the presence of GFP⁺ metastatic nodules in the GI tract, liver and peritoneum at Day 28 in the pericyte co-injected group while the same organs from the OVCAR-5 control group were free of metastasis (*Figure 4.6A-C*). By Day 35, metastasis had further spread to the omentum and lungs in the mice co-injected with pericytes while the OVCAR-5 injected mice still remained metastasis-free (*Figure 4.6D-H*). At Day 42, metastatic spread in the pericyte co-injected group was observed not only in the GI tract, peritoneum, liver, omentum, and lungs (*Figure 4.6I-M*), but also in the kidney and spleen (*Figure 4.6N-O*). Interestingly, at the rather late time-point of Day 42, GFP signal representing metastasis was also recorded in the GI tract of OVCAR-5 control mice (*Figure 4.6I*) thus validating the macroscopic observation in *Figure 4.5B*.

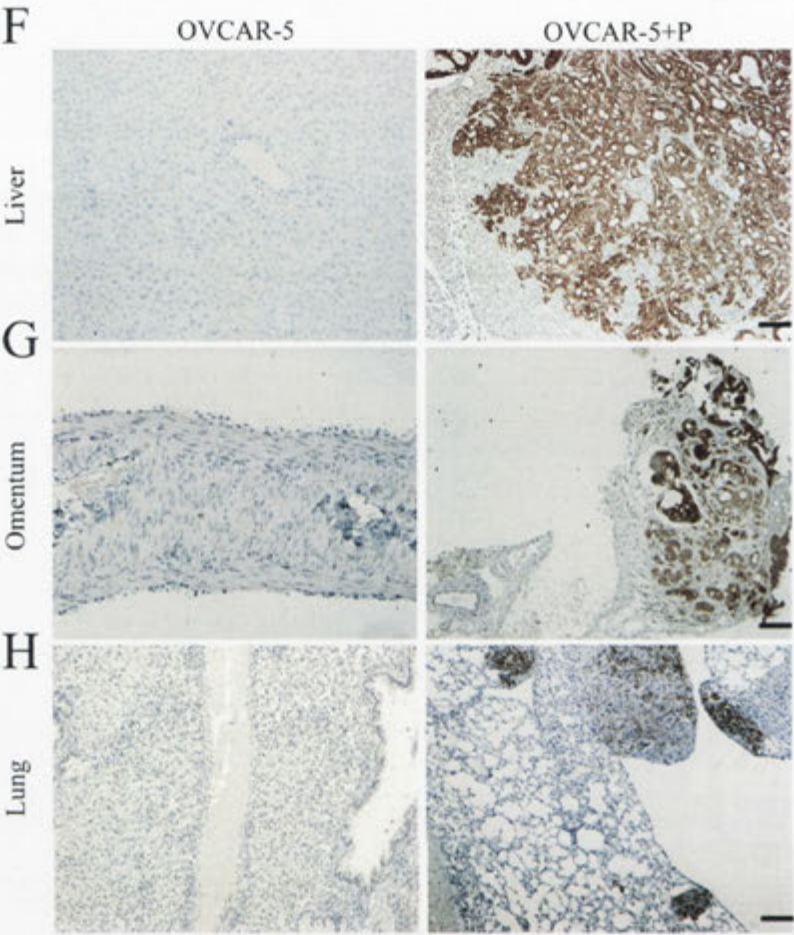
Day 28



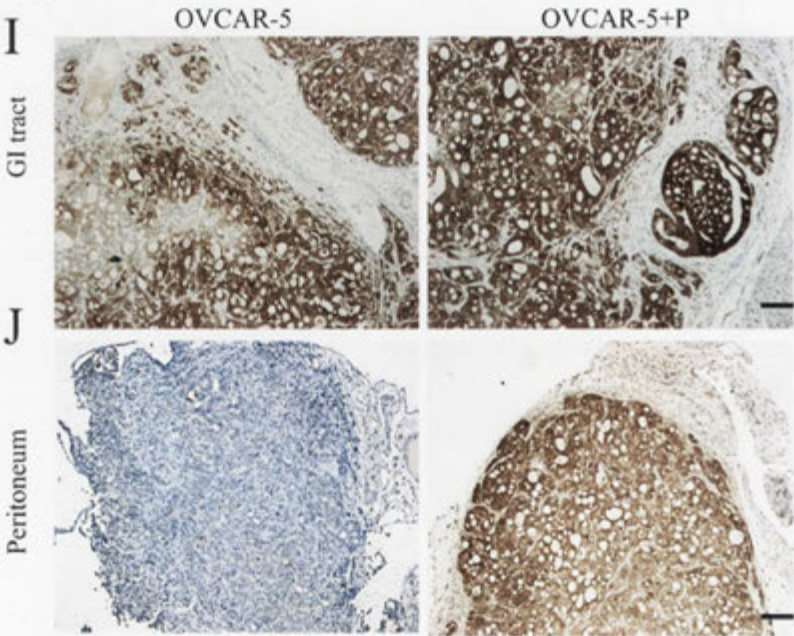
Day 35



Day 35 (continued)



Day 42



Day 42 (continued)

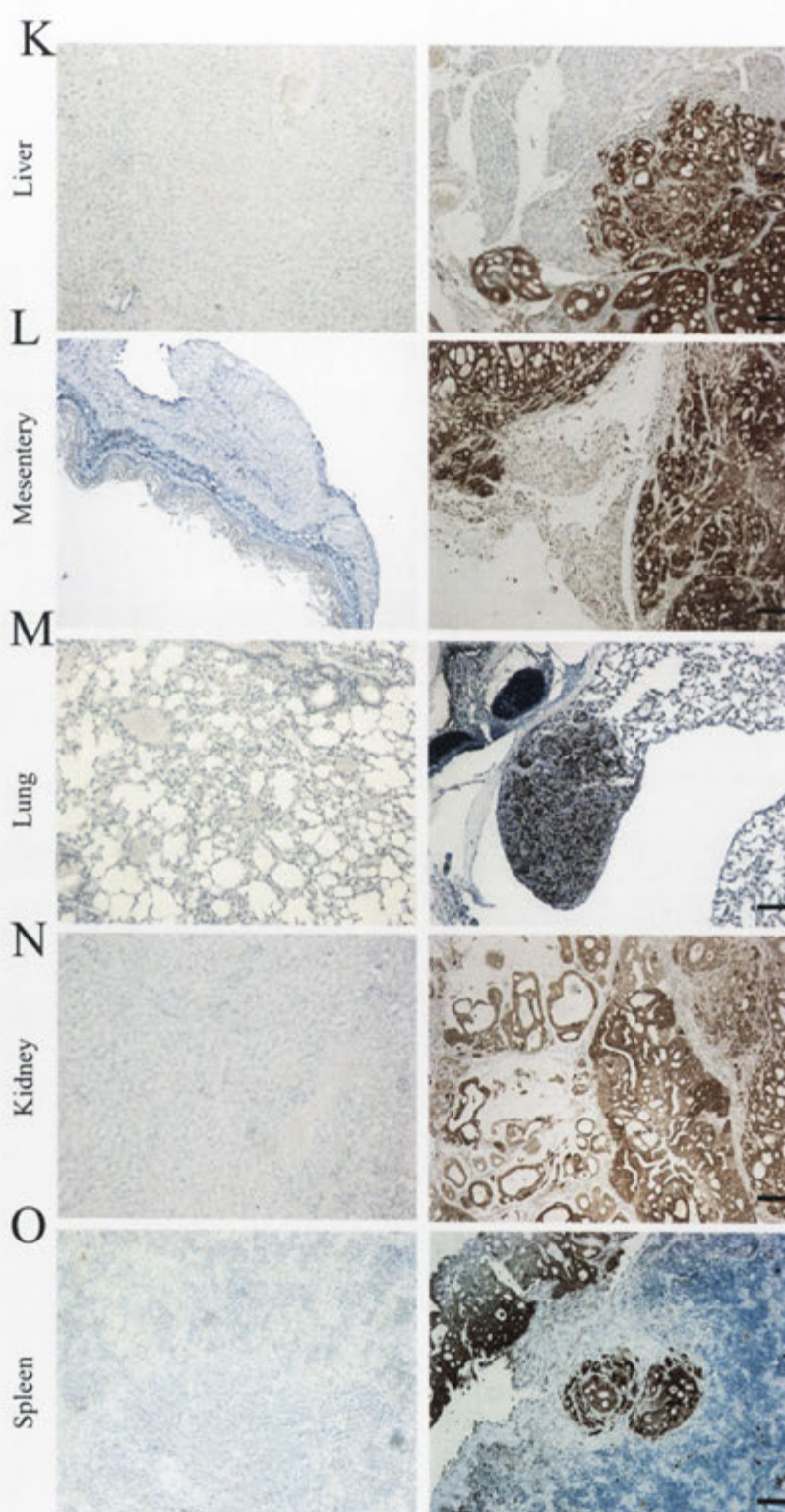


Figure 4.6. Histological validation of metastatic nodules observed in BLI images. (A-O) Histological validation of metastatic nodules showing immunohistochemical staining FFPE sections of local and visceral organs at (A-C) Day 28, (E-J) Day 35 and (K-R) Day 42 with an antibody against GFP to detect the presence of GFP⁺ OVCAR-5 cells (brown staining). (A-C) At Day 28, metastatic nodules in the (A) GI tract, (B) liver and (C) peritoneum were observed in the mice co-injected with pericytes whereas organs from mice injected with OVCAR-5 cells alone were free of metastasis. (D-H) At Day 35, OVCAR-5+P mice not only had metastasis in the (D) GI tract, (E) peritoneum and (F) liver but also showed further spread to the (G) omentum and (H) lung. (I-O) By Day 42, metastasis had spread to almost all vital organs including the (I) GI tract, (J) peritoneum, (K) liver, (L) omentum, (M) lung, (N) kidney and (O) spleen in the tumours co-injected with pericytes. Note that by this time-point, local spread to the GI tract was also observed in the control OVCAR-5 injected group of mice.

For number of mice per group per time-point in each experiment and the details of observations, please refer to *Table 4.1*. Haematoxylin was used as a counterstain. Scale bar=100 μ m.

4.2.1.4. *Pericytes promote tumour growth in the non-invasive OVCAR-8 cell line*

In addition to the experiments with the rather aggressive OVCAR-5 cell line, it was also investigated if pericytes were able to induce metastasis to visceral organs in a cell line that is non-aggressive. For this purpose, the OVCAR-8 cell line – derived from an early stage cisplatin-treated patient and known to form non-invasive xenograft tumours with long periods of latency (Godwin et al., 1992; Hamilton et al., 1984) was employed. OVCAR-8 cells were more epithelial in shape, had a relatively slower doubling time in culture (data not shown) and a much lower efficiency of lentiviral transduction (~40-50%, compared to ~90% transduction efficiency of the OVCAR-5 cells), as shown in *Figure 4.7A*. In a pattern very similar to that observed with the more aggressive OVCAR-5 cells, tumours generated from subcutaneous injection of OVCAR-8 cells with pericytes in a 10:1 ratio showed a significantly accelerated growth (*Figure 4.7B*) and larger end-point tumour volumes (*Figure 5.7C*). Interestingly, the pericyte co-injected tumours also had a reduced latency of ~15 days compared to the latency of ~35 days in the control OVCAR-8 tumours (*Figure 4.7B*).

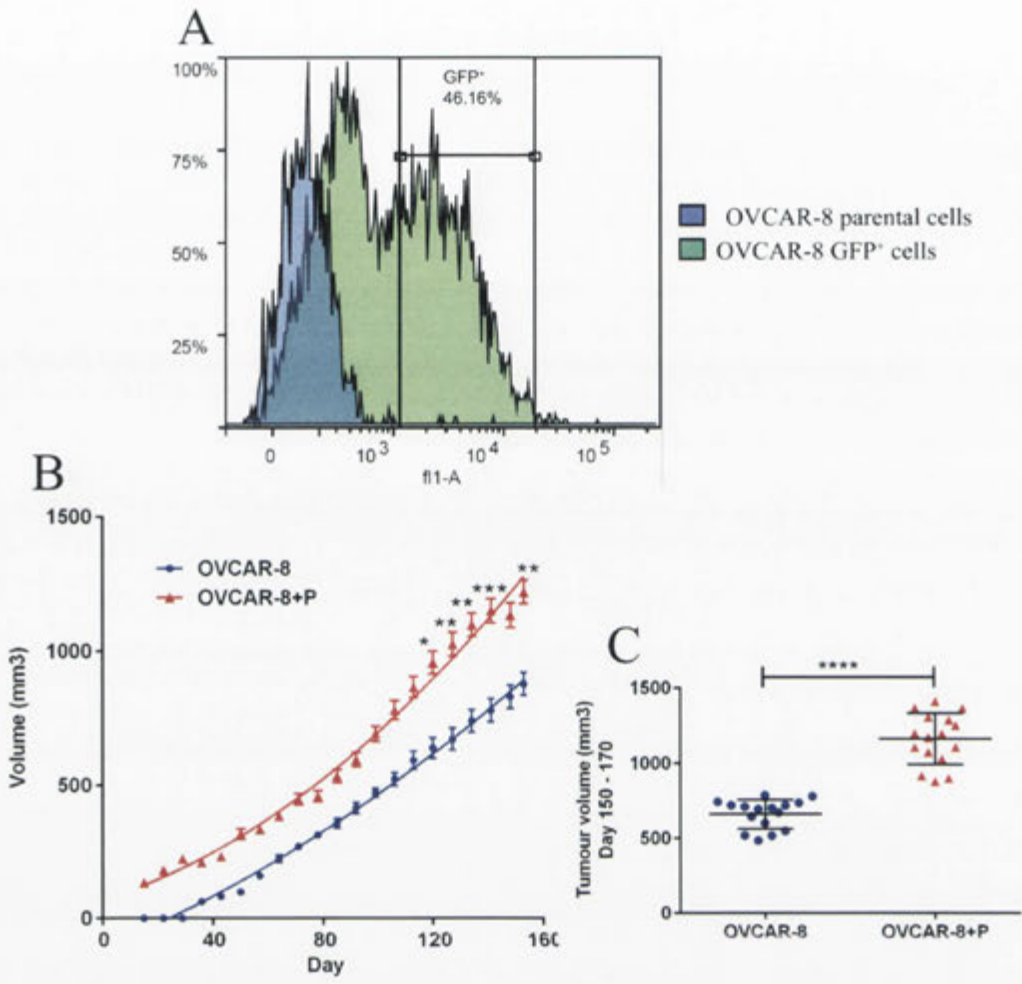


Figure 4.7. Pericytes accelerate the growth of the OVCAR-8 cells *in vivo*. (A) Flow cytometric analysis of parental OVCAR-8 (purple) and GFP-luciferase transduced OVCAR-8 cells (green). Gate on FL1 channel is indicative of GFP fluorescence and was used to sort for the GFP⁺ population. (B) Non-linear regression fit of tumour volumes of xenograft ovarian tumours generated from the injection of OVCAR-8 cells alone or together with 10:1 pericytes. Data represents mean $\pm$ SD of volume at each time-point of 16 mice per group from 2 independent experiments. Repeated measures data for each time-point was compared using two-way ANOVA. (C) Quantification of end-point tumour volume between Days 150-160. Data represented as mean volume $\pm$ SEM from 16 mice per group from 3 independent experiments. Statistical analysis performed using unpaired t-test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

4.2.1.5. Co-injection of pericytes initiates and promotes metastasis to distant organs in the non-metastatic OVCAR-8 cells

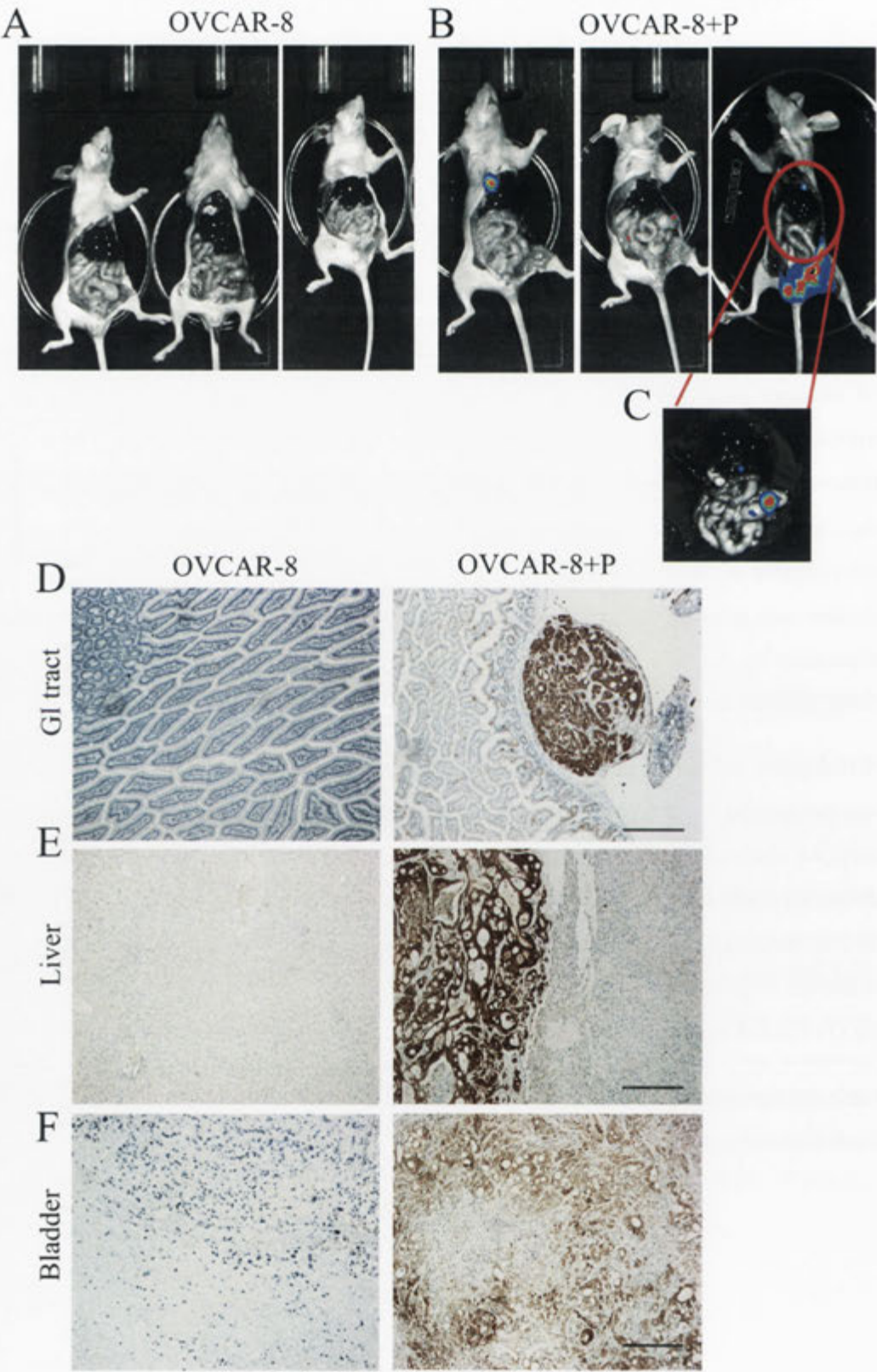
Metastatic spread in nude mice growing GFP-luciferase⁺ OVCAR-8 tumours injected with or without pericytes was similarly tracked using *in vivo* BLI. Signals from metastatic nodules in the gastrointestinal tract, liver, bladder and lung, were observed in the pericyte co-injected group between Days 150-160 post tumour cell injection; while the control OVCAR-8 injected group was completely free of metastasis (Figure 4.8 A-B).

Similar to the OVCAR-5 model, a recurrent observation while carrying out autopsy of tumours from the OVCAR-8 model was the close association of the primary tumour with the peritoneum and the adjacent tissue, often making it difficult to remove the primary tumour completely. On the other hand, the control tumours were tightly encapsulated in the subcutaneous region and hence relatively easier to excise. An example of this is illustrated in Figure 4.8C where the extent of primary tumour residue was substantial; the abdominal organs were therefore removed and imaged separately to confirm that the signals observed were exclusively from metastatic tissue and not the remnant primary tumour.

Histological validation of the metastatic signals observed in the BLI experiments was performed by immunohistochemical staining of sections from affected organs using an antibody against GFP. GFP staining confirmed the presence of metastases in the GI tract, liver, bladder and lung (Figure 4.8D-G) and revealed evidence of further metastasis in the form of smaller nodules in the kidney, peritoneum and omentum (Figure 4.8 H-J), in the pericyte co-injected group of mice. Organs from the OVCAR-8 injected control mice were free of metastasis (Figure 4.8 D-J).

Table 4.1 summarises the metastasis data obtained with the OVCAR-5 and OVCAR-8 cell lines in nude mice in this study.

Day 150-160 (imaged without primary tumour)



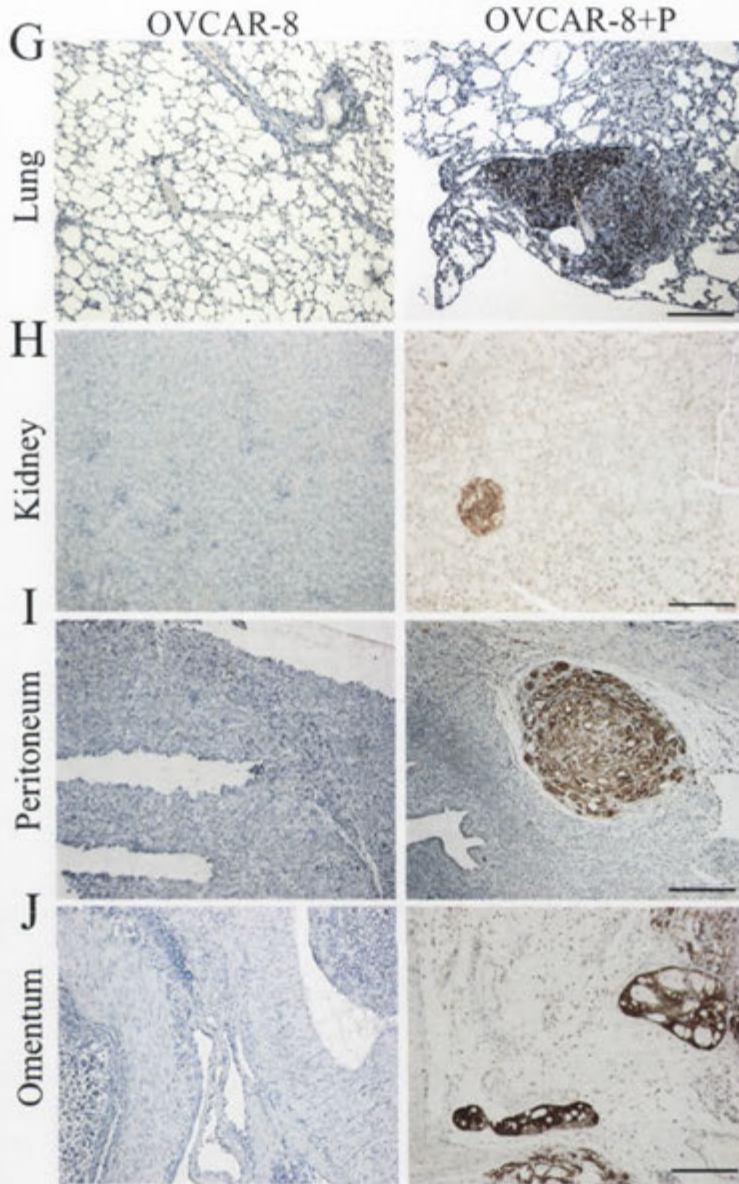


Figure 4.8. Pericytes promote metastasis in OVCAR-8 xenograft tumours in nude mice.

(A-C) Representative BLI images of nude mice carrying (A) GFP-luciferase⁺ OVCAR-8 control tumours and (B) those co-injected with pericytes between days 150-160 after excision of primary tumour in euthanised mice. (C) A representative case from the OVCAR-8+P group where organs were excised and imaged separately to avoid signal from the residual primary tumour. (D-E) Histological validation of metastatic nodules showing immunohistochemical staining with an antibody against GFP to detect the presence of GFP⁺ OVCAR-8 cells in local and visceral organs such as (D) GI tract, (E) liver, (F) bladder, (G) lung, (H) kidney, (I) peritoneum and (J) omentum. Both BLI and histology images are representative of 16 mice per experimental group from 2 independent experiments. Scale bar=100 μ m.

Table 4.1. Summary of metastasis data in OVCAR-5 and OVCAR-8 xenograft models of ovarian cancer in nude mice.

				Secondary organs							
Time	# expts [~]	#mice ^s	Group	GI tract	Liver	Peritoneum	Omentum	Lung	Kidney	Spleen	Bladder
OVCAR-5 model											
Day 28	n=6	n=43	OVCAR-5	-	-	-	-	-	-	-	-
	n=5	n=34	OVCAR-5+P (10:1)	✓	✓	✓	-	-	-	-	-
	n=2	n=10	OVCAR-5+P (1:1)	✓	✓	✓	-	✓	-	-	-
	n=2	n=10	OVCAR-5+F (10:1)	-	-	-	-	-	-	-	-
Day 35	n=3	n=22	OVCAR-5	-	-	-	-	-	-	-	-
	n=3	n=16	OVCAR-5+P (10:1)	✓	✓	✓	✓	✓	-	-	-
	n=2	n=10	OVCAR-5+P (1:1)	✓	✓	✓	✓	✓	-	-	-
	n=2	n=10	OVCAR-5+F (10:1)	-	-	-	-	-	-	-	-
Day 42	n=1	n=4	OVCAR-5	✓	-	-	-	-	-	-	-
	n=1	n=5	OVCAR-5+P (10:1)	✓	✓	✓	✓	✓	✓	✓	-
OVCAR-8 model											
Day	n=2	n=16	OVCAR-8	-	-	-	-	-	-	-	-
150 -	n=2	n=18	OVCAR-8+P (10:1)	✓	✓	✓	✓	✓	✓	-	✓
160											

[~]#expts denotes number of independent experiments
^s#mice denotes the total number of mice from all experiments for each group

4.2.2. Pericytes promote OVCAR-5 metastasis through increased invasion and EMT

4.2.2.1. OVCAR-5 tumours co-injected with pericytes bear invasive nodes as early as Day 11

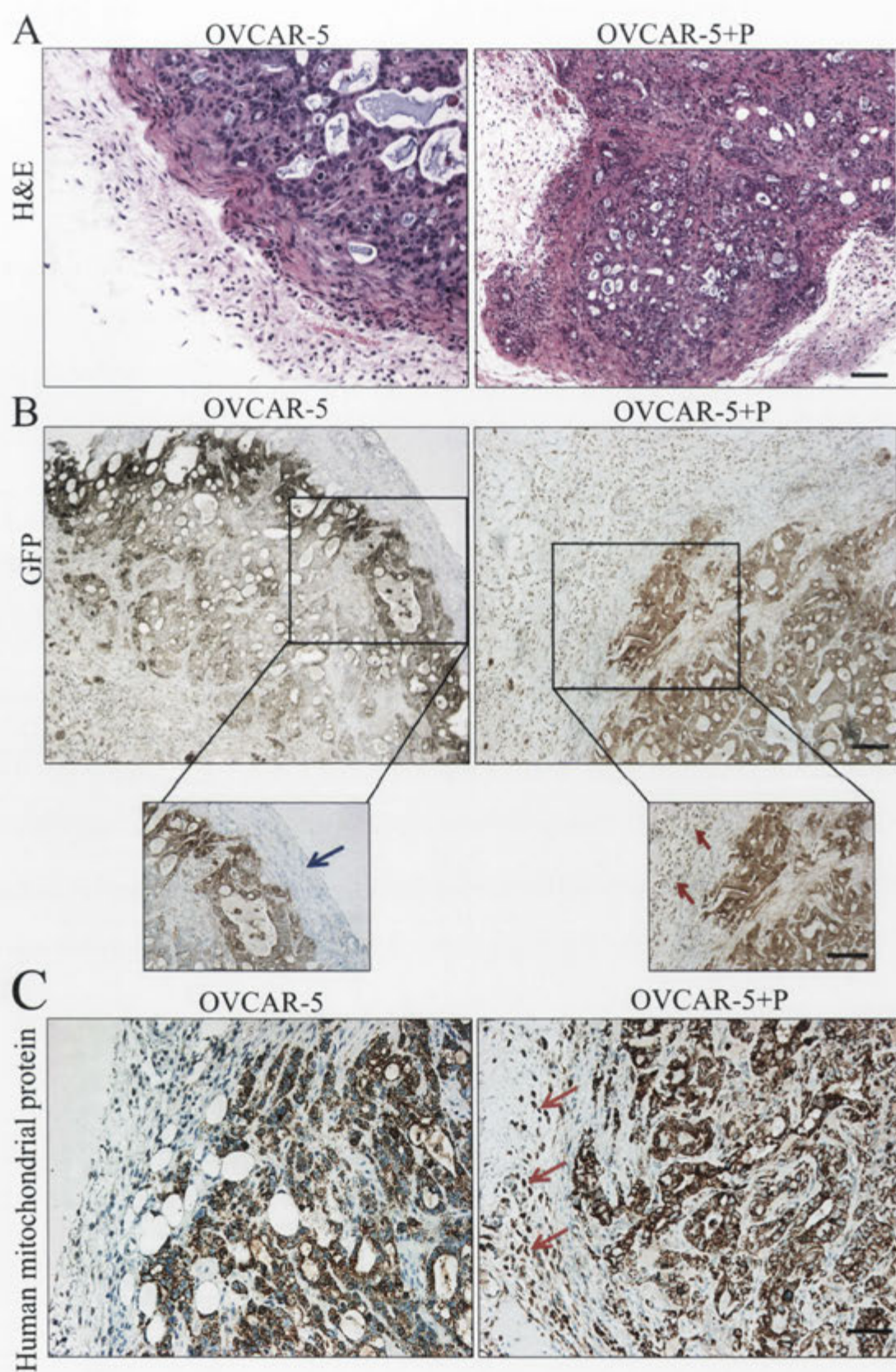
To recount from *Section 4.2.1.5*, one of the most prominent differences noticed while harvesting the xenograft tumours in both the OVCAR-5 and OVCAR-8 models was the close association of the pericyte co-injected tumours with the adjacent tissue. Further histological examination by H&E staining revealed that the pericyte co-injected tumours exhibited large invasive nodules at their leading edges as early as Day 11; in contrast, the OVCAR-5 control tumours presented smoother margins with no evidence of invasive nodules (*Figure 4.9A*).

To address if this was due to the invasion of tumour cells or influx of the murine stroma, sections from Day 11 primary tumours from both the GFP-luciferase⁺ OVCAR-5 control as well as pericyte co-injected tumours were stained with an antibody against GFP, and the morphology at the leading edges of the tumours examined. While control tumours bore a clear GFP⁺ stromal encapsulation, this was disrupted in tumours co-injected with pericytes by invading GFP⁺OVCAR-5 cells (*Figure 4.9B-C*).

Tumour sections were also stained with an antibody against a human-specific mitochondrial protein in order to further confirm the human origin of invading cells. Positive staining for this mitochondrial marker on single invasive cells disrupting the stromal lining at the tumour edges confirmed their human origin and further demonstrated that pericyte co-injection indeed rendered the OVCAR-5 xenograft tumours more invasive (*Figure 4.9D*).

Figure 4.9. Pericytes induce invasive & migratory phenotype of OVCAR-5 cells *in vivo*.

(A) Haematoxylin and eosin staining of invasive nodules in OVCAR-5+P tumour edges at Day 11 compared to the smooth margins of control OVCAR-5 tumours. (B) Immunohistochemical staining with an antibody against GFP showing single GFP⁺OVCAR-5 cells at the edge of OVCAR-5+P tumours (red arrow) compared to controls encapsulated within a GFP⁺ stromal lining (blue arrow). (C) Immunohistochemical staining with an antibody against a human-specific mitochondrial protein verifying human origin of single tumour cells at the edges of OVCAR-5+P tumours. Images are representative of 12-15 tumours per experimental group from 3 independent experiments. Scale bar=100µm.



4.2.2.2. Evidence of EMT at the invasive edges of OVCAR-5 tumours co-injected with pericytes

Epithelial mesenchymal transition is a key transformative process involved in the progression of a primary tumour to an invasive, disseminating state, the dynamics of which have been discussed in *Chapter 1, Section 1.2.2.2*.

In order to investigate if the tumour cells at the edges of the OVCAR-5+P tumours had undergone EMT, cryosections of Day 11 tumours were stained with antibodies against human E-cadherin and EpCam - given that loss of E-cadherin is widely associated with increased invasive capacity and EMT (Kalluri and Weinberg, 2009), while EpCam provides a marker for *all* epithelial OVCAR-5 cells irrespective of their EMT status. The results showed that tumours derived from co-injection of pericytes displayed loss of E-cadherin in EpCam⁺ tumour cells specifically at the leading edge of tumours, whereas control tumours co-expressed both markers uniformly. EpCam⁺E-cadherin⁻ cells in the pericyte co-injected tumours were also more motile, elongated and mesenchymal in appearance (*Figure 4.10A-B*).

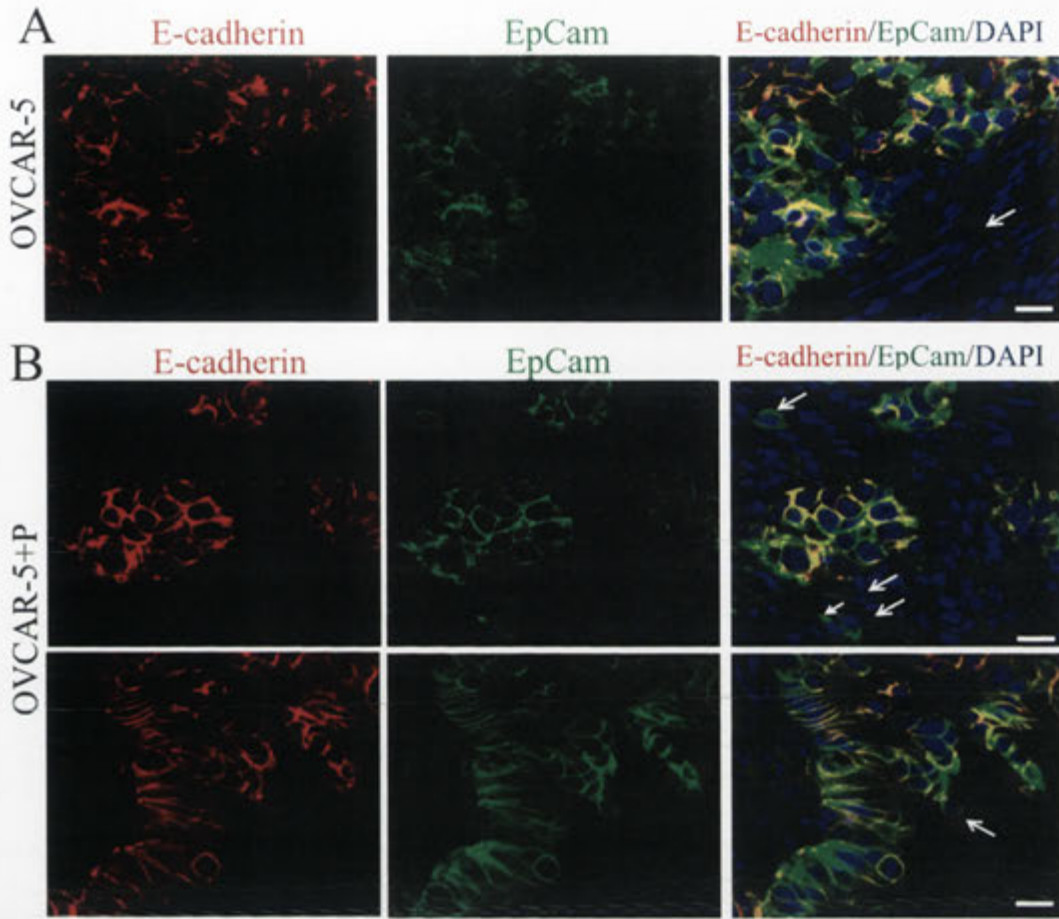


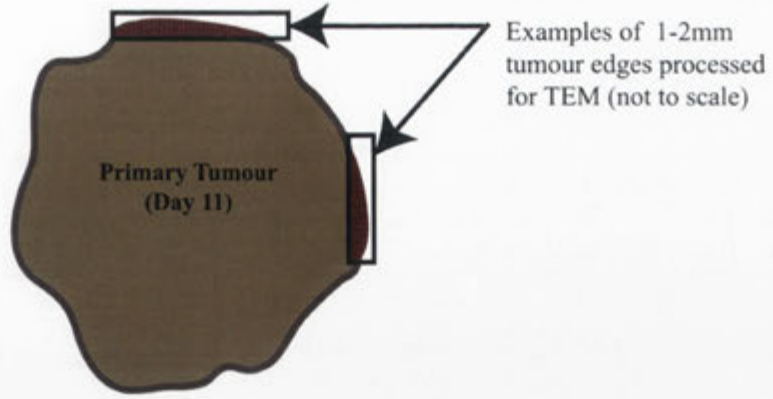
Figure 4.10. Pericytes induce EMT at the edges of ovarian xenograft tumours. (A-B) Immunofluorescent co-staining with antibodies against EpCam and E-cadherin on tumours generated from (A) OVCAR-5 control and (B) pericyte co-injected tumours at Day 11. While OVCAR-5 control tumours show clear encapsulation, tumours co-injected with pericytes show loss of E-cadherin and transformation to a motile, mesenchymal morphology in cells at the invasive edges (white arrows) of the tumours. Images are representative of 12 tumours per experimental group from 3 independent experiments. Nuclei counterstained with DAPI. Scale bar=20µm.

Further analysis of the ultrastructure of the tumour edges was carried out via transmission electron microscopy (TEM). 1-2 mm thin sections from the leading edges of OVCAR-5 and OVCAR-5+P tumours were processed for TEM analysis (*Figure 4.11A*). Results from TEM analysis further validated the previous observation of EMT in the presence of pericytes. Tumour cells at the edges of the OVCAR-5 control tumours had defined cell-cell junctions characteristic of their 'epithelial' state (*Figure 4.11B*). In contrast, tumour cells at the edges of pericyte co-injected tumours cells presented an elongated and motile shape and appeared to be in a more dynamic state, as early as Day 11 (*Figure 4.11C*).

Figure 4.11. Pericytes induce motile, invasive behaviour in OVCAR-5 tumour edges.

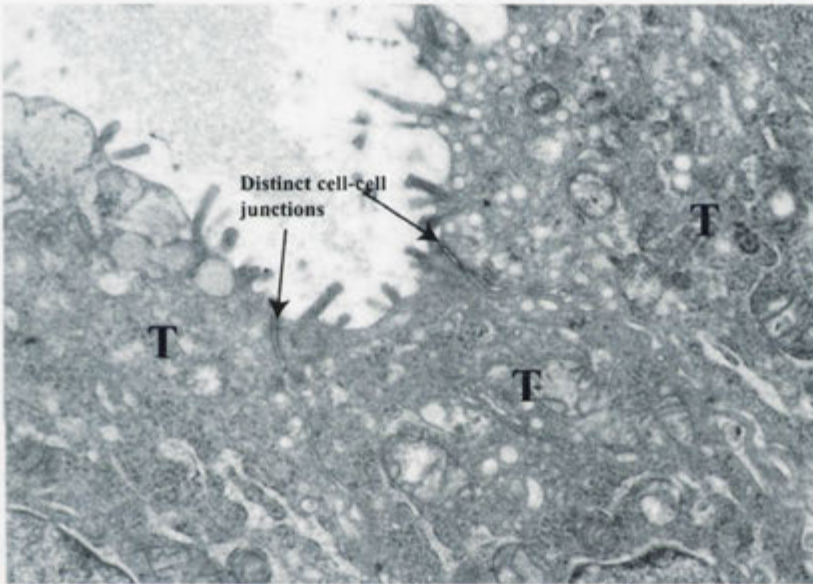
(A) Schematic illustration of the leading edges (1-2 mm) of tumours at Day 11 that were excised and processed for TEM. (B) A representative TEM image of the edge of an OVCAR-5 control tumour. Tumour cells are square-shaped and adhere to each other via cell-cell junctions. T (in dark blue) indicates tumour cells. Magnification= 150,000x. (C) In contrast, a representative TEM image of a pericyte co-injected tumour edge exhibit motile and elongated cells that have already undergone EMT (T in white). Note the absence of cell-cell junctions in the OVCAR-5+P tumour. Magnification= 400,000x. Images are representative of 5 tumours per experimental group from 2 independent experiments.

A



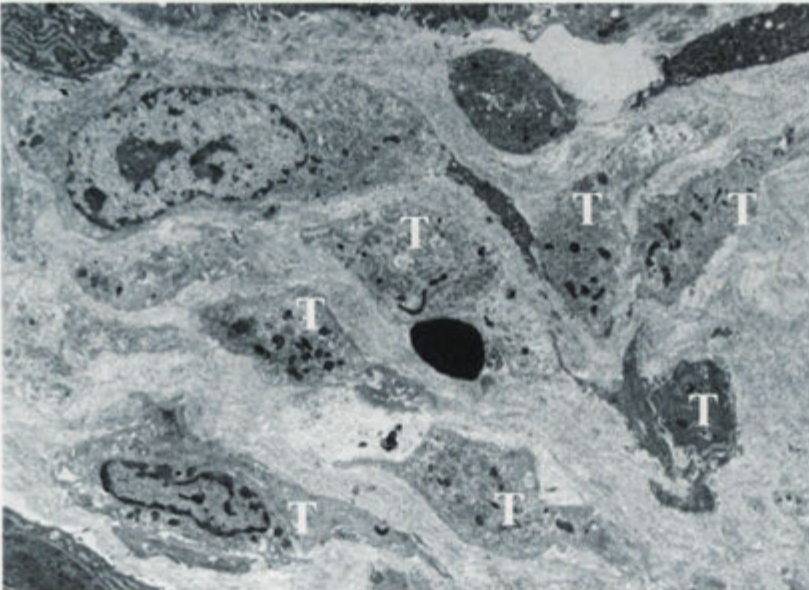
B

OVCAR-5



C

OVCAR-5+P



4.3. Discussion on key findings

The key findings of this Chapter are (i) Pericytes promote metastasis to distant organs in the OVCAR-5 co-injection model in nude and NOD/SCID mice; this effect is dose-dependent i.e. increasing the numbers of co-injected pericytes enhances metastatic burden; (ii) Pericyte reduce primary tumour latency and initiate metastasis in the non-metastatic OVCAR-8 cell line; (iii) Pericyte co-injected OVCAR-5 tumours have invasive leading edges characterised by EMT in the tumour cells at the tumour margins.

Data presented in this chapter are the first experimental evidence that leaving the tumour vasculature intact, yet permitting pericytes to interact with ovarian tumour cells at non-angiogenic sites, results in increased EMT, invasive behaviour and metastatic spread. Importantly, this is also the first evidence of spontaneous metastasis from subcutaneous tumours derived from OVCAR-5 as well as OVCAR-8 cells. In fact, to our knowledge, this study is the very first concrete example of pericytes driving metastasis in otherwise non-metastatic lines that were genetically un-manipulated. While there are no reports on OVCAR-8 metastasis irrespective of the route of tumour inoculation, previous studies have shown that OVCAR-5 cells can form lung metastases when directly injected into the blood stream through the tail vein or colonise the abdominal cavity when injected through the peritoneal layer; however, they were unable to metastasise spontaneously from solid subcutaneous tumours (Godwin et al., 1992; Mistry et al., 1991; Molpus et al., 1996). In fact, given the aggressive, invasive nature of the cancer from which the OVCAR-5 cell line was derived (Godwin et al., 1992), local gut spread at a considerably later time-point, as seen at Day 42 (*Figure 4.4 and 4.5*) was not disconcerting. Notably, a core clinical feature of aggressive serous ovarian cancer is local invasion in to the peritoneal and abdominal cavity (Khan et al., 2010; Lee et al., 2009).

Recent literature in the metastasis field in particular has focussed on the importance of orthotopic models for studying metastasis, specifically because local tissue environment not only dictates tumour behaviour but also affects organ tropism of disseminating tumour cells – a fact often cited as a limitation for studies that draw

conclusions from subcutaneous tumours, based on Paget's 'seed and soil' theory of metastasis (Bibby, 2004; Hoffman, 1999; Khanna and Hunter, 2005; Killion et al., 1998; Man et al., 2007). However, while many such well-characterised orthotopic models exist for breast cancer and melanoma in particular, ovarian cancer is one among many cancer types for which well-described orthotopic or syngeneic mouse models are quite rare. In fact, in patient-derived xenograft (PDX) models of ovarian cancer, parallel analyses using cells derived from the same patient tumours showed no differences in organ tropism for secondary disease in orthotopic versus subcutaneous injection models. Importantly, both models showed remarkable parity in the organs of metastasis between the mice and the affected patient (*personal communication of A/Professor Kaur with A/Professor Clare Scott, Epithelial Ovarian Cancer Program, The Walter and Eliza Hall Medical Research Institute, Melbourne*).

In an attempt to correlate organs of metastasis in our experimental data to clinical findings, visceral metastasis data from a subset of stage III/IV high-grade serous ovarian cancer patients, obtained through the AOCS, were analysed. A major limitation of this analysis was that despite advanced metastatic disease being a hallmark of stage III/IV ovarian cancer, information on specific sites of metastasis was not recorded in ~65% of the patients (63 out of 96). Nevertheless, it was reassuring to note that like the pericyte co-injected group of mice, organs affected in the patients also included the liver, lung, spleen and kidney, with the brain and mediastinal nodes additionally being affected in two separate patients. A list of patients with organ-specific details of metastasis is provided in *Appendix C*.

A correlation of larger primary tumour volume and increased metastasis has been suggested (linear progression model). However, there is an increasing body of evidence that has shown that metastatic dissemination happens *early* (Engel et al., 2003; Friberg and Mattson, 1997; Hüsemann et al., 2008), from primary tumours as small as 1 cm in diameter, and metastatic disease can develop in parallel to the development of the primary tumour (parallel progression model) (reviewed in Klein, 2009). It can be argued that the metastasis observed in our experimental model did not result from larger tumour volumes given that EMT and invasiveness of OVCAR-5 cells at the leading edges of pericyte co-injected tumours were observed as early as Day 11. Moreover, in the OVCAR-5 model, control and fibroblast co-injected

tumours do not metastasise to distant sites even after they eventually reach the volume at which metastasis from pericyte co-injected tumours was first observed ($\sim 600\text{-}700\text{ mm}^3$). Although local spread to the gastrointestinal tract was observed in the control tumours at the late time-point of Day 42, more distant organs like liver, lungs, spleen and kidney were still metastasis free, unlike the pericyte co-injected mice. Finally, the results presented here are also in agreement with previous *in vitro* data generated in our laboratory demonstrating that pericytes could directly affect OVCAR-5 cell migration and invasion in transwell Boyden chamber assays, a system devoid of vasculature. OVCAR-5 cells displayed enhanced migration ($p<0.05$) and invasion ($p<0.05$) in co-culture with pericytes, while fibroblasts did not have any significant effect (*Lynn Chong Ph.D. thesis, 2013; Appendix B, Figure 2*). Therefore it can be concluded that the metastasis observed in pericyte co-injected tumours was a result of direct interaction of the pericytes and tumour cells. However, an alternative way to address this in future experiments would be to surgically resect primary tumours at the same volume across all experimental groups, and then monitor metastasis.

Put together, experimental work described in *Chapters 3 and 4* demonstrate conclusive experimental evidence of an angiogenesis-independent action of pericytes in the tumour microenvironment - one that is clinically extremely relevant, given its substantial effect on metastasis and mortality.

Chapter 5

**Pericytes induce the infiltration of α SMA
cells in to the ovarian tumour
microenvironment – clinical relevance
of experimental findings**

5.1. Introduction and Rationale

As discussed previously (*Chapter 1, Section 1.1*), the stromal microenvironment of solid cancers is very crucial to tumour development, progression to secondary disease and determination of fatality. A bevy of recent studies have demonstrated the importance of stromal markers in the prognosis of solid tumour types. Specifically, Bacac et al., 2006 showed that stromal expression of matrix remodelling genes like lysosomal proteases, ADAMTS and MMPs was prognostic in breast and prostate cancer (Bacac et al., 2006). Finak et al. also showed that gene expression signature of a reactive stroma correlated with aggressive disease and poor survival in breast cancer (Finak et al., 2008). Further studies in breast (Witkiewicz et al., 2011), colorectal carcinoma (Petty et al., 2009) and cancers of the gastrointestinal tract (Saadi et al., 2010) also confirmed that a gene expression signature characteristic of a reactive, inflamed environment could indeed be used as early tools for patient prognosis.

The contribution of the stromal microenvironment to ovarian cancer pathogenesis has been discussed in *Chapter 1, Section 1.2.2.3 and Table 1.2*. A recently conducted *in vivo* study by Greenaway et al. elegantly illustrated the importance of the tumour microenvironment in ovarian cancer. Ascites resulting from i.p. injection of ID8, an epithelial ovarian cancer cell line, was compared with the establishment of a solid tumour in the ovarian bursa. Xenograft tumours from the bursa showed increased proliferation, decreased apoptosis and increased expression of growth and survival markers like PCNA, VEGF, VEGFR-2, activated phospho-Akt and the anti-apoptotic molecule Bcl-2, compared to tumour ascites resulting from i.p. injection. This was attributed to the stromal contribution to solid tumours in the ovarian bursa, which was absent in the i.p. model. Tumour cells derived from the solid tumours also showed increased *in vitro* migration ability and generated more aggressive tumours on re-implantation, establishing that the tumour stroma can influence tumour cell-intrinsic changes and is vital for sustenance and growth of ovarian tumours (Greenaway et al., 2008). Stromal contribution to ovarian cancer pathogenesis was also highlighted in an AOCS study that identified a novel ‘stromal’ gene expression signature that correlated with increased relapse and poor survival in serous ovarian cancer patients (Tothill et al., 2008). A recent study also showed that stromal

expression of the periostin (POSTN)/TGF β 1 axis in particular, is associated with poor prognosis in serous epithelial ovarian cancer (Karlan et al., 2014).

So far in this study, conclusive evidence has been presented to demonstrate that the co-injection of exogenous human pericytes has a substantial effect on tumour cells, affecting their proliferative and invasive capabilities resulting in increased tumour growth and metastasis (*Chapters 3 and 4*). Importantly, this did not result in increased angiogenesis – thereby providing a means to analyse the effects of pericytes localised at non-angiogenic sites. Therefore in this Chapter, further experiments were carried out to investigate if the addition of pericytes altered the recruitment of stromal cell types into the tumour microenvironment, in the hope that this might provide a clue to potential mechanisms of action utilised by pericytes to confer their effects.

Preliminary evidence from our laboratory on the OVCAR-5 xenograft tumours pointed towards this direction. Specifically, immunohistochemical staining combined with morphometric quantification revealed an increase in α SMA expression in the pericyte co-injected OVCAR-5 tumours, in comparison to the OVCAR-5 control and fibroblast co-injected groups, both at Day 11 and Day 35 (*Figure 5.1*) (*Lynn Chong Ph.D. thesis, 2013*).

Interestingly, α SMA⁺ BM MSCs have been reported as a potential source of CAFs that can mediate increased tumourigenesis in recent studies in various cancer models, including ovarian carcinoma (Cho et al., 2011; Mclean et al., 2011a; Quante et al., 2011; Spaeth et al., 2009) (*discussed in Chapter 1, Section 1.1.2.2.4*). Therefore, subsequent experiments in this Chapter were carried out with the goal of elucidating the origin and identity of the increased α SMA⁺ cells. ***The underlying hypothesis was that some of the α SMA⁺ cells observed in the pericyte co-injected tumours were of BM MSC origin and like pericytes, had angiogenesis independent sites of action within the ovarian tumour microenvironment.***

Hence, the specific aims of this the first part of this Chapter were to (i) characterise the microenvironment of pericyte co-injected xenograft tumours with respect to the key stromal marker, α SMA; and (ii) elucidate the involvement of BM MSCs in the microenvironment of the xenograft tumours.

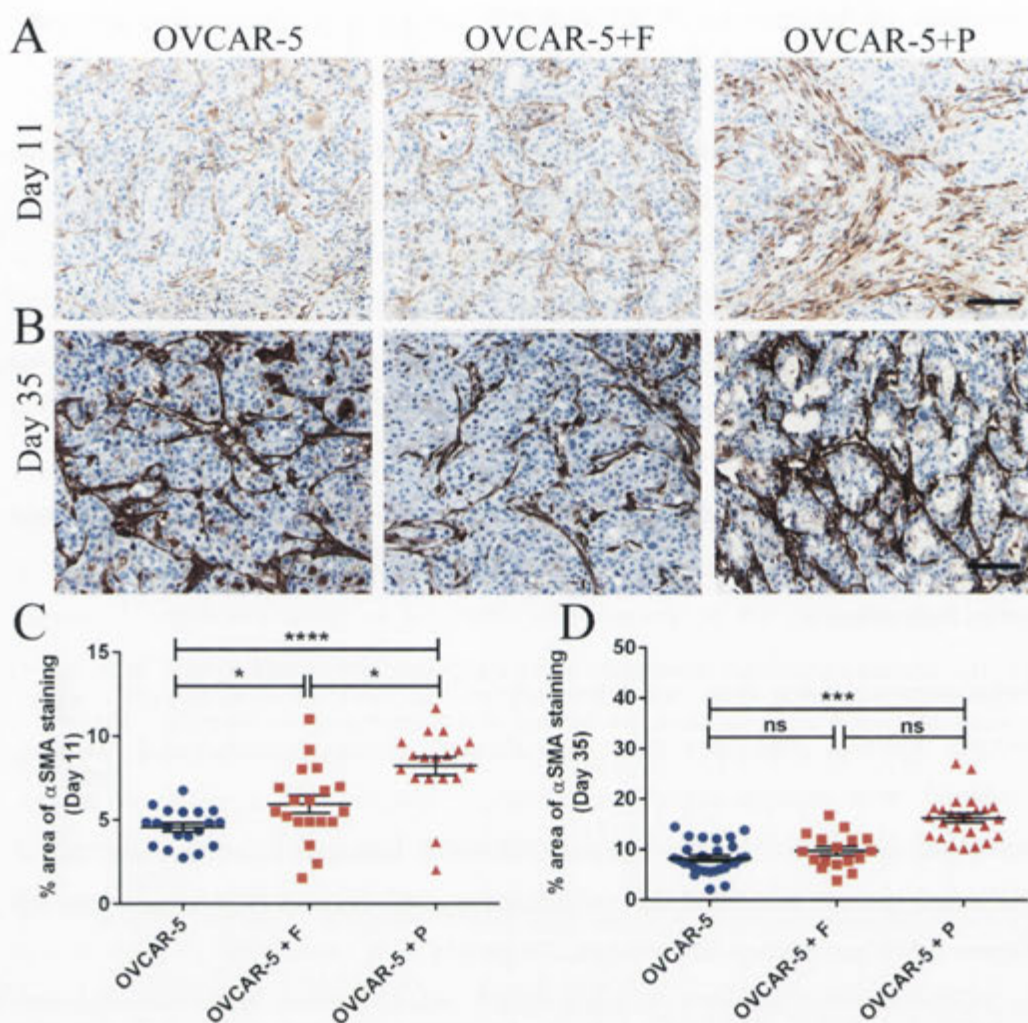


Figure 5.1. Pericytes induce the influx of host α SMA⁺ cells in the xenograft ovarian tumour microenvironment. (A-B) Immunohistochemical staining for α SMA, on xenograft tumour sections derived from the injection of OVCAR-5 cells alone or together with fibroblasts and pericytes at (A) day 11 and (B) day 35. Nuclei counterstained with haematoxylin. (C-D) Morphometric quantification of the staining data showing a significant increase in the percentage of α SMA stained area at (C) Day 11 and (D) Day 35 in the tumours co-injected with pericytes. Fibroblast co-injected tumours also show a slight increase in the percentage of α SMA staining at Day 11 but this difference is not maintained at the later stages of tumour progression (Day 35). Error bars represent mean $\pm$ SEM. Images are representative of 1-3 random fields from 7 tumours per experimental group from 3 independent experiments. Statistical analysis was performed using one-way ANOVA; * p <0.05, *** p <0.001, **** p <0.0001; ns = not significant. Scale bar = 100 μ m. (Data: Lynn Chong Ph.D. thesis, 2013).

5.2. Results

5.2.1. Co-injection of pericytes increase the expression of stroma-associated α SMA⁺ expression in xenograft tumours

Recruitment of the α SMA⁺ cells could be a possible mode of pericyte action in the tumour microenvironment. However, given that α SMA is a marker that is expressed on both pericytes and CAFs (*discussed in Chapter 1, Section 1.1.3 and Table 1.1*), it was important to carefully dissect the vessel association of the recruited α SMA⁺ cells.

Thus the relationship of the α SMA⁺ cells with respect to CD34⁺ blood vessels was evaluated by confocal microscopy. Cryosections of Day 11 control or pericyte co-injected OVCAR-5 tumours were co-stained with antibodies against α SMA and CD34 and confocal photomicrographs of random fields of the tumours acquired (*Figure 5.2A-B*). Numbers of CD34⁺ vessel-associated and dissociated (or stroma-associated) α SMA⁺ cells were counted and plotted as a function of the total number of α SMA⁺ cells per unit area. Results demonstrated that there was no difference in the percentage of vessel-associated α SMA⁺ cells (*Figure 5.2C*) between experimental groups. However, a significant number of α SMA⁺ cells in tumours co-injected with pericytes were stroma-associated ($p < 0.0001$; *Figure 5.2D*), demonstrating an accumulation of α SMA⁺ cells in the tumour microenvironment away from blood vessels in the presence of exogenous pericytes. Pie-charts in *Figure 5.2E and F* indicate that while almost three-quarters of α SMA⁺ cells in the OVCAR-5 control tumours were vessel-associated (*Figure 5.2E*), the relative proportion with the co-injection of pericytes showed an opposite distribution (*Figure 5.2F*). In addition, no difference in the numbers of vessel-associated α SMA⁺ cells implied that the net increase in α SMA expression resulted from stroma-associated α SMA⁺ cells (*Figure 5.2B, D, and F*). Given that the α SMA antibody used recognises cells from both human and murine origin, the possibility that a minor subset of α SMA⁺ cells were the co-injected human pericytes themselves, was considered. However, their rather low incidence (<0.5%, estimated from experiments with GFP-luciferase labelled pericytes, refer *Chapter 3, Figures 3.13 and 3.14*) indicated that the injected pericytes were unlikely to account for the substantial increase in the numbers of α SMA⁺ cells observed here (*Figure 5.1B and 5.2B*).

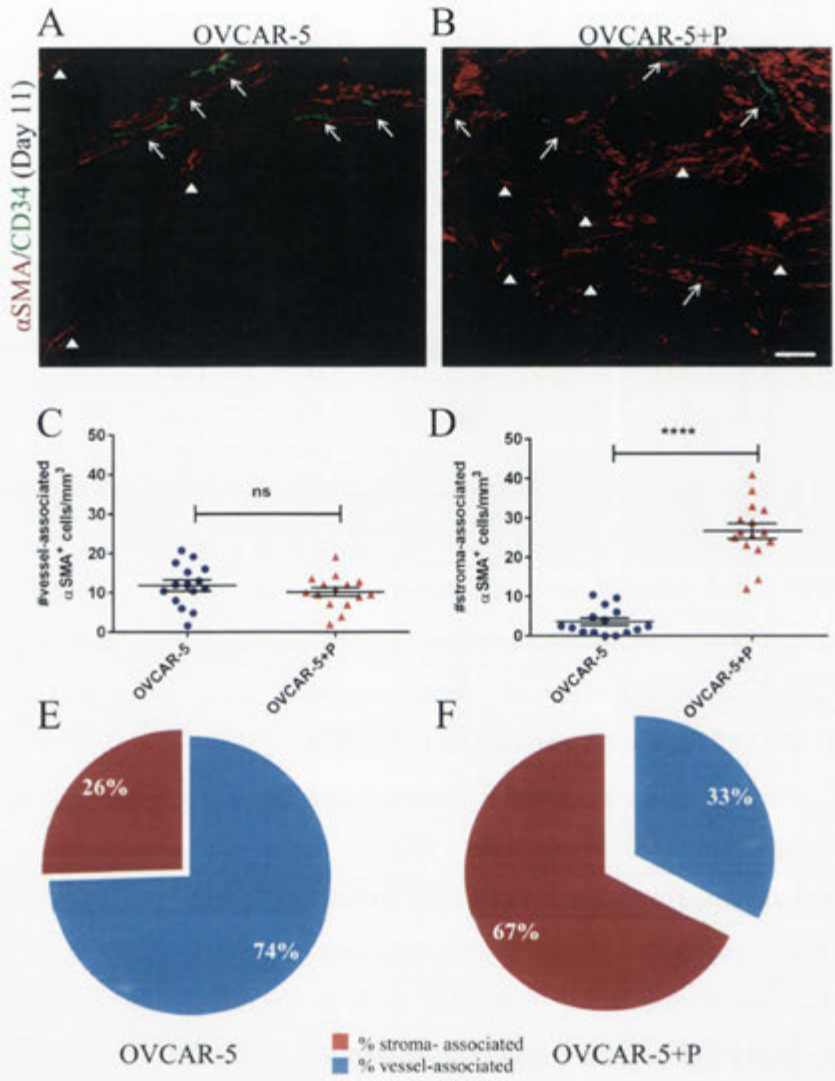


Figure 5.2. Pericytes induce the influx of stroma-associated host α SMA⁺ cells in the xenograft ovarian tumour microenvironment.(A-B) Immunofluorescence co-staining with antibodies against α SMA and the endothelial marker CD34 in tumours generated from the injection of (A) OVCAR-5 cells alone or together with (B) pericytes at Day 11. White arrows and arrowheads illustrate vessel-associated and tumour stroma-associated α SMA⁺ cells respectively. (C-D) Morphometric quantification of (C) vessel-associated and (D) stroma-associated α SMA⁺ cells in OVCAR-5 control versus pericyte co-injected tumours. (E-F) Pie-charts showing the relative proportions of vessel-associated and stroma-associated α SMA⁺ cells in (E) OVCAR-5 and (F) OVCAR-5+P tumour groups. Error bars represent mean $\pm$ SEM. Images and morphometric data are representative of 1-3 random fields from 7 tumours per experimental group from 3 independent experiments. Statistical analysis was performed using unpaired t-test; ****p<0.0001; ns = not significant. Scale bar = 20 μ m.

5.2.1.1. *Addition of exogenous pericytes induce the infiltration of α SMA⁺ BM MSCs to the xenograft tumour microenvironment*

To determine whether the α SMA⁺ cells observed in the pericyte co-injected tumours was a result of greater influx of α SMA⁺ cells or increased proliferation of the cells already recruited, tumour sections were co-stained with antibodies against α SMA and the proliferation marker Ki67 (*Figure 5.3A-C*). The absence of detectable α SMA⁺/Ki67⁺ cells in all the experimental groups suggested that the significant increase in the number of α SMA⁺ cells obtained with the co-injection of pericytes was a result of increased recruitment of host α SMA⁺ cells from extra-tumoural sites, rather than increased proliferation of cells already recruited to the tumour stroma.

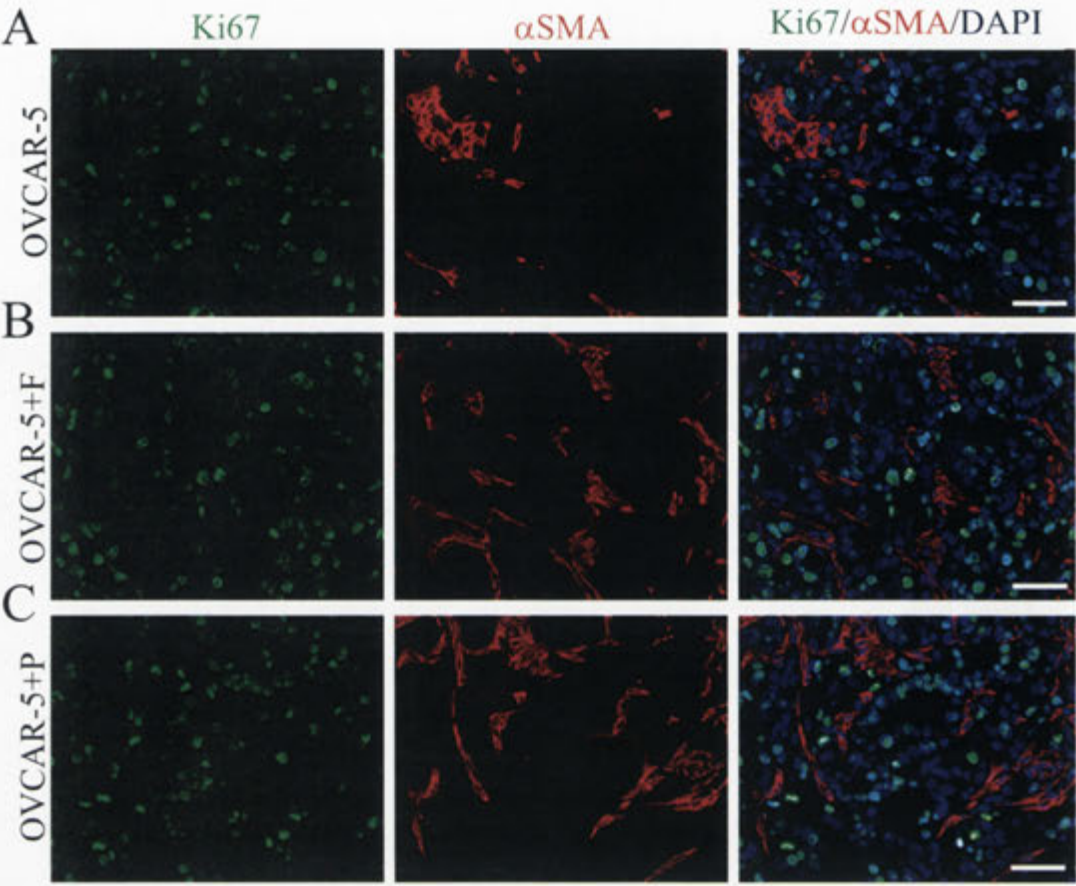


Figure 5.3. Host α SMA⁺ cells do not proliferate in the xenograft ovarian tumour microenvironment. Immunofluorescence co-staining with antibodies against alpha smooth muscle actin and mouse-specific Ki67 in tumours generated from the injection of (A) OVCAR-5 cells alone or together with (B) fibroblasts or (C) pericytes at Day 11. Ki67 negativity of α SMA⁺ cells indicated that these cells infiltrated the tumour microenvironment from extra-tumoural sites and did not originate as a result of increased proliferation of the resident α SMA⁺ cells. Nuclei counterstained with DAPI. Results are representative of 2-3 random sections from 7 tumours from 3 independent experiments. Scale bar = 50 μ m.

Previously, it has been reported that α SMA⁺ BM MSCs infiltrate the tumour microenvironment and differentiate into CAFs, thereby increasing tumour growth and invasion (McClean et al., 2011a; Quante et al., 2011; Spaeth et al., 2009). To determine whether some of the infiltrating α SMA⁺ cells observed in this study were BM MSCs, immunofluorescent staining for α SMA and *murine* MSC markers was performed. Antibodies against the murine BM MSC markers CD73 (5' nucleotidase) and Sca-1 (stem cell antigen -1/Ly6AE) (Boxall and Jones, 2012; Kurth et al., 2011; Peister et al., 2004; Tropel et al., 2004) were employed for this purpose.

CD73, a 70-kDa cell surface enzyme also known as ecto-5'-nucleotidase, is widely expressed in certain subsets of T and B cells, germinal centre follicular dendritic cells and thymic medullary reticular fibroblasts (Colgan et al., 2006; Zhang, 2010). In addition, CD73 is also a characteristic BM MSC marker in both human and mice (Dominici et al., 2006; Resta et al., 1998). Sca-1, on the other hand, is an exclusively murine progenitor cell marker and has been widely studied with respect to its role in self renewal, stem cell activation, lineage fate determination and as a bona fide marker for murine BM MSCs (Morikawa et al., 2009; Peister et al., 2004).

α SMA⁺CD73⁺ cells were detected only in the pericyte co-injected tumours (*Figure 5.4A-C*); however, not all α SMA⁺ cells were CD73. Notably, a few CD73⁺ cells were observed in the OVCAR-5 alone and fibroblast co-injected groups; it could be postulated that they were immune cells, given their negativity for α SMA (*Figure 5.4A-B*). In fact, α SMA⁻CD73⁺ cells were present in the pericyte co-injected group as well (*Figure 5.4C*).

Interestingly, α SMA⁺ cells in the pericyte co-injected group were also positive for Sca-1, yet completely absent from the remaining experimental groups (*Figure 5.5A-C*). Together, although the staining for CD73 and Sca-1 indicate that some of the α SMA⁺ cells in the pericyte co-injected group could be of BM MSC origin, further experiments by isolating these cells and prospectively testing marker expression and MSC properties *in vitro* might be required to convincingly establish their BM MSC origin.

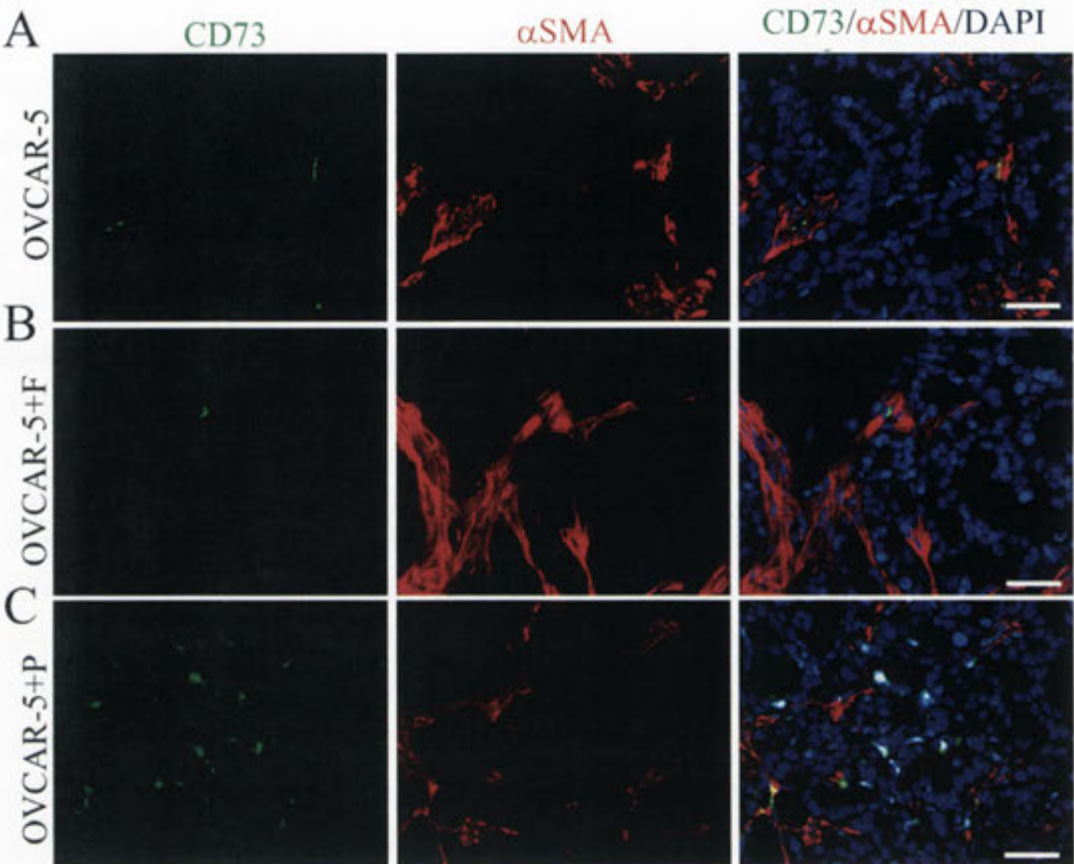


Figure 5.4. Some α SMA⁺ cells in the pericyte co-injected tumours co-express the MSC marker CD73. Immunofluorescence co-staining with antibodies against alpha smooth muscle actin and the MSC marker CD73 in tumours generated from the injection of (A) OVCAR-5 cells alone or together with (B) fibroblasts or (C) pericytes at Day 11. While minimal staining of CD73 was observed in the OVCAR-5 and the OVCAR-5+F tumours, the pericyte co-injected group showed substantial co-expression of α SMA and CD73, although the staining pattern was overall heterogeneous. Nuclei counterstained with DAPI. Results are representative of 2-3 random sections from 7 tumours from 3 independent experiments. Scale bar = 50 μ m.

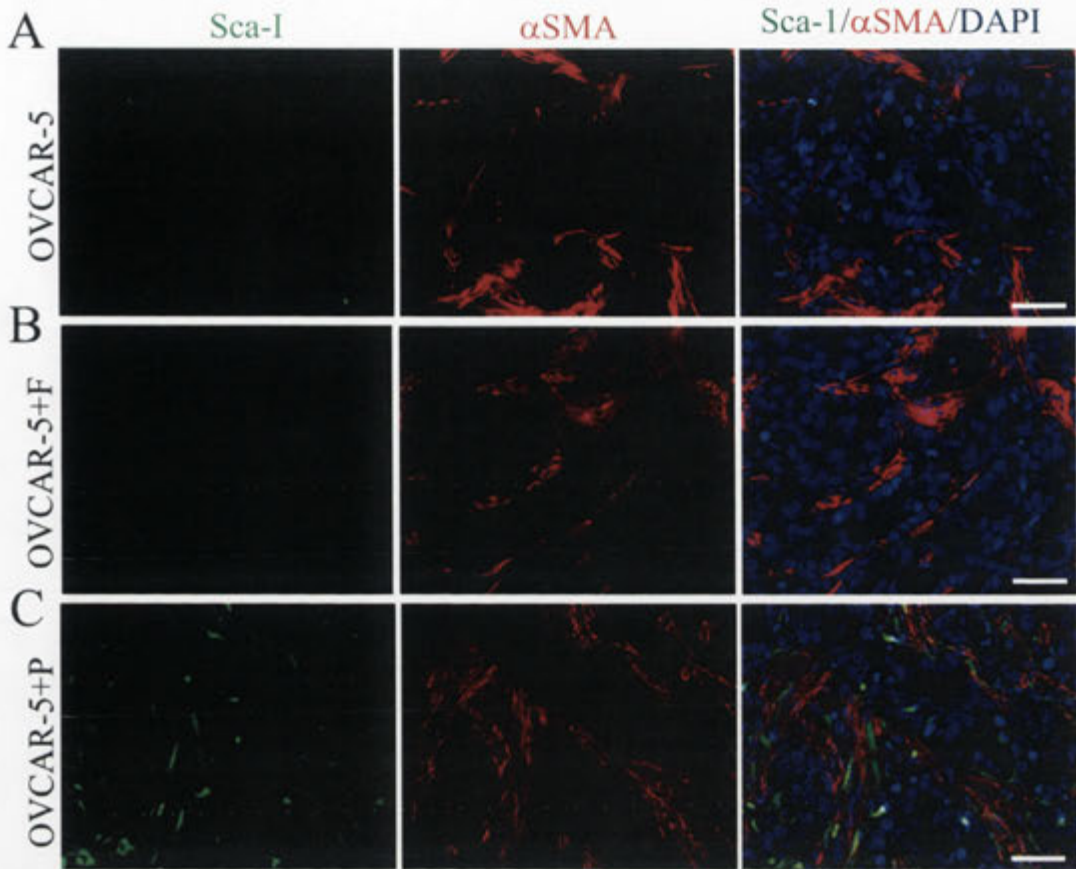


Figure 5.5. Some α SMA⁺ cells in the pericyte co-injected tumours co-express the MSC marker Sca-1. Immunofluorescence co-staining with antibodies against alpha smooth muscle actin and the MSC marker Sca-1 in tumours generated from the injection of (A) OVCAR-5 cells alone or together with (B) fibroblasts or (C) pericytes at Day 11. While minimal staining of CD73 was observed in the OVCAR-5 and the OVCAR-5+F tumours, almost all α SMA⁺ cells in the pericyte co-injected group showed co-expression of Sca-1. Nuclei counterstained with DAPI. Results are representative of 2-3 random sections from 7 tumours from 3 independent experiments. Scale bar = 50 μ m.

One of the major routes through which BM MSCs promote tumour growth and metastasis, thereby functioning as CAFs, is the CXCL12/SDF-1: CXCR-4 axis (Müller et al., 2001). Thus, a feedback loop between stromal CXCL12 and its receptor CXCR4 on the tumour cells, has been implicated as a potential mechanism tumours employ for increased invasion and metastasis in a variety of cancers types including ovarian cancer (reviewed in Cojoc et al., 2013). The pleiotropic action of CXCL12/CXCR4 signalling can also recruit BM MSCs to tumours (Eck et al., 2009). Therefore, to ascertain if the CXCL12/CXCR4 axis was active in the xenograft tumours being studied here, immunofluorescent staining was performed in the xenografts using an antibody that recognises both murine and human CXCL12.

Dual immunofluorescence for α SMA and CXCL12 revealed that while the OVCAR-5 cells showed abundant staining for this chemokine irrespective of the inclusion of pericytes, the α SMA⁺ cells were negative for CXCL12, across all experimental groups (*Figure 5.6A-C*). Notably, other studies have also shown that CXCL12 is expressed by ~70-90% of all epithelial ovarian cancer cells, as well as mesothelial cells on the peritoneal wall (Foussat et al., 2001; Jiang et al., 2006; Lau and Kane, 2010; Scotton et al., 2001). Thus, despite abundant CXCL12 expression across all experimental groups, aggressive tumour growth and metastasis was observed only in the pericyte co-injected group, indicating that tumour promotion and metastasis in this ovarian cancer model was not mediated through the CXCL12: CXCR-4 axis.

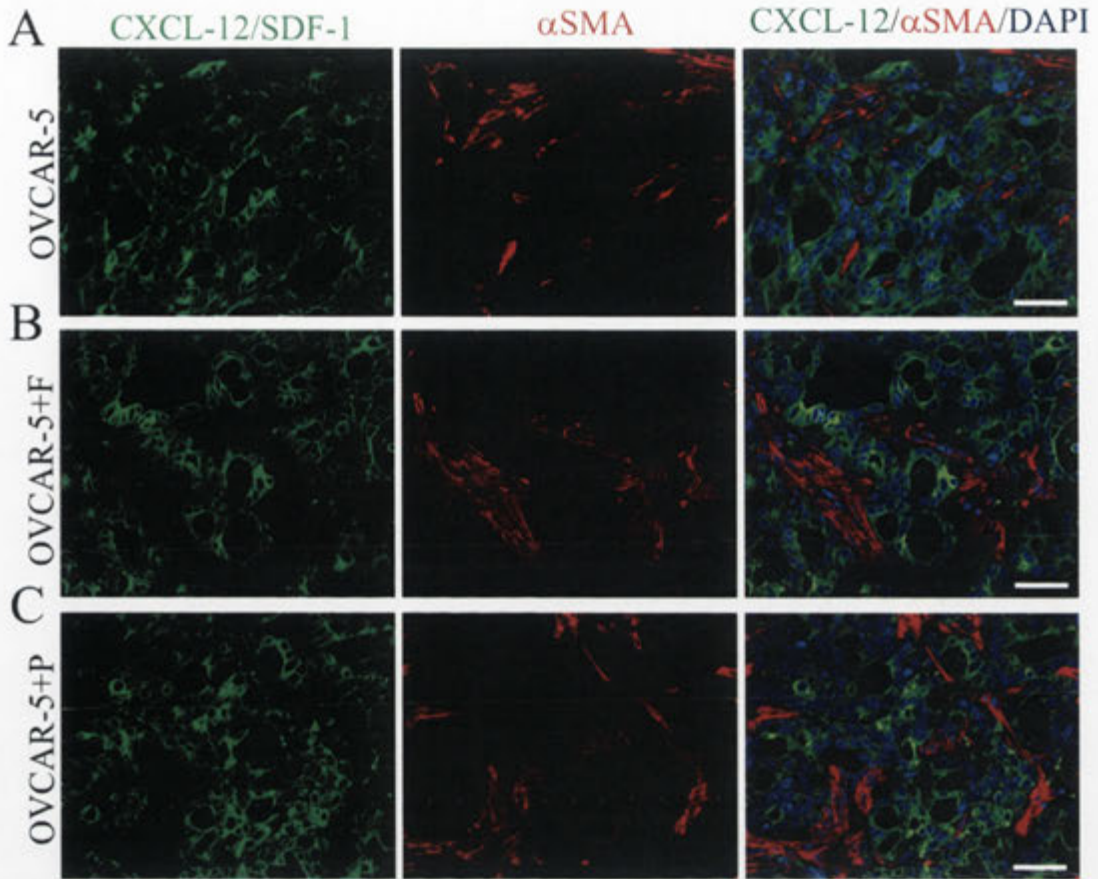


Figure 5.6. Host α SMA⁺ cells do not co-express CXCL12. Immunofluorescence co-staining with antibodies against alpha smooth muscle actin and CXCL12 in tumours generated from the injection of (A) OVCAR-5 cells alone or together with (B) fibroblasts or (C) pericytes at Day 11. α SMA⁺ cells were not positive for CXCL12 in all the experimental groups. CXCL12, however, was abundantly expressed in the OVCAR-5 tumour cells. Nuclei counterstained with DAPI. Results are representative of 2-3 random sections from 7 tumours from 3 independent experiments. Scale bar = 50 μ m.

5.2.2. Clinical correlation of experimental findings - Histological, morphometric & *in silico* analyses of patient samples

One of the major obstacles in epithelial ovarian cancer research is the lack of suitable *in vivo* models that mimic clinical progression of this fatally aggressive malignancy. The field is in need of advanced, clinically relevant mouse models to further understand the biology of the disease and explore novel therapeutic interventions and prognostic strategies. Given the availability of a well-characterised dataset of patient TMAs with known prognosis and gene expression data from AOCS, the second part of this Chapter focusses on exploring this dataset at protein and mRNA levels to determine the clinical relevance of experimental findings. Specifically, histological and morphometric analyses on the TMAs were performed to validate findings from the xenograft model and further, gene expression data was analysed to explore the prognostic capacity of stroma specific markers.

5.2.2.1. Introduction

Stage III and stage IV serous ovarian cancer patient TMAs containing FFPE sections of 215 core biopsy samples with known prognosis were obtained from AOCS as described in *Chapter 2, Section 2.1.4*. Notably, this was the same dataset used by Tothill et al. to determine that the molecular subtype of patients with stromal gene expression signature was prognostic in epithelial ovarian cancer (Tothill et al., 2008). Gene expression data from these patients was also employed at the beginning of this study to demonstrate that the VLA-1^{bri} pericyte gene expression signature was prognostic for poor survival (*Chapter 1, Figure 1.8*).

First, to investigate if stromal expression of key markers could be correlated with progression-free survival (PFS), samples were arranged in the order of time taken to relapse after surgical debulking following diagnosis. Next, 7 patients with the longest time to relapse were grouped as 'late relapse'; 7 patients with the lowest time to relapse were grouped as 'early relapse' while 7 randomly selected patients who did not relapse till the last date of record (five years since diagnosis) were grouped as 'control' or 'no relapse'. Further details of these patient samples are listed in *Table 5.1*.

Table 5.1. List of stage III/IV serous ovarian cancer patient grouped according to time taken to relapse into secondary disease.

Group	AOCS ID	Pericyte Score	PFS Time	Tumour Type	Weight	TMA
Early relapse	34168	0.20497	4.60274	Primary	0.919	V2
Early relapse	41264	-1.51111	8.77808	Metastasis	3.472	V4
Early relapse	22020	0.95136	9.07397	Primary	1.221	V4
Early relapse	41170	1.22014	9.60000	Primary	2.437	V4
Early relapse	70119	0.76773	9.99452	Primary	0.886	V2
Early relapse	34080	0.00604	10.32329	Metastasis	1.022	V2
Early relapse	70103	-0.63764	10.55342	Primary	2.63	V2
Late relapse	44087	-1.01633	24.98630	Primary	2.66	V5
Late relapse	60024	-1.52452	25.74247	Primary	0.81	V4
Late relapse	22012	1.12524	26.95890	Primary	1.877	V4
Late relapse	60066	0.85311	29.417	Metastasis	0.787	V4
Late relapse	41192	1.64607	28.50411	Metastasis	7.129	V5
Late relapse	32022	-0.66050	32.05479	Primary	1.76	V4
Late relapse	23102	-1.11282	32.54795	Primary	1.8	V4
No relapse	20019	-1.59803	NA	Primary	3.738	V2
No relapse	32032	-0.88466	NA	Primary	0.096	V4
No relapse	41307	-0.17305	NA	Primary	3.175	V4
No relapse	41010	-1.65156	NA	Primary	0.623	V5
No relapse	41203	-1.04889	NA	Primary	6.478	V5
No relapse	44110	-0.95020	NA	Primary	0.33	V5
No relapse	60107	-0.22646	NA	Primary	0.364	V5

The mean 'time to relapse', calculated as the average PFS time was 8.98 and 28.47 months for the 'early' and 'late' relapse groups respectively.

5.2.2.2. Increased number of stroma-associated α SMA⁺ cells correlate with early relapse

The presence of a significant number of infiltrating stroma-associated α SMA⁺ cells was observed in the pericyte co-injected xenograft tumours at the beginning of this Chapter (Figure 5.2). To verify the clinical relevance of this observation, FFPE sections of TMAs derived from patients from the three relapse groups described above (Table 5.1) were stained with an antibody against α SMA. Samples belonging to the 'early relapse' group showed high levels of α SMA staining (Figure 5.7A), compared to the 'late relapse' and 'no relapse' groups (Figure 5.7B-C). Closer microscopic examination at higher magnification revealed that the α SMA staining in the 'early relapse' group resembled aggregated cells not associated with the tumour vasculature, in a manner strikingly similar to the stroma-associated α SMA⁺ cells observed in the pericyte co-injected xenograft tumours (Figure 5.2).

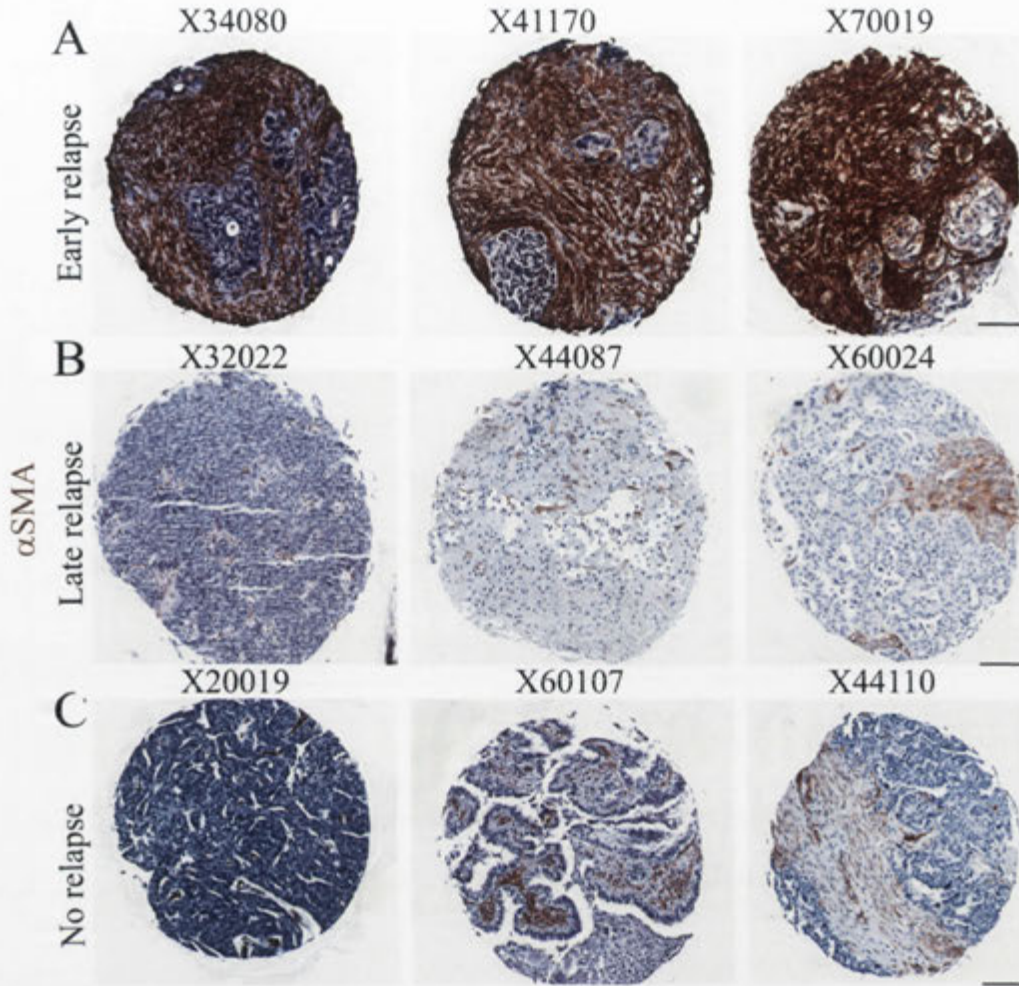


Figure 5.7. Early relapse patients exhibit increased expression of α SMA in their primary tumours. (A-C) α SMA expression in 3 representative serous ovarian cancer biopsy sections derived from the TMAs representing (A) early (B) late and (C) no relapse groups. Images indicate widespread expression of α SMA staining in the early relapse group. Haematoxylin was used as a counterstain. Results shown are representative of 7 patient samples per relapse group. Scale bar = 200 μ m. (Staining by Dr. Holger Schlüter)

Quantitative assessment of the localisation of αSMA^+ cells was performed by counting numbers of αSMA^+ cells with respect to their association with tumour blood vessels. A significantly increased number of stroma-associated αSMA^+ cells were observed in the 'early relapse' group of patients, compared to the 'late' and 'no' relapse groups (*Figure 5.8A*). Notably, while the numbers of blood vessel-associated αSMA^+ cells did not differ between the 'early' and the 'late relapse' groups, the 'no relapse' group contained significantly lower number of these cells compared to the 'early relapse' group (*Figure 5.8B*). Pie-charts in *Figure 5.8C-D* indicate that while ~80% of the αSMA^+ cells in the 'early relapse' group were localised to the tumour stroma the majority of αSMA^+ cells in the 'late' and 'no relapse' groups were blood vessel-associated. This was reminiscent of the comparative distribution of αSMA^+ cells observed in the xenograft tumours (*Figure 5.2E-F*). These results indicated that stromal αSMA could be prognostic for poor outcome in serous ovarian cancer patients.

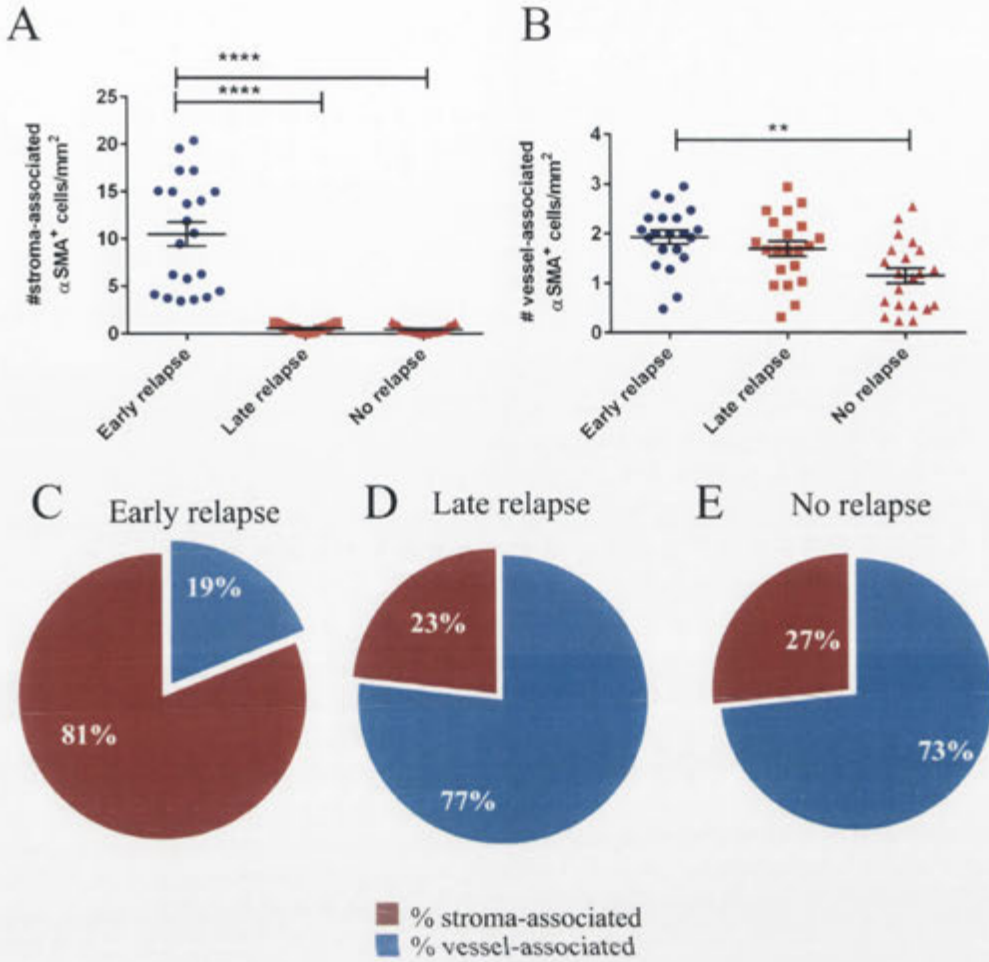


Figure 5.8. Increased α SMA expression observed in early relapse patients originates from stroma-associated cells. (A-B) Morphometric quantification of (A) stroma-associated versus (B) vessel-associated α SMA⁺ cells in early, late & no relapse patient groups showing significant increase in the numbers of stroma-associated α SMA⁺ cells in the early relapse group. (C-E) Pie-charts illustrating the relative proportions of vessel-associated and stroma-associated α SMA⁺ cells in (C) early, (D) late and (E) no relapse patient groups. The relative proportion of vessel-associated: stroma-associated α SMA⁺ cells was reversed in the early relapse group in comparison to the late and no relapse groups. Data represents 3 random fields from 7 patients per relapse group. Error bars represent mean $\pm$ SEM. Statistical analysis was performed using one-way ANOVA; **p<0.01, ****p<0.0001.

5.2.2.3. PDGFR β and CD34 expression does not correlate with relapse

Next, it was explored if α SMA expression alone could predict earlier relapse in serous ovarian cancer patients, or any other ‘pericyte’ marker would have a similar ability. PDGFR β , arguably the best known pericyte marker was chosen for this purpose. Thus, FFPE sections of patient TMAs were stained using an antibody against PDGFR β . Interestingly, PDGFR β expression showed no substantial variation between the three relapse groups qualitatively (*Figure 5.9A-C*). Moreover, the staining was perivascular in pattern irrespective of relapse status. Morphometric quantification of PDGFR β staining intensity further validated these observations, showing no significant differences in PDGFR β expression between the three groups (*Figure 5.9D*).

One of the major findings of this study is the angiogenesis-independent nature of the effect of pericytes, a novel observation contradicting the current dogma on the exclusively angiogenesis-related function of these cells in the tumour microenvironment (Bergers and Song, 2005). Thus, it was profoundly important to validate that this experimental observation had significant clinical relevance. Although, it was previously demonstrated that the mRNA expression of angiogenesis-related genes had no bearing on patient survival (*Chapter 3, Table 3.2*), assessment of endothelial markers at the protein level was essential to confirm this data.

Accordingly, FFPE sections of TMAs from the patients in the three relapse groups analysed earlier were stained with CD34, an endothelial marker employed earlier in the study to evaluate angiogenesis in the experimental xenograft model (*Chapter 3*). Predictably, no correlation was observed between the patterns of CD34 staining and relapse (*Figure 5.10A-C*). Moreover, morphometric quantification did not reveal any significant differences in percentage CD34 staining (*Figure 5.10D*) and microvessel density (*Figure 5.10E*) between the three groups.

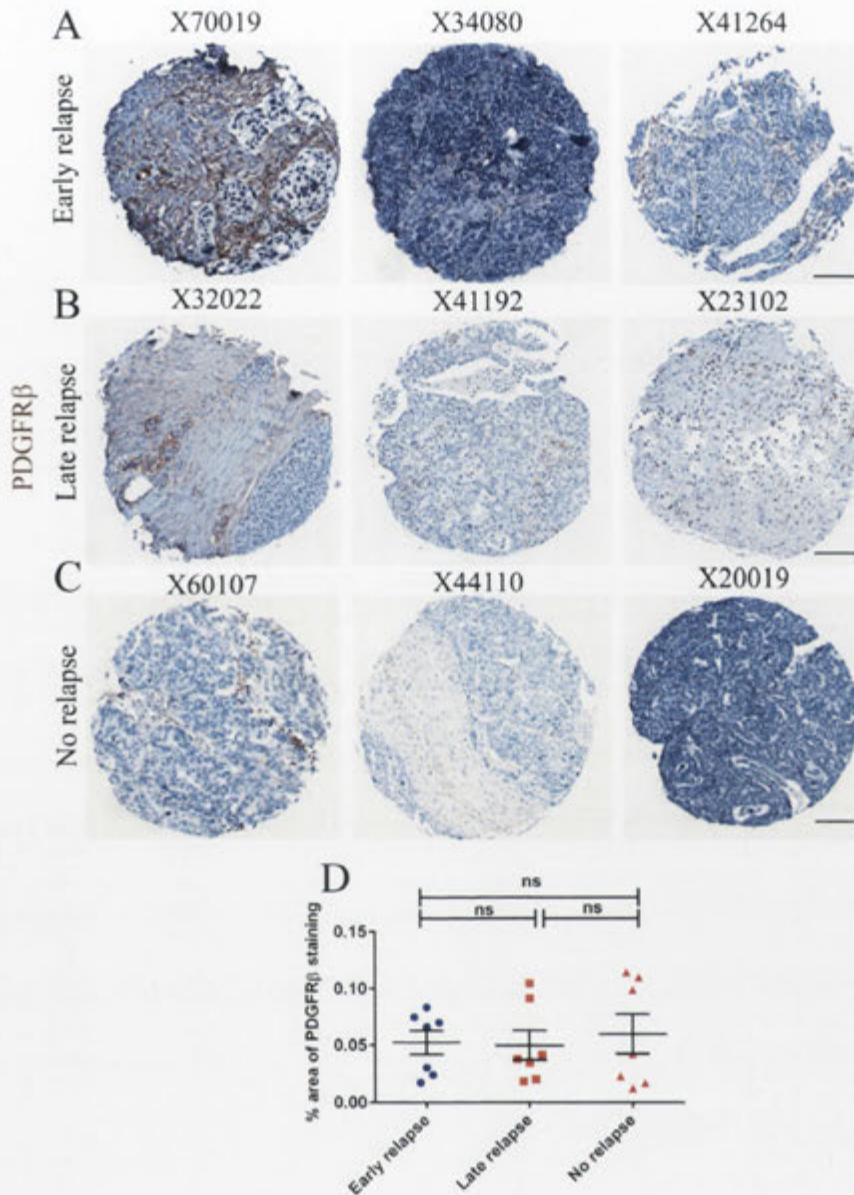


Figure 5.9. Serous ovarian cancer patient relapse status is independent of PDGFR β expression. (A-C) PDGFR β staining in 3 representative serous ovarian cancer biopsy sections derived from the TMAs representing (A) early (B) late and (C) no relapse groups. Staining indicates no qualitative differences between the three groups. Haematoxylin was used as a counterstain. (D) Morphometric quantification of the percentage area of PDGFR β staining showing no significant differences between the three groups of patients. Error bars represent mean $\pm$ SEM. Results shown are representative of 7 patient samples per relapse group. Statistical analysis was performed using one-way ANOVA; ns = not significant. Scale bar = 200 μ m. (Staining by Dr. Holger Schlüter)

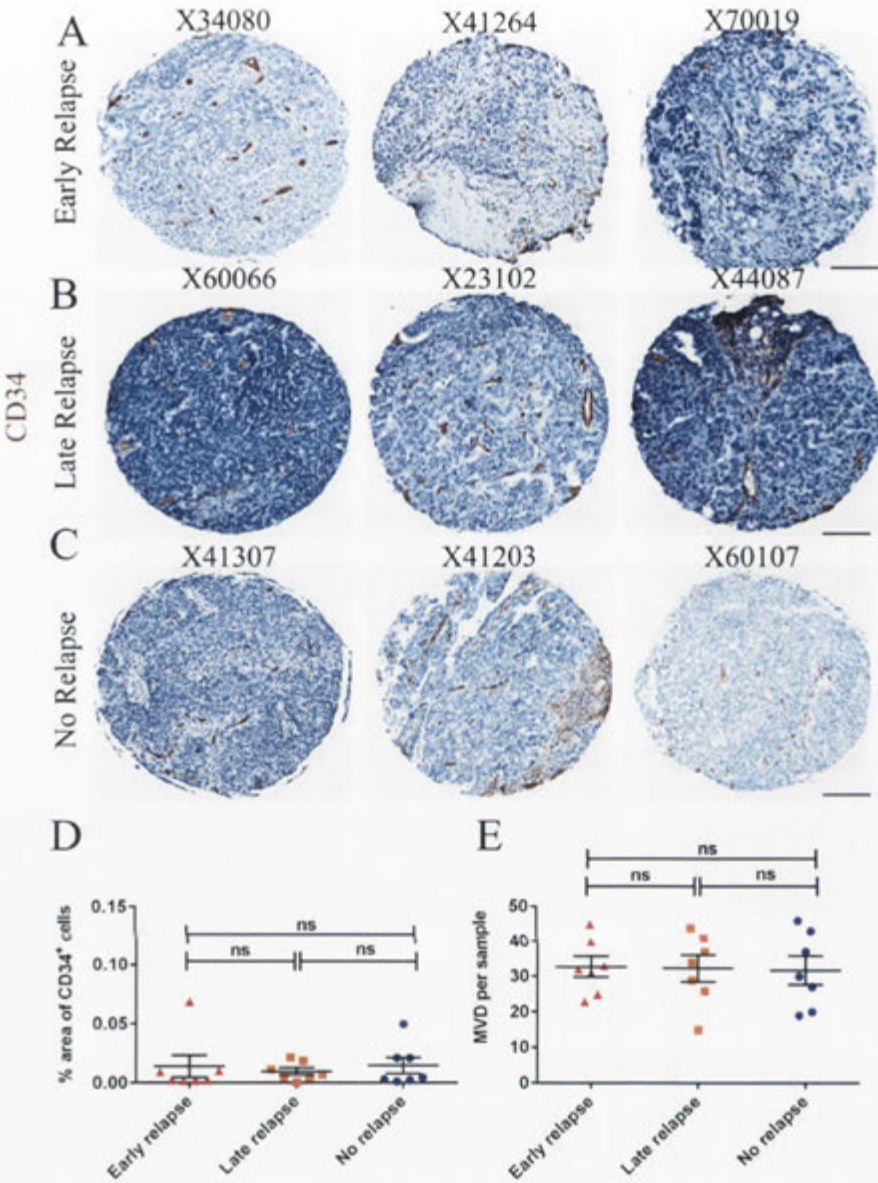


Figure 5.10. Relapse status of serous ovarian cancer patients is independent of angiogenesis. (A-C) CD34 staining in 3 representative serous ovarian cancer biopsy sections derived from the TMAs representing (A) early, (B) late and (C) no relapse groups. Staining indicates no qualitative differences in the extent of vasculature between the 3 groups. Haematoxylin was used as a counterstain. (D-E) Morphometric quantification of the (D) percentage area of CD34 staining and (E) microvessel density showing no significant differences between the 3 relapse groups indicating that the extent of angiogenesis in the primary tumour has no bearing on patient relapse. Error bars represent mean $\pm$ SEM. Results shown are representative of 7 patient samples per relapse group. Statistical analysis was performed using one-way ANOVA; ns = not significant. Scale bar = 200µm. (Staining by Dr. Holger Schlüter)

5.2.2.4. Correlation of α SMA, PDGFR β and CD34 expression

At the inception of this study, it was shown that the use of a pericyte-specific mRNA signature could significantly predict earlier relapse in high-grade serous ovarian cancer patients (*Chapter 1, Figure 1.8A*). Given the evidence of significant correlation of the presence of stroma-associated α SMA⁺ cells and patient survival, further studies were undertaken in our laboratory to determine if α SMA could be employed as a single marker at the protein level to predict relapse at the time of diagnosis. Thus, FFPE sections of all 215 patient TMAs were stained with an antibody against α SMA; after removing 'drop-outs' (sample with loss of tissue) and overstained specimens, the remainder were divided into 'high' and 'low' α SMA expression groups based on percentage stained area and their corresponding survival curves plotted against time. Higher levels of α SMA expression were significantly predictive of PFS ($p < 0.05$; *Figure 5.11A*) and consistent with the results from *Figures 5.9 and 5.10*, expression of PDGFR β and CD34 did not display any prognostic ability (*Figure 5.11B-C*) (*George, Schlüter and Kaur, unpublished data*).

To further explore the implications of the patient prognosis data described above, a series of correlation analyses were carried out in order to delineate the relationship between CD34, as a marker for angiogenesis, versus α SMA and PDGFR β . Interestingly, α SMA staining did not show any significant correlation with either PDGFR β (*Figure 5.12A*) or CD34 (*Figure 5.12B*) expression levels; in contrast, a significant correlation between the expression of PDGFR β and CD34 ($p < 0.05$; *Figure 5.12C*) was observed. This was also well-illustrated in a Principal Component Analysis (PCA) of 100 randomly selected α SMA, PDGFR β and CD34 stained TMA sections taken from the same patients (irrespective of relapse status), which showed a much closer clustering of PDGFR β and CD34 relative to the α SMA arm (*Figure 5.12D*). These analyses further established that α SMA⁺ and PDGFR β ⁺ cells represent distinct stromal populations within the tumour microenvironment and that the key to determining their precise roles lies in characterising their functions separately, especially given their remarkably different prognostic capacity for relapse.

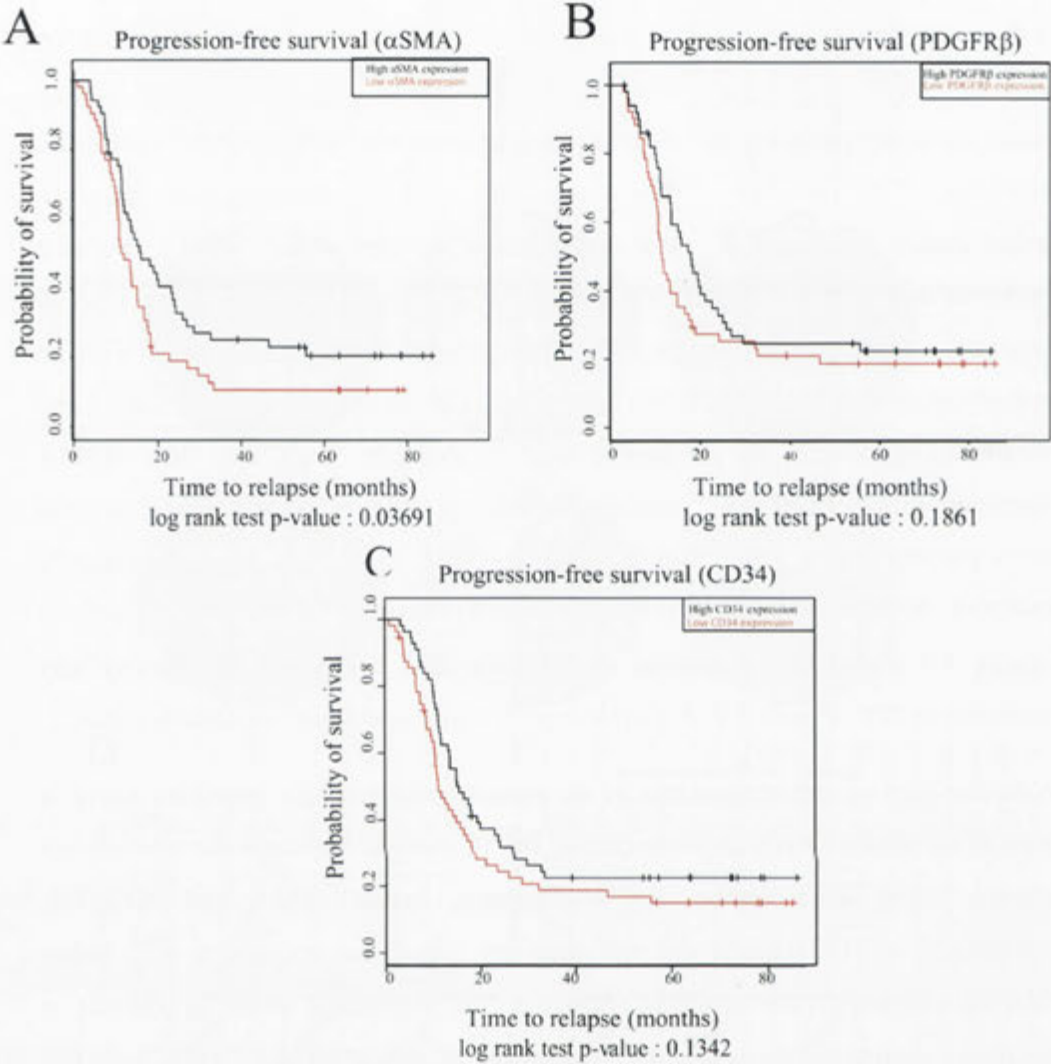


Figure 5.11. α SMA significantly predicts early relapse in serous ovarian cancer patients while PDGFR β and CD34 do not. (A-C) Kaplan-Meier survival curves for ovarian cancer patients using (A) α SMA, (B) PDGFR β and (C) CD34 expression as prognostic markers. Patients were categorised into 'high' and 'low' groups based on protein-level expression of markers. Expression of α SMA alone was able to significantly predict earlier disease recurrence and mortality. Analyses in (A-C) represent progression-free survival and gene expression data derived from 105, 102 and 112 patients respectively. Statistical analyses were performed using the log-rank (Mantel-Cox) test. (George, Schlüter and Kaur, unpublished data)

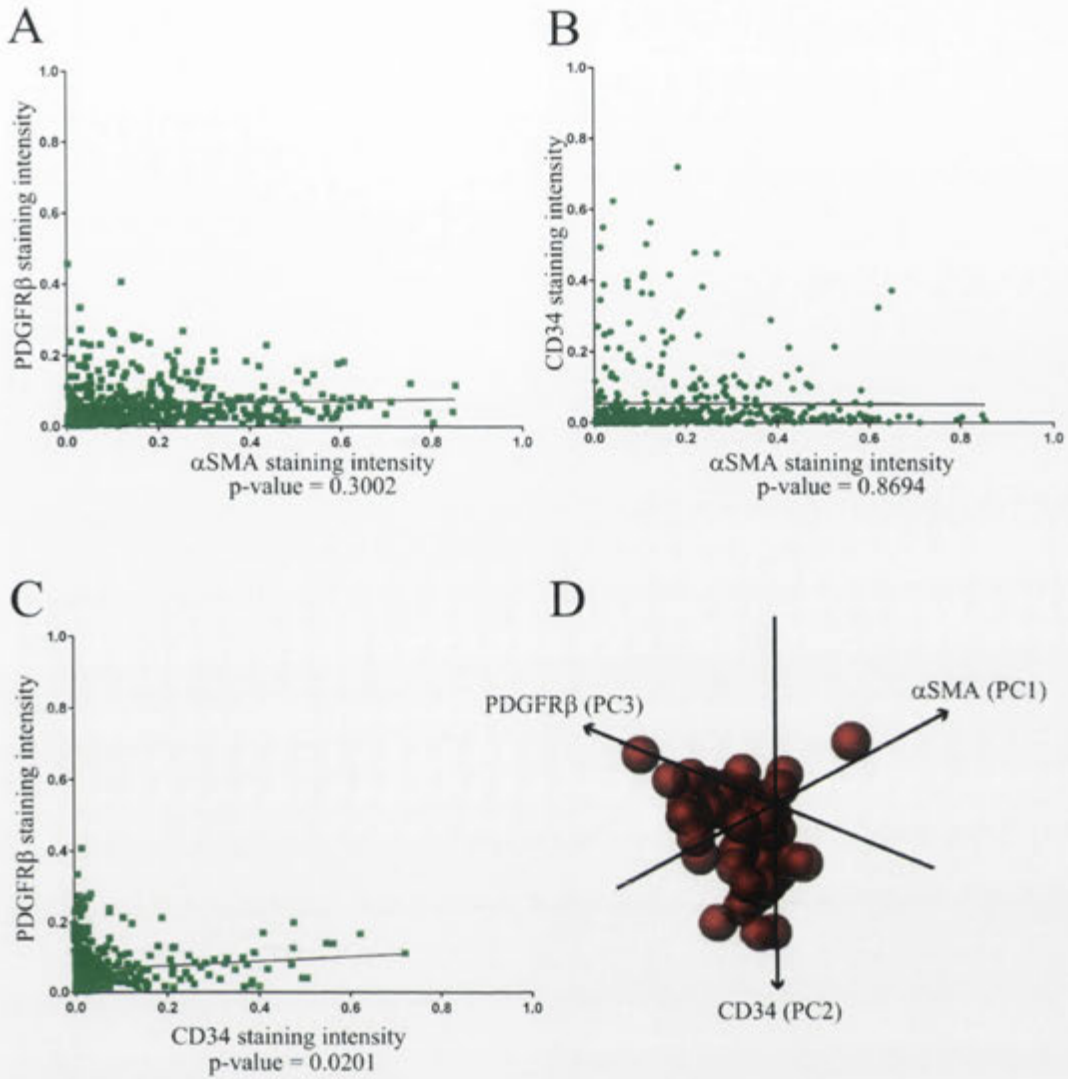


Figure 5.12. PDGFR β expression significantly correlates with CD34 while α SMA expression correlates with neither. (A-C) Correlation analysis showing no significant relationship in the degree of α SMA expression with either (A) PDGFR β or (B) CD34 staining intensities. (C) PDGFR β and CD34 expression showed a significantly positive correlation. Analyses in (A-C) represent 451, 462 and 444 tissue sections respectively from a total of 100 patient samples. (D) Principal Component Analysis on expression data derived from 100 matched patient TMA sections illustrating the relationship between α SMA (PC1), CD34 (PC2) and PDGFR β (PC3) expression levels, showing closer clustering between the CD34 and PDGFR β arms relative to the α SMA arm.

5.3. Discussion on key findings

The key findings of this Chapter are (i) Increased α SMA expression in pericyte co-injected xenograft tumours results from infiltration of α SMA⁺ cells into the microenvironment; (ii) Some α SMA⁺ cells in pericyte co-injected tumours are possibly of BM MSC origin but do not act to promote metastasis through CXCL12; (iii) The majority of α SMA⁺ cells in pericyte co-injected tumours are stroma-associated and, in parallel, majority of α SMA⁺ cells in the 'early relapse' group of patients are also stroma-associated; (iv) α SMA is a predictor of ovarian cancer relapse while PDGFR β and CD34 are not.

The most striking feature observed while characterising the stroma of the pericyte co-injected OVCAR-5 tumours was the significantly increased recruitment of host α SMA⁺ cells which formed a non-vascular network within the tumour microenvironment, some of which expressed the BM MSC markers Sca-1 and CD73 (Figures 5.1 – 5.5). Recruitment of BM MSCs could be a key step crucial for metastasis. However, the role of BM MSCs in ovarian tumour growth and spread remains controversial with a recent report showing that carcinoma-associated MSCs (CA MSCs) were more potent than BM MSCs at promoting SKOV-3 ovarian tumour cell growth mediated through BMP-2, although metastasis was not observed in this model (McLean et al., 2011b). Moreover, further experiments are required to concretely establish the BM MSC origin of the stroma-associated α SMA⁺ cells observed in the pericyte co-injected tumours in this study.

The correlation of increased stroma-associated α SMA⁺ cells with patient relapse implicated that α SMA is a critical functional component of the tumour microenvironment. Indeed, staining for α SMA in a large sample of patient TMAs did yield prognostic significance, indicating that either α SMA was functionally responsible for the tumour-promoting and metastasis-initiating properties that led to earlier relapse; or serves as a readout or biomarker of some other mechanism of action in the microenvironment. In fact, it can be argued that the accumulation of α SMA⁺ cells indicate a host response directed at containing the tumour, given that *in vitro* co-culture with pericytes alone could promote invasion and migration of the OVCAR-5 cells (Lynn Chong Ph.D. thesis, 2013; Appendix B, Figure 2). Notably,

the combined pericyte signature at mRNA level was much more effective at predicting relapse ($p=0.00067$; *Chapter 1, Figure 1.8A*) than the level of α SMA staining ($p=0.03691$; *Figure 5.11*). These data are consistent with the original AOCS study demonstrating poorer prognosis in patients with the C1 stromal subtype of serous ovarian cancer (Tothill et al., 2008) and a subsequent report in the literature showing that high-grade tumours contain a larger stromal α SMA⁺ component (Schauer et al., 2011). Further, high α SMA has also been reported to be of prognostic value in colorectal cancer (Tsujino et al., 2007) and pancreatic adenocarcinoma (Fujita et al., 2010; Infante et al., 2007).

Notably, the pericyte marker PDGFR β was not able to subset serous ovarian cancer patients based on relapse ($p=0.1861$; *Figure 5.11B*), leading to the conclusion that while α SMA and PDGFR β identify pericytes, these markers are also present in other stromal cells in the tumour microenvironment; whereas the combined pericyte signature is more specific for pericytes. Consistent with this, both PDGFR β and α SMA up-regulation was shown in cultured fibroblasts (*Chapter 3*), although they are both preferentially expressed in pericytes *in vivo* (Paquet-Fifield et al., 2009). Based on the correlation of PDGFR β and CD34 expression demonstrated in *Figure 5.12*, it could be postulated that PDGFR β identifies the vessel-associated subset of α SMA⁺ cells, a population that did not have any bearing on patient relapse, a possibility that could be addressed in future experiments by co-staining for both markers on patient TMAs.

Further, *in silico* exploration of biological pathways expressed by patients with early recurrence of disease was undertaken (*in collaboration Susann Mönchgesang*), aimed at understanding what processes were responsible for early relapse and mortality in serous ovarian cancer patients. Specifically, analysis of Gene Ontology (GO) biological processes (*Appendix D, Table 1*) showed that genes associated with altered cell adhesion and actin cytoskeleton reorganisation were up-regulated in patients that exhibited early recurrence of disease. Importantly, angiogenesis-related pathways ('angiogenesis' and 'regulation of angiogenesis') ranked 136th and 251st out of 802 gene sets and did not pass any significance threshold to be considered as enriched in the 'early relapse' group of patients, again reiterating that angiogenesis

as a process, although definitely key to the sustenance of the primary tumour, was not instrumental in predicting relapse at the very advanced stage of disease analysed here.

Subsequent analyses using the GO cellular component (*Appendix D, Table 2*) and GO molecular function (*Appendix D, Table 3*) pathways revealed further evidence for significant enrichment of genes involved in matrix remodelling, ECM degradation and actin cytoskeleton organisation related processes in patients with who relapsed earlier. Analysis using the KEGG pathway revealed over-expression of genes related to focal adhesion, TGF β signalling pathway, cytoskeleton organisation and cell-cell adhesion. The top 20 KEGG and GO pathways enriched in patients who relapsed earlier are enlisted in Table 5.2.

Table 5.2: List of Top 20 KEGG and GO pathways enriched in the early versus late relapse patients (*analysis by Susann Mönchgesang*)

#	KEGG pathways	GO pathways
1	KEGG_FOCAL_ADHESION	ACTIN_FILAMENT_BASED_PROCESS
2	KEGG_MELANOMA	ACTIN_CYTOSKELETON_ORGANIZATION_AND_BIOGENESIS
3	KEGG_TGF_BETA_SIGNALING_PATHWAY	ACTIN_FILAMENT_ORGANIZATION
4	KEGG_AXON_GUIDANCE	REGULATION_OF_CYTOSKELETON_ORGANIZATION_AND_BIOGENESIS
5	KEGG_VASCULAR_SMOOTH_MUSCLE_CONTRACTION	CELL_MATRIX_ADHESION
6	KEGG_GAP_JUNCTION	CELL_SUBSTRATE_ADHESION
7	KEGG_REGULATION_OF_ACTIN_CYTOSKELETON	REGULATION_OF_ORGANELLE_ORGANIZATION_AND_BIOGENESIS
8	KEGG_ADHERENS_JUNCTION	NEGATIVE_REGULATION_OF_CELL_ADHESION
9	KEGG_ADIPOCYTOKINE_SIGNALING_PATHWAY	SKELETAL_DEVELOPMENT
10	KEGG_ECM_RECEPTOR_INTERACTION	REGULATION_OF_CELLULAR_COMPONENT_SIZE
11	KEGG_MELANOGENESIS	G_PROTEIN_SIGNALING_COUPLED_TO_IP3_SECOND_MESSENGERPHOSPHOLIPASE_C_ACTIVATING
12	KEGG_PROSTATE_CANCER	G_PROTEIN_SIGNALING_COUPLED_TO_CAMP_NUCLEOTIDE_SECOND_MESSENGER
13	KEGG_GLIOMA	ENDOSOME_TRANSPORT
14	KEGG_PATHWAYS_IN_CANCER	ACTIN_POLYMERIZATION_AND_OR_DEPOLYMERIZATION

#	KEGG pathways	GO pathways
15	KEGG_PANCREATIC_CANCER	CELL_MIGRATION
16	KEGG_WNT_SIGNALING_PATHWAY	CAMP_MEDIATED_SIGNALING
17	KEGG_DILATED_CARDIOMYOPATHY	AXON_GUIDANCE
18	KEGG_ACUTE_MYELOID_LEUKEMIA	REGULATION_OF_CELL_MIGRATION
19	KEGG_CALCIIUM_SIGNALING_PATHWAY	G_PROTEIN_SIGNALING_COUPLED_TO_CYCLIC_NUCLEOTIDE_SECOND_MESSENGER
20	KEGG_GLYCOSAMINOGLYCAN_BIOSYNTHESIS_CHONDROITIN_SULFATE	REGULATION_OF_CELLULAR_COMPONENT_ORGANIZATION_AND_BIOGENESIS

The findings of these *in silico* analyses point towards ECM degradation and matrix remodelling, not increased angiogenesis, as the chief biological pathways up-regulated in patients with early recurrence of disease. Given that pericytes are major effectors of these processes even in homeostasis (Bergers and Song, 2005), the clinical observations, experimental findings and *in silico* analyses reported in this Chapter collectively point towards a direct role for ‘pericytes’ located at non-angiogenic sites in degrading the ECM, thereby causing the tumour cells to undergo EMT and disseminate to secondary sites, resulting in increased fatality from secondary disease.

Chapter 6

General discussion

7.1. Introduction

Over the past decade there has been increased recognition of the fact that cells in the microenvironment of solid tumours play a significant role in cancer development, growth, metastasis and fatality. Recent scientific interest in CAFs has demonstrated their central role in tumour formation and cancer progression in models of pancreatic, breast and prostate carcinoma (Kalluri and Zeisberg, 2006). Although certain studies have demonstrated MSCs to be a major sources of CAFs, MSCs and their related cells precise role in tumour progression remains unclear. Previously, our laboratory showed that MSC-like pericytes, an important component of the tumour microenvironment, directly promote the proliferation and differentiation of normal epithelial cells in a novel, paracrine manner. This function is independent of angiogenesis - a role pericytes are most associated with (Bergers and Song, 2005). The ambiguity in the literature on the identification and lineage of various perivascular and 'fibroblast'-like populations under different environments, especially in the cancer context, their function, and some compelling clinical data from ovarian cancer patients, called for a thorough investigation into the specific role of pericytes in the ovarian tumour microenvironment. Hence, the underlying hypothesis of the work undertaken in this thesis was that *tissue resident pericytes bearing MSC properties, when hijacked away from the blood vessels, can promote tumour growth, invasion and metastasis in a CAF-like, angiogenesis-independent manner.*

7.2. Summary of work undertaken in this study

The study undertaken in this thesis sought to explore and characterise novel roles for an important but under-appreciated cell type present within the tumour microenvironment unanimously across all cancer types, i.e. the pericytes. At the inception of this study lies the pivotal clinical finding that the gene expression signature of normal, untransformed pericytes derived from healthy human neonatal tissue could predict relapse in a dataset of 215 high-grade serous ovarian cancer patients (*Chapter 1, Section 1.3.2*). Importantly, this predictive capacity was exclusive to pericytes, given that the gene expression signature of fibroblasts derived from the same tissue of origin was not prognostic. Moreover, the pericyte signature used to interrogate the patient gene expression dataset showed minimal overlap with an angiogenesis gene signature.

To further investigate the tumour-promoting properties of pericytes, an *in vivo* study focused on a xenograft model of ovarian cancer was undertaken in *Chapter 3*. A subcutaneous co-injection model was adopted where an aggressive serous ovarian cancer cell line, OVCAR-5, was injected alone or together with pericytes or fibroblasts. Consistent with their prognostic capacity in ovarian cancer patients, the *in vivo* xenograft model showed significantly increased tumour growth only in the pericyte co-injected group of mice. Given that pericytes are known to strengthen blood vessels, next, it was important to examine whether the increased tumour growth observed was a result of increased angiogenesis.

Initial evidence supporting the hypothesis that pericytes can promote tumour growth without affecting the vasculature were derived from CD31 and CD34 staining of xenograft tumours. Percentage staining area for both these key endothelial cell markers revealed that co-injection of pericytes (or fibroblasts) did not result in significant differences in the extent or distribution of tumour blood vessels. Subsequent analyses showed that there were no detectable differences in microvessel pericyte coverage index (MPI), hypoxia, necrosis or vascular permeability despite the co-injection of pericytes. It was particularly critical to explore the effect of pericytes in this context, given that these parameters and processes are commonly

implicated in various cancer types for disruption of angiogenesis leading to increased metastasis (Lu et al., 2008). Together, these data indicated that the introduction of exogenous pericytes did not alter the stability, structure and distribution of the tumour microvessels.

Interestingly, *in vivo* BLI imaging showed that the exogenous pericytes persisted but did not proliferate in the xenograft microenvironment, with a gradual decrease in their numbers with tumour progression. Another striking observation was that the injected pericytes did not migrate towards the tumour vasculature as expected. Instead, the pericytes lodge within pockets of tumour cells. This enabled the establishment of an ideal *in vivo* model where the impact of pericyte mislocalisation at non-angiogenic sites could be investigated.

Subsequently, the effect of adding exogenous pericytes on metastasis to secondary organs was explored in detail (*Chapter 4*). Co-injection of pericytes induced metastasis in the aggressive OVCAR-5 line as well the non-invasive and non-aggressive, OVCAR-8 cell line. Importantly, co-injection of fibroblasts under similar experimental conditions did not result in metastasis. Histological analyses revealed that the increased metastasis resulted from increased invasion of tumour cells at the leading edges of the pericyte co-injected tumours, a process characteristic of ovarian cancer histopathology (Auersperg et al., 2008; Cannistra, 2004). Subsequent examination of the OVCAR-5 xenograft tumours using confocal and electron microscopy techniques confirmed that tumour cells at the edges of the pericyte co-injected tumours were undergoing epithelial mesenchymal transition (EMT), presenting an elongated, motile and more mesenchymal morphology as early as Day 11. This indicated that pericytes were inducing an inherent change within the microenvironment which directly resulted in modulation of the stromal matrix, loss of adhesion and increased invasive and migratory behaviour of the tumour cells leading to metastasis. This finding reaffirmed our central hypothesis of a novel, vasculature-independent role of pericytes in the ovarian cancer stroma.

While attempting to further characterise changes in the ovarian cancer stroma brought about by the co-injection of pericytes, *Chapter 5* focused on deciphering the role of a particularly important but under-appreciated component of the microenvironment, i.e. α SMA⁺ cells. In fact, one of the major differences in the

tumours co-injected with pericytes was found to be a huge accumulation of host derived αSMA^+ cells. Experiments undertaken in this study further revealed that the pericyte-recruited αSMA^+ cells were mostly vasculature-dissociated (or stroma-associated), while numbers of vessel-associated αSMA^+ cells did not differ across the experimental groups. Dual immunofluorescent analysis revealed that some of the αSMA^+ cells infiltrating into the tumour in the pericyte co-injected tumours were BM MSCs – a population reported to promote tumour growth and metastasis in various cancer types (*Chapter 1, Section 1.1.2.2.4*). Next, it was observed that the ‘early relapse’ group of patients also showed significant increase in the number of stroma-associated infiltrating αSMA^+ cells, while the numbers of vessel-associated αSMA^+ cells remained unaltered across relapse groups.

Importantly, αSMA expression in patient primary tumours was a potent predictor of early relapse and mortality, while a seemingly more ‘pericyte-specific’ marker, PDGFR β , and the endothelial marker CD34, failed to predict relapse. This not only highlighted that the route taken by pericytes to promote tumour growth was indeed independent of vasculature, but also demonstrated remarkable clinical relevance of the xenograft mouse model developed in this study - a powerful tool in cancer research, especially given the rarity of such models in the study of ovarian cancer (Lengyel et al., 2013). Moreover, the staining pattern and expression levels of PDGFR β coincided significantly with that of the angiogenesis marker CD34, while αSMA expression correlated with neither. Thus, it could be argued that αSMA and PDGFR β represent overlapping but distinct populations of cells with contrasting effects on relapse; and that a simplistic view of these proteins as markers for pericytes/CAFs/MSCs can be erroneous. Further *in silico* analyses of the patient gene expression data revealed ECM remodelling, focal adhesion and actin cytoskeleton reorganisation to be the most up-regulated biological processes in patients who relapsed at a relatively earlier time-point.

7.3. General discussion

To review from *Chapter 1, Section 1.1.3.3*, the best known functions of pericytes under homeostatic conditions are primarily related to stabilising blood vessel structure and function (Bergers and Song, 2005). However, the role of pericytes in the tumour context has not been explored beyond their pro-angiogenic properties. Based on the current understanding of the role of pericytes in stabilising blood vessels, it has been proposed that destroying the vasculature by targeting both the endothelial cells and pericytes would result in tumour regression (Bergers et al., 2003). In fact, a recent report showing that stripping blood vessels of pericytes results in hypoxia within the tumour, induced EMT and increased metastatic dissemination of tumour cells to the lungs, in two different mouse models of breast cancer (4T1 xenografts and MMTV-Polyoma middle T transgenic mice), the B16 melanoma mouse model and the 786-O human renal cell carcinoma model (Cooke et al., 2012). However, while Cooke et al., 2012 obliterated NG2⁺ ‘pericytes’ using a thymidine kinase promoter, instead of eliminating a cell type from the environment which is unlikely to happen without therapeutic intervention, our study focussed on the effects of mislocalisation of pericytes away from blood vessels. Data shown here present conclusive evidence that leaving the tumour vasculature intact, yet permitting pericytes to interact with ovarian tumour cells at a non-angiogenic site results in increased tumour cell proliferation, invasion and metastatic spread through previously unrecognised means. Importantly, to reiterate from *Chapter 4, Section 4.3*, this is the first evidence of spontaneous metastasis from subcutaneous tumours of OVCAR-5 and OVCAR-8 cells.

One of the most interesting features of the pericyte co-injected tumours in this study was the recruitment of infiltrating α SMA⁺ cells (*Chapter 5*). Intriguingly, another line of investigation in the laboratory showed that the co-injected fibroblasts, in contrast, led to a similarly increased infiltration of CD45⁺ haematopoietic (immune) cells (Lynn Chong Ph.D. thesis, 2013; *Appendix B, Figure 3*). In fact, in a recent study Fearon and colleagues has shown that FAP α ⁺ α SMA⁺ stromal cells (interestingly not referred to as ‘CAFs’) render a survival advantage to tumours through immune suppression (Kraman et al., 2010). Given that the fibroblasts injected in our experiments were indeed FAP α ⁺ α SMA⁺ (*Chapter 3, Section 3.2.1*),

this observation further highlights the ambiguity regarding fibroblasts/myofibroblasts/CAFs and pericytes/perivascular MSCs – showing that the two cell types attract the infiltration of inherently different populations thus making up two very different stromal environments. The fact that this brings about a big difference in patient prognosis and relapse further accentuates the importance of duly recognising the scale of effects these cell types can have on the tumour microenvironment. Notably, ‘true’ pericytes, like most stem cell populations, possibly exist at rather low numbers and yet exert a substantial physiological effect, as demonstrated by the intratumoural persistence of GFP-luciferase⁺ pericytes *in vivo* in Chapter 3. In fact, due to the unavailability of specific markers, this ‘true’ pericyte population is probably obscured in the conundrum of α SMA⁺ and PDGFR β ⁺ cells.

It was also rather interesting to note the differential relapse predicting capacities of PDGFR β , α SMA and CD34, and the correlation between these markers, all adding to the ‘specific’ marker conundrum – a recurring theme in this study (*Chapters 1, 3, and 5*). CD34 was not prognostic, consistent with the lack of correlation between microvessel density and progression-free survival. Thus, while angiogenesis is obviously critical for the development and sustenance of the primary tumour, it may not be as relevant to cancer progression at the advanced stages of malignancy analysed here. Hence, patient datasets from earlier stages of malignancy are essential for analysing the role of pericytes in the initial stages of malignant transformation. However, given that most ovarian cancer cases are clinically presented at rather advanced stages, obtaining samples from earlier stages of malignancy can prove to be quite challenging.

In summary, this study provides a new view of the ovarian tumour microenvironment, showing that pericytes, when in close proximity to the vascular wall, are associated with non-aggressive and well encapsulated tumours. However, when placed at non-angiogenic sites within the microenvironment, pericytes initiate a cascade of events, which includes infiltration of BM MSCs, matrix remodelling, loss of adhesion and EMT of neighbouring cancer cells - all contributing towards a dynamic and invasive environment resulting in increased tumour growth, subsequent metastasis to distant organs and relapse (*Figure 6.1*).

THE OVARIAN TUMOUR MICROENVIRONMENT

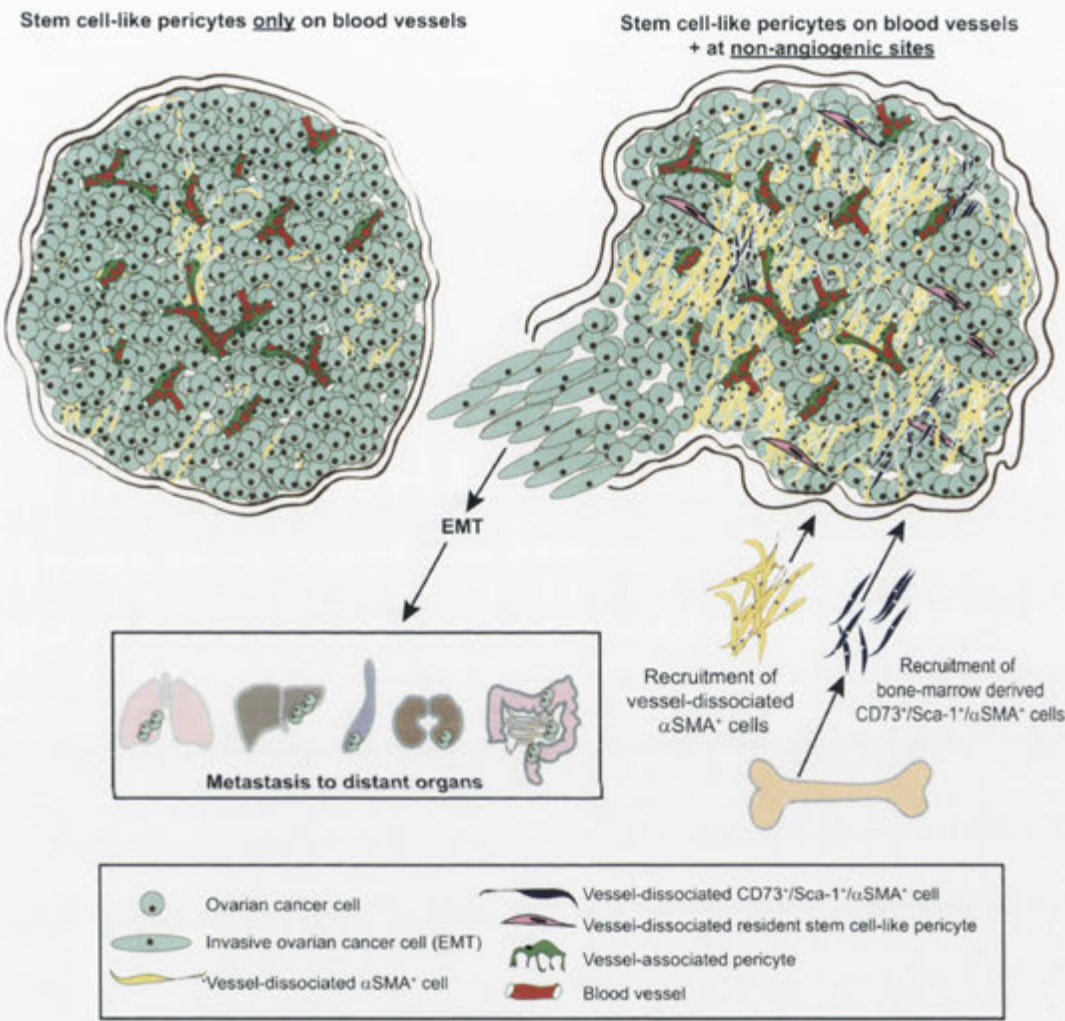


Figure 6.1. Schematic model of the role of pericytes in promoting malignant ovarian cancer malignancy. Localisation of pericytes in intimate association with blood vessels results in a tightly encapsulated tumour (left) while placing pericytes directly within the tumour microenvironment leads to increased tumour cell proliferation, increased recruitment of α SMA⁺ stromal cells including cells expressing markers of BM MSCs, induction of EMT and invasion, and metastatic spread to distant organs.

7.4. Future directions

Based on the results obtained in this study, it can be postulated that pericytes represent a resident pool of MSC-like cells that can respond rapidly to local tissue damage – a process hijacked by the tumour to the detriment of the patient. Therefore, a target of further work is to explore how pericytes become dissociated from blood vessels – a process that clearly takes place during tissue re-modelling under physiological conditions (Bergers and Song, 2005).

The other avenue for future studies is to perform further *in silico* analyses to stratify particular prognostic candidates over-expressed by pericytes that are mechanistically relevant. However, given the ability of pericytes to regulate several processes including tumour cell proliferation, migration, invasion and EMT, it is rather unlikely that any one marker would bear substantial predictive capacity. Given the concerted up-regulation of ECM remodelling and matrix degradation pathways in both pericytes as well as patients with early relapse, exploring candidate factors within these pathways could provide important clues behind the action of pericytes in the ovarian tumour microenvironment.

TGF β signalling, one of the major pathways up-regulated in patients with early relapse (Chapter 5, Table 5.2) is one such pathway that can arguably affect the tumour microenvironment at multiple points - tissue remodelling, actin cytoskeleton reorganisation, ECM receptor interaction, focal adhesion and regulation of cell migration being the more relevant ones in this context (Bierie and Moses, 2006; Hanahan and Weinberg, 2011; Pietras and Ostman, 2010). Importantly, the TGF β pathway has also been implicated as a major inducer of EMT, arguably postulated to be the single most co-ordinated axis in the multi-step process of metastasis (Bhowmick et al., 2004; Massagué, 2008; Xu et al., 2009). Interestingly, the VLA-1^{bri} pericytes show a significant up-regulation of two key factors from this pathway – BMP2 and TGF β 3 (Paquet-Fifield et al., 2009). A few preliminary *in silico* analyses were carried out to investigate if either factor was responsible or prognostic for relapse in the patient data set studied, and strikingly, while BMP2 failed to segregate

patients by relapse based on its expression (*Figure 6.2A*), there was a remarkably significant correlation between high TGF β 3 expression and patient relapse ($p < 0.001$; *Figure 6.2B*). Given that TGF β can affect a myriad of physiological processes, it was important to show that this effect came from TGF β 3 specifically in this analysis. Hence next, it was shown that TGF β 2 and TGF β 1 did not bear a similar ability to predict patient relapse and mortality (*Figure 6.2 C-D*). There is indeed evidence in the literature that TGF β 3 can rapidly activate RhoA and stimulate the formation of actin stress fibres resulting in EMT and increased cancer cell motility (Kaartinen et al., 2002). Given this rather strong preliminary evidence, future studies focussing on building *in vivo* knock-out models of TGF β 3 and studying the effects of pericytes not just in cancer, but also in processes like wound healing, might yield a lot of much-needed insight into the specific molecular mechanisms by which pericytes exert physiological effects.

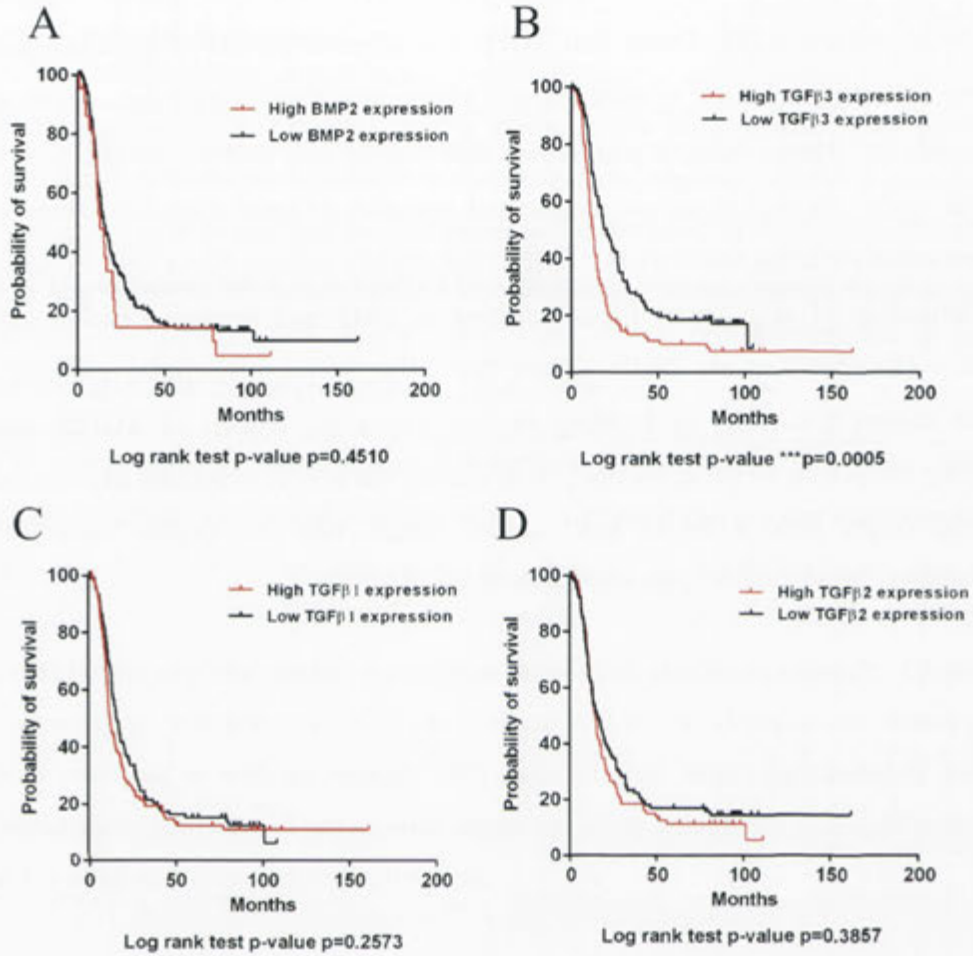


Figure 6.2. TGFβ3 predicts serous ovarian cancer relapse. (A-D) Kaplan-Meier survival curves for ovarian cancer patients using (A) BMP2, (B) TGFβ3, (C) TGFβ1 and (D) TGFβ2 as prognostic markers. TGFβ3 alone was able to predict earlier disease recurrence and mortality significantly. Analyses in (A-D) represent progression-free survival and gene expression data derived from 213, 208, 198 and 209 patients respectively. Statistical analyses were performed using the log-rank (Mantel-Cox) test. $***p<0.001$.

Another focus of future investigation would be to define markers that can distinguish bona fide pericytes from other stromal/fibroblastic/CAFs/MSC-like cells in the tumour microenvironment. It will also be important to identify markers that distinguish the pericytes that have detached from blood vessels and interact more closely with tumour cells, from vessel-associated pericytes. The co-expression of some markers by both pericytes and MSCs poses further challenge; however, this could be addressed using well-designed lineage tracing techniques in mice genetically manipulated to generate labelled pericyte progeny.

In conclusion, work reported in this thesis is the first clinical and experimental evidence for a novel angiogenesis-independent role for pericytes in promoting ovarian cancer progression. This novel finding bears significant implications for understanding the development of solid tumours in many organs given that pericytes are found in all vascularised tissues. Moreover, it is interesting to speculate that pericytes can act as a potent population of cancer-promoting MSCs and can be recruited away from blood vessels causing aggressive malignant progression. This data also draws attention to the under-appreciated fact that just like CSCs, potent stem cells in tumour stroma can also have far-reaching effects in determining malignant fatality. Thus, data presented here represents a shift from the current thinking about the contribution of pericytes to the tumour microenvironment while providing an effective means of identifying those patients that are at significantly greater risk of early relapse and mortality. These results also present the possibility of discovering novel 'pericyte-specific' markers that could be used at the protein level for prognosis in the clinic in the diagnosis of ovarian cancer. In addition, this newly-discovered role of pericytes could also be further investigated and applied in other epithelial cancer models, e.g. gastric, cervical and squamous cell carcinoma, to further understand the biology of pericytes. Moreover, general scientific understanding of the origin, lineage and the molecular mechanism of action of pericytes and related paracrine interactions within the stroma would bring us a step closer to further understanding of the epithelial tumour microenvironment.

Appendix A

**List of genes up-regulated in both
pericytes and ovarian stroma**

Appendix A- List of probe sets for the 146 overlapping genes up-regulated in the pericytes and ovarian stroma. (George, Bowtell and Kaur, unpublished data)

[#]Fc denotes fold change of expression in patients and the corresponding p-values denote the significance of this expression. The Fc values are in log scale, thus a value of 1 means 2-fold higher expression in early relapse versus late relapse samples. A score >0 denotes increased expression, while a score <0 denotes decreased expression in patients with early relapse.

Unique probe ID	Gene Name	Fc#	p-value
203980_at	FABP4	3.20952	0.0014
227140_at	NA	2.43023	0.00048
204914_s_at	SOX11	2.40931	0.00595
204913_s_at	SOX11	2.33465	0.00846
226695_at	PRRX1	2.18938	0.00024
201150_s_at	TIMP3	2.09981	0.00016
210511_s_at	INHBA	1.9063	0.00146
204915_s_at	SOX11	1.83973	0.01294
201147_s_at	TIMP3	1.75255	0.00054
221541_at	CRISPLD2	1.72277	0.00355
232523_at	MEGF10	1.69442	0.00337
204463_s_at	EDNRA	1.67891	0.00062
228821_at	ST6GAL2	1.66861	0.0007
204464_s_at	EDNRA	1.65923	0.00057
228057_at	DDIT4L	1.64139	0.0012
203868_s_at	VCAM1	1.56713	0.00114
240135_x_at	TIMP3	1.56208	0.00079
222450_at	TMEPAI	1.54708	0.0018
237802_at	XKR4	1.52385	0.00096
205991_s_at	PRRX1	1.51849	0.00066
212353_at	SULF1	1.51411	0.01152
243140_at	ACTA2	1.47495	0.00411
238852_at	PRRX1	1.44332	0.00205
225328_at	NA	1.40331	5.94E-05
201149_s_at	TIMP3	1.38918	0.00357
212354_at	SULF1	1.38045	0.01045
228766_at	CD36	1.37845	0.01359
201148_s_at	TIMP3	1.35071	0.00062
231213_at	PDE1A	1.34972	0.00396
225688_s_at	PHLDB2	1.30311	0.00071
201348_at	GPX3	1.28432	0.01424
214091_s_at	GPX3	1.27336	0.01916
225782_at	MSRB3	1.26285	0.00159
204777_s_at	MAL	1.23615	0.03622
229580_at	NA	1.23404	0.00284
213844_at	HOXA5	1.21749	0.03179
211597_s_at	HOPX	1.21114	0.01019

Unique probe ID	Gene Name	Fc#	p-value
213496_at	LPPR4	1.21034	0.00152
206488_s_at	CD36	1.19461	0.02801
200974_at	ACTA2	1.19002	0.00452
208396_s_at	PDE1A	1.18948	0.00285
209869_at	ADRA2A	1.15779	0.00932
225803_at	FBXO32	1.11007	0.00093
205547_s_at	TAGLN	1.08185	0.00962
227646_at	NA	1.07354	0.00858
218087_s_at	SORBS1	1.0718	0.00443
203548_s_at	LPL	1.06316	0.01946
226047_at	MRVI1	1.04419	0.00078
225496_s_at	SYTL2	1.02599	0.0086
203865_s_at	ADARB1	1.01886	0.0002
219463_at	C20orf103	0.99576	0.06504
202177_at	GAS6	0.9903	0.00196
201215_at	PLS3	0.98971	0.0002
230214_at	MRVI1	0.98494	0.00452
201645_at	TNC	0.9585	0.03551
201579_at	FAT	0.95093	0.00406
214023_x_at	TUBB2B	0.93647	0.18937
214053_at	ERBB4	0.93302	0.04687
1555724_s_at	TAGLN	0.93273	0.01353
201616_s_at	CALD1	0.92199	0.00375
236029_at	FAT3	0.90678	0.05495
209301_at	CA2	0.89261	0.11606
232914_s_at	SYTL2	0.89214	0.02017
204379_s_at	FGFR3	0.88916	0.06658
214803_at	NA	0.88269	0.02837
229310_at	KLHL29	0.88213	0.01907
201617_x_at	CALD1	0.88147	0.01932
225540_at	MAP2	0.86436	0.05233
212412_at	PDLIM5	0.86433	5.03E-05
232204_at	EBF1	0.8636	0.02738
203571_s_at	C10orf116	0.85273	0.18418
212077_at	CALD1	0.83668	0.0065
225987_at	STEAP4	0.83402	0.10039
229613_at	NA	0.83375	0.01856
225790_at	MSRB3	0.83127	0.00219
228333_at	NA	0.82912	0.00783
205943_at	TDO2	0.82769	0.20406
229530_at	GUCY1A3	0.82543	0.03196
201117_s_at	CPE	0.81771	0.00432
203549_s_at	LPL	0.81186	0.03909
203243_s_at	PDLIM5	0.8114	0.00014

Unique probe ID	Gene Name	Fc#	p-value
1598_g_at	GAS6	0.80039	0.0024
216235_s_at	EDNRA	0.78909	0.01295
225275_at	EDIL3	0.78592	0.10402
209183_s_at	C10orf10	0.78494	0.01833
223316_at	CCDC3	0.78138	0.01475
202273_at	PDGFRB	0.78006	0.00287
203323_at	CAV2	0.77258	0.00671
229947_at	PII5	0.77086	0.04347
223499_at	C1QTNF5	0.76232	0.00207
209656_s_at	TMEM47	0.75563	0.06694
201058_s_at	MYL9	0.74703	0.00147
227235_at	GUCY1A3	0.73968	0.03714
228776_at	GJC1	0.73249	0.03699
204894_s_at	AOC3	0.72802	0.03972
235046_at	NA	0.72292	0.02206
221942_s_at	GUCY1A3	0.72242	0.05409
204584_at	L1CAM	0.72124	0.15576
204072_s_at	FRY	0.7212	0.02017
218353_at	RGS5	0.71736	0.00733
203819_s_at	IGF2BP3	0.71148	0.33576
205609_at	ANGPT1	0.70469	0.00719
236234_at	PDE1A	0.70262	0.02501
203766_s_at	LMOD1	0.70101	0.01588
225728_at	SORBS2	0.69641	0.09968
205303_at	KCNJ8	0.69364	0.01471
204870_s_at	PCSK2	0.68757	0.08482
229487_at	NA	0.68749	0.03242
219895_at	FAM70A	0.68706	0.0195
210986_s_at	TPM1	0.68598	0.00681
201615_x_at	CALD1	0.68098	0.00631
211538_s_at	HSPA2	0.68027	0.03691
203695_s_at	DFNA5	0.68002	0.03143
202551_s_at	CRIM1	0.6743	0.00505
202274_at	ACTG2	0.67061	0.17223
205440_s_at	NPY1R	0.65367	0.0459
225911_at	NPNT	0.65245	0.10045
210605_s_at	MFGE8	0.64981	0.04486
202998_s_at	LOXL2	0.64932	0.11995
201830_s_at	NET1	0.64455	0.00996
227498_at	NA	0.64091	0.13807
205608_s_at	ANGPT1	0.64085	0.00946
204083_s_at	TPM2	0.63983	0.02102
209866_s_at	LPHN3	0.63923	0.21027
225895_at	SYNPO2	0.63671	0.13196

Unique probe ID	Gene Name	Fc#	p-value
209655_s_at	TMEM47	0.63662	0.06198
203697_at	FRZB	0.63612	0.27613
222379_at	KCNE4	0.63508	0.00718
210987_x_at	TPM1	0.63329	0.01244
204803_s_at	RRAD	0.6324	0.10178
242828_at	FIGN	0.62533	0.19334
230482_at	ST6GALNAC5	0.62458	0.16748
218870_at	ARHGAP15	0.62221	0.04592
203698_s_at	FRZB	0.62182	0.2795
218717_s_at	LEPREL1	0.62103	0.09038
45297_at	EHD2	0.61727	0.00282
236656_s_at	NA	0.61528	0.02044
212473_s_at	MICAL2	0.61076	0.10202
209324_s_at	RGS16	0.6104	0.05221
201116_s_at	CPE	0.60884	0.02397
235368_at	ADAMTS5	0.60589	0.14357
216804_s_at	PDLIM5	0.60131	0.00125
229168_at	COL23A1	0.59535	0.09477
202552_s_at	CRIM1	0.59453	0.00776
227367_at	SLCO3A1	0.59268	0.05087
1554127_s_at	MSRB3	0.58866	0.00832
213413_at	GTF2A1L	0.58764	0.11386
40148_at	APBB2	0.58713	0.0338
233547_x_at	PDE1A	0.58438	0.03064
221870_at	EHD2	0.58256	0.00702
201829_at	NET1	0.57924	0.01162
203650_at	PROCR	0.57856	0.00419
205304_s_at	KCNJ8	0.57763	0.03549
209071_s_at	RGS5	0.57633	0.02088
230692_at	LOC157503	0.57598	0.019
209200_at	MEF2C	0.57376	0.01029
204802_at	RRAD	0.57186	0.1715
209325_s_at	RGS16	0.57099	0.08653
228310_at	ENAH	0.57094	0.03558
209747_at	TGFB3	0.56908	0.00458
239710_at	NA	0.56828	0.22813
204720_s_at	DNAJC6	0.56796	0.07586
205111_s_at	PLCE1	0.56478	0.08529
227480_at	SUSD2	0.56356	0.15432
209487_at	RBPMS	0.56235	0.04906
204939_s_at	PLN	0.55728	0.19886
217546_at	MT1M	0.55634	0.24171
244655_at	LOC644192	0.55319	0.14626
203498_at	RCAN2	0.54464	0.04598

Unique probe ID	Gene Name	Fc#	p-value
209576_at	GNAI1	0.54396	0.14624
219935_at	ADAMTS5	0.54012	0.25025
227497_at	NA	0.53961	0.09178
215306_at	STON1	0.5331	0.08536
215768_at	SOX5	0.53169	0.17533
202732_at	PKIG	0.52896	0.00289
222824_at	SEC61A2	0.52095	0.02786
213075_at	OLFML2A	0.51958	0.12309
228771_at	ADRBK2	0.51934	0.03033
242447_at	LOC285382	0.51675	0.12245
226223_at	PAWR	0.51558	0.04103
227662_at	SYNPO2	0.5153	0.33443
219743_at	HEY2	0.51195	0.21813
213125_at	OLFML2B	0.51188	0.22566
212472_at	MICAL2	0.51143	0.09527
219985_at	HS3ST3A1	0.50803	0.16997
212364_at	MYO1B	0.508	0.09394
219230_at	TMEM100	0.50496	0.39053
205265_s_at	SPEG	0.50377	0.0137
205376_at	INPP4B	0.50004	0.12001
206898_at	CDH19	0.49942	0.05534
229357_at	ADAMTS5	0.49669	0.22675
208999_at	SEPT8	0.49571	0.01492
238009_at	NA	0.49192	0.11678
228214_at	NA	0.49164	0.10957
229515_at	PAWR	0.49151	0.00427
205501_at	NA	0.49108	0.15339
225163_at	FRMD4A	0.48679	0.0832
209199_s_at	MEF2C	0.48416	0.02021
205352_at	SERPINI1	0.48087	0.1335
227196_at	RHPN2	0.47675	0.18427
204639_at	ADA	0.4755	0.01356
208636_at	ACTN1	0.47544	0.01111
204099_at	SMARCD3	0.47282	0.15419
228900_at	NA	0.46685	0.05304
1552508_at	KCNE4	0.46222	0.00051
224836_at	TP53INP2	0.45944	0.00606
203835_at	LRRC32	0.45494	0.05052
225502_at	DOCK8	0.4517	0.16926
204288_s_at	SORBS2	0.44711	0.19419
242680_at	NA	0.44664	0.12259
221796_at	NTRK2	0.44654	0.06157
212463_at	CD59	0.442	0.02855
224823_at	MYLK	0.43891	0.11176

Unique probe ID	Gene Name	Fc#	p-value
235593_at	ZEB2	0.43858	0.09163
226632_at	CYGB	0.43307	0.01523
235561_at	NA	0.4312	0.09826
202555_s_at	MYLK	0.43062	0.10527
210150_s_at	LAMA5	0.4296	0.07177
204869_at	PCSK2	0.4295	0.0807
52255_s_at	COL5A3	0.42939	0.02629
209488_s_at	RBPMS	0.42776	0.11839
238592_at	PDLIM3	0.42659	0.1658
222008_at	COL9A1	0.42486	0.36946
228731_at	NA	0.42368	0.30302
218330_s_at	NAV2	0.42345	0.12361
238751_at	SORBS2	0.4152	0.26835
233599_at	hCG_2003663	0.41511	0.16973
206116_s_at	TPM1	0.41458	0.08562
209781_s_at	KHDRBS3	0.41319	0.22785
225258_at	FBLIM1	0.4118	0.05524
214954_at	SUSD5	0.41142	0.0309
203820_s_at	IGF2BP3	0.41128	0.56244
227856_at	C4orf32	0.40874	0.16166
205112_at	PLCE1	0.40871	0.23921
209070_s_at	RGS5	0.40754	0.10713
214428_x_at	C4A	0.40754	0.20762
215073_s_at	NR2F2	0.40692	0.24106
205463_s_at	PDGFA	0.40677	0.20572
1569003_at	TMEM49	0.40305	0.15666
221748_s_at	TNS1	0.40226	0.18688
230158_at	DPY19L2	0.40169	0.19021
227088_at	PDE5A	0.40066	0.08703
209343_at	EFHD1	0.39974	0.23201
227826_s_at	SORBS2	0.39826	0.27857
226326_at	PCGF5	0.39697	0.06623
227618_at	FLJ44635	0.39502	0.07207
47550_at	LZTS1	0.39458	0.0142
200601_at	ACTN4	0.39301	0.06713
218975_at	COL5A3	0.39229	0.04421
1555904_at	NA	0.3905	0.00311
204273_at	EDNRB	0.39044	0.34226
203642_s_at	COBLL1	0.38838	0.10661
212993_at	NA	0.38818	0.14754
208637_x_at	ACTN1	0.38699	0.05244
201474_s_at	ITGA3	0.38621	0.19096
220975_s_at	C1QTNF1	0.3847	0.03557
229092_at	NR2F2	0.38222	0.09636

Unique probe ID	Gene Name	Fc#	p-value
225720_at	SYNPO2	0.38201	0.24533
236264_at	LPHN3	0.38034	0.28816
205532_s_at	CDH6	0.38005	0.37203
229830_at	NA	0.3789	0.26745
228108_at	NA	0.37556	0.16729
205824_at	HSPB2	0.37498	0.07525
212646_at	RFTN1	0.37358	0.20582
217966_s_at	FAM129A	0.37347	0.23265
1558828_s_at	LOC728264	0.37323	0.29109
236396_at	NA	0.37218	0.09205
227272_at	C15orf52	0.37185	0.23884
223595_at	TMEM133	0.37054	0.19227
228496_s_at	CRIM1	0.3677	0.11283
238784_at	DPY19L2	0.36728	0.28209
222717_at	SDPR	0.36658	0.27182
1555725_a_at	RGS5	0.36563	0.18167
219654_at	PTPLA	0.36489	0.32263
205582_s_at	GGT5	0.36433	0.0064
235085_at	PRAGMIN	0.36408	0.15275
226731_at	PELO	0.364	0.12342
212950_at	GPR116	0.36352	0.14635
203303_at	DYNLT3	0.36104	0.01042
209874_x_at	CNNM2	0.36089	0.00243
227099_s_at	LOC387763	0.35652	0.41486
227945_at	TBC1D1	0.35504	0.04363
228748_at	CD59	0.35432	0.06445
242033_at	RNF180	0.35413	0.09066
213317_at	CLIC5	0.35402	0.51096
208383_s_at	PCK1	0.35105	0.59314
202887_s_at	DDIT4	0.35058	0.26317
210657_s_at	SEPT4	0.3496	0.05601
217967_s_at	FAM129A	0.34789	0.38902
231987_at	LOC728264	0.34529	0.08579
221747_at	TNS1	0.34463	0.17246
219279_at	DOCK10	0.34381	0.26094
238868_at	UACA	0.34375	0.20532
208562_s_at	ABCC9	0.34264	0.01628
222760_at	ZNF703	0.34146	0.20071
205935_at	FOXF1	0.33734	0.02137
204004_at	PAWR	0.3373	0.10842
220559_at	EN1	0.3359	0.17832
201957_at	PPP1R12B	0.33382	0.04082
210078_s_at	KCNAB1	0.33378	0.07783
225056_at	SIPA1L2	0.32649	0.20964

Unique probe ID	Gene Name	Fc#	p-value
227758_at	RERG	0.32528	0.45344
228456_s_at	LOC149832	0.32488	0.04913
226743_at	SLFN11	0.32267	0.169
220936_s_at	H2AFJ	0.32119	0.20145
229518_at	FAM46B	0.32055	0.22009
238835_at	NA	0.31758	0.11594
219825_at	CYP26B1	0.31592	0.21389
217897_at	FXVD6	0.31311	0.45546
219229_at	SLCO3A1	0.31169	0.25782
232843_s_at	DOCK8	0.31103	0.25082
226523_at	TAGLN	0.31097	0.0174
205289_at	BMP2	0.31029	0.16367
1554547_at	FAM13C1	0.30981	0.27379
217757_at	A2M	0.30965	0.13696
63825_at	ABHD2	0.30871	0.13209
1555480_a_at	FBLIM1	0.30614	0.08555
206818_s_at	CNNM2	0.3047	0.02859
225894_at	SYNPO2	0.30169	0.15717
241879_at	NA	0.30015	0.30621
1552507_at	KCNE4	0.29835	0.02165
224811_at	NA	0.29775	0.07932
204271_s_at	EDNRB	0.29711	0.44962
219454_at	EGFL6	0.29598	0.42386
225809_at	DKFZP564O0823	0.29563	0.34956
212511_at	PICALM	0.29441	0.07553
228563_at	GJC1	0.29399	0.18012
236335_at	NA	0.29326	0.48179
212951_at	GPR116	0.29217	0.15274
209765_at	ADAM19	0.29186	0.22256
201037_at	PFKP	0.29177	0.21812
200878_at	EPAS1	0.29088	0.22394
209120_at	NR2F2	0.28953	0.33686
209121_x_at	NR2F2	0.28946	0.26573
224588_at	XIST	0.289	0.28823
220276_at	RERGL	0.28636	0.05512
225721_at	SYNPO2	0.28547	0.24135
213924_at	MPPE1	0.28537	0.12463
213438_at	NFASC	0.28469	0.19501
1557374_at	ABCC9	0.28239	0.1004
218094_s_at	DBNDD2	0.28195	0.18475
1554690_a_at	TACC1	0.27951	0.14565
208078_s_at	ZEB1	0.27905	0.32268
244745_at	RERG	0.27833	0.52057
225585_at	RAP2A	0.27756	0.09487

Unique probe ID	Gene Name	Fc#	p-value
235355_at	NA	0.27718	0.50421
212365_at	MYO1B	0.27435	0.34201
205290_s_at	BMP2	0.27361	0.19216
235017_s_at	NA	0.27318	0.44428
221830_at	RAP2A	0.27267	0.09092
226856_at	TMEM110	0.27149	0.10295
225763_at	RCSD1	0.27088	0.25484
204844_at	ENPEP	0.26944	0.37848
213714_at	CACNB2	0.26868	0.12664
203413_at	NELL2	0.26858	0.39185
226436_at	RASSF4	0.26831	0.42709
227827_at	SORBS2	0.26757	0.41864
229670_at	NA	0.26675	0.21382
209119_x_at	NR2F2	0.26674	0.32541
217721_at	NA	0.26572	0.10069
206762_at	KCNA5	0.26471	0.11991
219358_s_at	CENTA2	0.26385	0.20417
222876_s_at	CENTA2	0.26355	0.19226
208561_at	ABCC9	0.26094	0.12964
226828_s_at	HEYL	0.25848	0.06404
203951_at	CNN1	0.257	0.22291
222921_s_at	HEY2	0.25575	0.49588
228202_at	PLN	0.25378	0.3306
229811_at	NA	0.25293	0.37277
212565_at	STK38L	0.25269	0.29731
233587_s_at	SIPA1L2	0.25243	0.35542
228875_at	C6orf189	0.25127	0.19864
212848_s_at	C9orf3	0.25116	0.31079
210319_x_at	MSX2	0.25101	0.02415
220574_at	SEMA6D	0.24997	0.35092
205934_at	PLCL1	0.24832	0.10491
212976_at	NA	0.24657	0.09202
209867_s_at	LPHN3	0.24644	0.54224
204726_at	CDH13	0.24616	0.16466
203499_at	EPHA2	0.24428	0.33411
221795_at	NTRK2	0.24358	0.21312
206374_at	DUSP8	0.24209	0.11753
211964_at	COL4A2	0.24046	0.4984
220462_at	FAM130A2	0.23207	0.50954
212326_at	VPS13D	0.22941	0.16081
1560359_at	PELO	0.22598	0.1943
221815_at	ABHD2	0.22423	0.30754
215311_at	NA	0.22409	0.14822
205257_s_at	AMPH	0.22403	0.24166

Unique probe ID	Gene Name	Fc#	p-value
202609_at	EPS8	0.22225	0.3045
212197_x_at	M-RIP	0.22183	0.0867
211980_at	COL4A1	0.22092	0.50113
228732_at	NA	0.22076	0.30738
208460_at	GJC1	0.22011	0.14486
204029_at	CELSR2	0.21731	0.3385
214771_x_at	M-RIP	0.21515	0.07845
222810_s_at	RASAL2	0.21514	0.19492
204916_at	RAMP1	0.215	0.23523
36499_at	CELSR2	0.21486	0.32084
211981_at	COL4A1	0.21442	0.51775
204940_at	PLN	0.21195	0.48677
207836_s_at	RBPMS	0.21094	0.42289
243555_at	EDNRA	0.21087	0.10149
213050_at	COBL	0.20871	0.52208
236304_at	NA	0.20815	0.03695
221246_x_at	TNS1	0.20709	0.2195
226342_at	SPTBN1	0.20621	0.26149
206030_at	ASPA	0.20442	0.23306
211926_s_at	MYH9	0.20173	0.22558
202822_at	LPP	0.2008	0.24395
209031_at	CADM1	0.20044	0.5157
220065_at	TNMD	0.19881	0.05089
218418_s_at	KANK2	0.19837	0.29787
218779_x_at	EPS8L1	0.19811	0.386
213151_s_at	SEPT7	0.19572	0.09295
227929_at	NA	0.19406	0.17918
220329_s_at	RMND1	0.19405	0.33508
213436_at	CNR1	0.19351	0.65833
222108_at	AMIGO2	0.19342	0.60359
209321_s_at	ADCY3	0.19035	0.45385
226137_at	NA	0.19015	0.26727
209994_s_at	ABCB1	0.18842	0.29844
1565823_at	SEPT7	0.18788	0.3448
40560_at	TBX2	0.18777	0.5631
202011_at	TJP1	0.18755	0.23475
232217_at	FAM26E	0.18711	0.18858
209082_s_at	COL18A1	0.1859	0.36624
238332_at	ANKRD29	0.18468	0.52836
213960_at	NA	0.18384	0.05497
237169_at	NA	0.18351	0.26541
242738_s_at	NA	0.17979	0.24917
44783_s_at	HEY1	0.17932	0.44023
226829_at	AFAP1L2	0.17831	0.62341

Unique probe ID	Gene Name	Fc#	p-value
227750_at	KALRN	0.17829	0.22802
206496_at	FMO3	0.1782	0.48026
225806_at	JUB	0.17653	0.54126
202517_at	CRMP1	0.17548	0.24417
1556185_a_at	NA	0.17474	0.59728
225278_at	PRKAB2	0.17436	0.36074
225337_at	ABHD2	0.17436	0.26786
1553764_a_at	JUB	0.17385	0.57139
229936_at	GFRA3	0.17185	0.04335
223623_at	C2orf40	0.16892	0.78064
202547_s_at	ARHGEF7	0.16683	0.24967
214660_at	ITGA1	0.16593	0.27847
212135_s_at	ATP2B4	0.16563	0.4385
227862_at	LOC388610	0.16462	0.57274
210542_s_at	SLCO3A1	0.16428	0.44073
219073_s_at	OSBPL10	0.16148	0.52067
201162_at	IGFBP7	0.16142	0.43322
238419_at	PHLDB2	0.16082	0.04517
203881_s_at	DMD	0.16077	0.62982
227533_at	RALGPS2	0.15967	0.42966
209167_at	GPM6B	0.15945	0.6323
235562_at	LOC285382	0.1593	0.01696
212711_at	CAMSAP1	0.15811	0.25793
205347_s_at	TMSL8	0.15719	0.74345
201497_x_at	MYH11	0.15666	0.61354
242186_x_at	LPHN3	0.15645	0.38504
206307_s_at	FOXD1	0.15609	0.74752
213746_s_at	FLNA	0.15461	0.49441
218472_s_at	PELO	0.15424	0.28001
209448_at	HTATIP2	0.15413	0.5632
212823_s_at	PLEKHG3	0.15392	0.20043
235874_at	PRSS35	0.15335	0.67475
204938_s_at	PLN	0.15212	0.54466
203756_at	ARHGEF17	0.15086	0.12025
223315_at	NTN4	0.15012	0.78049
91826_at	EPS8L1	0.14903	0.51701
230254_at	NA	0.14722	0.23951
243502_at	GJC1	0.14667	0.21997
214752_x_at	FLNA	0.14635	0.44999
213435_at	SATB2	0.14618	0.53456
212551_at	CAP2	0.14472	0.64264
200859_x_at	FLNA	0.14129	0.45196
226610_at	PRR6	0.13914	0.69312
205080_at	RARB	0.13893	0.44797

Unique probe ID	Gene Name	Fc#	p-value
201603_at	PPP1R12A	0.13817	0.42965
225308_s_at	TANC1	0.1379	0.45559
214297_at	CSPG4	0.1377	0.24458
209566_at	INSIG2	0.13596	0.5112
239058_at	NA	0.13508	0.40787
244832_at	EDNRA	0.13457	0.05729
210471_s_at	KCNAB1	0.13435	0.15982
213417_at	TBX2	0.13405	0.67812
212830_at	MEGF9	0.13377	0.47033
203296_s_at	ATP1A2	0.13208	0.84821
208690_s_at	PDLIM1	0.13192	0.49762
207961_x_at	MYH11	0.13063	0.6138
205132_at	ACTC1	0.13022	0.13788
219719_at	HIGD1B	0.12862	0.23229
202759_s_at	AKAP2	0.12744	0.58912
201177_s_at	UBA2	0.12677	0.38969
218839_at	HEY1	0.12419	0.47184
219506_at	C1orf54	0.1227	0.39499
229308_at	NA	0.12246	0.55226
200985_s_at	CD59	0.11968	0.52752
242342_at	NA	0.11876	0.73392
206118_at	STAT4	0.11575	0.46554
208476_s_at	FRMD4A	0.11562	0.54465
204687_at	DKFZP564O0823	0.11477	0.36192
203072_at	MYO1E	0.11271	0.45447
208712_at	CCND1	0.11266	0.8086
228060_at	SLC35F1	0.11191	0.35078
235721_at	DTX3	0.11185	0.60237
232270_at	C9orf3	0.11165	0.63698
244461_at	SPECC1	0.10795	0.61498
212136_at	ATP2B4	0.10749	0.63344
1558881_at	LOC145820	0.10368	0.43073
202834_at	AGT	0.10305	0.57282
219167_at	RASL12	0.10087	0.65949
225167_at	FRMD4A	0.10037	0.71585
205152_at	SLC6A1	0.09936	0.11059
221665_s_at	EPS8L1	0.09799	0.67925
207819_s_at	ABCB4	0.0979	0.56154
221009_s_at	ANGPTL4	0.09562	0.58645
216080_s_at	FADS3	0.09486	0.66617
212099_at	RHOB	0.09423	0.73086
220170_at	FHL5	0.09269	0.53621
226272_at	NA	0.09121	0.73053
205679_x_at	ACAN	0.09111	0.27193

Unique probe ID	Gene Name	Fc#	p-value
209169_at	GPM6B	0.09001	0.73849
215241_at	TMEM16C	0.08854	0.29709
229373_at	NA	0.08796	0.45334
216874_at	DKFZp686O1327	0.08789	0.11235
214321_at	NOV	0.08703	0.82834
235639_at	NA	0.08649	0.27022
230596_at	NA	0.08649	0.70965
201602_s_at	PPP1R12A	0.08633	0.71244
239580_at	GUCY1A3	0.08603	0.4237
244885_at	EBF2	0.08569	0.44745
225896_at	M-RIP	0.08507	0.33366
203726_s_at	LAMA3	0.08308	0.88666
217161_x_at	ACAN	0.0827	0.34799
231048_at	NA	0.08154	0.11296
206167_s_at	ARHGAP6	0.08043	0.78488
221211_s_at	C21orf7	0.0802	0.62786
227821_at	LGI4	0.0801	0.17432
213847_at	PRPH	0.07952	0.51936
221272_s_at	C1orf21	0.07952	0.64159
1555419_a_at	ASAH1	0.07784	0.74762
1552667_a_at	SH2D3C	0.07503	0.54068
225807_at	JUB	0.07426	0.79144
40665_at	FMO3	0.07387	0.75561
210108_at	CACNA1D	0.07203	0.75402
210524_x_at	NA	0.07195	0.79125
209081_s_at	COL18A1	0.07194	0.77918
211966_at	COL4A2	0.0711	0.84174
228579_at	NA	0.06797	0.69633
206117_at	TPM1	0.06564	0.73522
214369_s_at	RASGRP2	0.06548	0.63403
204845_s_at	ENPEP	0.06525	0.75751
229145_at	C10orf104	0.06519	0.7024
225665_at	ZAK	0.06507	0.81392
215115_x_at	NTRK3	0.06479	0.38954
218678_at	NES	0.06356	0.73496
230595_at	LOC572558	0.06339	0.77179
202910_s_at	CD97	0.06153	0.7762
229587_at	UBA2	0.05996	0.62953
241859_at	NA	0.05901	0.30568
209170_s_at	GPM6B	0.05872	0.85432
206194_at	HOXC4	0.05819	0.80643
222106_at	PRND	0.05699	0.4004
206819_at	DKFZP434P211	0.05692	0.53512
226188_at	HSPC159	0.05672	0.82618

Unique probe ID	Gene Name	Fc#	p-value
1558017_s_at	PAWR	0.05576	0.65806
241442_at	DAAM2	0.05554	0.4495
201496_x_at	MYH11	0.05509	0.80542
223519_at	ZAK	0.05489	0.84828
212841_s_at	PPFIBP2	0.05304	0.81307
225662_at	ZAK	0.05277	0.85576
206210_s_at	CETP	0.05271	0.57689
224049_at	KCNK17	0.0526	0.46449
242500_at	NA	0.05228	0.68116
229382_at	C1orf183	0.0512	0.61543
236300_at	NA	0.05071	0.8811
213138_at	ARID5A	0.05009	0.56096
209099_x_at	JAG1	0.05004	0.8543
241558_at	NA	0.04898	0.30086
228783_at	BVES	0.04862	0.83485
202205_at	VASP	0.04759	0.7759
232424_at	PRDM16	0.04719	0.81217
218711_s_at	SDPR	0.04654	0.82004
235924_at	NA	0.04578	0.88852
203641_s_at	COBLL1	0.04499	0.83415
241255_at	NA	0.04481	0.68043
219282_s_at	TRPV2	0.0437	0.7895
209030_s_at	CADM1	0.0428	0.88223
222924_at	SLMAP	0.04244	0.68083
208072_s_at	DGKD	0.04181	0.70161
212730_at	DMN	0.04142	0.84935
233301_at	OXCT2	0.04127	0.54589
236576_at	NA	0.03958	0.54294
237056_at	INSC	0.03882	0.39162
202890_at	MAP7	0.03854	0.85572
225889_at	AEBP2	0.03635	0.87759
226653_at	MARK1	0.03577	0.87077
231166_at	GPR155	0.03491	0.9055
225102_at	MGLL	0.03411	0.92241
205414_s_at	RICH2	0.0331	0.91441
224998_at	CMTM4	0.03292	0.87925
235147_at	SATB2	0.0328	0.87166
212285_s_at	AGR1	0.03245	0.84015
206528_at	TRPC6	0.03109	0.66784
219958_at	C20orf46	0.03104	0.84388
212554_at	CAP2	0.02982	0.91595
207317_s_at	CASQ2	0.02912	0.81676
213332_at	PAPPA2	0.02868	0.92377
230574_at	SPIRE2	0.02683	0.66737

Unique probe ID	Gene Name	Fc#	p-value
210869_s_at	MCAM	0.02669	0.90582
203315_at	NCK2	0.02608	0.87967
238891_at	NA	0.02601	0.68005
243409_at	FOXL1	0.02227	0.76805
230047_at	FLJ32810	0.02162	0.92926
218804_at	TMEM16A	0.02141	0.96294
210602_s_at	CDH6	0.02024	0.95996
204736_s_at	CSPG4	0.01899	0.79207
1555993_at	CACNA1D	0.01873	0.909
209086_x_at	MCAM	0.01871	0.89979
204257_at	FADS3	0.01696	0.91772
220392_at	EBF2	0.01654	0.8183
1557570_a_at	LOC285084	0.01638	0.63499
219058_x_at	TINAGL1	0.01462	0.91802
209087_x_at	MCAM	0.01376	0.95064
230788_at	GCNT2	0.0137	0.97666
1555191_a_at	FHL5	0.01359	0.83997
217455_s_at	SSTR2	0.01236	0.8614
228507_at	NA	0.01201	0.96791
205555_s_at	MSX2	0.01078	0.89026
204311_at	ATP1B2	0.00988	0.93485
230249_at	KHDRBS3	0.00642	0.9755
225369_at	ESAM	0.00533	0.97108
236359_at	SCN4B	0.0043	0.97326
210246_s_at	ABCC8	0.00244	0.97024
201288_at	ARHGDIB	0.00195	0.99434
231431_s_at	NA	-0.0016	0.99015
210300_at	REM1	-0.0023	0.97622
206701_x_at	EDNRB	-0.0025	0.99342
216867_s_at	PDGFA	-0.0026	0.98067
200621_at	CSRP1	-0.0031	0.98566
228849_at	NTRK3	-0.0031	0.97555
211340_s_at	MCAM	-0.004	0.98542
218224_at	PNMA1	-0.0047	0.97821
209032_s_at	CADM1	-0.0048	0.98534
231183_s_at	JAG1	-0.0054	0.98466
220301_at	CCDC102B	-0.006	0.97625
209481_at	SNRK	-0.0103	0.93739
239230_at	HES5	-0.0115	0.77097
1554008_at	OSMR	-0.0122	0.961
223126_s_at	C1orf21	-0.0124	0.95308
226438_at	MTBP	-0.0131	0.95429
225567_at	NA	-0.0135	0.95316
206090_s_at	DISC1	-0.0145	0.94339

Unique probe ID	Gene Name	Fc#	p-value
217377_x_at	NTRK3	-0.0147	0.83542
206826_at	PMP2	-0.017	0.6404
233882_s_at	SEMA6D	-0.0171	0.95976
203410_at	AP3M2	-0.0172	0.90372
203710_at	ITPR1	-0.0177	0.93013
223610_at	SEMA5B	-0.0184	0.93444
206252_s_at	AVPR1A	-0.02	0.66015
206209_s_at	CA4	-0.0209	0.9259
225243_s_at	SLMAP	-0.0214	0.88925
226673_at	SH2D3C	-0.0216	0.85708
205610_at	MYOM1	-0.0223	0.88649
206462_s_at	NTRK3	-0.0229	0.68271
216887_s_at	LDB3	-0.0237	0.61209
227204_at	PARD6G	-0.0242	0.89922
217997_at	PHLDA1	-0.0245	0.92991
225757_s_at	CLMN	-0.0246	0.88064
242670_at	LGI4	-0.0247	0.73809
224301_x_at	H2AFJ	-0.025	0.92474
236281_x_at	HTR7	-0.0258	0.57279
226231_at	PAWR	-0.026	0.85872
234563_at	PDE3A	-0.027	0.93516
212350_at	TBC1D1	-0.0277	0.83308
201604_s_at	PPP1R12A	-0.0277	0.85264
205577_at	PYGM	-0.0279	0.84833
209695_at	PTP4A3	-0.028	0.88334
225168_at	FRMD4A	-0.0285	0.90178
1568760_at	MYH11	-0.0289	0.73513
222870_s_at	B3GNT2	-0.0296	0.89339
217287_s_at	TRPC6	-0.0299	0.70644
213371_at	LDB3	-0.0306	0.66669
209842_at	SOX10	-0.0307	0.47657
205967_at	HIST1H4C	-0.0307	0.88583
212323_s_at	VPS13D	-0.0313	0.82068
226911_at	EGFLAM	-0.0327	0.87868
236121_at	OR51E2	-0.0339	0.48088
225699_at	SNORA9	-0.0376	0.80521
226364_at	HIP1	-0.0407	0.87205
220987_s_at	NUAK2	-0.0427	0.8446
219728_at	MYOT	-0.0437	0.39194
225245_x_at	H2AFJ	-0.045	0.8659
206208_at	CA4	-0.0457	0.76048
216268_s_at	JAG1	-0.0469	0.8712
1553614_a_at	FLJ25694	-0.0474	0.54975
206927_s_at	GUCY1A2	-0.0482	0.75018

Unique probe ID	Gene Name	Fc#	p-value
225009_at	CMTM4	-0.05	0.82593
207066_at	HRC	-0.0523	0.49818
228724_at	NA	-0.0523	0.62412
229615_at	NA	-0.0523	0.75441
241612_at	FOXD3	-0.0547	0.30428
230665_at	NA	-0.0571	0.75827
228434_at	BTNL9	-0.0583	0.85701
206414_s_at	DDEF2	-0.0586	0.77237
225589_at	SH3RF1	-0.0609	0.81244
229952_at	SPTB	-0.0622	0.76316
218051_s_at	NT5DC2	-0.0627	0.7989
226690_at	ADCYAP1R1	-0.0629	0.84061
230964_at	FREM2	-0.0633	0.83985
209948_at	KCNMB1	-0.0637	0.57575
216331_at	ITGA7	-0.0641	0.79419
209914_s_at	NRXN1	-0.0649	0.45169
207695_s_at	IGSF1	-0.0649	0.70309
203143_s_at	KIAA0040	-0.0663	0.76021
225320_at	CCDC109A	-0.0667	0.60913
207692_s_at	ACAN	-0.0682	0.26789
229768_at	OR51E1	-0.0724	0.44462
229781_at	CAMTA1	-0.0724	0.29911
218076_s_at	ARHGAP17	-0.0741	0.5259
240402_at	KIRREL3	-0.0744	0.43735
237719_x_at	RGS7BP	-0.0766	0.15823
220460_at	SLCO1C1	-0.0767	0.68915
228570_at	BTBD11	-0.0787	0.7955
218864_at	TNS1	-0.0791	0.62693
206858_s_at	HOXC6	-0.0802	0.85964
232406_at	JAG1	-0.0823	0.69901
212864_at	CDS2	-0.0841	0.61558
205207_at	IL6	-0.0859	0.7173
238613_at	ZAK	-0.0865	0.68837
228133_s_at	NDE1	-0.091	0.24818
219543_at	PBLD	-0.0917	0.64902
228547_at	NRXN1	-0.0919	0.40557
235183_at	NA	-0.0922	0.82298
204032_at	BCAR3	-0.0927	0.60538
232305_at	HMGCLL1	-0.0944	0.38928
231337_at	DKFZp686O1327	-0.0946	0.48941
205081_at	CRIP1	-0.0955	0.82437
243584_at	PRSS23	-0.0986	0.81008
231936_at	HOXC9	-0.1002	0.66857
1554299_at	NPAS4	-0.1019	0.03042

Unique probe ID	Gene Name	Fc#	p-value
212978_at	LRRC8B	-0.1022	0.52889
227224_at	RALGPS2	-0.1056	0.59605
238763_at	RBM20	-0.1057	0.45723
203817_at	GUCY1B3	-0.1079	0.71758
231800_s_at	DMRT3	-0.1109	0.2223
201365_at	OAZ2	-0.1116	0.27651
214479_at	GFRA3	-0.1133	0.1036
209292_at	ID4	-0.1137	0.84001
204005_s_at	PAWR	-0.1158	0.51204
221031_s_at	APOLD1	-0.1173	0.63875
236373_at	NA	-0.1192	0.53546
1555812_a_at	ARHGDIB	-0.1207	0.67687
226492_at	SEMA6D	-0.1211	0.7734
214319_at	FRY	-0.1217	0.49054
214168_s_at	TJP1	-0.123	0.63191
203006_at	INPP5A	-0.123	0.45116
205110_s_at	FGF13	-0.1231	0.70624
227167_s_at	RASSF3	-0.1255	0.59513
209826_at	PPT2	-0.1256	0.46735
217996_at	PHLDA1	-0.1266	0.73222
209198_s_at	SYT11	-0.1279	0.64626
218484_at	NDUFA4L2	-0.1289	0.70421
209168_at	GPM6B	-0.1294	0.61458
201674_s_at	AKAP1	-0.1332	0.39022
204529_s_at	TOX	-0.1338	0.72656
235004_at	RBM24	-0.1346	0.65967
218081_at	C20orf27	-0.1375	0.33302
209195_s_at	ADCY6	-0.1376	0.26117
235275_at	BMP8B	-0.1409	0.38404
218833_at	ZAK	-0.1483	0.17833
204028_s_at	RABGAP1	-0.1485	0.2675
239262_at	PRSS23	-0.151	0.6875
201952_at	ALCAM	-0.1542	0.63363
239921_at	COL28A1	-0.1589	0.04866
225755_at	KLHDC8B	-0.1638	0.25788
213248_at	LOC730101	-0.1655	0.46824
241951_at	NA	-0.1662	0.4392
228802_at	RBPM5	-0.1663	0.27449
202080_s_at	TRAK1	-0.1671	0.14502
201675_at	AKAP1	-0.1677	0.33188
202761_s_at	SYNE2	-0.1679	0.45452
227396_at	PTPRJ	-0.1687	0.42741
202893_at	UNC13B	-0.169	0.29497
213721_at	SOX2	-0.1706	0.19262

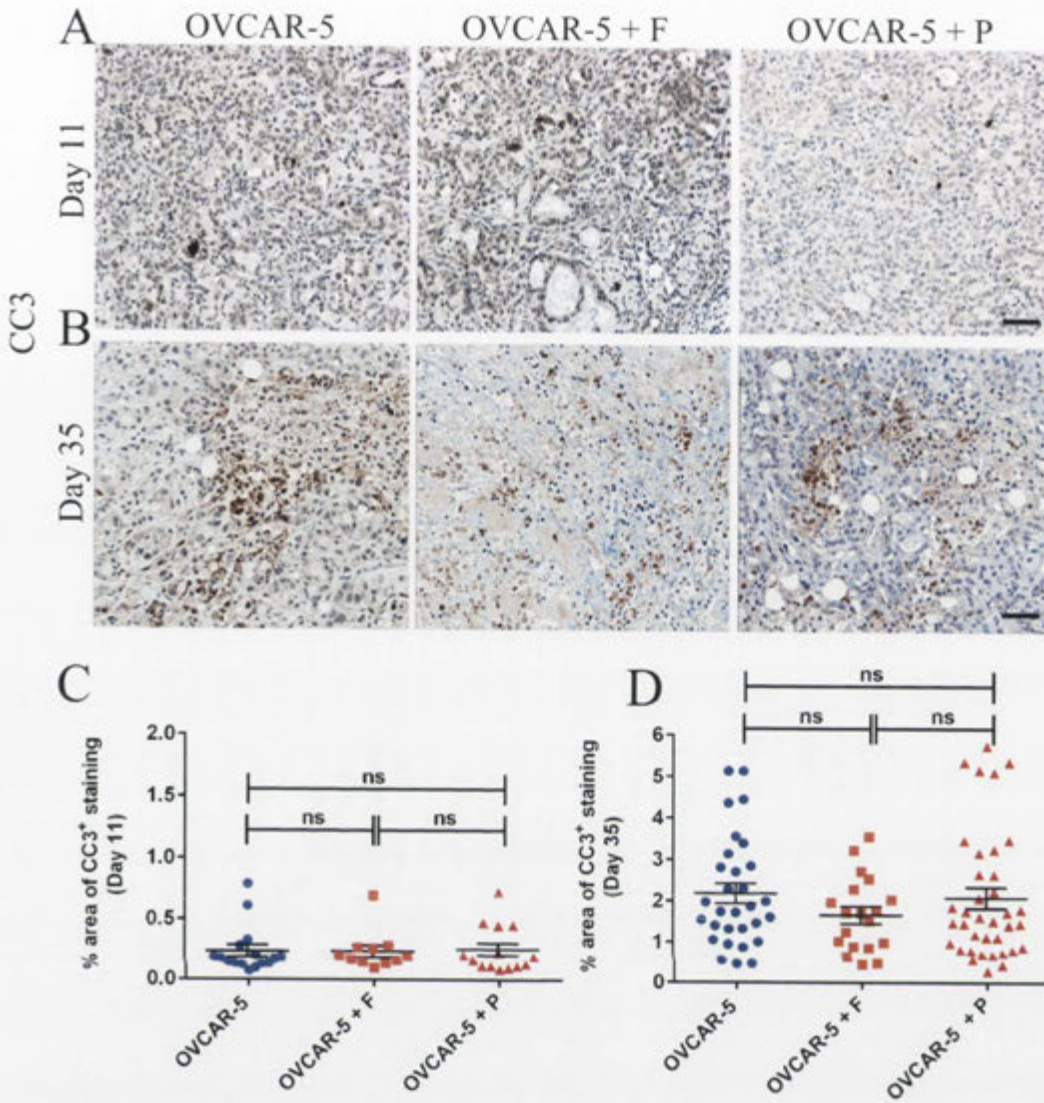
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224818_at	SORT1	-0.1731	0.41474
222486_s_at	ADAMTS1	-0.1735	0.55509
206078_at	KALRN	-0.1746	0.21694
214044_at	RYS2	-0.1759	0.1396
219882_at	TTLL7	-0.176	0.37636
205483_s_at	ISG15	-0.1802	0.6488
211026_s_at	MGLL	-0.1842	0.61061
213839_at	KIAA0500	-0.1848	0.52807
206655_s_at	GP1BB	-0.1856	0.09578
208820_at	PTK2	-0.187	0.26407
1555797_a_at	ARPC5	-0.1884	0.16433
225516_at	SLC7A2	-0.1888	0.59643
206191_at	ENTPD3	-0.1901	0.47327
213093_at	PRKCA	-0.1908	0.56437
206574_s_at	PTP4A3	-0.1914	0.43285
226446_at	HES6	-0.2008	0.24264
204022_at	WWP2	-0.2012	0.12234
213720_s_at	SMARCA4	-0.2069	0.20617
201951_at	ALCAM	-0.2093	0.49541
204714_s_at	F5	-0.2107	0.19692
212666_at	SMURF1	-0.2122	0.18555
200950_at	ARPC1A	-0.2133	0.15988
232676_x_at	MYEF2	-0.2153	0.30575
215785_s_at	CYFIP2	-0.2171	0.41709
222757_s_at	ZAK	-0.2175	0.29755
225260_s_at	MRPL32	-0.2189	0.05989
224634_at	GPATCH4	-0.2203	0.10332
213313_at	RABGAP1	-0.2235	0.13509
201596_x_at	KRT18	-0.2282	0.30437
205229_s_at	COCH	-0.23	0.64694
209663_s_at	ITGA7	-0.2309	0.37
229339_at	NA	-0.2312	0.24172
242794_at	MAML3	-0.2336	0.324
236291_at	RDH5	-0.2353	0.09577
218942_at	PIP4K2C	-0.2417	0.09763
205572_at	ANGPT2	-0.2456	0.42289
1565951_s_at	CHML	-0.248	0.32308
237261_at	NA	-0.2496	0.15664
214578_s_at	ROCK1	-0.2528	0.07144
230087_at	PRIMA1	-0.2566	0.34048
1555216_a_at	LOC645722	-0.2586	0.37698
205659_at	HDAC9	-0.2596	0.35325
204469_at	PTPRZ1	-0.2619	0.17988
209098_s_at	JAG1	-0.2625	0.46944

Unique probe ID	Gene Name	Fe#	p-value
213610_s_at	KLHL23	-0.263	0.32197
209803_s_at	PHLDA2	-0.2641	0.56008
226350_at	CHML	-0.2662	0.30303
206128_at	ADRA2C	-0.2702	0.1752
230720_at	RNF182	-0.2709	0.57116
212793_at	DAAM2	-0.2766	0.2935
230053_at	NA	-0.2799	0.18726
207821_s_at	PTK2	-0.2824	0.0785
207169_x_at	DDR1	-0.2824	0.09181
215143_at	DPY19L2P2	-0.2863	0.40833
235173_at	MBNL1	-0.2891	0.02625
205449_at	SAC3D1	-0.2949	0.1603
226403_at	TMC4	-0.295	0.45384
201364_s_at	OAZ2	-0.295	0.06576
212817_at	DNAJB5	-0.2975	0.06767
219478_at	WFDC1	-0.2978	0.10901
206395_at	DGKG	-0.3004	0.07272
219412_at	RAB38	-0.3028	0.31205
227006_at	PPP1R14A	-0.3033	0.30017
210625_s_at	AKAP1	-0.3097	0.10112
222240_s_at	ISYNA1	-0.3097	0.25563
823_at	CX3CL1	-0.3165	0.21328
219327_s_at	GPRC5C	-0.3182	0.23239
226013_at	TRAK1	-0.3193	0.09994
232181_at	LOC153346	-0.324	0.12957
202079_s_at	TRAK1	-0.3241	0.0347
212807_s_at	SORT1	-0.3253	0.22611
208978_at	CRIP2	-0.3286	0.21593
203238_s_at	NOTCH3	-0.3321	0.29701
214038_at	CCL8	-0.3372	0.44729
235843_at	DGKG	-0.3385	0.15897
229893_at	FRMD3	-0.3462	0.17057
225759_x_at	CLMN	-0.3491	0.10167
211673_s_at	MOCS1	-0.3499	0.02632
211555_s_at	GUCY1B3	-0.3501	0.18038
212573_at	ENDOD1	-0.3508	0.12673
222889_at	DCLRE1B	-0.3511	0.03633
211148_s_at	ANGPT2	-0.3589	0.19532
228375_at	IGSF11	-0.3632	0.11461
221042_s_at	CLMN	-0.3675	0.25985
203680_at	PRKAR2B	-0.3745	0.33413
204904_at	GJA4	-0.3746	0.08672
221489_s_at	SPRY4	-0.3773	0.16969
237206_at	MYOCD	-0.3798	0.28489

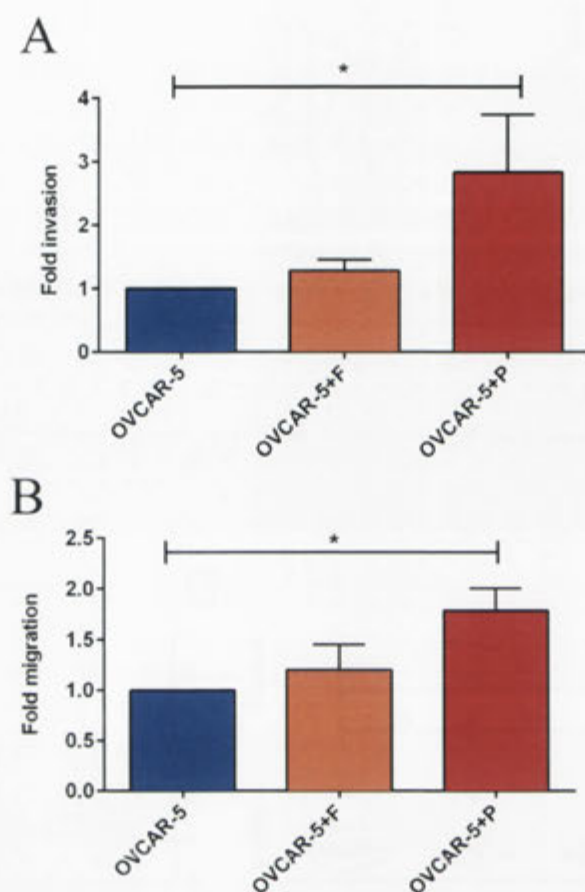
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232298_at	hCG_1806964	-0.3924	0.01513
209291_at	ID4	-0.3962	0.38746
222735_at	TMEM38B	-0.4011	0.08544
227443_at	C9orf150	-0.4052	0.08699
230645_at	FRMD3	-0.4066	0.2116
218719_s_at	GINS3	-0.4159	0.01525
201761_at	MTHFD2	-0.4193	0.04629
214510_at	GPR20	-0.4221	0.04414
210106_at	RDH5	-0.4267	0.04809
234725_s_at	SEMA4B	-0.4277	0.02116
226590_at	ZNF618	-0.4286	0.01359
227347_x_at	HES4	-0.4394	0.04593
226933_s_at	ID4	-0.4432	0.26568
218772_x_at	TMEM38B	-0.4523	0.03914
40687_at	GJA4	-0.458	0.0569
241682_at	KLHL23	-0.4638	0.16263
210983_s_at	MCM7	-0.4679	0.03626
209513_s_at	HSDL2	-0.4747	0.06337
202495_at	TBCC	-0.4772	0.00197
225355_at	LOC54492	-0.4812	0.05192
226499_at	NRARP	-0.4845	0.00679
227702_at	CYP4X1	-0.4974	0.22937
1554020_at	BICD1	-0.4981	0.15534
209293_x_at	ID4	-0.5004	0.21548
202454_s_at	ERBB3	-0.5056	0.22006
227372_s_at	BAIAP2L1	-0.5358	0.04164
200657_at	SLC25A5	-0.5426	0.00644
226213_at	ERBB3	-0.5621	0.18731
205632_s_at	PIP5K1B	-0.5732	0.06804
213181_s_at	MOCS1	-0.5764	0.00144
205088_at	MAMLD1	-0.5797	0.01231
229242_at	NA	-0.5806	0.06658
204510_at	CDC7	-0.5889	0.00716
206453_s_at	NDRG2	-0.6261	0.04482
212909_at	LYPD1	-0.6438	0.24946
209512_at	HSDL2	-0.6589	0.01546
202613_at	CTPS	-0.6719	0.00316
213547_at	CAND2	-0.6758	0.00117
214433_s_at	SELENBP1	-0.7723	0.02212
203126_at	IMPA2	-0.7918	0.00069
202870_s_at	CDC20	-0.8214	0.0204
221245_s_at	FZD5	-0.8311	0.04977
229551_x_at	ZNF367	-1.0658	0.00071

Unique probe ID	Gene Name	Fc#	p-value
202350_s_at	MATN2	-1.0931	0.00204
224428_s_at	CDCA7	-1.1655	0.00581
241803_s_at	TTC7B	-1.3017	0.00065

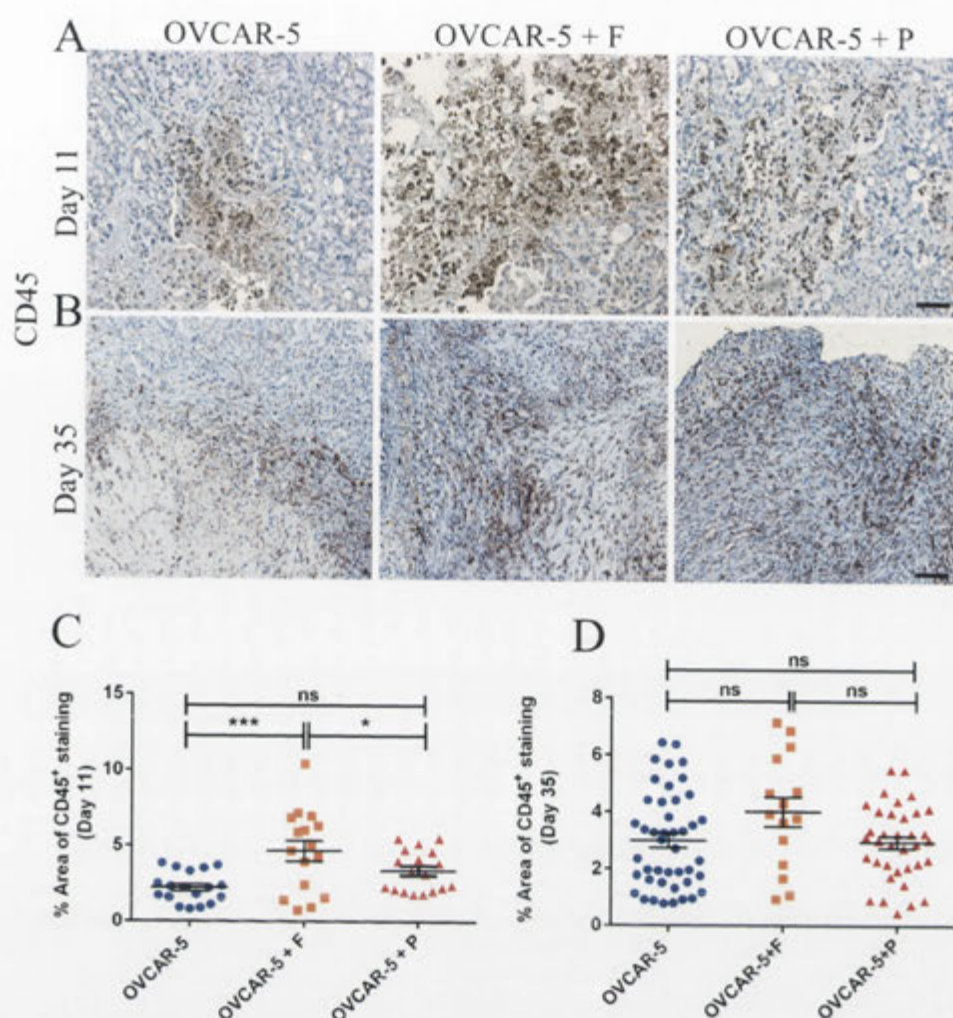
Appendix B
Previous OVCAR-5 Data



Appendix B, Figure 1. Co-injection of fibroblasts and pericytes does not alter the level of apoptosis in the OVCAR-5 xenograft tumours. (A-B) Immunohistochemical staining with an antibody against the apoptosis marker cleaved caspase 3 (CC3) on tumour sections derived from mice injected with OVCAR-5 cells alone or together with fibroblasts or pericytes at (A) Day 11 and (B) Day 35. (C-D) Morphometric quantification of CC3 stained area both at (C) Day 11 and (D) Day 35 showing no significant differences in the extent of apoptosis. Images and staining data for Day 11 are representative of 2-3 random fields of 3 tumours from each experimental group from a total of 3 independent experiments; Similarly, Day 35 data are representative of 2-3 random sections from 10 tumours from OVCAR-5 and OVCAR-5+P groups, and 6 tumours from OVCAR-5+F group respectively, from 3 independent experiments. Haematoxylin was used as a counterstain. Error bars represent mean $\pm$ SEM. Statistical analysis performed using one-way ANOVA; ns=not significant. (Data: Lynn Chong Ph.D. thesis, 2013)



Appendix B, Figure 2. Pericytes significantly increases the invasive and migratory capacity of OVCAR-5 cells *in vitro*. (A-B) Quantification of transwell migration; and (A) transwell invasion through Matrigel™ and (B) transwell migration of OVCAR-5 cells towards pericytes or fibroblasts, normalised to OVCAR-5 alone control. Data represents mean+SD from 3 independent experiments each performed in triplicate. Statistical analysis was performed using one-way ANOVA; * $p < 0.05$. (Data: Lynn Chong Ph.D. thesis, 2013)



Appendix B, Figure 3. Co-injection of fibroblasts increases the influx of CD45⁺ cells in OVCAR-5 xenograft tumours. (A-B) Immunohistochemical staining with an antibody against the haematopoietic cell marker CD45 on tumour sections derived from mice injected with OVCAR-5 cells alone or together with fibroblasts or pericytes at (A) Day 11 and (B) Day 35. (C-D) Morphometric quantification of CD45 stained area both at (C) Day 11 showing a significant increase in CD45 staining in the group injected with fibroblasts the effect of which decreases by (D) Day 35. Images and staining data for Day 11 are representative of 2-3 random fields of 3 tumours from each experimental group from a total of 3 independent experiments; similarly data for Day 35 is representative of 2-3 random sections from 10 tumours from OVCAR-5 and OVCAR-5+P groups, and 6 tumours from OVCAR-5+F group respectively, from a total of 3 independent experiments. Haematoxylin was used as a counterstain. Error bars represent mean±SEM. Statistical analysis performed using one-way ANOVA; *p<0.05, ***p<0.001, ns=not significant. (Data: Lynn Chong Ph.D. thesis, 2013)

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CHARLES THE FIRST

BY

JOHN BURNET

OF THE UNIVERSITY OF OXFORD

IN TWO VOLUMES

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Appendix C
AOCS metastasis data

Appendix C – Visceral metastasis data of serous ovarian cancer patients

AOCS ID	Pericyte Score [#]	PFS time	PFS event	TMA	Evidence of Visceral Metastasis?	SITE2	SITE3
20019	-1.59803	NA	0	V2	No		
34019	-2.0607	10.61918	1	V2	No		
23165	-1.848	16.60274	1	V5	No		
41010	-1.65156	NA	0	V5	No		
60024	-1.52452	25.74247	1	V4	No		
41264	-1.51111	8.778082	1	V4	No		
32103	-1.48267	18.83836	1	V4	No		
70049	-1.47607	27.64932	1	V2	Yes	brain	
44286	-1.46734	7.331507	1	V4	Yes	liver	spleen
70054	-1.37316	11.0137	1	V2	Yes	lung	
34078	-1.30191	31.52877	0	V2	Yes	liver	
41325	-1.2744	21.36986	0	V5	No		
23102	-1.11282	32.54795	1	V4	No		
60065	-1.06897	17.45753	1	V5	No		
41203	-1.04889	NA	0	V5	No		
34125	-1.02345	29.81918	0	V2	Yes	liver	spleen
44087	-1.01633	25.9863	1	V5	Yes	Not reported	Not reported
23143	-0.96653	23.86849	1	V5	Yes	liver	mediastinal nodes
34186	-0.95904	20.97534	0	V4	No		
44110	-0.9502	NA	0	V5	No		
34102	-0.92194	9.665753	1	V2	No		
32032	-0.88466	NA	0	V4	No		
60135	-0.88409	11.30959	1	V5	Yes	liver	brain
23204	-0.84617	9.534247	0	V5	No		
23106	-0.83068	10.68493	0	V5	No		
70118	-0.78331	11.47397	1	V2	No		
60318	-0.7819	5.720548	0	V5	Yes	liver	lung
60175	-0.73412	28.40548	0	V2	No		
41165	-0.73349	23.40822	1	V4	No		
32034	-0.69208	10.5863	0	V5	No		
70148	-0.68772	22.12603	0	V2	No		
70005	-0.68123	15.23562	1	V4	No		
32022	-0.6605	32.05479	1	V4	No		
51125	-0.63952	8.021918	1	V5	Yes	pancreas	liver
70103	-0.63764	10.55342	1	V2	Yes	lung	

AOCS ID	Pericyte Score [#]	PFS time	PFS event	TMA	Evidence of Visceral Metastasis?	SITE2	SITE3
23182	-0.60682	18.27945	0	V5	Yes	liver	
60174	-0.58188	11.14521	1	V4	No		
32055	-0.5812	7.594521	1	V4	No		
44090	-0.5003	19.33151	1	V5	No		
51062	-0.45601	15.09041	0	V4	No		
60102	-0.43472	NA	1	V5	Yes	lung	
60258	-0.41905	11.50685	0	V4	Yes	liver	
23187	-0.36469	23.17808	0	V4	Yes	liver	spleen
44303	-0.36335	11.27671	1	V4	No		
51171	-0.35535	17.29315	0	V2	No		
34117	-0.31939	11.67123	1	V2	Yes	Liver	
27098	-0.28327	22.52055	1	V5	Yes	chest	
60169	-0.23583	33.30411	0	V2	No		
60107	-0.22646	NA	0	V5	No		
60049	-0.1894	8.120548	0	V5	No		
70039	-0.18874	18.14795	1	V2	Yes	liver	
44377	-0.18039	20.02192	1	V5	Yes	lung	spleen
41307	-0.17305	NA	0	V4	No		
41358	-0.14349	10.61918	1	V5	No		
70125	-0.129	18.11507	1	V2	No		
23128	-0.06128	13.28219	1	V5	No		
23197	-0.02479	7.791781	0	V5	Yes	lung	
70107	0.160395	13.34795	1	V2	Yes	liver	
60048	0.181311	16.33973	1	V2	No		
22013	0.201247	46.68493	0	V4	No		
34168	0.204973	5.60274	1	V2	No	para-tracheal nodes	
70100	0.217025	12.69041	1	V2	Yes	lung	
44080	0.245094	6.476712	1	V4	No		
23221	0.267528	5.208219	1	V5	No		
34103	0.294269	15.99178	1	V2	Yes	liver	
70077	0.350987	7.364384	1	V2	No		
23172	0.434376	8.153425	1	V4	No		
60188	0.484202	10.55342	1	V5	No		
44341	0.519387	5.142466	1	V5	No		
44349	0.539971	6.641096	1	V4	No		
44285	0.555958	11.17808	1	V5	No		
34066	0.661274	13.57808	1	V2	No		
32013	0.66384	2.235616	1	V4	No		
70119	0.76773	9.994521	1	V2	Yes	liver	spleen

AOCS ID	Pericyte Score [#]	PFS time	PFS event	TMA	Evidence of Visceral Metastasis?	SITE2	SITE3
60091	0.809888	8.843836	0	V5	No		
41310	0.833226	13.34795	1	V5	No		
60066	0.853108	29.4170	1	V4	Yes	liver	
44234	0.862476	9.830137	1	V5	No		
51086	0.90948	7.2	1	V4	No		
44366	0.913723	12.95342	1	V5	No		
60067	0.935274	15.55068	1	V5	No		
22020	0.951363	9.073973	1	V4	No		
60020	1.040669	NA	1	V4	No		
44288	1.076837	8.515068	1	V5	No		
70124	1.077369	13.61096	1	V2	No		
23212	1.111133	3.057534	0	V5	No		
22012	1.125241	26.9589	1	V4	Yes	Liver	Spleen
41300	1.151316	6.476712	1	V4	Yes	liver	
41170	1.220135	9.6	1	V4	Yes	liver	
34202	1.386035	11.0137	1	V2	No		
22047	1.482249	9.30411	1	V5	Yes	Liver	
34801	1.541383	NA	0	V5	Yes	liver	
41192	1.64607	28.50411	1	V5	No		
23170	1.670849	12	0	V4	Yes	liver	
32098	2.403248	0.920548	1	V4	No		
70115	3.09692	16.53699	1	V5	No		

[#]A score >0 denotes increased expression, while a score <0 denotes decreased expression of pericyte genes from the VLA-1^{hi} signature

Appendix D
**GSEA data on pathways enriched in
patients with early recurrence of
disease**

Appendix B

GSEA data on pathways enriched in patients with early recurrence of disease

Appendix D, Table 1: List of top 20 GO (biological processes) pathways enriched in the GSEA of early versus late relapse patients.

GO biological process	Size	NES ^a	FDR ^b	Core Genes	Fraction	Top10 genes in core
actin_filament_based_process	114	1.93	0.116	45	39%	PRKG1, CXCL12, WASF3, WIPF1, ADRA2A, CCDC88A, NEBL, DOCK2, MRAS, GST
actin_cytoskeleton_organization_and_biogenesis	104	1.91	0.089	41	39%	PRKG1, CXCL12, WASF3, WIPF1, ADRA2A, CCDC88A, NEBL, DOCK2, MRAS, GST
regulation_of_cytoskeleton_organization_and_biogenesis	30	1.83	0.176	14	47%	NEXN, CXCL12, CCDC88A, NEBL, LIMA1, CDC42EP5, APC, RASAI, CAPG, TSC1
cell_matrix_adhesion	38	1.82	0.151	22	58%	ECM2, COL13A1, ITGA2, CDK6, ITGA11, PKD2, THY1, FBLN5, PTEN, ACVRL1
cell_substrate_adhesion	39	1.82	0.121	22	56%	ECM2, COL13A1, ITGA2, CDK6, ITGA11, PKD2, THY1, FBLN5, PTEN, ACVRL1
actin_polymerization_and_or_dimerization	23	1.80	0.128	13	57%	CXCL12, WASF3, WIPF1, LIMA1, WASF1, CDC42EP5, RASAI, CAPG, EVL, ANG
skeletal_development	102	1.80	0.118	36	35%	COL11A1, INHBA, IGF1, POSTN, FBN1, COL10A1, AEBP1, GHR, COL13A1, COL12A1
g_protein_signaling_coupled_to_gq_protein_activation	63	1.78	0.132	12	19%	EDNRA, PTGER4, NPY1R, ADRA2A, ADORA3, EDNRB, GABBR1, FPR1, RGS1, NF1
actin_filament_organization	24	1.77	0.126	10	42%	LIMA1, FSCN1, KPTN, RHOF, TSC1, SORBS1, EVL, ARHGEF10L, SORBS3, DLG1

GO biological process	Size	NES ^a	FDR ^b	Core Genes	Fraction	Top10 genes in core
camp_mediated_signaling	64	1.77	0.120	12	19%	EDNRA, PTGER4, NPY1R, ADRA2A, ADORA3, EDNRB, GABBR1, FPR1, RGS1, NFI
endosome_transport	23	1.76	0.116	13	57%	DOPEY1, ZFYVE16, LYST, ABCA1, TOM1, ANKRD27, RHOB, EEA1, ADRB2, VTIIA
regulation_of_organelle_organization_and_biogenesis	40	1.76	0.107	22	55%	NEXN, CXCL12, PPARGC1A, CCDC88A, NEBL, LIMA1, CDC42EP5, APC, RASA1, CAPG
axon_guidance	22	1.76	0.102	9	41%	CNTN4, KALI, SLIT2, FEZ1, SPON2, UNC5C, NRP1, NRP2, NRXN3
cell_migration	94	1.75	0.103	27	29%	SCG2, NEXN, VEGFC, CNTN4, KALI, SLIT2, ENPEP, SPON2, TGFB2, LAMB1
regulation_of_cell_migration	27	1.73	0.120	12	44%	NEXN, LAMB1, EGFR, THY1, PTEN, SPHK1, ACVRL1, NF1, CLIC4, ANGPTL3
negative_regulation_of_cell_adhesion	18	1.72	0.135	8	44%	TGFB1, PR47, PTEN, ACVRL1, RASA1, ARHGDIB, ADAM10, CDH13
regulation_of_cell_differentiation	58	1.68	0.191	18	31%	INHBA, DTX1, RUNX1, CDK6, TGFB2, MAFB, BMP4, NPHP3, ACVR2A, TWIST2
negative_regulation_of_cell_differentiation	28	1.67	0.191	11	39%	INHBA, CNTN4, CDK6, MAFB, BMP4, NPHP3, TWIST2, NF1, TBX3, NOTCH1
wound_healing	53	1.67	0.185	16	30%	CD36, ITGA2, SERPINE1, F13A1, TFPI, TGFB2, PR47, ENTPD1, PROS1, ACVRL1
positive_regulation_of_cell_differentiation	23	1.65	0.207	9	39%	INHBA, RUNX1, TGFB2, BMP4, ACVR2A, IGFBP3, PPARG, ETS1, FOXO3

^aNES=normalised enrichment score
^bFDR=false discovery rate

Appendix D, Table 2: List of Top10 enriched GO terms (cellular component) enriched in the early versus late relapse patients.

GO:ID CC	Term	Annotated [#]	Significant [†]	Expected [‡]	p-value (BH) [§]	Top10 genes [§]
GO:0031012	extracellular matrix	284	26	1.36	5.70E-28	SFRP2, EPYC, COL11A1, POSTN, COL8A1, TIMP3, ASPN, CCDC80, COL5A2, LUM
GO:0044421	extracellular region	745	30	3.56	5.10E-22	SFRP2, EPYC, COL11A1, POSTN, COL8A1, INHBA, NTM, TIMP3, ASPN, GREM1
GO:0005578	proteinaceous extracellular matrix	237	21	1.13	5.40E-22	EPYC, COL11A1, POSTN, COL8A1, TIMP3, ASPN, CCDC80, COL5A2, LUM, COL5A1
GO:0005576	extracellular region	1404	36	6.7	8.10E-21	SFRP2, EPYC, COL11A1, POSTN, COL8A1, INHBA, THBS2, NTM, TIMP3, ASPN
GO:0005581	collagen	32	10	0.15	1.50E-16	COL11A1, COL8A1, COL5A2, LUM, COL5A1, LOX, COL10A1, DCN, COL12A1, COL3A1
GO:0044420	extracellular matrix part	99	13	0.47	6.10E-16	COL11A1, COL8A1, TIMP3, CCDC80, COL5A2, LUM, COL5A1, LOX, FBN1, COL10A1
GO:0005583	fibrillar collagen	10	5	0.05	5.00E-10	COL11A1, COL5A2, LUM, COL5A1, COL3A1
GO:0005615	extracellular space	586	17	2.8	7.90E-10	SFRP2, INHBA, NTM, GREM1, CTSK, LUM, IGF1, VCAN, LOX, FBN1
GO:0005604	basement membrane	67	5	0.32	1.60E-05	COL8A1, TIMP3, CCDC80, COL5A1, FBN1
GO:0005588	collagen type V	3	2	0.01	6.70E-05	COL5A2, COL5A1

[#] Annotated= number of genes on chip with specific GO term;[†] Significant= number of overexpressed genes with specific GO term;[‡] Expected= annotated/ total number of genes on chip, expected value if randomly distributed;[§] p-val(BH)= p-value corrected according to Benjamini-Hochberg (Benjamini, Yoav; Hochberg, 1995)[§] Top10 genes= gene symbols of overexpressed genes with specific GO annotation in the order of declining fold-change

Appendix D, Table 3: List of Top10 enriched GO terms (molecular function) enriched in the early versus late relapse patients.

GO.ID MF	Term	Annotated [#]	Significant [~]	Expected [^]	p-value (BH) [*]	Top10 genes ^{\$}
GO:0097367	carbohydrate derivative binding	128	11	0.62	1.80E-11	EPYC, POSTN, THBS2, CCDC80, COL5A1, VCAN, FBN1, CRISPLD2, DCN, THBS1
GO:0005539	glycosaminoglycan binding	115	10	0.56	1.40E-10	EPYC, POSTN, THBS2, CCDC80, COL5A1, VCAN, CRISPLD2, DCN, THBS1, ECM2
GO:0005201	extracellular matrix structural constituent	65	7	0.32	2.30E-08	COL11A1, COL5A2, LUM, COL5A1, FBN1, COL12A1, COL3A1
GO:0008201	heparin binding	87	7	0.42	1.80E-07	POSTN, THBS2, CCDC80, COL5A1, CRISPLD2, THBS1, ECM2
GO:1901681	sulphur compound binding	120	7	0.58	1.60E-06	POSTN, THBS2, CCDC80, COL5A1, CRISPLD2, THBS1, ECM2
GO:0001968	fibronectin binding	9	3	0.04	8.80E-06	SFRP2, CCDC80, THBS1
GO:0005178	integrin binding	60	5	0.29	9.80E-06	SFRP2, COL5A1, THBS1, ECM2, COL3A1
GO:0005518	collagen binding	30	4	0.15	1.20E-05	LUM, DCN, THBS1, ECM2
GO:0019838	growth factor binding	75	5	0.36	2.90E-05	COL5A1, THBS1, COL3A1, WISP1, CD36
GO:0070052	collagen V binding	3	2	0.01	6.90E-05	THBS1, ECM2

[#]Annotated= number of genes on chip with specific GO term;

[~]Significant= number of overexpressed genes with specific GO term;

[^]Expected= annotated/ total number of genes on chip, expected value if randomly distributed;

^{*}p-val (BH)= p-value corrected according to Benjamini-Hochberg (Benjamini, Yoav; Hochberg, 1995)

^{\$}Top10 genes= gene symbols of overexpressed genes with specific GO annotation in the order of declining fold-change

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