Implications of MEK and VEGFR2 Inhibition on Melanoma CXCR4-CXCL12 Chemotaxis, Survival and Tumour Progression

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Abstract:

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Abstract

Both uveal and cutaneous metastatic melanoma remain incurable, largely due to the deregulation of signalling networks that facilitate drug resistance and tumour dissemination for which CXCR4-CXCL12 chemotaxis, MAPK signalling and angiogenesis play key roles. While hyper-activating mutations in MAPK signalling promote tumour survival/drug resistance, CXCR4-CXCL12 chemotaxis and angiogenesis, promote melanoma migration. Known individually to drive melanoma escape from the localised microenvironment, how these pathways interact remains largely undefined. The principle aim of the present study was thus to define the role and crosstalk of CXCR4-CXCL12, MAPK and VEGFR2 signalling in uveal and cutaneous melanoma, to inform on more efficacious targeted approaches to prevent metastasis.

Results revealed increased CXCR4 expression as a putative prognostic biomarker for AJCC stage II cutaneous melanomas, while CXCL12 expression within the epidermis likely prevents metastasis. Additionally, CXCL12 secretion by melanomas was associated with autocrine CXCR4-CXCL12 signalling and MAPK activation. VEGFR2 was expressed by primary melanomas and associated with cutaneous metastasis to lymph nodes, with VEGF treatment promoting melanoma cell migration. Analysis of CXCR4-CXCL12-mediated chemotaxis demonstrated enhanced migration of melanoma cells towards CXCL12, either recombinant of derived from primary dermal fibroblast supernatants, the effect of which was inhibited by both the MEK inhibitor, trametinib, and the VEGFR2 inhibitor, pazopanib. Interestingly, the concurrent increase in autophagy observed in response to trametinib, prevented by co-treatment of uveal/cutaneous melanoma cells with chloroquine, suggest the efficacy of MEK inhibition may be potentiated with dual inhibition of pro-survival autophagy.

Given that both CXCR4-CXCL12 and VEGF-VEGFR2 signalling activate MAPK pathway, and alone promote melanoma migration. Results demonstrated combined trametinib and pazopanib, potentiated both inhibition of uveal and cutaneous melanoma cell viability as well as CXCR4-CXCL12-mediated migration. Collectively these studies confirm the intimate relationship between CXCR4-CXCL12/VEGFR2/MAPK signalling, highlighting potential novel combinational approaches through which to prevent melanoma survival and migration.

Dedication

This work is dedicated to Bryan McConnell

My Dad who taught me importance of education, and that you can achieve anything in life if you put your mind to it, and work hard.

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Finally, Aaron, I finish this journey without you and it is with a heavy heart. I thank you for encouraging me to make the career change and supporting me to the best of your ability, I am so very grateful for everything you have done for me.

Declaration

This thesis is submitted for the degree of Doctor of Philosophy at Newcastle University. The research was performed in Dermatological Sciences in the Institute of Cellular Medicine under the main supervision of Professor Penny Lovat. This thesis is my own work unless otherwise stated within the text. I certify that none of the material offered in this thesis has been previously submitted by me for a degree or any other qualification at this, or any other University.

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Abbreviations

µl - microliter

µm – micrometre

µM – micro molar

˚C – Degrees Centigrade

1˚ - Primary

Akt – Ak Thymoma

AJCC - American Joint Committee on Cancer

ALK- Anaplastic lymphoma kinase

α-MSH - α-melanocyte stimulating hormone

Ambra 1 - Activating Molecule in Beclin-1 Regulated Autophagy Protein 1

AMO - Antisense Morpholino Oligonucleotides

ANOVA - Analysis of Variance

ARAF - V-Raf Murine Sarcoma Viral Oncogene Homolog A1

Atg - Autophagy Related Protein

BAP-1 - BRCA1 Associated Protein-1

BCG vaccine - Baculillus Calmette-Guerin Vaccine

BECN-1 - Beclin-1

BP - Base pair

BPE - Bovine Pituitary Extract

B-RAF - v-Raf Murine Sarcoma Viral Oncogene Homolog B1

BSA - Bovine Standard Albumin

cAMP - Cyclic adenosine monophosphate

CDK4 – Cyclin-Dependent kinase 4

CDKN2A - Cyclin-Dependent Kinase Inhibitor 2A

cDNA – Complementary DNA

CEACAM - Carcinoembryonic Antigen-Related Cell Adhesion Molecule

CHO - Chinese Hamster Ovary

cm - Centimetre

CNR1 - Cannabinoid Receptor Gene 1

CO_2 - Carbon dioxide

CRAF - v-Raf Murine Sarcoma Viral Oncogene Homolog C1

CREB - cAMP response element-binding protein

Ct – Cycle Threshold

CTLA-4 - Cytotoxic T Lymphocyte Associated Antigen-4

Ctrl - Control

CQ - Chloroquine

DAPI - 4',6-diamidino-2-phenylindole

DMEM - Dulbecco's Modified Eagle's Medium

DMSO - Dimethyl Supfoxide

DNA - Deoxyribonucleic Acid

dNTP - Deoxyribonucleotide triphosphate

EDTA - Ethylenediaminetetraacetic Acid

EIF1AX - Eukaryotic Translation Initiation Factor 1A

ELISA – Enzyme Linked Immunosorbent Assay

EMT - Epithelial to Mesenchymal Transition

ER – Endoplasmic Reticulum

ERK - Extracellular Signal-Regulated Kinase

EVMM - Extravascular Migratory Metastasis

FCS - Fetal Calf Serum

FDA - Food and Drug Administration

FFPE - Formalin-Fixed Paraffin-Embedded

FISH - Fluorescence *in situ* Hybridization

FOXP3 - Forkhead P3

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

GM-CSF - Granulocyte-Macrophage Colony-Stimulating Factor

GNA11 - Guanine Nucleotide-Binding Protein Subunit Alpha-11

GNAQ - Guanine Nucleotide-Binding Protein Subunit Alpha- Q

GTP - Guanosine Triphosphate

HCl - Hydrochloric Acid

HCQ – Hydroxychloroquine

HGF/SF - Hepatocyte Growth Factor-Scatter Factor

HIF-1α - Hypoxia-Inducible Factor 1-Alpha

HLP - Hyperthermic Isolated limb Perfusion

Hr - Hour

HRAS - v-Ha-ras Harvey Rat Sarcoma Viral Oncogene Homolog

HUVEC – Human Umbilical Vein Endothelial Cell

IB - Immunoblotting

IHC – Immunohistochemistry

IF – Immunofluorescence

ILP - Isolated limb Perfusion

INF- α - Interferon-Alpha

IL-2 – Interleukin-2

IGF - Insulin Growth Factor

IGF1R - Insulin-Like Growth Factor 1 (IGF-1) Receptor

kDa – Kilo Dalton

KDR - Kinase Insert Domain Receptor

Ki67- Antigen KI-67

KRAS - V-Ki-ras2 Kirsten Rat Sarcoma Viral Oncogene Homolog

LC3 - Microtubule-Associated Protein Light Chain 3

LDH - Lactate Dehydrogenase

LN – Lymph Node

MAPK - Mitogen-Activated Protein Kinase

MC1R - Melanocortin 1 Receptor Mcl-1 - Myeloid Cell Leukemia 1

MCAM - Melanoma Cell Adhesion Molecule

MCSF-1 - Macrophage Colony-Stimulating Factor

MEK - Mitogen Activated Protein Kinase Kinase

MET - MET proto-oncogene, receptor tyrosine kinase

MITF - Microphthalmia-Associated Transcription Factor

Min - Minutes

ml – Millilitre

MLPA - Multiple Ligation Probe Amplification

mM – Millimolar

MMP - Matrix Metalloproteinases

mRNA - Messenger Ribonucleic Acid

mTOR - Mammalian Target of Rapamycin

MTS – 3-(4,5-Dimethylthiazol-2-yl)-5-(3-Carboxymethoxyphenyl)-2-(4-Sulfophenyl)-2H-Tetrazolium

NaCl - Sodium Chloride

NF-κB - Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells

nM - Nanomolar

NRAS - Neuroblastoma Rat Sarcoma Viral Oncogene Homolog

NT – Non-targeting

OS - Overall Survival

P or Paz - Pazopanib

p53 - Protein 53

p62 - Nucleoporin p62

PAS - Pre-Autophagosome Structure

PBS - Phosphate Buffered Saline

PBS/T - Phosphate Buffered Saline and Tween

PCR - Polymerase Chain Reaction

PCD-1 - Programmed Cell Death Receptor-1

PDGF - Platelet-Derived Growth Factor

PDGFR - Platelet-Derived Growth Factor Receptor

PE – Phosphatidylethanolamine

PEDF - Pigment Epithelium-Derived Factor

PFS – Progression Free Survival

PI3K - Phosphatidylinositol 3-Kinase

PKA – Protein Kinase A

PLGF - Placental Like Growth Factor

PMA - Phorbol 12-myristate 13-acetate

POMC - Pro-Opiomelanocortin

P/S – Penicillin Streptomycin

PTEN - Phosphatase and Tensin Homolog Deleted on Chromosome 10

q-PCR - Quantitative Real-time Polymerase Chain Reaction

rCXCL12 – Recombinant CXCL12

RAF - V-Raf Murine Sarcoma Viral Oncogene Homolog

RAS - Rat Sarcoma

Rap – Rapamycin

Rb - Retinoblastoma

REMARK - Reporting Recommendations for Tumour Marker Prognostic Studies

RGP – Radial Growth Phase

ROS - Reactive Oxygen Species

RTK – Receptor Tyrosine Kinase

RT-PCR – Reverse Transcription Polymerase Chain Reaction

SD - Standard Deviation

SDS - Sodium Dodecyl Sulfate

SEM - Standard Error of the Mean

Shc - Src Homology 2 Domain Containing

shRNA - Short-Hairpin Ribonucleic Acid

Sin1 - Short Integument 1

siRNA - Short Interfering Ribonucleic Acid

SF3B1 - Splicing Factor 3b Subunit 1

STAT3 - Signal Transducer and Activator of Transcription 3

SWI/SNF - SWItch/Sucrose Non-Fermentable

T- Trametinib

T+P – Trametinib and pazopanib

TAM – Tumour Associated Macrophage

TBS – Tris Buffered Saine

Temoz - Temozolomide

TERT - Telomerase Reverse Transcriptase

TGF-β - Transforming Growth Factor β

THC – Tetrahydrocannabinol

TNF-α - Tumour Necrosis Factor-α

TNM – Tumour, Node, Metastasis Staging

TP53 – p53 Gene

Tram - Trametinib

ULK1/2 - Unc-51-Like Kinase 1/2

UV – Ultraviolet

UVA – Ultraviolet A Radiation

UVB - Ultraviolet B Radiation

UVR – Ultraviolet Radation

UVRAG - Ultraviolet Radiation Resistance-Associated Gene Protein

VEGF – Vascular Endothelial Growth Factor

VEGFR – Vascular Endothelial Growth Factor Receptor

Vps34 - Vacuolar Protein Sorting 34

VGP - Vertical Growth Phase

WT - Wild-Type

YAP - Yes-Associated Protein

Chapter 1

Introduction

Chapter 1 Introduction

1.1 Melanogenesis: From Melanocyte to Melanoma

1.1.1 Melanocyte Origin, Differentiation and Function

Melanocytes, our pigment producing cells within the skin reside dispersed along the dermal to epidermal border, surrounded by fibroblasts within the dermis, and keratinocytes in the epidermis. Originating from undifferentiated and un-pigmented precursor melanoblasts in the neural crest during the early stages of embryonic development, which migrate dorsolaterally and invade the ectoderm, differentiated melanocytes migrate to different destinations including the skin epidermis and the iris or choroid of the eye (Figure 1.1) (Bertolotto, 2013).

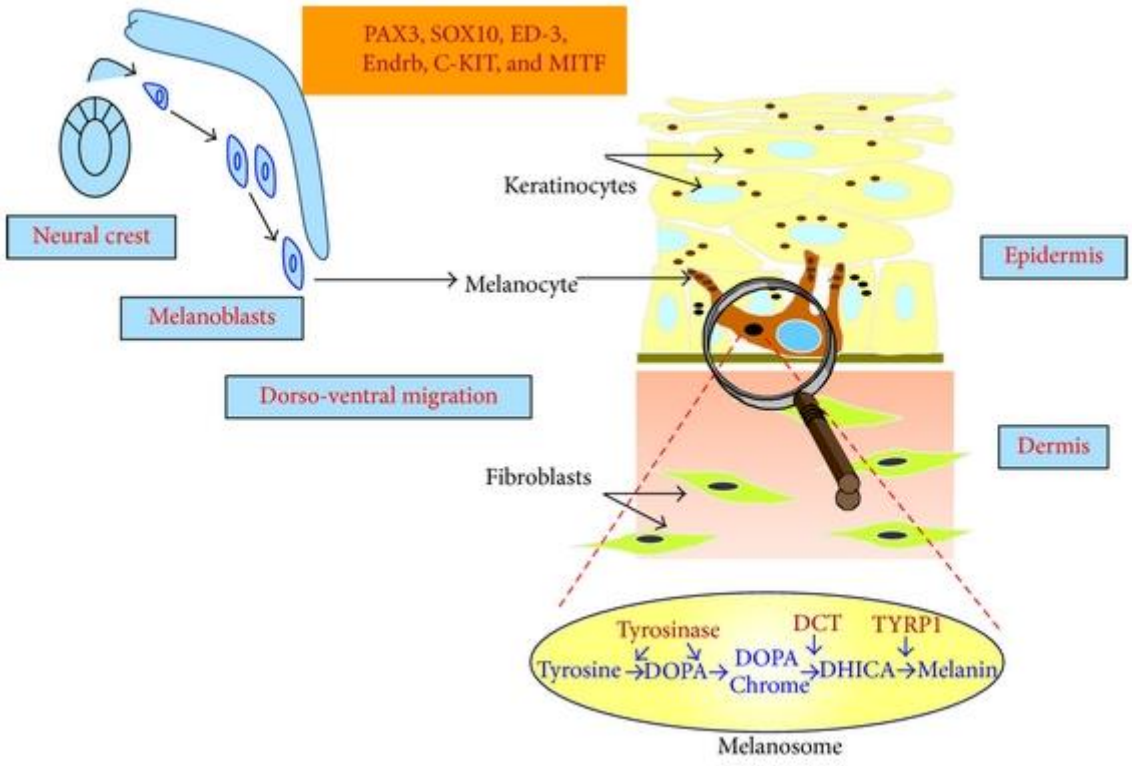

Figure 1.1 Overview of Melanocyte Development and Distribution Within the Skin.
Melanocytes are derived from the neural crest melanoblasts where transcription factors and signalling molecules such as microphthalmia associated transcription factor (MITF), paired domain and homeodomain containing transcription factor (Pax3), and the sry related transcription factor Sox10 determine cell commitment to a melanocytic fate (Thomas and Erickson, 2008). Melanoblasts differentiate into pigmented cells and migrate to the epidermis where they synthesise melanin from tyrosine within their melanosomes. Melanosomes are then transferred to neighbouring keratinocytes through their protruding dendrites, offering vital UV protection to the skin. Image taken from (Bertolotto, 2013).

Melanocytes in the epidermis interact through their many dendrites with up to 30-40 keratinocytes, and transfer matured melanosomes by excocytosis and cytophagocytosis to

keratinocytes (Park *et al.*, 2009). Melanosomes containing the pigmented melanin of which there are two forms eumelanin and pheomelanin, are then positioned over the keratinocyte cell nucleus to prevent DNA damage and genomic instability induced by UV radiation, by absorbing and scattering the radiation, converting it into the less genotoxic energy source, heat (Tsatmali *et al.*, 2002; Lin and Fisher, 2007; Park *et al.*, 2009).

Although both cutaneous and uveal melanocytes are derived from the neural crest and synthesise both eumelanin and pheomelanin pigments, they differ substantially in terms of their interactions and stimulatory effects. Unlike epidermal melanocytes, uveal melanocytes do not transfer melanin to any other cell types (Hu, 2000). Furthermore, epidermal melanocytes respond to both endogenous an exogenous stimulation, with the ability to adapt to such stimuli, whereas uveal melanocytes remain unaffected and hence changes in iris pigmentation are very rare (Imesch PD *et al.*, 1997). These differences may illustrate the discord between these two cell types, or highlight the very different microenvironment of uveal melanocytes in the eye compared to cutaneous melanocytes in our skin.

Melanogenesis describes the process by which the pigmented polymer melanin, is produced by melanocytes, which acts as a vital natural photo-protection that augments the accumulation of DNA damage leading to skin aging and malignant transformation. The process of melanogenesis is multifaceted and is tightly regulated by multiple genetic factors, and cellular signalling pathways, that respond to both endogenous and exogenous stimulation. Interestingly, uveal melanocytes do not respond to stimulation by α-MSH or adrenocorticotropic hormone, strong stimulators of cutaneous melanogenesis, which is thought to be attributable to the lack of MC1R receptors present on uveal melanocytes (Li *et al.*, 2006).

The greatest activator of melanogenesis is exposure to UV radiation (UVR), and although melanocytes are specialised to protect against and repair UV induced DNA damage, not everyone's skin acts alike, with constitutional differences in skin photo type and regulation of melanogenesis increasing an individual's risk of developing melanoma. Interestingly it is proposed that the increased incidence of melanomas in fair skinned individuals may be due to germline mutations in the MCR1 gene that causes disruption in the key cAMP, Protein Kinase A, CREB melanogenesis pathway increasing risk of melanoma 2-4 fold (Kennedy *et al.*, 2001). Individuals with this skin photo type have propensity to produce mainly pheomelanin,

which has weaker UV protective properties than eumelanin (Marrot *et al.*, 1999). Recently the direct genotoxic effects of UV radiation on melanocytes have been demonstrated by the identification of UVB-induced cyclobutane pyrimidine dimers and 6-pyrimidine photoproducts in DNA that result in C to T or G to A transitions, and activating mutations in melanoma genes (De Fabo *et al.*, 2004; Hodis *et al.*, 2012b). UVA and B have also been shown to indirectly trigger DNA mutations via the generation of oxidative stress (Filipe *et al.*, 2013). Therefore, effective DNA damage prevention by melanogenesis and DNA repair mechanisms is essential to prevent genomic instability and UV-induced melanocytic transformation.

1.1.2 Melanomagenesis

Cutaneous melanoma arises from the malignant transformation of melanocytes derived from normal skin or a pre-existing melanocytic naevi. DNA damage can be induced by both endogenous (e.g ROS production) and exogenous (e.g UV radiation) sources, initiating tumourigenesis of melanocytes, a result of genetic or epigenetic changes in genes that control critical cellular processes such as proliferation, apoptosis, senescence and DNA repair (Bertolotto, 2013).

Figure 1.2 Benign Naevi Development into a Melanoma
A benign naevus (mole) (http://www.melanoma.net.au/Melanoma/melanoma.htm) or dysplastic naevi (http://www.cancerresearchuk.org) or a melanoma (Melanoma Diagnostic Centre). Adapted from (McKee, 2013).

In line with the multistep process of carcinogenesis, a stepwise model for melanoma progression has been proposed, whereby genetic mutations cause disruption of critical cellular processes regulating melanocyte proliferation and survival leading to a pre-malignant melanoma, with benign naevi (common mole) or dysplastic naevi formation (Figure 1.2) (Chin *et al.*, 1998).

The consecutive acquisition of further genetic mutations, promotes tumour progression to *in situ* disease with the melanoma expanding radially within the epidermis (radial growth phase), before breaching the basement membrane and vertically invading within the dermis (vertical

growth phase). This invasive disease may further progress to metastatic disease if the melanoma is able to invade microvasculature and lymphatics of the dermis, allowing spread to lymph nodes and other organs (Figure 1.3).

Recently studies have further defined the succession of genetic alterations during melanoma progression demonstrating the evolutionary paths of melanoma subtypes (Maressa C. Criscito *et al.*, 2016). The following section will discuss the most prevalent and key molecular events in melanoma initiation and progression by germline and sporadically formed mutations but is not exhaustive.

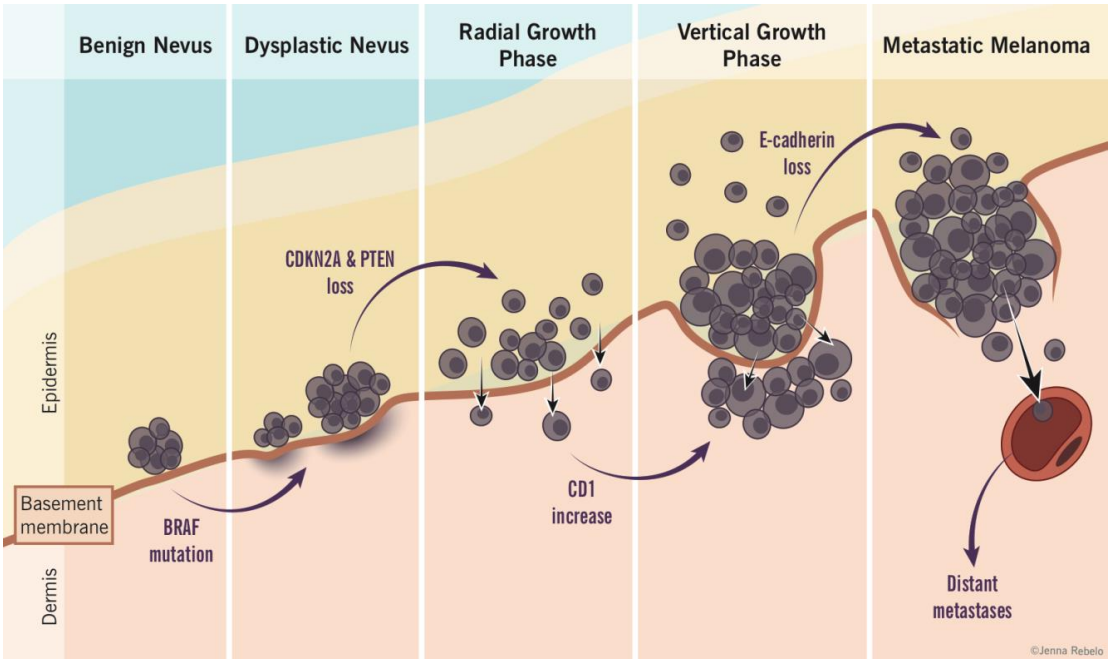

Figure 1.3 Melanoma Progression
Melanocytes within the basal layer of the epidermis proliferate forming naevi of which some become dysplastic. Radial growth phase proceeds and the melanoma spreads horizontally within the epidermis. Vertical growth phase the melanoma breaches the basement membrane and invades the dermis. Infiltration into lymphatics or blood vessels precedes metastatic spread to lymph nodes and distant organs. Adapted from (Abbasi et al., 2004).

A seminal study of the evolution of melanoma, suggests that the acquisition of melanocytic naevi is primarily induced by oncogenic mutations causing hyperactivation of the MAPK signalling pathway (as discussed in section 1.2.3). Unequivocally, benign naevi have been found to possess B-RAF^{V600E} mutations in 85% of cases, suggesting that B-RAF^{V600E} is the major initiating event (Shain *et al.*, 2015). It is therefore proposed that an activating point mutation in N-Ras, B-RAF, GNAQ and GNA11 in a mutually exclusive manner, is the driving event for the

development of melanocytic naevi in both cutaneous and uveal melanoma (Pollock *et al.*, 2003; Van Raamsdonk *et al.*, 2009b).

However, given the large spectrum of mutations in melanoma, distinguishing between driver and passenger events has been a difficult process that large genetic sequencing studies have aimed to uncover. TERT (human telomerase reverse transcriptase) promoter mutations, result in increased TERT expression and a concomitant increase in telomerase activity that can promote tumourigenesis by overcoming replicative senescence executed by telomere shortening (Chiba *et al.*, 2015). Recent studies have identified TERT promoter mutations as the earliest secondary mutations frequently identified with B-RAF or N-Ras mutations in 77% of "likely benign" intermediate melanocytic tumours, suggesting that neither are sufficient for malignant transformation (Lian CG and Murphy GF, 2016).

Additional early, but not initiating molecular events in naevi include the epigenetic modification of ALK (anaplastic lymphoma kinase) and biallelic loss of BAP-1 (BRCA-1 associated protein). The novel isoform of ALK present in 11% of melanomas, is initiated from a *de novo* alternative transcription initiation (ATI) site in ALK intron 19, and translates a kinase domain of ALK without the extracellular or transmembrane regions resulting in constitutively activation of MAPK, AKT, and STAT3 signalling (Wiesner *et al.*, 2015). BAP-1 is a tumour suppressor gene that encodes a histone deubiquitinase, involved in cell cycle progression, growth and DNA repair (Machida *et al.*, 2009b; Eletr and Wilkinson, 2011). Although biallic loss of BAP-1 is common in early stages of uveal melanoma progression and associated with GNAQ/11 mutations and a worse prognosis (Kalirai *et al.*, 2014), the cutaneous counterpart presents with B-RAF or N-Ras mutations and is found in a distinct epithelioid low risk intermediate naevi (Wiesner *et al.*, 2012; Yeh *et al.*, 2014).

As naevi progress to melanomas *in situ* and invasive melanomas, chromosome and copy number aberration become frequent, reflecting the ongoing genomic instability. Biallelic inactivation of CDKN2A has been found to be an exclusive signature of invasive melanomas and present in up to 50% of all tumours (Bennett, 2008; Lian CG and Murphy GF, 2016), with further mutations in SWI/SNF chromatin remodelling genes associated with the development of invasive melanomas (Lian CG and Murphy GF, 2016).

At later stages of tumour progression, tumour evolution becomes more heterogeneous in terms of genomic alterations, with Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) and TP53 tumour suppressor loss characteristic of advanced primary melanomas (Lian CG and Murphy GF, 2016). PTEN is reported to be deleted in 37% of cutaneous melanomas but not in naevi, allowing cells to bypass senescence via negative regulation of the PI3K/AKT pathway (Stahl *et al.*, 2004; Bertolotto, 2013), while the nuclear transcription factor P53, is functionally defective in 90% of advanced melanomas (Vogelstein *et al.*, 2000; Lu *et al.*, 2013).

1.2 Cutaneous Melanoma

1.2.1 Cutaneous Melanoma: Risk Factors, Staging and Prognosis

Cutaneous melanoma is one of the most aggressive and therapeutically challenging forms of skin cancer, with a continually growing world-wide incidence that affects individuals from all ethnicities, socioeconomic groups, geographic locations and stages of life. In the UK alone, 15.9 cases per 100,000 men and 16.5 cases per 100,000 women were reported in 2014 and metastatic melanoma continues to increase more than any other malignancy in the last 40 years (Cancer Research U.K www.cancerresearchuk.org, 2014).

Undoubtedly the greatest environmental risk factor contributing to melanoma development is UV exposure, with sun exposure both cumulative and chronic, and sun burn episodes, positively correlating with melanoma incidence (Gandini *et al.*, 2005; Chang *et al.*, 2010; Russak and Rigel, 2012). Host susceptibility to UVR-induced risk of melanoma is also associated with individuals with fair skin and hair complexion, light coloured eyes, freckles and a tendency to burn, as well as geographical location (Elwood *et al.*, 1990; Bishop *et al.*, 2002). Unsurprisingly due to its location with a high UV index, and a predominantly Caucasian population Australia has the highest rate of melanoma, 12 fold the average world-wide rate (Thompson *et al.*, 2005; National Cancer Institute, 2013). In support of the role of UVR exposure in melanomagenesis, many studies have now linked sun bed use with the development of melanoma (Westerdahl *et al.*, 2000; Ting *et al.*, 2007; Russak and Rigel, 2012). Further, increased molecular insight into mechanisms mediating melanomagenesis additionally reveals UVR-induced mutations in TP53 accelerate mutant B-RAF driven melanoma (Viros *et al.*, 2014). As well as environmental factors, the risk of developing melanoma encompasses genetic factors. These may include a previous family history of

melanoma, increasing melanoma risk 3-8 fold, also linked to the number of relatives affected, and suggestive of heritable risk factors (Hemminki *et al.*, 2003; Amundadottir *et al.*, 2004; Florell *et al.*, 2005). Genome wide association studies have also highlighted mutations in high risk melanoma loci that are often found within families in an autosomal dominant pattern (Eggermont *et al.*, 2014), including mutations in the CDKN2A gene observed in up to 40% of hereditary cases of melanoma (Tsao *et al.*, 2012). The presence of multiple or large congenital naevi is also a risk factor (Green A *et al.*, 1985), where giant congenital naevi over 20cm in diameter can increase lifetime melanoma risk by up to 10% (Arneja and Gosain, 2007).

Although appreciation of risk factors for the development of melanoma may assist disease prevention, many individuals nevertheless develop melanoma, at which point clinical disease staging provides vital information on likely outcome as well as informing treatment stratification. In 2009 the American Joint Committee on cancer (AJCC) Melanoma Staging Committee lead by Charles Balch incorporated data from worldwide institutions analysing over 30,000 patients to develop what is now considered the most comprehensive evidence based and widely used staging system for cutaneous melanoma, currently in its 7th version (Balch *et al.*, 2009a).

The AJCC staging system is based on TNM (tumour node metastasis) cancer staging, that takes into account both tumour depth (Breslow thickness) as well as the degree of tumour spread to lymph nodes 'N' or distant sites of metastasis 'M' (Table 1.1). Breslow thickness forms the basis of the tumour 'T category' where thin melanomas with a breslow thickness < 1mm (T1) have a good prognosis and 5-year survival rates of 95%, whereas tumours with breslow thickness >4mm, fall into the T4 category with a considerably worse prognosis (Balch *et al.*, 2009a) (Table 1.1). Additionally, the presence of tumour ulceration and mitosis is incorporated into the 'T' categorisation. Ulceration defined as the loss of epidermal integrity overlying resected melanomas is one of the worst prognostic indicators (Balch *et al.*, 2009b) although a tumour defined by a higher T category in absence of ulceration may have a similar poor prognosis (Balch *et al.*, 1980; Grande Sarpa *et al.*, 2006; Balch *et al.*, 2009a). Mitotic rate, has been shown also to be a strong predictor of prognosis in thin melanomas where mitosis under or over 1mm determines T1a or T1b staging respectively (Balch *et al.*, 2009a). Mitotic rate, has been shown also to be a strong predictor of prognosis in thin melanomas where mitosis under or over 1mm determines T1a or T1b staging respectively (Balch *et al.*, 2009a).

Classification	Thickness	Ulceration/Mitoses
T0 (*in situ*)	N/A	N/A
T1	≤ 1mm	a. without ulceration and mitosis <1mm^2 b. with ulceration or mitoses ≥ 1mm^2
T2	1.01-2mm	a. without ulceration b. with ulceration
T3	2.01-4mm	a. without ulceration b. with ulceration
T4	>4mm	a. without ulceration b. with ulceration
Classification	**Number of Metastatic nodes**	**Nodal metastatic Burden**
N0	0	N/A
N1	1	a. micrometastasis b. macrometastasis
N2	2-3	a. micrometastasis b. macrometastasis c. in transit metastases, without metastatic nodes
N3	4+ metastatic nodes, or in transit metastases	N/A
Classification	**Site**	**Serum LDH**
M0	No distant metastases	N/A
M1a	Distant skin, subcutaneous or nodal metastases	Normal
M1b	Lung metastases	Normal
M1c	All other visceral metastases Any distant metastases	Normal Elevated

Table 1.1 Tumour Node Metastasis (TNM) Staging in Melanoma

The greatest prognostic factor influencing reduced overall survival for melanoma is the presence or number of lymph node metastasis (Gershenwald *et al.*, 1999; Scolyer *et al.*, 2008) with the 'N' category denoting the number of regional nodal metastatic sites, and the final 'M'

category, defining the region of metastasis and the presence of normal or elevated LDH levels (used to stratify M1 stage melanomas into a, b or c category, Table 1.1)

AJCC Stage	TNM Stage	Disease Characteristics	5 year Survival (%)
0	Tumour *in situ*	Confined to epidermis	100
IA	T1a	Tumour ≤ 1mm, level II/II, without ulceration	95
IB	TIb-T2a	Tumour ≤ 1mm, level IV/VI, or with ulceration, or tumour1.01-2mm without ulceration	89-91
IIA	T2b-T3a	Tumour1.01-2mm with ulceration, or tumour 2.01-4mm without ulceration	77-79
IIB	T3b-T4a	Tumour 2.01-4mm with ulceration, or >4mm without ulceration	63-67
IIC	T4b	Tumour >4mm with ulceration	45
IIIA	T1-4a N1a/N2a	Any thickness without ulceration and 1-3 pos LN	63-70
IIIB	T1-4b N1a/N2a T1-4b N1b/N2b/N2c	Any thickness with ulceration and 1-3 LN or any thickness and 4+ LN or in transit metastasis	46-59
IIIC	T1-4b N1/N2b/N2c/N3	Any thickness with ulceration and 1-3 LN or any thickness and 4+ LN or in transit metastasis	24-29
IV	T-Any, N-Any M1a/1b/1c	Any distant metastasis regardless of thickness or LN status	7-19

Table 1.2 AJCC Staging in Cutaneous Melanoma and 5 year Survival
The AJCC staging in cutaneous melanoma and 5-year survival. Adapted from (Balch et al., 2009a). 'LN' lymph node, 'a' = micrometastases, 'b' = macrometastases, 'c' = in-transit metastases without metastatic lymph nodes.

Overall, patients diagnosed with AJCC stage Ia melanomas (breslow thickness ≤ 1mm, no ulceration and mitotic count ≤1mm^2), are usually cured by surgical excision with a resulting 5 year survival of 95% (Table 1.2) (Balch *et al.*, 2009a). However, for those patients with AJCC stage IV melanomas, 5 year survival falls to <20 % (Table 1.2) (Green *et al.*, 2012), emphasising

the acute need for novel biomarkers able to predict disease progression as well as more effective precision based treatment strategies for metastatic disease.

1.2.2 Current Biomarkers

Although AJCC staging provides a useful means of melanoma classification guiding to a certain extent treatment strategy, it is unable however, to predict the risk of disease progression for early stage, seemingly low risk melanomas. Despite the introduction of sentinel node biopsy to AJCC staging to improve accuracy of prognostication, 15% of patients with thin melanomas (≤1mm) with a negative sentinel node still metastasise and die (Gimotty *et al.*, 2004). Similarly 62% of patients with a positive sentinel node do not progress to distant metastasis (van der Ploeg *et al.*, 2011; Morton *et al.*, 2014), highlighting shortfalls in current prognostication.

Additional biomarkers have therefore been considered including clinico-pathological factors such as anatomical location (Garbe *et al.*, 1995), the presence of infiltrating lymphocytes (Azimi *et al.*, 2012), and specific histological tumour subtypes (Ettl *et al.*, 2014). In addition biological markers of cellular processes critical to tumour metastasis including markers of tumour proliferation such as Ki67 (Gimotty *et al.*, 2005), survivin (Schramm and Mann, 2011), P16/INK4A (Schramm and Mann, 2011), B-RAF activating mutations (Nagore *et al.*, 2014) and TERT promoter mutations (Griewank *et al.*, 2014), have all been used to aid the prediction of melanoma survival.

Markers of tissue invasion such as cell adhesion molecules CEACAM and MCAM (Rothberg *et al.*, 2009), or regulators of epithelial-mesenchymal transition such as Twist transcription factor (Zhang *et al.*, 2014) maybe predictive of melanoma progression as well as markers of immunocompetence, where the presence of programmed death receptor-1 (PD-1) (Cooper *et al.*, 2014), and T-regulatory cell marker forkhead P3 (FOXP3) (Mohos *et al.*, 2013) are associated with worse prognosis. Further, markers of angiogenesis and lymphangiogenesis such as tumour micro-vessel density and peri-tumoural lymphatic density have also been used to predict prognosis and disease free survival respectively (Pastushenko *et al.*, 2014a).

However, to date, no single biomarker or combinational marker has added to the current AJCC staging system, with most potential candidates only tested within a single cohort, without adherence to REMARK criteria, performed with inappropriate analytical methods, and without validation across populations to authenticate prognostic clinical value (Rowe and

Khosrotehrani, 2016). Hence there is still an urgent need for fully validated prognostic biomarkers in both cutaneous and uveal melanomas.

1.2.3 MAPK Signalling Activation and Therapeutic Targets

Early excision of cutaneous melanoma can be curative for 90% of patients with early stage primary lesions, however, there is currently no effective therapeutic modality for the treatment of advanced metastatic disease. Once established, the intrinsic or acquired resistance of metastatic melanoma to current strategies results in 5 year survival rates of less than 20%, thus emphasising the urgent need for novel treatments with sustained clinical benefit (Altekruse S.F *et al.*, 2010).

Molecular genetics have eluted distinct patterns of common mutational changes within signalling pathways and cell cycle events that can drive tumourigenesis of melanocytes, providing the first opportunity of targeted therapy. Perhaps the best defined of these deregulating cell signalling mechanisms in melanoma, is the Ras-RAF-MEK-ERK signalling pathway. Mutations within the Ras signalling network are particularly important in melanoma as signalling cascades to both the mitogen activated protein kinase (MAPK) and phosphatidylinositide 3-kinases (PI3K) pathways (Hocker *et al.*, 2008). Ras is a small GTPase protein that transmits signals from extracellular growth factors by conversion into an active GTP bound formation mediated by receptor tyrosine kinases. Mutated oncogenic forms of Ras include N-Ras, K-Ras and H-Ras of which N-Ras is most commonly mutated in 15-20% of all melanomas resulting in the constitutive activation of downstream MAPK signalling independent of external stimuli (Downward, 2003; Dumaz *et al.*, 2006). However drugs selectively targeting Ras, have not come to fruition with most therapeutic efforts alternatively concentrated on downstream elements of the MAPK pathway, fuelled by the seminal identification of a gain of function mutation in B-RAF present in up to 90% of all melanomas (Davies *et al.*, 2002b) (Figure 1.4).

RAF is an intermediary kinase for which 3 members A-RAF, B-RAF and C-RAF are phosphorylated by active Ras. Activated RAF subsequently participates in the MAPK signalling cascade by phosphorylating MEK 1/2 MAP-kinase which consequently phosphorylates ERK 1/2 mitogen activated kinases, leading to the activation of fos and jun transcription factors with pleiotypic effects (Platz *et al.*, 2008). Of more than 50 known mutations discovered in B-RAF, 80% of those in melanoma, contain a miss-sense substitution of thymine to adenine, resulting

in a valine to glutamic acid substitution at codon 600 (V600E) (Davies *et al.*, 2002b; Garnett and Marais, 2004). This results in, increased B-RAF kinase activity, enhanced proliferation, and the increased likelihood of melanocyte transformation (Davies *et al.*, 2002b).

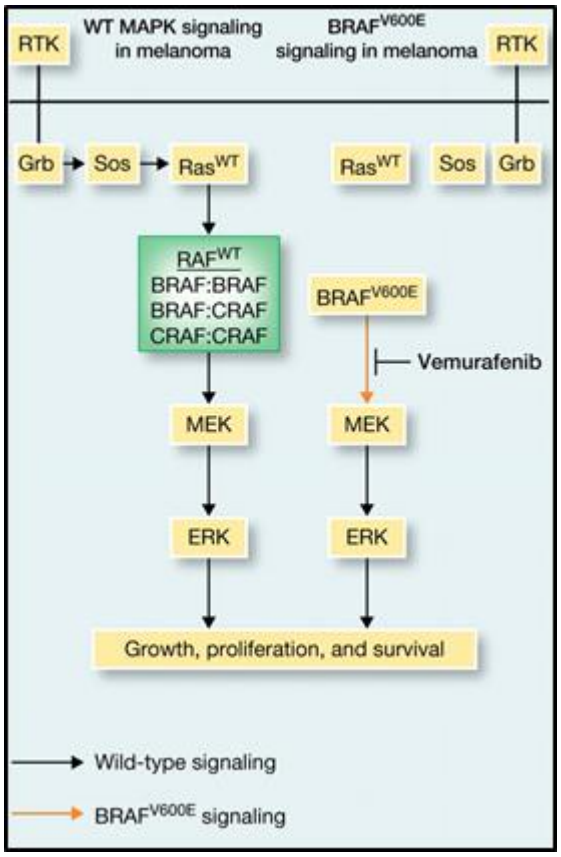

Figure 1.4 Signalling Events Mediating Melanomagenesis and Targeted Therapeutic Strategies

In normal melanocytes the Ras-RAF-MEK-ERK signalling pathway is tightly regulated to promote growth, proliferation and cell survival. However, in melanoma the Ras signalling pathway often harbours mutations that are central to melanoma development. Of the most prolific is B-RAF V600E mutant that causes oncogenic constitutive signalling and cell transformation. Vemurafenib is a small molecule inhibitor that has been shown to cause death of B-RAF mutant cells. Adapted from (Luke and Hodi, 2012).

Interestingly B-RAF V600E mutations are observed in over 80% of benign naevi (Dankort *et al.*, 2009), suggesting mutant B-RAF alone is insufficient to induce oncogenic transformation and that additional mutagenic events are required (Michaloglou *et al.*, 2005). Nevertheless the fact that mutant B-RAF promotes anchorage independent growth (Smalley, 2009), melanocyte survival (Cartlidge *et al.*, 2008), invasion and metastasis (Sharma *et al.*, 2005; Klein and Aplin, 2009; Arozarena *et al.*, 2011) as well as tumour escape from immune surveillance (Kono *et al.*, 2006; Sumimoto *et al.*, 2006) confirms the potential for targeting B-RAF in melanoma

therapeutically, and hence has pathed the way for an influx of drugs specifically targeting B-RAF V600E into clinical trials (Figure 1.4).

In fact the B-RAF V600E small molecule inhibitor vemurafenib, that prevents signal transduction between B-RAF and MEK has yielded the greatest response rates in patients with metastatic melanomas harbouring mutant B-RAF V600E compared to any other drug in the last 20 years, increasing progression free survival by up to 6 months compared to single agent dacarbazine (Flaherty *et al.*, 2010; Chapman *et al.*, 2011). Unfortunately, however, the response to vemurafenib is short lived with the development of acquired resistance observed in the majority of patients after 6 month or so, and hence impairing any improvement in long term survival for patients with malignant melanoma (Flaherty *et al.*, 2010; Chapman *et al.*, 2011) (Figure 1.5).

Some melanomas are intrinsically resistant to vemurafinib from the onset, with mechanisms of resistance proposed to include an increase in cyclin D1 expression resulting in deregulation of cell cycle machinery, as well as a switch or diversion in signalling through A or C-RAF causing the reinstatement of MAPK signalling (Figure 1.5) (Montagut *et al.*, 2008; Smalley *et al.*, 2008; Villanueva *et al.*, 2010). Intrinsic resistance may also be mediated by loss of the PTEN tumour suppressor gene, seen in over 10% of melanomas and leading to a compensatory increase in AKT signalling (Boisvert-Adamo and Aplin, 2008; Paraiso *et al.*, 2011).

Alternatively acquired resistance to vemurafenib may result from the emergence of secondary mutations in B-RAF preventing vemurafenib binding to its target site (Whittaker *et al.*, 2010), or the advance of a B-RAF variant lacking a Ras binding site (p61B-RAF^{V600E}) enabling increased dimerization with A-RAF and C-RAF and the potentiation of drug-induced resistance (Poulikakos *et al.*, 2011; Shi *et al.*, 2012). Furthermore the amplification of B-RAF itself or C-RAF may also enhance resistance increasing downstream signalling (Poulikakos *et al.*, 2011; Shi *et al.*, 2012). Vemurafenib-mediated inhibition of MAPK signalling may also be abrogated by acquired upstream activating mutations in N-Ras leading to C-RAF-dependant activation of MEK-ERK signalling (Johannessen et al., 2010; Nazarian et al., 2010), or downstream mutations in MEK1/2 (MEK1^{C1251}) (Nazarian *et al.*, 2010). Finally the activation or upregulation of other receptor tyrosine kinase receptor signalling pathways in response to vemurafenib treatment including, joint IGF1Rβ and PDGFRβ signalling via the PI3K/AKT pathway, may result in a reduction of cell dependence on active ERK signalling (Nazarian *et al.*, 2010; Villanueva *et al.*,

2010; Wagle *et al.*, 2011). Increased activation of the MEK by either its direct activator COT serine/threonine protein kinase or via MET activation by stromal secreted hepatocyte growth factor (Straussman *et al.*, 2012), can mediate RAF-Independent MEK signalling and hence can also offer a mode of resistance to B-RAF specific inhibition (Johannessen *et al.*, 2010) (Figure 1.5).

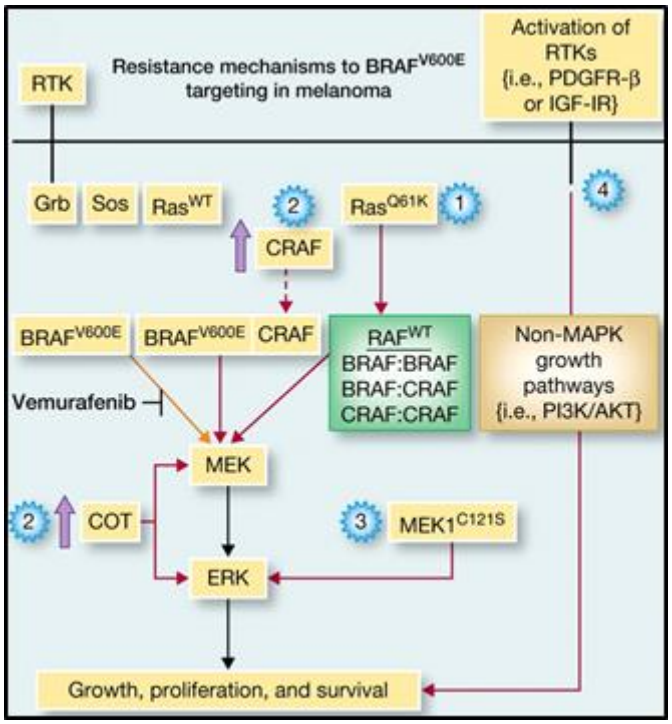

Figure 1.5 Possible Mechanisms of Vemurafenib Resistance.
Resistance to Vemurafenib may be mediated by reactivation of MAPK signalling via (1) Mutations in upstream Ras, (2) C-RAF or COT over expression increasing MEK/ERK activation, (3) activating mutations in MEK or (4) Non MAPK growth pathways such as insulin-like growth factor receptor or Platelet derived growth factor receptors promoting growth, proliferation and survival of melanocytes independent of MAPK signalling. Modified from (Luke and Hodi, 2012).

Dabrafenib a newer B-RAF inhibitor has yielded similar increases in progression free survival and response rates as vemurafenib, with remarkable efficiency in intracranial metastases and with a slightly more favourable toxicity profile (Falchook *et al.*; Long *et al.*, 2012), and hence its use was therefore FDA approved for the treatment of unresectable melanoma harbouring B-RAF mutation in 2013.

Additional concern for the use of B-RAF specific inhibition is for melanomas that do not harbour a B-RAF V600E mutation, but which display paradoxical activation of MAPK in response, possibly accounting for the acquisition of new carcinomas and melanomas with B-RAF inhibition (Poulikakos *et al.*, 2010; Robert *et al.*, 2011; Su *et al.*, 2012). To tackle this

problem intra and inter MAPK pathway inhibitors are being evaluated to overcome these issues.

Collectively research highlights the dependence of melanoma on hyperactive MAPK signalling, and hence supports the targeting of MEK 1/2 as a promising alternative therapeutic strategy, applicable therefore not only to patients harbouring B-RAF mutant melanomas, but also for those with N-Ras mutant or indeed N-Ras/B-RAF mutant melanomas. MEK is a particularly attractive target given that, activation of RAF becomes amplified at MEK1/2 due to its much greater abundance in cells (Gray *et al.*, 2001), the fact that MEK1/2 is rarely mutated, and finally by its downstream convergence for both mutant Ras and RAF (McCubrey *et al.*, 2007). To this aim, small molecule inhibitors to MEK, including trametinib (GSK 1120212) have been developed. *In vivo* pre-clinical data demonstrated the complete abrogation of tumour growth in B-RAF mutant xenografts in response to MEK-specific inhibition, providing proof of concept for the use of single-agent trametinib and in combination with new generation B-RAF specific inhibitors (Solit *et al.*, 2006; InfanteJR *et al.*, 2010; Gilmartin *et al.*, 2011).

As a single agent in a phase III trials of trametinib demonstrated improved progression free survival and overall survival, resulting in FDA approval in 2013 and the use of trametinib as first line treatment for unresectable B-RAF mutated melanoma (Flaherty *et al.*, 2012c). Likewise as with B-RAF inhibition, some individuals displayed resistance to MEK inhibition mediated via mechanisms disrupting ERK1/2 negative feedback loops, overexpression of cyclin D1, increased wnt or PKA signalling and amplification of Ras and RAF family members to name but a few (Little *et al.*, 2012). In a bid to overcome resistance gained by either B-RAF or MEK inhibitor alone subsequent approaches were taken to target both mutant B-RAF and MEK as a strategy to abrogate reactivation of MAPK signalling. Phase I/II trials demonstrated impressive results with improved patient free survival to 10 months with the combination of trametinib and dabrafenib compared to 5.6 months with dabrafenib alone, leading to the rapid approval of combinational therapy in 2014 (Flaherty *et al.*, 2012b). Subsequent phase III trials have further confirmed improved progression free survival and overall responses with combined MEK and B-RAF inhibition, and hence is now the standard of care for patients with metastatic melanoma bearing either mutant B-RAF V600E or V600K (Robert *et al.*, 2015).

A promising new area in melanoma therapeutics includes immunotherapy, which targets the patient's defective immune responses to melanoma. The cytotoxic T lymphocyte associated

antigen (CTLA-4) down regulates T cell activation and promotes immune tolerance of melanoma cells. To counteract this, trials of a monoclonal antibody to CTLA-4, ipilimumab to promote T cell activation and the reinstatement of anti-tumour immunity have been initiated (Fong and Small, 2008). A phase III trial of ipilimumab demonstrated increased overall survival by 3.6 months compared to gp100 vaccine alone (Hodi *et al.*, 2010), which has led to its adoption into clinical practice. Programmed cell death receptor 1 (PDCD1) and its ligand CD274 are similarly immunotherapy targets in melanoma, with engagement of the ligand to the receptor resulting in the downregulation of T cell activation. Re-stimulation of the immune system by anti-PDCD1 antibodies nivolumab and lambrolizumab has proven to be superior to ipilimumab with responses in up to 50% of individuals and outstanding survival rates of 66% at 1 year compared to only 25% with systemic chemotherapies (Hamid *et al.*, 2013; Mario Sznol *et al.*, 2013). A newer anti-PD-1 monoclonal antibody Pembrolizumab, was FDA approved in 2014 for advanced melanoma and demonstrates impressive 1 year survival as a monotherapy, at 74.1% compared to 58.2% with ipilimumab (Robert *et al.*, 2015). Collectively these data thus suggest that immunomodulatory therapy is likely to make a great impact in the future treatment of advanced stage melanomas with other emerging strategies including toll-like receptor activation by imiquimod, adoptive T-cell therapy and oncolytic viral therapy.

Activating mutations or amplification of KIT have been reported in 39% of mucosal and 36% of acral melanomas, suggesting that in certain melanoma phenotypes targeting KIT maybe advantageous (Curtin *et al.*, 2006). The KIT inhibitor nilotinib and imatinib have shown efficacy in phase 2 trials of advanced melanoma trials and further trials with these inhibitors compared to systemic chemotherapies are ongoing (Guo *et al.*, 2011a; Carvajal *et al.*, 2015).

Epigenetic modulations are implicated in melanoma pathogenesis, and may include methylation of promoter regions, histone modification, and chromatin remodelling providing possible new therapeutic targets (Lee *et al.*, 2014), with early phase trials of decitabine a DNA-methyltransferase inhibitor and vorinostat a histone deacetylase inhibitor currently under way (Tang *et al.*, 2016).

In an era of personalised and precision based medicine, clearly it will be important to define which deregulated signalling mechanisms are best targeted within individual patients with cutaneous metastatic melanoma as well as their cross talk in order to optimally define the

best therapeutic approach and how to circumvent the development of acquired drug resistance.

1.3 Uveal melanoma

1.3.1 Uveal Melanoma Characteristics and Prognosis

Uveal melanoma is the most common primary intraocular cancer in adults accounting for 5% of all melanomas. Uveal melanoma originates from neural crest derived melanocytes of the uveal tract and arises in 90% of cases, from the choroid where tumours are dome shaped and acquire a typical mushroom shape once rupturing the Bruch's membrane (Figure 1.6). 7% of uveal melanomas arise in the ciliary body, with 3% in the iris (Damato, 2006). Unlike cutaneous melanoma, the incidence of uveal melanoma has remained relatively constant and is still rare in the UK, affecting only 6 per million individuals (Singh *et al.*, 2005; Coupland *et al.*, 2013). Although uveal melanomas may occur at any age usual presentation is around 50-60 years of age with a slight preponderance for larger tumours in males (Frenkel *et al.*, 2009). Risk factors for uveal melanoma development include light skin and eye colour, red or blonde hair, and inability to tan (Weis *et al.*, 2006). However, unlike cutaneous melanoma there is no firm evidence to suggest a linkage to UV exposure and only a few reported cases of Mendelian inheritance (Damato, 2012). Choroid naevi are relatively frequent in Caucasians and undergo malignant transformation at a very slow rate, however giant choroid naevi (over 10mm) are more likely to transform with 18% transforming into melanomas within 10 years (Li *et al.*, 2010).

50% of uveal melanomas metastasise, almost exclusively by a haematogenous route, due to lack of lymphatic drainage to the ocular interior, and resulting in liver metastasis in 95% of cases (Buzzacco *et al.*, 2012). Once metastasis to the liver occurs this is unvaryingly fatal with a median survival of only 4-5 month and 1 year survival rates less than 15% (Lamba *et al.*, 2009; Besaratinia and Pfeifer, 2011). On the other hand, extrahepatic metastasis to the lung, bone or skin are less common and are associated with a slightly longer median survival of 19-18 months (Bedikian, 2006).

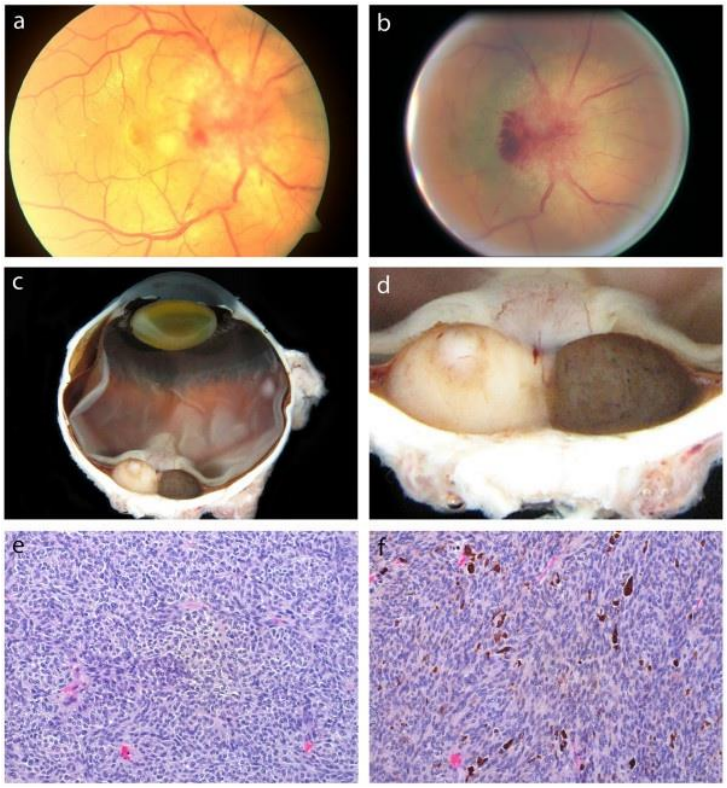

Figure 1.6 Uveal Melanoma.
A and B.) example of a fundus microscope examination used to detect the presence of ocular tumours. C.) and D.) uveal melanoma with both pigmented and non-pigmented halves. E.) histological staining of a uveal melanoma with an epithelioid cell type and F.) histological staining of a spindle cell type uveal melanoma with melanin pigmentation. Taken from (Lim et al., 2014).

The inability to predict the timescale to metastasis development makes prognostication of uveal melanoma challenging. Staging is largely based on the 2010 updated criteria by the AJCC criteria, which similarly to cutaneous melanoma, is based on the tumour, node, metastasis (TNM) staging model for anatomical staging (Figure 1.7), with prognosis determined according to primary tumour size, thickness, ciliary body involvement and extraocular spread (Damato, 2012). Based on the fact that larger tumours are more likely to metastasise, basal tumour diameter is the most widely used prognostic indicator within AJCC staging, with, relative ease of detection making this the most clinically useful factor (Shields *et al.*, 2009).

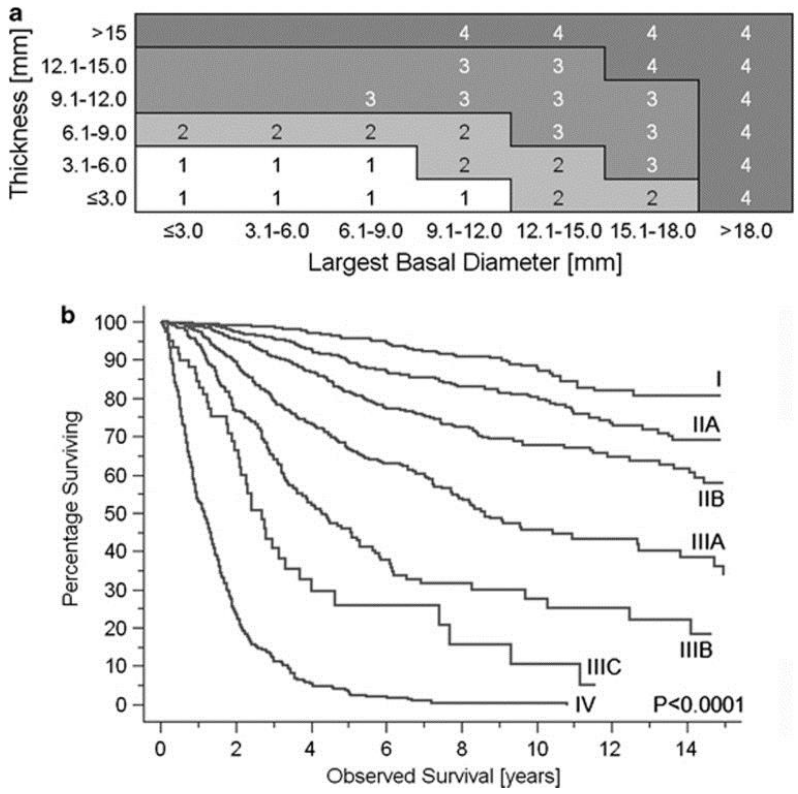

Figure 1.7 AJCC Staging in Uveal Melanoma.
A.*) Determination of T stage by tumour thickness and basal diameter. B.) Survival curve of uveal melanomas at all AJCC stages. Taken from (Harbour, 2012; Kivela and Kujala, 2013).*

The only histopathological feature that is incorporated into the AJCC staging is cell type; with tumours recorded as being of spindle cell phenotype, mixed cell (spindle and epithelioid) or solely of epithelioid phenotype, and for which spindle cell morphology is associated with a more favourable prognosis (S.B. Edge *et al.*, 2010). Other histopathological features that correlate with worse prognosis include the presence of extravascular closed loops and high mitotic count (Damato *et al.*, 2011). Prognosis ranges from a 5 year survival of 96% for patients with AJCC stage I uveal melanomas to just 3% survival for patients with stage IV tumours, further highlighting the aggressive nature of metastatic disease (Kujala *et al.*, 2013).

Genetic prognostication is thought to surpass histological prognostication for uveal melanoma, with loss of one copy of chromosome 3 has been the strongest prognostic indicator to date with metastasis and metastatic death almost exclusive in these individuals (Sisley K *et al.*, 1992; Tschentscher *et al.*, 2000).

1.3.2 MAPK Signalling Activation, Genetic Alterations and Therapeutic Targets

The constitutive activation of the MAPK/ERK pathway is a common event in both uveal and cutaneous melanomas, leading to cell cycle progression and autonomous cell proliferation

(Denhardt, 1996). Interestingly, and in contrast to cutaneous melanoma, uveal melanoma lacks mutations in the typical oncogenes commonly mutated in cutaneous disease, with mutations in B-RAF, N-Ras and KIT extremely rare (Landreville *et al.*, 2008; Hofmann *et al.*, 2009). The driving force of MAPK hyper-activation in uveal melanoma however, is the existence of somatic mutations in the guanine nucleotide-binding protein G(q) subunit (GNAQ) or guanine nucleotide-binding protein subunit alpha-11 (GNA11) (Figure 1.8), present in 83% of primary uveal melanomas and 90% of metastatic uveal melanomas (Van Raamsdonk *et al.*, 2010). GNAQ and GNA11 encode members of the q class of heterotrimeric G-protein α subunits, with mutations in either the Gαq or Gα11 subunits preventing intrinsic GTPase activity required to maintain the protein in its inactive configuration and enabling constitutive G-protein activation (Harbour, 2012). G-protein activation leads to downstream signalling events including hyper-activation of phospholipase C and MAPK signalling driving tumorigenesis. The GNAQ mutation has 5 known variants arising at codon 209 where a glutamine is substituted at this point, with $GNAQ^{Q209L}$ or $GNAQ^{Q209P}$ most common (Van Raamsdonk *et al.*, 2009b). Mutations at the same codon 209 in GNA11 may also occur with both GNAQ or GNA11 mutations at codon 183 additionally observed, albeit less commonly, but in each case are mutually exclusive events (Van Raamsdonk *et al.*, 2010). GNAQ or GNA11 mutations are also present in naevi and in uveal melanomas at all stages, so it is likely these are also early events in uveal melanoma pathogenesis, but which may not be entirely necessary for malignant transformation (Van Raamsdonk *et al.*, 2009a; Van Raamsdonk *et al.*, 2009b). In addition to hyper-activation of MEK, mutant GNAQ/11 may also lead to the downstream activation of the yes-associated protein (YAP), which is the major effector of the Hippo tumour suppressor pathway, reported to be essential for the transduction of oncogenic activity and promotion of growth of GNAQ/11 mutated uveal melanomas (Yu *et al.*, 2014).

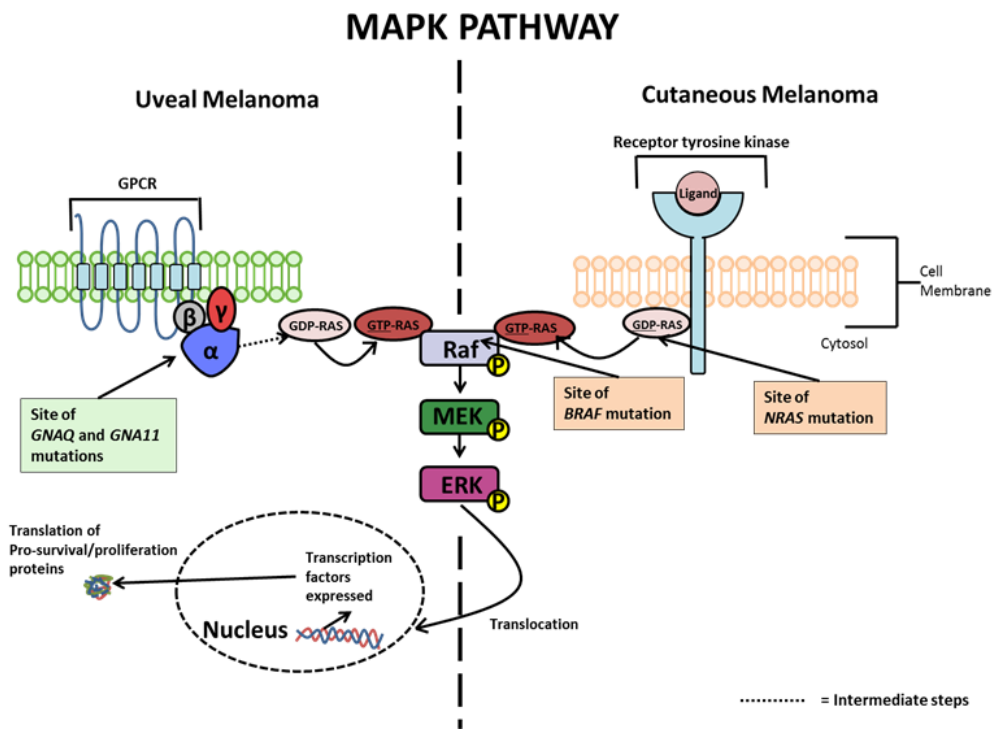

Figure 1.8 Constitutive Activation of MAPK Signalling in Uveal and Cutaneous Melanoma
GNAQ/11 or B-RAF/N-Ras mutations present in uveal or cutaneous melanomas results in the hyper-activation of Ras-RAF-MEK-ERK signalling that leads to enhanced tumour proliferation and survival. Figure illustrated by Barnaby Pathy Bsc project dissertation, Newcastle University.

In the majority of uveal melanomas the tumour suppressor proteins retinoblastoma (Rb) and p53 are dysfunctional and associated with poorer prognosis, although mutations in the cognate genes are very rare (Coupland et al., 1998; Brantley Jr and Harbour, 2000). Rb is inactivated in 65% of uveal melanomas, mediated by the over expression of cyclin D1 or less frequently by hyper-methylation of the promoter CDKN2A (Coupland *et al.*, 1998; Brantley and Harbour, 2000; Coupland *et al.*, 2000; van der Velden *et al.*, 2001), while p53 is likely supressed by overexpression of MDM2, also a common feature of uveal melanomas (Brantley Jr and Harbour, 2000; Coupland *et al.*, 2000).

Studies have also demonstrated the constitutive activation of PI3K/AKT pathway signalling is associated with a poorer prognosis in uveal melanoma (Saraiva *et al.*, 2005). Furthermore, loss of heterozygosity within the PTEN tumour suppressor locus can arise in 76% of primary uveal melanomas leading to increased aneuploidy and poor prognosis (Abdel-Rahman MH *et al.*, 2006; Ehlers *et al.*, 2008).

In addition to oncogenic changes in signalling pathways, uveal melanoma hosts some unique chromosomal alterations likely specific to tumour progression, and distinct from those

observed in cutaneous melanoma. As adhered to above, the most common chromosomal alterations include loss on 1p,3,6q, and 9p with a gain at 1q, 6p and 8q (Harbour, 2012). Chromosome 3 monosomy or partial loss is the strongest prognostic indicator to date and is associated with metastasis and a striking reduction in 5 year survival from 100 to 50% (Bornfeld *et al.*, 1996). Interestingly, chromosome 3 is home to the BRCA1-associated protein-1 (BAP-1) tumour suppressor gene. The BAP-1 gene encodes an ubiquitin carboxy-terminal hydrolase that binds to BRCA1 forming a tumour suppressor complex among other roles in cell cycle regulation, transcription and histone ubiquination (Machida *et al.*, 2009a; Scheuermann *et al.*, 2010; Laurent *et al.*, 2011). The loss of one copy of BAP-1 mediated by monosomy of chromosome 3 or mutations in the other BAP-1 gene is observed in almost 50 % of uveal melanomas, suggesting a recessive 'two hit' scenario that results in the loss of cell differentiation and the emergence of stem-like features promoting uveal melanoma dissemination into a metastatic phenotype (Matatall *et al.*, 2013). The amplification of the long arm of chromosome 8 that occurs in 40% of uveal melanomas also correlates with reduced survival and metastasis (Sisley *et al.*, 1997) . Conversely a 6p gain in a third of uveal melanomas is associated with a much better prognosis compared to chromosome 3 loss with each event tending to be mutually exclusive (White *et al.*, 1998).

Gene expression profiling has enabled researchers to understand not only the pathobiology of uveal melanoma but also provide prognostic information about two classes of tumour that differ markedly in their metastatic potential (Onken *et al.*, 2004). In both cases early molecular initiating events are thought to be GNAQ/11 mutations in normal uveal melanocytes, with progressing tumours dividing into two distinct classes (Figure 1.9). Class 1 tumours remain differentiated resembling uveal melanocytes or naevi, often have a 6p and 8q gain, less aneuploidy with less than 5% of tumours going on to metastasise (Chang *et al.*, 2008). Class 2 uveal melanomas on the other hand, have a 90% risk of metastasis are dedifferentiated resembling primitive neural or ectodermal stem cells (Chang *et al.*, 2008). Class 2 tumours have chromosomal abnormalities, commonly chromosome 3 loss and/or amplification of 8q, with 84% harbouring inactivating mutations in BAP-1, and suggesting biallelic loss of BAP-1 maybe a key step in uveal melanoma progression (Harbour *et al.*, 2010). Loss of BAP-1 has more recently been shown to be predictive of death from metastasis post enucleation, emphasising the importance of this chromosomal aberration in prognostication (van Essen *et al.*, 2014). Although BAP-1 mutations have been noted in cutaneous melanoma, these are

however, not thought that they play such an important role in disease progression, as they do in uveal melanoma (Wiesner *et al.*, 2011).

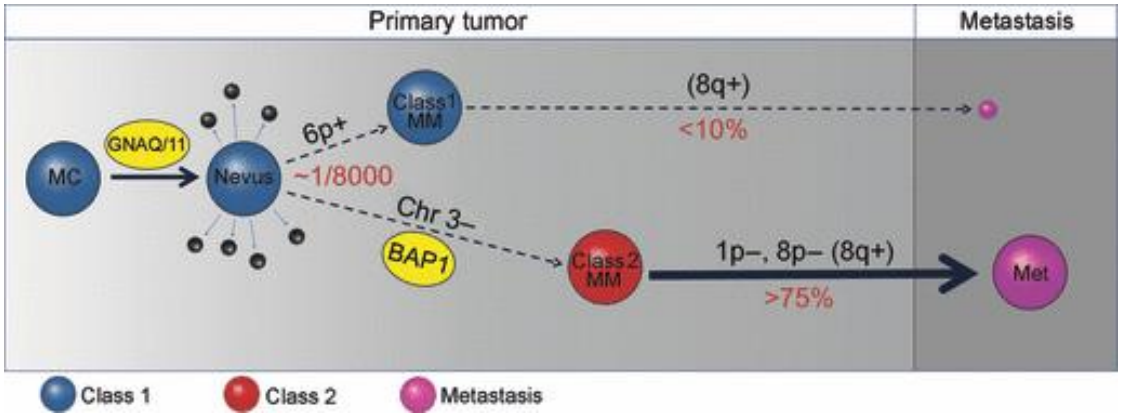

Figure 1.9 Molecular Events in Uveal Melanoma Progression.
Gene expression profiling has identified the early events in uveal melanoma progression. The earliest events are thought to be activating mutations in GNAQ or 11 that triggers MAPK signalling and entry into a senescent state such as that of a nevus tumour. It is thought that from a naevi uveal melanomas can progress down one of two molecular pathways. 10% of uveal melanomas are Class 1 tumours that gain 6p and 8q with low risk of metastasis. Most uveal melanomas however acquire BAP-1 mutations and loss of chromosome 3 falling into the class 2 tumours where they also commonly exhibit 1p and 8p loss and gain of 8q and almost exclusively metastasise. Taken from (Harbour, 2012).

Treatment options for primary uveal melanoma include the radical treatment of enucleation for advanced large local disease, with exoresection with plaque brachytherapy or radiotherapy for medium size tumours, or an conservative approach of plaque brachytherapy, photodynamic therapy, or external ionizing radiation for more cosmetically pleasing results and increased chance of saving vision in smaller tumours (Damato, 2010; Spagnolo *et al.*, 2012). Interestingly both radical and conservative treatments show similar impact on survival and metastatic development. Despite successful treatment of the primary tumour, and advances in systemic chemotherapies, there are still however, no effective systemic therapies for uveal melanoma with many clinical trials of differing chemotherapeutics failing to demonstrate any effective response and at best only improving median survival just under 10 months in a handful of individuals (Bedikian *et al.*, 2003; Kivelä *et al.*, 2003; Schmittel *et al.*, 2005; O'Neill *et al.*, 2006; Schmittel *et al.*, 2006; Bedikian *et al.*, 2008; Homsi *et al.*, 2010). Compared to other agents some positive responses has been observed with fortemustine and although limited, this remains the standard agent for the treatment of uveal melanoma in Europe (J C Becker et al., 2002; Pons et al., 2011; Patricia Rusa Pereira et al., 2013). Although only few individuals with liver metastasis are eligible for liver resection this may prolong survival (Hsueh *et al.*, 2004), and hence Loco-regional treatments have been developed to

target the liver metastasis including radiofrequency ablation, hepatic intra-arterial chemotherapy, immunoemobilisation, chemoembolization and hepatic arterial perfusion (Sato, 2010).

Given the aggressive and resistant nature of metastatic uveal melanoma to systemic therapies and targeted treatment for liver metastasis, it is clear that there is an urgent need for more effective precision based therapies. In this context, there is increasing interest in the potential for new generation MEK specific inhibitors as a targeted therapeutic strategy for GNAQ/GNA11 mutant uveal melanomas. A multicentre randomised phase II clinical trial of the MEK inhibitor selumetinib (AZD6244) compared to single agent temozolomide, in 101 uveal melanoma patients has revealed promising preliminary outcomes, with observations of increased progression free survival to 15.9 weeks following treatment with selumetinib compared to 11.8 weeks with temozolomide (Carvajal *et al.*, 2014).

Other targeting options may include inhibition of the PI3K/AKT pathway, targeting at the level of AKT or mTOR, or possibly targeting BAP-1 mutation/loss. Furthermore, given recent discoveries that mutant GNAQ/11 mediated activation of YAP, verteporfin a YAP transcriptional activity inhibitor has already been FDA approved for macular degeneration, but which also shows promising ability to inhibit uveal melanoma tumour growth, and may thus also represent a therapeutic target in uveal melanoma (Feng *et al.*, 2014; Yu *et al.*, 2014). As in cutaneous melanoma, the use of immunotherapy in the treatment of uveal melanoma is gaining considerable clinical interest, with the initiation of trails of ipilimumab (anti-CTLA-4). However, although phase III trials of ipilimumab have demonstrated improved survival for cutaneous metastatic melanoma (Hodi *et al.*, 2010), such results were not reflected in trials for metastatic uveal melanoma (Zimmer *et al.*, 2015). Although to date, studies of the potential efficacy for PD-1 blockade in metastatic uveal melanoma are limited, interestingly, a single centre trial of 7 patients with pembrolizumab (humanised monoclonal antibody to PD-1) reported increased progression free survival in 2 patients (Kottschade et al., 2016), suggesting this may also be a viable future therapeutic strategy for uveal melanoma.

1.4 Melanoma Metastasis

1.4.1 The Metastatic Cascade

Cancer metastasis is one of the main clinical parameters that determines prognosis in all forms of cancer, typically associated with resistance to treatment, high risk of relapse and lower survival rates (Valastyan and Weinberg, 2011). Metastasis is a biological multistep process or cascade that describes the propagation of cancer cells from the primary tumour, to distant sites in the periphery where they form micro metastasis and eventual secondary tumours (Figure 1.10).

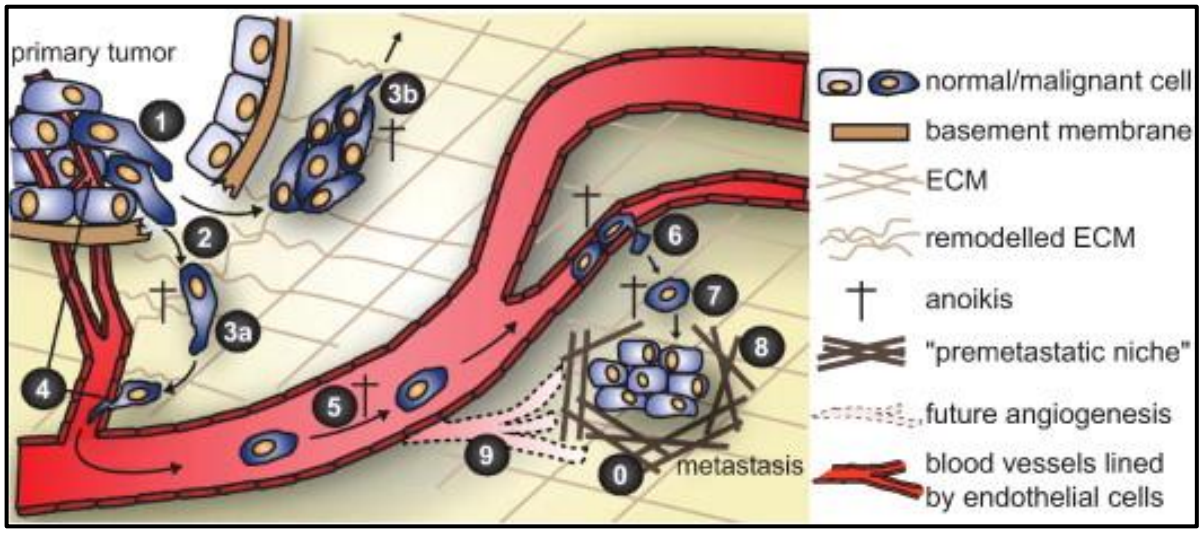

***Figure 1.10** Metastatic Cascade*
The metastatic cascade (0.) A distant site of metastasis being formed from a pre-metastatic niche. (1.) Primary melanoma cells undergo epithelial to mesenchymal transition to acquire an invasive phenotype. (2.) Metastatic melanoma cells migrate through the basement membrane aided by secretion of metalloproteinases to degrade and remodel the extracellular matrix. (3a) A single metastatic cancer cells invades local tissue or (3b.) a group of metastatic cells invade together into the extracellular matrix. (4.) New blood vessels are formed by angiogenesis supplying the primary tumour allowing cancer cells to intravast through endothelial cells lining the blood vessels into general circulation. Alternatively, angiotropic tumour cells that do not invade the vasculature undergo EMT/pericyte mimicry and migrate along the blood vessel without entering it towards a secondary metastatic site (5.) Once detached cancer cells must avoid death by anoikis being transported through circulation. Location of metastasis is often determined by cancer cell migration driven by chemokine gradients secreted from the target organ. (6.) The cancer cells attach to capillary endothelial cells and extravast into the tissue. (7.) The cancer cell may remain dormant for years but may gain further ability to remodel the extracellular matrix and induce angiogenesis to support the formation of a micrometastasis and a secondary tumour. Taken from (Geiger and Peeper, 2009).

Metastatic cascade events start in the primary tumour with underlying events often described as 'epithelial to mesenchymal' (EMT) in nature, and are a prerequisite to melanoma metastasis. EMT is characterised firstly by loss of cell–cell adhesion via the suppression of E-

Cadherin and gain of N-Cadherin expression on the cell surface. EMT is often induced by transforming growth factor β, Wnt, NOTCH, hedgehog and NFκb signalling pathways, in concert with Ras signalling and under the influence of factors within the tumour microenvironment (Oft *et al.*, 1996; Huber *et al.*, 2005). Such changes allow escape of melanoma cells from tight linkage with adjacent keratinocytes in the epidermis, facilitating invasion and new interaction with stromal cells, including fibroblasts and other cancer cells in the immediate tumour microenvironment (Haass NK *et al.*, 2004). Cancer cell metastatic potential is not only determined by its own phenotype but is also greatly influenced by the tumour microenvironment, which comprises many cell types including; cancer associated fibroblasts and immune cells. Together these cells have the potential to shape cancer cell behaviour by providing oncogenic signalling stimuli, growth factors, cytokines, chemokines and pro or anti-angiogenic factors, in addition to generating an inflammatory or immunosuppressive environment promoting cancer cell immune evasion (Spano *et al.*, 2012).

Next in the metastatic cascade universal to all cancers, cells invade through endothelial cell basement membranes lining blood or lymphatic vessels in a process called intravasation (Figure 1.10). Intravasation allows for the transport of cancer cells away from the primary tumour site. Once the cancer cell has migrated through the basement membrane and no longer adheres to the extracellular matrix via integrins, metastasis maybe prevented by induction of cell death by anoikis (Geiger and Peeper, 2009). Cancer cells must acquire the ability to prevent anoikis induced death which may occur at many stages of the metastatic cascade (Zhu *et al.*, 2001). Circulating tumour cells then travel via blood or lymph adhering to the vascular endothelium at the site of metastasis. In the extravasation process the cancer cell crosses from circulation over the endothelium, and extracellular matrix of the targeted tissue. The location of the site of metastasis is dependent on adhesion and chemokine ligands and receptors expressed by the cancer cell and endothelium (Felding-Habermann *et al.*, 2001). Finally once the cancer cells have transendothelially migrated into the tissue the process of micro metastasis establishes a peripheral niche of cancer cells, which in favourable microenvironmental conditions, often after a period of dormancy may form a secondary tumour completing the metastatic cascade (Bustelo, 2012).

More recently, a new paradigm has emerged in the field of melanoma metastasis namely ‘Extravascular Migratory Metastasis’ (EVMM) an alternative mechanism by which melanoma cells disseminate from the primary tumour, to sites of metastasis without intravasation into

blood vessels (Lugassy *et al.*, 2014). EVMM describes the process in which tumour cells from the advancing front of the primary tumour adhere to abluminal vasculature and migrate in a crawling fashion along the external surfaces of the vasculature to metastatic sites without entering any vascular channels. Studies of these 'angiotropic' melanoma cells suggests they express EMT and mesenchymal stem cell associated genes and have migratory properties mirroring what is seen in embryonic development where neural crest cells travel along external surfaces (Lugassy *et al.*, 2013a; Lugassy *et al.*, 2013b). The angiotropic melanoma cells occupy a pericyte location on blood vessels, upregulating the pericyte receptor displaying 'pericyte mimicry' (Lugassy *et al.*, 2013b). Angiotropism has been demonstrated in several studies to be a prognostic factor in melanoma spread both locally and to distant metastasis (Van Es *et al.*, 2008; Wilmott *et al.*, 2012). This phenomenon of angiotropism and EVMM therefore should be considered an alternative mechanism in the metastatic cascade, alongside the widely excepted mechanism of cancer cell intravasation in to blood vessels.

Although the general events and mechanisms by which cancer cells facilitate metastasis is shared by most forms of cancer, there are some ways by which uveal melanoma and cutaneous melanomas differ in their metastatic spread. The first difference owes to the contrasting anatomical location of the melanomas. Cutaneous melanomas must undergo epithelial to mesenchymal transition and invade the skins basement membrane for progression, whereas uveal melanoma has no such requirement in the uveal layer of the eye (Onken *et al.*, 2006). Secondly the absence of lymphatic structures in the eye large enough to allow cell passage, means that cutaneous melanoma may spread by both lymphatic's and haematogenously to secondary sites including the lymph nodes, skin, lung, liver, small intestine, brain and bone, whereas uveal melanoma may only disseminate haematogenously with greatest propensity to metastasise to the liver and rarely to the lung, bone or skin (Yücel *et al.*, 2009).

Why most uveal melanomas metastasise to the liver is unknown, but recently it has become clearer that many factors may influence the favouritism of uveal melanoma seeding in the liver. The presence of the chemokine receptor CXCR4 and c-Met on the surface of uveal melanoma cells are thought to promote uveal melanoma migration to the liver, as the liver secrets the respective ligands CXCL12 chemokine and hepatocyte growth factor (HGF). Uveal melanoma cells forming small micro metastasis of only a few cells are thought to lie dormant within the liver for years before metastatic progression is triggered and the metastasis

enlarges and macro metastasises are formed (Ossowski and Aguirre-Ghiso, 2010). It is likely that the microenvironment within the liver also plays a key role in determining the progression of uveal melanoma metastasis. Stromagenesis occurs in the liver, and involves the activation of hepatic stellate cells and the production of extracellular matrix components such as collagens, that provide a scaffold for the development of blood vessels during angiogenesis, supporting increases in tumour burden (Vidal-Vanaclocha, 2008). Pigment epithelium-derived factor (PEDF) is a factor that is secreted from hepatocytes and is a potent inhibitor of stromagenesis and angiogenesis. PEDF may be inhibited by hypoxia and degraded by metalloproteinases MMP-2/9 which are often secreted from tumour cells, suggesting that hypoxic tumour microenvironments can downregulate PEDF and induce a more favourable metastatic microenvironment (He *et al.*, 2015). It is thought that the ratio of VEGF:PEDF may be key to the angiogenic switch in uveal melanoma liver micro metastases. Uveal melanoma cells have been found to express PEDF and overexpression can decrease uveal melanoma tumour growth and liver metastasis (Lattier *et al.*, 2013). Similarly using a mouse model of uveal melanoma, PEDF-null mice were found to have an increased number of metastases, enhanced hepatic stellate cell activity, collagen III production, and greater microvessel density, compared to control PEDF producing mice (Lattier *et al.*, 2013). Taken as a whole this study illustrates a hypothesis where under normal homeostasis hepatocytes secrete PEDF that inhibits angiogenesis. However, in the hypoxic environment of a liver micro metastasis where VEGF and MMP's are produced by tumour cells, PEDF is degraded leading to hepatocyte activation, increased production of stroma and blood vessels, leading to metastatic progression. This indicates that PEDF may control angiogenesis and stromagenesis in uveal melanoma liver metastases and maybe a key regulator of rapid metastatic advancement.

1.5 The CXCR4-CXCR7-CXCL12 Chemokine Axis

1.5.1 The Chemokine System and Cancer: CXCR4

Chemotaxis refers to the process in which cells direct their movement in reaction to a chemical stimulus, and in general in response to chemo-attractant chemokines, a group of small secreted proteins which act together with their cell surface receptors to control the migration of cells during normal processes of immune regulation, tissue maintenance or developmental processes. Cancer cells however, acquire the ability to subvert the chemokine system, such that these molecules and their receptors become important regulators of cell movement in

and out of the tumour microenvironment and hence are major players in the process of metastasis.

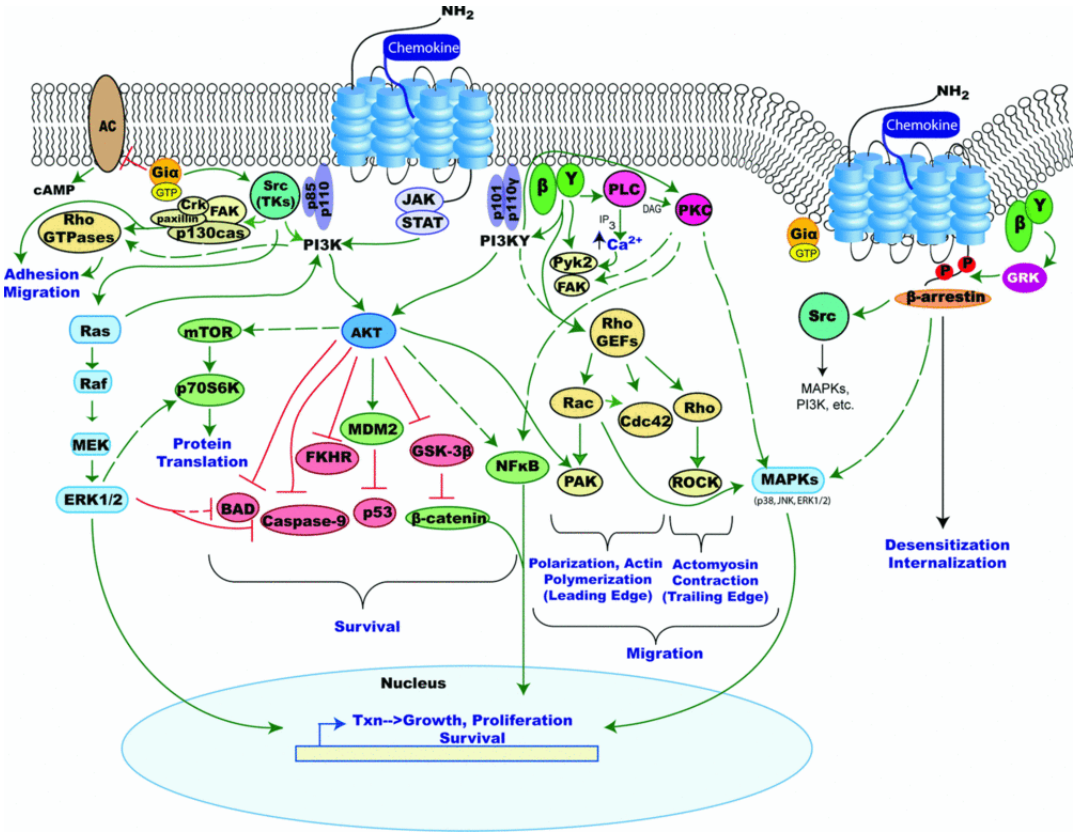

Figure 1.11 Chemokine Receptor Signalling Pathways Promoting Cell Migration, Growth and Survival.

The G-protein coupled cell surface chemokine receptors belong to class A rhodopsin-like G protein coupled seven transmembrane spanning cell surface receptors which homo or heterodimerise on ligand binding (Mellado et al., 2001). The dimerised receptor is then internalised via clatherin, dynamin dependant or a caveolin dependant routes differential to individual receptors (Kiefer and Siekmann, 2011). Intracellular signalling is propagated by the release of GDP from the G proteins Gα subunit allowing for the association with GTP and allosteric changes in the receptor allowing for signal transduction. For the cell to migrate the cell must become polarised with accumulation of PI-3 Kinases, Rho GTPases, Rac and Cdc42 at the leading edge causing actin reorganisation, within the trailing edge PTEN phosphatase counteracting PI3 Kinase activity allowing for tail retraction (Balkwill, 2012). Further downstream signalling pathways include activation of many MAPK pathways including RAS and AKT, promoting cell growth, survival and proliferation. The internalised receptor may be degraded or recycled back to the cell surface which is dependent on the particular chemokine allowing for chemokines to alter the level of receptor expression on the cell (Kiefer and Siekmann, 2011). Taken from (O'Hayre et al., 2008).

Chemokines are the largest cytokine superfamily comprising 48 small secreted protein ligands that act with their 20 or so cognate G-protein coupled cell surface receptors to activate downstream signalling and activation of effectors such as MAPK, PI3K, AKT signalling, integrin activation, increased cell adhesion, and polarization of the actin skeleton, resulting in directional sensing and cell migration along chemokine gradients (Figure 1.11) (Kaibuchi *et al.*,

1999; Serrador *et al.*, 1999; Bajetto *et al.*, 2001; Balkwill, 2012). The chemokine system therefore generates 'cellular highways' in our bodies by which any cells expressing chemokine receptors can home to specific locations in the body to the source of chemokine ligand secretion (Zlotnik *et al.*, 2011).

The chemokine system can be divided into 4 subsets based on their chemokine expression patterns and functional roles (Figure 1.12). Homeostatic chemokines are constitutively expressed by certain tissues and cells, and are involved in development and normal human physiology. Inflammatory chemokines are induced during inflammation and are involved in coordination of immune responses, with viral chemokines enabling pathogens to modulate immune responses against them, with atypical chemokines acting as decoys to negatively regulate chemokine availability.

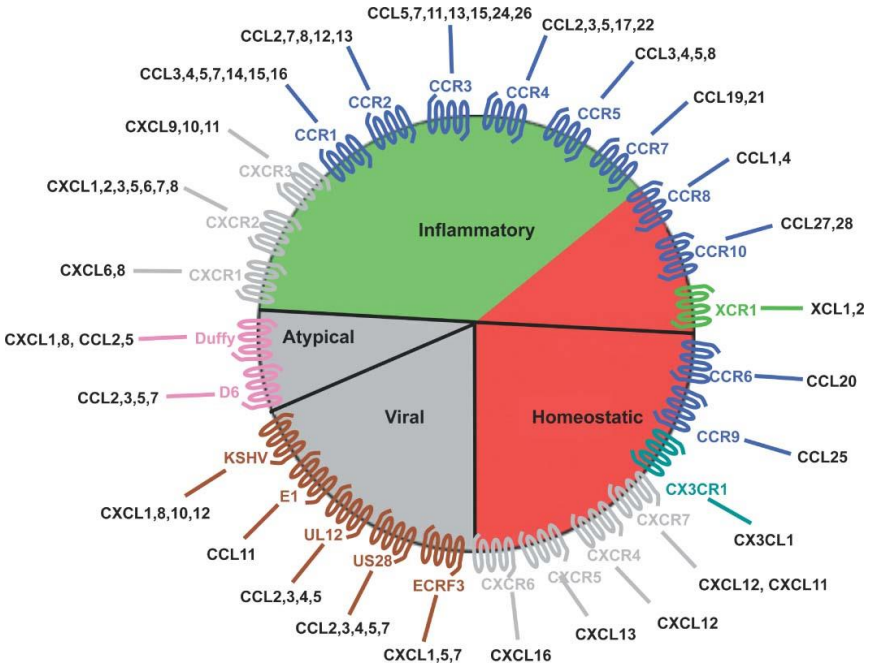

Figure 1.12 The Chemokine System.
The chemokine system is made up of the 4 chemokine receptor groups broadly determined by their physiological function and their constitutive ligands. Inflammatory chemokine receptors dominate immune responses. Homeostatic chemokine receptors, regulate cell migration during developmental processes. Viral chemokine receptors coordinate the immune response during pathogenic responses (Seet and McFadden, 2002). Atypical chemokine receptors are often termed 'decoy' receptors as they dampen chemokine responses by being non responsive in nature (Balkwill, 2012). Taken from (Balkwill, 2012).

Given cancers evolutionary ability to hijack normal cellular controlled processes for its own propagation and the pivotal role of chemokines in tissue homeostasis and cell migration, it is

not surprising therefore that the chemokine system plays an integral part in cancer progression and metastasis.

Deregulation of molecular mechanisms in the pathogenesis of cancer results in the upregulation of chemokine receptor expression, the most common being CXCR4 which is overexpressed in over 20 different cancers including cutaneous and uveal melanoma (Scala *et al.*, 2007; Kim *et al.*, 2008). Chemokine receptor expression by cancer cells not only has profound effects on the cells ability to migrate, but engagement also leads to internal cell signalling that may increase the cells resistance to apoptosis and enhance its proliferation, induced in particular by chemokine receptor mediated activation of downstream MAP/ERK signalling , demonstrated in many cancers including melanoma (Balkwill, 2004). Anti-apoptotic protection has also been seen by chemokine activation of PI3K pathways (Murakami *et al.*, 2003). 80% of melanoma death mediated by FAS ligand activation can be prevented by prior exposure to CCL27 highlighting the ability of chemokines to prevent apoptosis and also aid immune evasion (Murakami *et al.*, 2003).

Although oncogenic changes may be responsible for chemokine receptor up regulation, the tumour microenvironment and hypoxia may also play a role. In breast cancer VEGF and oestradiol can up regulate CXCR4 expression in an autocrine manner (Bachelder *et al.*, 2002; Hall and Korach, 2003). Furthermore, studies in uveal melanoma have shown that both CXCR4 and VEGF expression correlates with liver metastasis, which likewise suggests that chemokine receptor expression and metastasis is influenced by VEGF with intercalating pathways (Franco *et al.*, 2010). Additionally, hypoxic conditions such as those in the tumour microenvironment have also been shown to cause CXCR4 upregulation, reinforcing the pivotal role of the tumour microenvironment in tumour metastasis (Evemie Schutyser *et al.*, 2007).

Cunningly, cancer cells often express both the chemokine and its receptor creating autocrine loops aiding its survival and progression (Balkwill, 2012). Although it is often presumed that autocrine chemokine secretion would have to be reduced at later stages of progression to allow directional metastasis, interestingly autocrine mediated secretion of chemokines may provide a means through which to facilitate tumour migration and invasion in the absence of a strong paracrine chemokine secreting source. It has been shown that under the influence of interstitial fluid flow, autocrine secretion of chemokines can cause cell migration in the direction of flow, a term conned ‘autologous chemotaxis’. It is hypothesised that interstitial

flow alters the concentration of the cancer cell secreted chemokine to create a gradient that allows the cell to become polarised and directionally migrate, a concept that has been demonstrated in many cancers (Shields *et al.*, 2007; Shah *et al.*, 2015).

It has long been hypothesised that the immune system has the potential to recognise and destroy cancer cells or hinder tumour progression. The amount and type of chemokine produced by the tumour can profoundly determine the constituents of inflammation in the tumour microenvironment, making a cancer chemokine profile either detrimental or beneficial to tumour evolution (Viola *et al.*, 2012)(Figure 1.13).

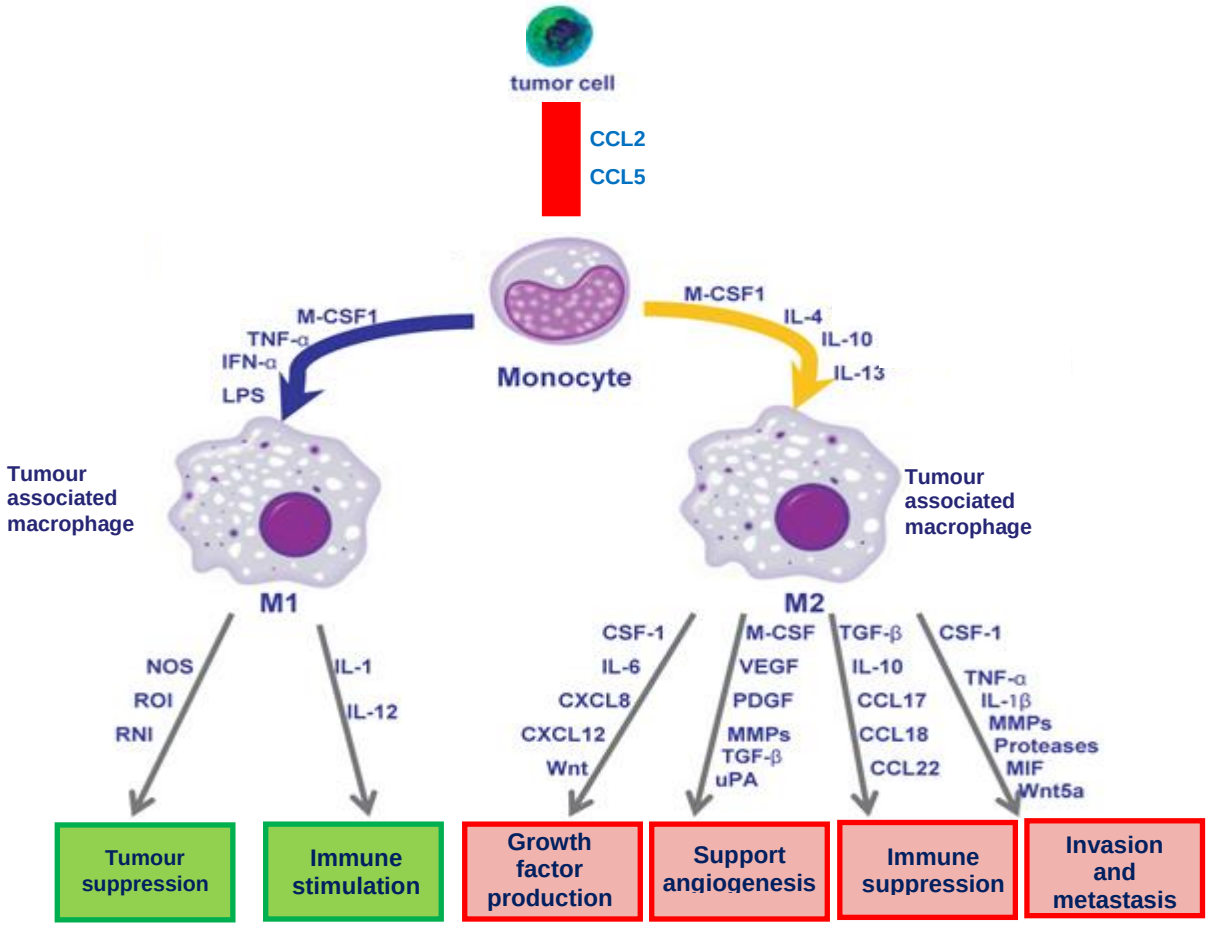

Figure 1.13 The Role of Tumour Associated Macrophages in Tumour Progression
Chemokines CCL2 and CCL5 are secreted by tumour cells and recruit monocytes to the tumour microenvironment. Here the tumour cells have ability to polarise monocyte differentiation into M1 or M2 macrophages, with opposing effects on tumour progression. M1 macrophages secrete IL-12 that stimulates a cytotoxic cancer eradicating response. M2 macrophages on the other hand secrete many cytokines and chemokines supporting tumour progression. Of note CXCL12 in melanoma supports tumour growth, and VEGF secretion promotes angiogenesis. TGF-β, IL-10 and chemokines CCL17, 18 and 22 secreted by tumours promote immune tolerance promoting the invasion and metastasis of melanoma. Adapted from (Fukuda K et al., 2012).

Tumour secretion of chemokines including CXCL9,10, or 11 induces innate and adaptive immune responses by attracting CXCR3 positive Natural Killer cells, and CD4+/CD8+ tumour infiltrating lymphocytes to the microenvironment, which correlates with both better prognosis and anti-tumour responses (Villegas *et al.*, 2002; Pan *et al.*, 2006). Pro-inflammatory chemokines CCL3, CCL4 and CCL5 also recruit CCR5 expressing immune cells with anti-tumour activity (Viola *et al.*, 2012).

However the majority of infiltrating immune cells are tumour associated macrophages (TAMs) also named kuppfer cells if resident in the liver, and are attracted by CCL2 and CCL5 produced by both tumour and stromal cells (Figure 1.13)(Talmadge, 2011). Classically activated macrophages with a M1 phenotype respond to Th1 helper T cell responses exerting pro-inflammatory activity and anti-tumoural effects via secretion of reactive oxidative species (Kiefer and Siekmann, 2011). Conversely, macrophage colony-stimulating factor (MCSF-1) and IL-10 secreted by tumour cells may induce differentiation of monocytes towards M2 macrophages which appear exclusively in growing tumours, and by suppressing the adaptive immune response are equipped to generate a pro-tumorigenic microenvironment (Hagemann *et al.*, 2006; Qian and Pollard, 2010). M2 macrophages themselves also secrete chemokines, that attract Th2 and T regulatory cells devoid of anti-tumoural cytotoxic action (Balkwill, 2012).

In addition to shaping the nature of the inflammatory infiltrate, the chemokine system can also directly or indirectly contribute to tumour angiogenesis. Chemokines can promote tumour angiogenesis by acting on endothelial cells surrounding the tumour expressing chemokine receptors. Chemokines that contain a conserved ELR amino acid motif (ELR+), such as CXCL5 and CXCL8 are pro-angiogenic and stimulate CXCR1/CXCR2 receptors on local endothelial cells inducing survival and proliferation required for the induction of angiogenesis. Although CXCL12 is a chemokine without a conserved ELR amino acid motif (ELR-) which typically promotes angiostasis, it nevertheless has potent angiogenic properties and acts on CXCR4 receptors on endothelial cells synergistically with VEGF inducing proliferation and cell migration (Kryczek *et al.*, 2005). In contrast, Interferon-γ induces the expression of CXC chemokines lacking the ELR motif and in contrast is highly anti-angiogenic, inhibiting endothelial cell proliferation, or secretion of VEGF and FGF (Strieter *et al.*, 1995; Romagnani *et al.*, 2001). Due to the opposing effects of ELR+ and ELR- chemokines it is hypothesised that angiogenesis may in part be determined by the balance between ELR+ and ELR- chemokine

secretion and consequently affects patient outcome (Allavena *et al.*, 2011) (Figure 1.14). Indirectly, the chemokine system may further contribute to angiogenesis by the recruitment of leukocytes, TAMs, myeloid-derived suppressor cells, dendritic cells to the tumour site that release pro-angiogenic factors further contributing to the microenvironment (Lewis and Pollard, 2006; Mantovani *et al.*, 2006; Sozzani *et al.*, 2007).

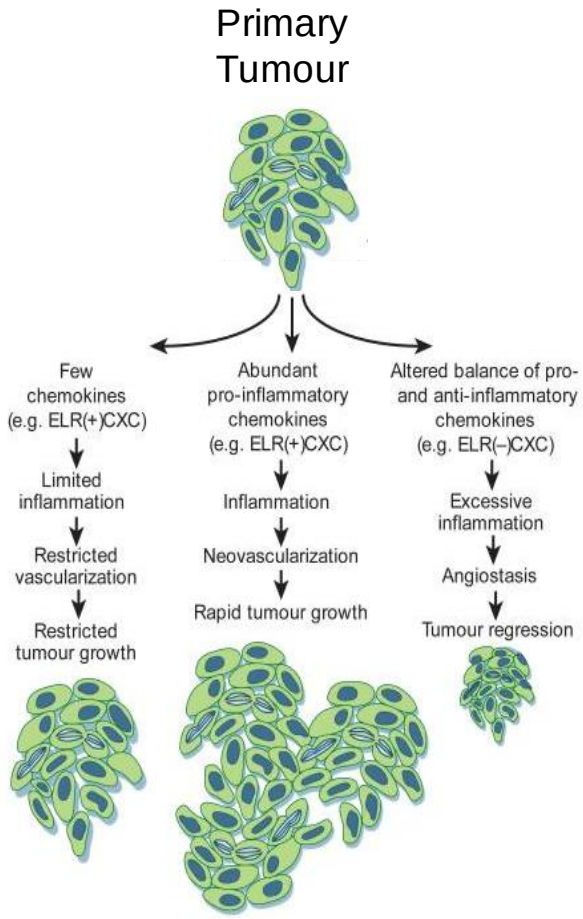

Figure 1.14 The Differing Tumour Outcomes Determined by Chemokine Regulation of Angiogenesis.

Angiogenesis is a result of an imbalance of chemokines favouring overexpression of ELR+ CXC pro-inflammatory chemokines with lack of ELR- CXC anti-inflammatory chemokines. Low chemokine secretion by the tumour may restrict tumour growth. Excessive ELR- CXC chemokines induces inflammation and angiostasis where as excessive ELR+ chemokine secretion tips the angiogenic balance towards neovascularization and tumour growth. Adapted from (Coussens and Werb, 2002).

However, cancers greatest manipulation of the chemokine system is the up-regulation of chemokine receptors enabling tumour migration, a pre-requisite for metastasis. Tumour cells preferentially metastasise to specific regions, a concept first coined by Stephen Paget, based on his observations that breast cancer preferentially spreads to the liver but not the spleen (Paget, 1889). This idea of cancer organ specific metastasis has developed into the 'seed and

soil hypothesis' which theorises that the destination of metastasis is determined by the cancer cell itself (seed) and development of secondary metastasis, also determined by environmental conditions at the metastatic site (soil) (Fidler, 2003). One explanation for the 'seed and soil' hypothesis may lie with the ability of cancer to manipulate the chemokine system, as chemokine receptor expression on tumour cells and chemokine gradients generated from specific organs may define the organ specific destination of the tumour and the preferential site of metastasis. CXCL12, is secreted by lymph nodes, bone marrow, liver and lungs under normal physiological processes to attract haematopoietic cells (Nagasawa T, 2000). However the upregulation of CXCR4 by melanoma cells generates an attraction towards peak CXCL12 secreting sites, unsurprisingly the common sites of metastasis (Furusato *et al.*, 2010). More recently studies indicate the expression of CXCL12 by liver myofibroblasts promotes the migration of CXCR4 tumour cells to the liver and this may be inhibited by use of the CXCR4 inhibitor AMD-11070 (O'Boyle *et al.*, 2013).

Given the multifaceted role chemokines play in cancer cell progression, immune evasion, angiogenesis and migration, CXCR4 expression may represent a powerful independent biomarker in melanoma, in particular given the correlation of high expression in primary cutaneous melanomas with tumour ulceration, increased Breslow tumour thickness, and a worse prognosis (Longo-Imedio *et al.*, 2005; Scala *et al.*, 2005; Tucci *et al.*, 2007). Further, CXCR4 expression in liver metastatic cutaneous melanoma tumours correlates with reduced patient survival (Joseph Kim *et al.*, 2006). Interestingly, increased CXCR4 expression in uveal melanomas correlates with an epithelioid phenotype, a prognostic indicator of metastasis (Scala *et al.*, 2007). Furthermore, CXCR4 expression, both *in vitro* and *in vivo*, predisposes the migration of uveal melanoma cells towards CXCL12 which may be inhibited by both CXCR4 inhibitors and RNAi mediated knockdown (Di Cesare *et al.*, 2007a; Li *et al.*, 2008; Li *et al.*, 2009). Studies therefore suggest that as in cutaneous melanoma, CXCR4 expression in uveal melanoma maybe a bad prognostic feature and that uveal melanoma may use the CXCR4/CXCL12 chemotaxis axis to mediate organ specific homing to the liver. Although, additional factors have been implicated in the preferential homing of uveal melanoma to the liver including; hepatocyte growth factor-scatter factor (HGF/SF) (Rusciano D *et al.*, 1993; Rusciano *et al.*, 1994; Rusciano *et al.*, 1998), and Insulin like growth factor 1 (IGF-1) secreted by the liver (Bakalian *et al.*, 2008), targeting the CXCR4-CXCL12 chemokine axis may

nevertheless represent a viable therapeutic strategy through which to prevent the metastasis of cutaneous or uveal melanoma.

1.5.2 CXCR7

The activation of CXCR4 receptor by its cognate ligand CXCL12 results in the activation of ERK and AKT controlling multiple downstream cellular effects such as chemotaxis, proliferation, survival and adhesion, hence the CXCR4/CXCL12 axis an attractive anti-tumour target. However the maintained binding of CXCL12 to cells without CXCR4 expression, coupled with the observation of incomplete cessation of cell chemotaxis towards CXCL12 with use of CXCR4 inhibitors, highlights the possibility of another CXCL12 binding receptor (Burns *et al.*, 2006). Recently this receptor has been identified as CXCR7 or RDC-1 a seven transmembrane protein, that binds to CXCL12 with 10 times the affinity of CXCR4 (Balabanian *et al.*, 2005). Additionally CXCR7 also binds CXCL11 (I-TAC) chemokine but with much lower affinity (Burns *et al.*, 2006). Despite having many features of classical G-protein coupled chemokine receptors, CXCR7 has a small alteration in the typical chemokine receptor DRYLAIV motif which is essential for recruitment and activation of G proteins and subsequent calcium mobilisation (Thelen and Thelen, 2008). This lack of G-protein mediated signalling and inability to induce calcium mobilization, leads CXCR7 to be regarded as a 'decoy' receptor that, 'scavenges' (removal) monomeric CXCL12 to dampen, inhibit or regulate CXCR4-CXCL12 signalling (Naumann *et al.*, 2010). Chemotaxis models in zebra fish have demonstrated CXCR7 mediated scavenging of CXCL12 from the extracellular space, internalising bound CXCL12, trafficking it to the lysosome for degradation, hence generating a chemokine gradient required for a differential CXCR4 mediated chemotactic response (Boldajipour *et al.*, 2008). The potent ability of CXCR7 to modulate circulating chemokine levels, has been further demonstrated in CXCR7 knock out mice, as well as by the use of a CXCR7 inhibitor *in vivo*, where plasma CXCL12 levels increased up to 5 fold (Berahovich *et al.*, 2014). CXCR7's modulation of circulating CXCL12 levels has also been shown to effect leucocyte CXCL12 homing, and so may indirectly modulate tumour infiltration (Berahovich *et al.*, 2014).

Chemokine receptors are known to dimerise and oligomerise depending on the expression level of the receptors, with CXCR4 and CXCR7 forming both homo- and hetero-dimers with equal efficiency (Levoye *et al.*, 2009). CXCR7 has the ability to modulate CXCR4 G-protein signalling, with CXCR4-CXCR7 heterodimers found to enhance CXCL12 induced calcium

signalling, and delay ERK activation and cell migration compared to CXCR4 signalling alone (Sierro *et al.*, 2007). The co-expression of CXCR7 and CXCR4 has been found to reduce the ability of CXCR4 to interact with G proteins, suggesting that there is a shift from G-protein to β-arrestin signalling in CXCR4-CXCR7 heterodimers, highlighting CXCR7 as a potential modulator CXCR4-CXCL12 signalling (Décaillot *et al.*, 2011a). Further, discoveries demonstrate that CXCR7 alone can activate the AKT, PKC and MAP-kinase pathway independent of G-protein signalling, via β-arrestin recruitment in a ligand dependant manner, suggesting CXCR7 has functions other than that of a decoy receptor, and which may affect cell proliferation and survival (Rajagopal *et al.*, 2010).

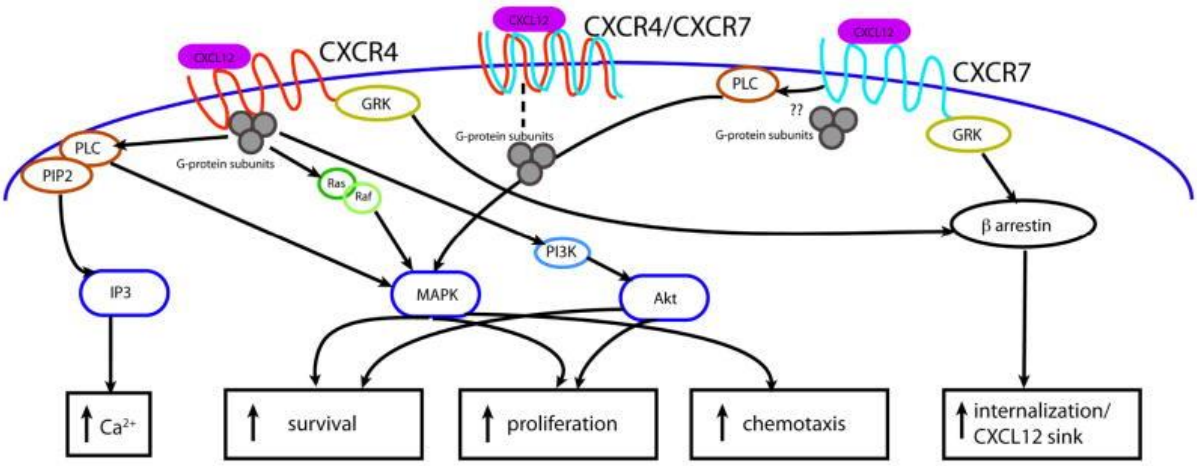

Figure 1.15 The CXCL12 Signalling Pathway.
CXCR4 activation by CXCL12 leads to cell survival, proliferation and cell chemotaxis by activation of cell signalling through PI3K/AKT, IP3 and MAPK pathways. CXCR7 signals through β arrestin to internalise and sink CXCL12, or in some circumstances signals through AKT, PLC/MAPK to promote cell survival, proliferation, adhesion and chemotaxis of CXCR4 negative cells (Wang et al., 2008b). Heterodimerisation of CXCR4 and CXCR7 is thought to recruit β arrestin and induce conformational rearrangements to the CXCR4/G-protein complex abrogating CXCR4 cell signalling. Taken from (Duda et al., 2011).

Given the large influence the chemokine system plays in tumourigenesis and metastasis and the newly identified biological functions of CXCR7, efforts have been made to elute the role of CXCR7 in cancer. CXCR7 is expressed in normal human tissues and commonly restricted to vascular endothelial cells of the kidney, heart, brain, lung, thyroid and spleen, however CXCR7 may be upregulated by many cancer cells including breast, lung, prostate and glioma, suggesting an advantageous role in tumorigenesis (Burns *et al.*, 2006; Miao *et al.*, 2007; Wang *et al.*, 2008b; Berahovich *et al.*, 2014). In support of this, the tumour suppressor gene, hypermethylated in cancer 1 (HIC1) supresses CXCR7 expression, consistent with CXCR7 upregulation during tumourigenesis (Van Rechem *et al.*, 2009). Further, a role of CXCR7 in

cancer growth and survival has been demonstrated in breast cancer, where tumour cells overexpressing CXCR7, proliferated more rapidly into larger tumours than control cells, and in lung cancer where the use of a CXCR7 inhibitor reduced tumour growth (Décaillot *et al.*, 2011b; Hernandez *et al.*, 2011). In prostate cancer, CXCR7 is highly expressed and correlates with disease aggressiveness, associated with increased cell proliferation, adhesion and migration, suggesting CXCR7 expression may be of prognostic value in some cancers (Wang *et al.*, 2008b). CXCR7 has also been postulated to have a role in differentiation demonstrated by exclusive CXCR7 expression in mature and differentiated glioblastomas or neuroblastomas (Hattermann *et al.*, 2010; Liberman *et al.*, 2012). Interestingly, it has been reported that melanoblast cell migration is determined by CXCL12-CXCR4 signalling however mature melanocytes express both CXCR4 and CXCR7 receptors and migrate, signalling via CXCR7 and β-arrestin-MAPK pathways, suggesting a switch between receptor function during differentiation (Belmadani *et al.*, 2009; Lee *et al.*, 2013). In general, accumulating evidence proposes CXCR7 and CXCR4 may have differential functions where CXCR7 signalling is associated with increased cell adhesion and survival and CXCR4 determines cell migration enhancing cancer metastasis, although co-expression of both receptors has also demonstrated contradictory effects including reduced invasiveness and metastasis (Mazzinghi *et al.*, 2008; Levoye *et al.*, 2009; Décaillot *et al.*, 2011a).

Although there have been no reports of CXCR7 expression by melanoma tumour cells, notably CXCR7 is highly expressed by most tumour associated blood vessels including in melanoma and hence, proposed as a marker for tumour vasculature (Sánchez-Martín *et al.*, 2011a). CXCR7 expression on tumour endothelial cells is upregulated by hypoxia (HIF-1α induction) and by factors secreted in the immediate tumour microenvironment, including VEGF and CXCL8 (Maksym *et al.*, 2009b). Interestingly, in prostate cancer CXCR7 has been shown to increase the secretion of angiogenic factors such as VEGF and IL-8 and its overexpression has resulted in higher density of neovessels in tumours (Wang *et al.*, 2008b). It has also been demonstrated that CXCR7 expression on endothelial cells results in the redistribution of platelet endothelial cell adhesion molecule-1 (PECAM-1) altering endothelial cell barrier function, hence effecting vascular homeostasis (Totonchy *et al.*, 2014). Paradoxically, CXCR4-CXCL12 signalling by endothelial cells has been demonstrated to supress endothelial permeability (Kobayashi *et al.*, 2014). Overall this suggests a role of CXCR7 in tumour angiogenesis and highlights CXCR7 as a possible anti-angiogenic therapeutic target, however

it must be noted that a direct link between CXCR7 expression and stimulation of endothelial cell chemotaxis has not yet been established.

It is likely that the role of CXCR7 in cancer maybe dynamic, differing between tumour types, cells within tumours, stages of progression or expression by tumour associated vasculature. It is clear the distinct or intercalating roles of CXCR4 and CXCR7 are yet to be established in melanoma. Given the potential contribution of the CXCL12-CXCR7 axis to processes that were previously thought to be monogamously CXCL12-CXCR4 driven, CXCR7 expression deserves some consideration.

1.5.3 CXCL12

Chemokines are small 7-12 kDa chemoattractant peptides, that have 4 conserved cysteine residues that form 2 disulphide bonds, classified into 4 subfamilies, CXC, CC, CX_3C and C, based on the arrangement of the first 2 cysteine's (where X is any amino acid). The CXC chemokine ligand CXCL12 also named stromal cell derived factor-1 (SDF-1) or pre-B cell growth stimulating factor (PBSF), is constitutively expressed by bone marrow cells, and binds to the G-protein coupled chemokine receptors CXCR4, CXCR7 and glycosaminoglycans. CXCL12 is expressed by many different tissues and organs including lymph nodes, liver and lungs, attracting CXCR4 expressing cells. CXCL12 exists in 2 isoforms generated by alternative splicing of the single CXCL12 gene on chromosome 10, generating an 89 amino acid CXCL12α peptide or a CXCL12β peptide with 4 additional amino acids at its carboxy terminal end (Kryczek *et al.*, 2007). In our bodies CXCL12 exists in a monomer-dimer equilibrium, of which both states have been shown to activate CXCR4 (Veldkamp *et al.*, 2005). Interestingly, the half-life of CXCL12 is only minutes, as the ligand may be degraded by numerous biological enzymes including matrix metallioproteinase-2 (MMP-2) (McQuibban *et al.*, 2001).

CXCL12 was the first chemokine demonstrated to be critical for development processes, with CXCL12/CXCR4 knock out mice having deficient hematopoiesis, cardiogenesis, vascular formation and neurogenesis precluding embryonic viability (Nagasawa *et al.*, 1996; Ma *et al.*, 1998; Tachibana *et al.*, 1998; Zou *et al.*, 1998). CXCL12 is a strong chemoattractant for immature hematopoietic stem cells and progenitor cells, and has been shown to be critical for their embryonic migration and homing to bone marrow during ontogeny (Ara *et al.*, 2003). Importantly CXCL12 also plays a key role in immune cell regulation, promoting B cell precursor

proliferation and migration of mature B cells and plasma cells to the bone marrow (Tokoyoda *et al.*, 2004).

Although the physiological function of CXCL12 during development is well established, its role within skin is much less well defined. In normal skin CXCL12 is documented to be present in the basal layer of epidermis, papillary dermis, small capillary vessels of the sub papillary plexus ,endothelial layer of blood vessels, pericytes, fibrous sheath of hair follicles, sweat glands, mature sebocytes, axons, and small blood vessels in nerve tissue (Pablos *et al.*, 1999; Avniel *et al.*, 2006). Keratinocytes and dermal fibroblasts however, are not believed to express CXCL12 under normal physiological conditions, but may display increased expression in inflamed and wounded skin with dermal fibroblasts additionally reported to secrete CXCL12 under stress during hypoxia, irradiation and burns (Avniel *et al.*, 2006). It is postulated that CXCL12 in injured skin promotes the attraction of immune cells to the damage site including epidermal stem cells, encouraging the re-epithelialisation of the epidermis (Guo *et al.*, 2015). Further, the CXCL12 gradient generated between the epidermis and dermal skin layers promotes epidermal mature Langerhans cells (highly CXCR4 positive) to migrate into the dermis for effective antigen presentation in the immune response to injured skin (Ouwehand *et al.*, 2008).

Though it is well established that the secretion of CXCL12 by endothelial cells, fibroblasts, and osteoblasts in organs determines the migration and specific metastasis of CXCR4 positive cancer cells, the role of CXCL12 in the primary tumours remains poorly understood. Tumoural expression of CXCL12 has been documented in a plethora of cancers including prostate, breast and pancreatic cancer (Koshiba *et al.*, 2000; Bachelder *et al.*, 2002; Darash-Yahana *et al.*, 2004). In breast and prostate cancer CXCL12 expression has been strongly correlated with lymph and bone marrow metastasis, and for which secretion of CXCL12 by tumour stromal fibroblasts is reported to contribute to primary tumour vascularisation or transformation (Muller *et al.*, 2001a; Taichman *et al.*, 2002; Orimo *et al.*, 2005). Hypoxia is thought to be a common trigger of CXCL12 upregulation by cancer cells, via induction of hypoxia inducible factor (HIF-1) of which there are 2 potential binding sites in the promoter region of the CXCL12 gene. There is evidence that CXCL12 has the ability to transduce tumour cell proliferation through activation of AKT and ERK cell signalling pathways in many cancers (Julie and Kenneth, 2003; Marchesi *et al.*, 2004). Furthermore, CXCL12 has also been reported to regulate tumour cell apoptosis through activation of anti-apoptotic pathways, such as activation of NF-κB

signalling, or indirectly by activating integrins such as integrin α4, resulting in increased tumour cell adherence, and preventing chemotherapy induced death (Helbig *et al.*, 2003; Hartmann *et al.*, 2005). Tumoural CXCL12 secretion may also facilitate the escape of tumour cells from immune surveillance since CXCL12 is able to recruit CXCR4+CD4+ T regulatory cells from the bone marrow (Zou *et al.*, 2004b). However, some beneficial effects of CXCL12 in the tumour microenvironment are perceived by the recruitment of CXCR4+ cytotoxic T cells that promote tumour immunity (Zhang *et al.*, 2005). The greatest effects of CXCL12 in cancer are most frequently demonstrated during the process of metastasis. CXCL12 can encourage the dissemination of cancer cells from the primary site or deeper invasion not only by CXCR4-CXCL12 chemotaxis but through CXCL12 induction of metalloproteases, enzymes that degrade the extracellular matrix assisting in the migration of cancer cells (Figure 1.16) (Burger *et al.*, 2003; Singh *et al.*, 2004). Given that many cancer cells express CXCR4 and upregulate the secretion of CXCL12 or have CXCL12 expressed in the tumoural microenvironment, logic suggests that the expression of both CXCR4 and CXCL12 would retain tumour cells at the primary site. However, CXCL12 isn't always expressed throughout the whole tumour (Kryczek *et al.*, 2007), and tumour cells may nevertheless become sensitised to CXCL12 in the local environment, thereby preventing such retention. Another explanation maybe that cancer cells may become desensitised to CXCL12 in the local environment. Cancer cells can regulate their sensitivity to CXCL12 by modulating their expression of CD26 or dipeptidyl peptidase IV (DPPIV) an extracellular peptidase (Kryczek *et al.*, 2007). The absence of CD26 leads to increased homing of HSCs towards CXCL12 gradients (increased sensitivity) (Christopherson *et al.*, 2004). CD26 is expressed on endothelial cells but is often lost by cancer cells, yet can be upregulated in response to hypoxia (Wesley *et al.*, 2004; Wesley *et al.*, 2005). The presence of CD26 by tumour cells in response to hypoxia could desensitise them to local CXCL12 thereby aiding movement from the CXCL12 rich primary site. Overall, the effects of CXCL12 on tumour proliferation, survival, angiogenesis, immunity, and organ specific metastasis suggest that CXCL12 in the tumour microenvironment is a detrimental effect and hence maybe a viable therapeutic target.

In the context of melanoma, the role of CXCL12 in tumourigenesis has yet to be fully elucidated. The largest immunohistochemical study of CXCL12 expression in 107 primary cutaneous melanomas suggested lack the of CXCL12 tumoural expression as a poor prognostic indicator (Mitchell *et al.*, 2014). A further small study demonstrated a positive correlation

between CXCR4 expression and CXCL12, however this was contradicted by a larger study in which no such correlation or any association with CXCL12 expression and tumour B-RAF mutational status was observed (Toyozawa *et al.*, 2012). Moreover, this contradicts the findings of an interesting study where the induced expression of mutated B-RAF V600E in wild-type melanoma cells enhanced the secretion of cytokines IL-1β, IL-6 and IL-8 and MMP-1, and in which the derived supernatants promoted the secretion of CXCL12 in dermal fibroblasts, suggesting a link between melanoma mutational status and CXCL12 secretion by dermal fibroblasts (Whipple and Brinckerhoff, 2014).

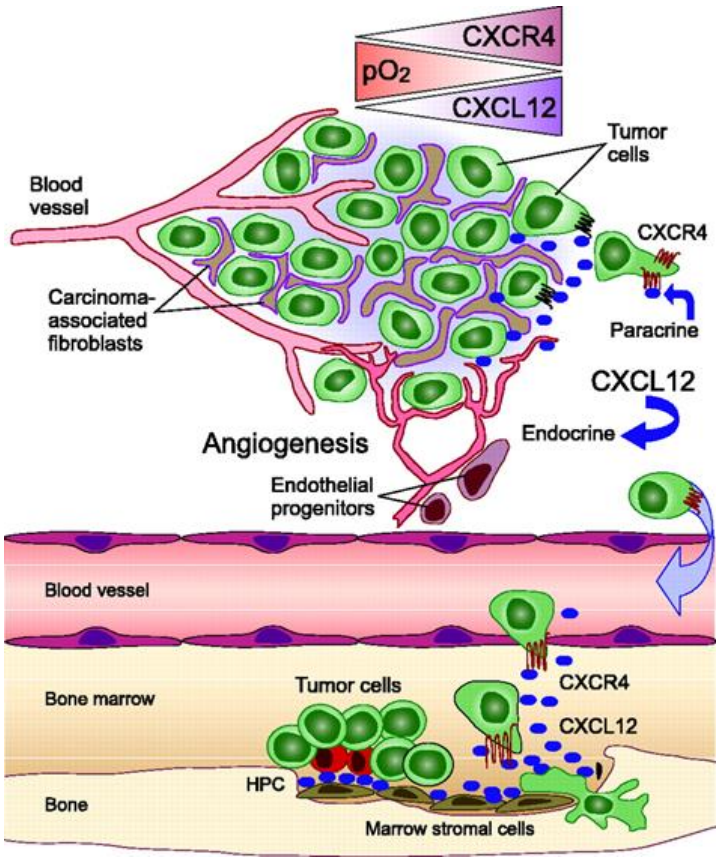

Figure 1.16 Effects of CXCR4 and CXCL12 within the Tumour Microenvironment
Hypoxic areas of solid tumours induce the expression of CXCR4 on tumour cells and CXCL12 secreted from fibroblast stimulating tumour progression. CXCL12 stimulates tumour growth by activating CXCR4 on tumour cells, and angiogenesis by recruiting endothelial progenitor cells to the tumour microenvironment. CXCR4 positive tumour cells hijack the CXCR4-CXCL12 migratory path used by haematopoietic progenitor cells and travel to CXCL12 secreting sites such as the bone marrow, where they invade into tissues forming a secondary metastasis (Burger and Kipps, 2006).

In terms of uveal melanoma, studies of CXCL12 expression are limited with just one study reporting expression in 27% of primary tumours, and the expression of the chemokines CXCL19 and CXCL21 which bind to CCR7, the predominantly expressed chemokine receptor (Dobner *et al.*, 2012). Nevertheless, the eye is likely a CXCL12 rich environment with

documented expression of CXCL12 within the retina (Crane *et al.*, 2000; Hasegawa *et al.*, 2008; Lai *et al.*, 2008; Otsuka *et al.*, 2010). How such expression may affect uveal melanoma tumorigenesis, or the interplay of tumour cells with the stroma within the eye is unknown. Given the perceived diverse role of CXCL12 within tumours and the immediate microenvironment, a defined role of CXCL12 expression in both cutaneous and uveal melanoma progression, or use as a prognostic indicator is not fully established.

1.6 Angiogenesis and Vascular Endothelial Growth Factor Signalling

1.6.1 The Role of VEGF and Receptors in Angiogenesis

Tumour invasion alone aids local tumour spread but angiogenesis is an important prerequisite for tumour growth, deeper invasion and distant melanoma metastasis. Angiogenesis is the term applied to describe the formation of new blood vessels from existing vessels and is essential for oxygen, nutrient and growth factor supply, and for the removal of waste metabolic products from the growing tumour, subsequently aiding its metastasis (Hanahan and Weinberg, 2011). Briefly angiogenesis involves the induction of vessel sprouting by endothelial cell proliferation and migration, with eventual formation into tubular structures and maturation of new functional vessels (Figure 1.17) (Hicklin and Ellis, 2005). Although this is the traditional 'sprouting' mechanism of angiogenesis, in melanoma it is now recognised that angiogenesis may occur by additional mechanisms (Pastushenko *et al.*, 2014b). Concisely these involve intussusceptive angiogenesis where an interstitial column is inserted into a pre-existing vessels lumen, vascular co-option where tumour cells migrate along existing vessels, mosaic vessels where melanoma cells become part of the vessel walls, vasculogenic mimicry where the melanoma cells upregulate endothelium associated genes and form vascular like networks, and finally bone marrow derived vasculogenesis where tumour cells recruit endothelial progenitors from bone marrow to form vessel walls (Pastushenko *et al.*, 2014b).

The process of angiogenesis is thought to be regulated by a similar set of oncogenic mutations that drive melanoma progression, including abnormal cell signalling of MAPK, PI3K, NFκB pathways and generation of reactive oxygen species all of which have been shown to enhance the secretion of pro-angiogenic and principally vascular endothelial growth factor (VEGF) (Arbiser *et al.*, 1997; Amiri and Richmond, 2005; Pastushenko *et al.*, 2014b).

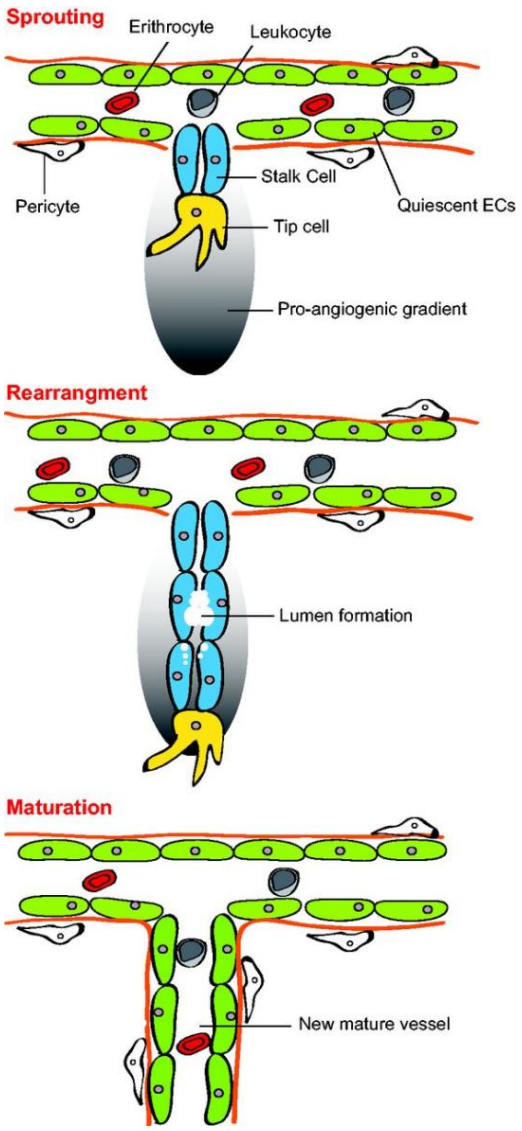

Figure 1.17 Sprouting Angiogenesis

There are 4 major sequential steps of angiogenesis that turn a vascular sprout from a pre-existing vessel into a mature vessel supplying blood. (1) Sprouting: Pro-angiogenic stimuli (e.g VEGF) initiates sprouting and induces quiescent endothelial cells to proliferate and migrate. There are two types of activated endothelial cells, tip cells that are located at the head of the sprout and are more migratory and stalk cells that elongate and proliferate rapidly causing sprouting. In response to angiogenic stimuli pericytes detach from the endothelium and migrate through the basement membrane. (2) Tube Morphogenesis: After migrating from the original vessel the stalk cells form vacuoles by pinocytosis, which conjoin with each other to form an interstitial vessel column. (3) Adaptation: The endothelial tubes will then regress or go on to mature into permanent vessels. The decision is mediated by pro-angiogenic factors. If the pro-angiogenic stimuli are lost the endothelial tubes will dismantle and the cells die. Maintained pro-angiogenic and growth factor stimuli the vessel will be sustained. (4) Maturation: Endothelial cells cease proliferation and become quiescent once more. Circulatory anastomosis provide the new vessel with a blood supply. Tight junctions form and pericytes encase the new vessel providing pro-survival signals to maintain homeostasis of the endothelial cells. (Taken from (Irvin et al., 2014)).

Angiogenesis is tightly regulated by the opposing balance between pro- and anti-angiogenic factors. It is thought that dysregulation in this balance, favouring pro-angiogenesis factors

within the tumour microenvironment causes an “angiogenic switch” and the evolution of a phenotype characteristic of the metastatic disease (Hanahan and Weinberg, 2011). Pro-angiogenic regulators include VEGF, basic fibroblast growth factor (bFGF), placental like growth factor (PLGF), angioprotein-2, angiogenin, interleukin-8 and endostatin, with VEGF signalling a key factor to cancer progression.

The VEGF family consists of 5 secreted angiogenic and lymphangiogenic glycoprotein ligands VEGF A, B, C, D and placental growth factor (PIGF). VEGF A is the most characterised ligand and exists in seven pro-angiogenic isoforms and five anti-angiogenic isoforms as a result of alternative RNA splicing, with the VEGF 165 predominantly secreted by cancer cells and infiltrating immune cells (Ferrara and Davis-Smyth, 1997). VEGF C and D are synthesised as precursor proteins with long N and C terminal pro-peptides that are removed under proteolytic cleavage by pro-peptidases to their mature forms. The ligands bind and activate 3 class III receptor tyrosine kinases with different specifics and functions termed VEGF receptor 1, 2, 3 (VEGFR1, 2, 3) (Figure 1.18). VEGF binds with greatest affinity to VEGFR1 with nearly 10 fold greater affinity than to VEGFR2, however the complex has weak tyrosine kinase activity with strongest intercellular signalling response mediated by VEGFR2 (Koch and Claesson-Welsh, 2012). VEGFR1 can also exist as a soluble isoform that can act like a decoy receptor sequestering VEGF in the local environment, and so may in part regulate VEGFR signalling (Marika J Karkkainen and Petrova, 2000).

VEGFR2 exists in an immature form located intracellularly within the golgi apparatus and translocates to the endoplasmic reticulum where it undergoes N-linked glycosylation in a secretory pathway to produce a fully mature form, before transport to the plasma membrane. When ligand activates the VEGF receptor the receptor undergoes conformational changes that include homo or hetero-dimerisation exposing the tyrosine kinases ATP binding site. Trans-autophosphorylation of the tyrosine kinase site generates a docking site for SH2-domain adaptor molecules and activation of downstream signalling cascades. Inactivation of the receptor is under the strict control and mediated by ubiquitination, followed by receptor internalisation, dephosphorylation and degradation by protein tyrosine phosphatases. VEGFR2 becomes ubiquitinated after activation and is internalised into early endosomes, where it may be recycled back to the plasma membrane or cleaved of its C-terminus before trafficking to late endosomes where it will undergo lysosomal processing and terminal degradation. Interestingly VEGFR2 is able to continue to transduce cell signalling cascades

once internalised until it is committed to either a recycling or degradation pathway (Murdaca *et al.*, 2004). VEGF receptor cell signalling may also be modulated in part by the promiscuous interactions of VEGF with other receptors including MET receptor or by VEGF mediated activation of additional receptors such as neuropilin receptor 2 (Cao *et al.*, 2012; Lu *et al.*, 2012).

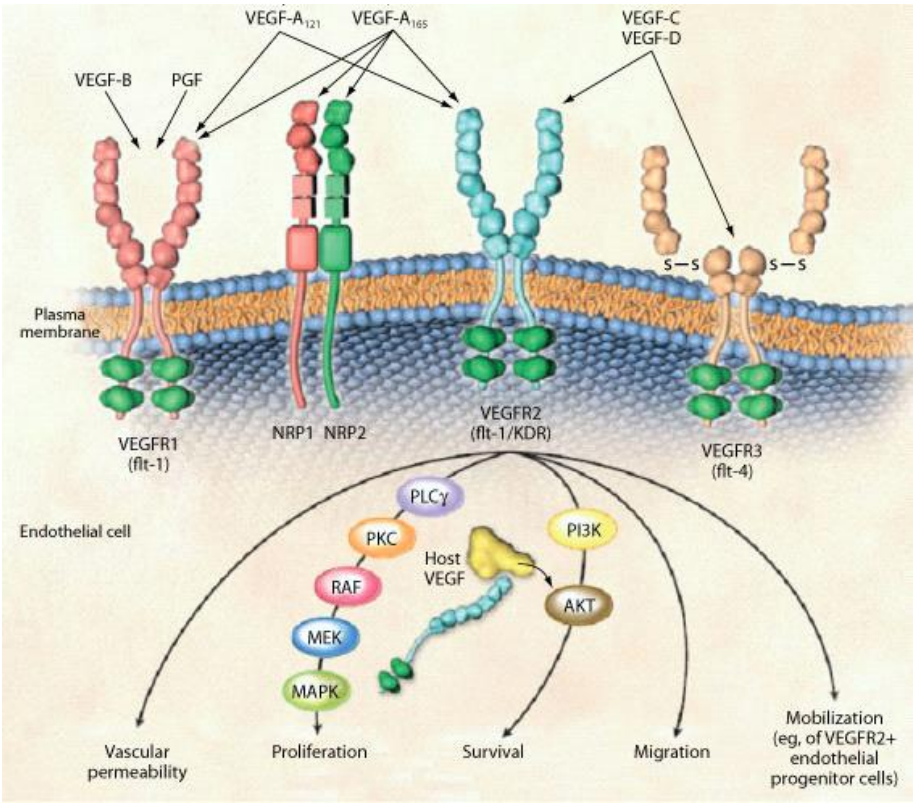

Figure 1.18 Vascular Endothelial Growth Factor Ligands and Receptors
The five mammalian VEGF ligands have specific receptor binding specificities. VEGF-B and Placental growth factor bind exclusively to VEGF receptor 1. VEGF-A binds to both VEGF receptors 1 and 2, and can form heterodimers with PGF also activating VEGFR2 (Ellis and Hicklin, 2008b). VEGF-C and D bind VEGF receptor 3 and after proteolytic cleave may bind VEGF receptor 2 additionally (Ellis and Hicklin, 2008b). VEGF signalling can cause many functional changes in endothelial cells. VEGF can promote vascular permeability by inducing endothelial fenestrations, opening junctions between adjacent endothelial cells and recruitment of vesicovascuolar organelles (Hicklin and Ellis, 2005). Endothelial cell proliferation is induced by activation of mitogen activated protein kinases, and cell survival via induction of PI3K/AKT, Bcl-2, surviving, XIAP or inhibition of caspases (Hicklin and Ellis, 2005). Migration of endothelial cells occurs by activation of FAK, P38 and nitric oxide, with invasion promoted by initiation of metalloproteinase secretion (Hicklin and Ellis, 2005). Adapted from (Veena Shankaran, 2008).

VEGF secreted from cancer cells or host stromal cells mediates angiogenesis in a number of different ways through stimulation of its cognate receptors expressed by local endothelial cells, lymphatic endothelial cells or haematopoietic cells. A key microenvironmental regulator of VEGF expression is oxygen deprivation, that activates the hypoxia inducible factor-1 (HIF-1)

(Iliopoulos *et al.*, 1996). In hypoxic conditions such as in the avascular core of solid tumours, HIF-1α and HIF-β are not degraded by the ubiquitin proteasome pathway, leading to their dimerization and translocation to the VEGF promoter, increasing VEGF transcription as a tumour survival mechanism (Iliopoulos *et al.*, 1996).

One of VEGF's most prominent angiogenic features is its potent ability to increase local microvascular permeability which is thought to be mediated by induction of vesicovascular organelles, endothelial fenestrations, or by the opening of inter-endothelial cell junctions (Ferrara, 1999; Dvorak, 2002). VEGF further promotes angiogenesis by inducing local endothelial cell migration, by inducing genes involved in integrin expression and cell cytoskeleton changes aiding cell movement (Dvorak, 2002). The migration of endothelial cells through the basement membrane is further aided by the ability of VEGF to induce metalloproteinases and serine proteases secretion, important in the degradation process of the extracellular matrix (Zachary and Gliki, 2001). Further, an important feature of angiogenesis is the promotion of endothelial cell survival and proliferation mediated by VEGF dependent activation of key signalling mechanisms including PI3K-Akt signalling, upregulation of anti-apoptotic BCL-2 family protein bcl-2, and inhibitors of apoptosis proteins, survivin and XIAP (Zachary and Gliki, 2001).

In addition to stimulating angiogenesis, VEGF-C and D secreted from cancer cells are thought to induce lymphangiogenesis (lymphatic endothelial proliferation), as a way to expand the lymphatics and make a more favourable pre-metastatic niche for cancer cell arrival, enhancing the likelihood of distant metastasis (Hirakawa *et al.*, 2005; Harrell *et al.*, 2007; Hirakawa *et al.*, 2007). This maybe particularly evident in melanoma, as high rates of lymphangiogenesis are a biomarker for sentinel node metastasis (Dadras *et al.*, 2005).

In addition to the effect VEGF has on endothelial cells, many more cell types are affected by VEGF signalling within the immediate tumour microenvironment. Notably immune cells may express VEGF receptors and respond to VEGF, of particular importance are T regulatory cells that supress anti-tumour immune responses (Hansen *et al.*, 2012). Macrophages and stromal fibroblasts both secrete VEGF and their presence in the tumour microenvironment may contribute to angiogenesis (Galdiero *et al.*, 2013).

1.6.2 VEGF and Receptor Expression in Melanoma

As cutaneous and uveal melanomas are highly vascularised cancers, the VEGF ligand and receptor axis forms a critical angiogenic pathway. It has been demonstrated that angiogenesis is often induced early in melanoma tumour development and in tumours over $1mm^3$ in size require angiogenesis to avoid hypoxia (Folkman, 1971). In cutaneous melanoma increased secretion of pro-angiogenic regulators are evident in the serum of patients, and have been correlated with shorter progression free survival and lower overall survival (Donnini *et al.*, 1999; Ugurel *et al.*, 2001; Kurschat *et al.*, 2007; Helfrich *et al.*, 2009). Immunohistochemical studies have additionally shown VEGF secretion by 20-70% of primary cutaneous melanomas, with expression correlating with transition from radial to vertical growth indicating angiogenesis as an essential step of melanoma progression (Erhard H *et al.*, 1997; Potti A *et al.*, 2003).

The clinical progression of uveal melanomas is often slower than for cutaneous melanomas with metastasis developing even 10 years after primary tumour treatment, suggesting a period of disease dormancy (el Filali *et al.*, 2010). The rate limiting step in uveal melanoma metastasis is often the development of tumour vasculature, so it is possible that the angiogenic switch is required for the haematological dissemination and the development of metastasis. Needless to say the angiogenic profile of uveal melanoma has therefore attracted a lot of attention owing to in part to the capillary rich tissue in which uveal melanoma develops (Notting *et al.*, 2006). Studies have demonstrated primary uveal melanomas harbour areas of high vascular density and tumour growth within the lumen of tumour associated blood vessels correlating with poor prognosis (Foss *et al.*, 1996; Mäkitie *et al.*, 1999; Ly *et al.*, 2010). VEGF-A levels are also elevated in the serum, aqueous and vitreous humour of the eyes in uveal melanoma patients, correlating with patient survival, and tumour size (Sheidow *et al.*, 2000; el Filali *et al.*, 2010). Interestingly, two studies have shown that VEGF levels are raised in patients only when metastasis develops, and in a murine uveal melanoma model VEGF A serum concentrations correlated with metastatic frequency and lower survival, further supporting the role of VEGF in the dissemination of metastatic disease (Boyd *et al.*, 2002a; Missotten *et al.*, 2006; el Filali *et al.*, 2010; Barak *et al.*, 2011; Crosby *et al.*, 2011a).

Of equal importance in angiogenesis is VEGF receptor expression. It is well known that VEGF stimulates VEGF receptors on tumour endothelium in a paracrine fashion, but more recently

accumulating evidence suggests VEGF secretion can also act in an autocrine manner stimulating VEGF receptors on the surface of tumour cells themselves (Lacal *et al.*, 2000). Microarray tissue studies have additionally revealed the secretion of VEGF and expression of all 3 VEGF receptors by melanomas, with greater VEGFR2 expression detected in metastatic compared to primary cutaneous melanomas (Mehnert *et al.*, 2010). Cutaneous melanomas expressing high levels of VEGFR2 also demonstrate increased propensity to invade, with inhibition of VEGFR1 receptors reducing melanoma growth by 90%, thus supporting an autocrine role for VEGF signalling, in the promotion of tumour growth and invasion (Pedro Miguel Lacal *et al.*, 2005; Frank *et al.*, 2011). In addition studies have shown that tumours may use VEGF signalling to promote their survival as evidenced by the secretion of VEGF from VEGF receptor expressing tumour cells and the induction of MAPK dependant survival (Graells *et al.*, 2004). Interestingly, a recent paper has also shown that uveal melanoma cell lines not only express an active VEGF receptor (VEGF-R2) but they also secrete VEGF-A, postulating that uveal melanomas also use autocrine VEGF cell signalling in the same way as cutaneous melanomas (Patrick Logan *et al.*, 2013). Collectively these data provide evidence for an extended role of VEGF beyond angiogenesis and its contribution to metastatic progression and the potentiation of tumour survival, through the induction of MAPK signalling. This hypothesis predicts a vicious circle where by aberrant MAPK signalling characteristic of both cutaneous and uveal melanoma, drives VEGF secretion which in an autocrine manor, further potentiates MAPK hyperactivity, cell proliferation, survival, angiogenesis and metastasis, making VEGF signalling both tumour driving and tumour driven. Thus there is a strong biological rational for targeted VEGF and VEGFR2 inhibition as a therapeutic strategy for both cutaneous and uveal melanomas.

1.6.3 Anti-angiogenesis Therapy in Melanoma

Based on the importance of angiogenesis in local and metastatic tumour development, anti-angiogenic therapies have been trailed in both cutaneous and uveal melanomas. The premise for this therapeutic approach is to prevent tumour growth and metastasis by inhibiting new blood vessel formation, starving tumours of nutrients, oxygen and growth factors required for survival, growth and metastasis. Current therapeutic strategies involve prevention of all isoforms of VEGF binding to its receptor by use of monoclonal anti-VEGF antibodies, or inhibition of VEGF receptor signalling by use of tyrosine kinase inhibitors (Figure 1.19). So far bevacizumab a humanised monoclonal antibody targeting VEGF and tyrosine kinase inhibitors

with selectivity for VEGF receptors sorafenib and sunitinib are U.S.A food and drug administration (FDA) approved for clinical use in melanoma.

Initial studies with Bevacizumab administered as a monotherapy in cutaneous melanoma patients, only resulted in disease stabilisation in a quarter of patients, with effects deemed only modest, however in combination with cytotoxic chemotherapy, more favourable results have been demonstrated (Varker *et al.*, 2007a). Phase II trials of combined bevacizumab, carboplatin and paclitaxel have shown a trend for improved survival (Perez *et al.*, 2009). A phase III trial of bevacizumab in combination with nanoparticle albumin bound paclitaxel as a first line therapy for metastatic cutaneous melanoma demonstrated a 12 month survival rates in 83% of patients (P. Boasberg *et al.*, 2009). The apparent improvement in anti-tumour activity of cytotoxics in combination with bevacizumab is thought to be due to the reversal of the cytotoxics induction of pro-survival VEGF secretion and bevacizumab's normalisation of tumour vasculature from the chaotic hyper-permeable vasculature that often hinders cytotoxic drug delivery (Jain, 2001). In the case of uveal melanoma, bevacizumab has also shown efficacy to reduce primary tumour size and the number of micro metastasis, in models, resulting in current clinical trials of intra-vitreal bevacizumab injections, and a phase II trial in combination with temozolomide (Hua Yang *et al.*, 2010; Piperno-Neumann S *et al.*, 2012). In addition the decoy VEGF receptor drug aflibercept (VEGF Trap, an antibody with a VEGF-A binding domain of VEGFR1 and 2 fused to a Fc domain) has been used in a phase II trial of cutaneous and uveal melanoma patients with stage III/VIII inoperable melanoma, in which 4 months progression free survival was observed in 50% of uveal melanoma patients (Tarhini et al., 2011), and further supporting the use of anti-angiogenesis therapies to treat uveal melanoma.

Despite initially promising results targeting VEGF ligand with bevacizumab, this has had no effect on improving overall survival for patients with metastatic melanoma, leading to studies focusing on targeting VEGF receptors mainly with tyrosine kinase inhibitors. Tyrosine kinase inhibitors are a particularly attractive modality for inhibition of VEGF signalling, preventing signal transduction of VEGF receptors on tumour blood vessel endothelial membrane and tumour cells themselves. While some inhibitors are highly specific others display multiple tyrosine kinase specificity affecting numerous signalling pathways concurrently. Sorafenib originally developed as a B-RAF inhibitor, selectively inhibits VEGFR2,3 with some specificity for platelet derived growth factor receptor. Unfortunately sorafenib has proved of little value

for melanoma care either as a monotherapy or in combination with cytotoxics as first or second line treatment (Eisen *et al.*, 2006; Hauschild *et al.*, 2009).

Pazopanib (GSK GW786034) is a novel second generation receptor tyrosine kinase inhibitor which targets many angiogenic pathways including VEGF receptors 1, 2, 3, PDGF receptors α and β, and c-KIT, demonstrating anti-angiogenic activity *in vivo* in many types of tumours and greater potency than sunitinib or sorafenib, and with fewer serious side effects (Podar *et al.*, 2006). The additional kinase targets of pazopanib may also afford bonus therapeutic benefits, for example through its ability to inhibit PDGF receptors it may have display the additional anti-angiogenic function of disassociating pericytes from tumour vascular rendering the vascular endothelial cells more sensitive to therapy without the protective cover from pericytes (Bergers and Hanahan, 2008). The additional potential for pazopanib to inhibit c-KIT, may also be advantageous, given 28% of melanomas that arise from acral, mucosal or chronically sun damaged sites exhibit amplification or mutations in KIT receptor tyrosine kinase (Becker *et al.*, 2007). Furthermore, pazopanib has been shown to significantly improve progression free survival in metastatic renal cell cancer and soft tissue sarcoma culminating in its approval as monotherapy in the U.S.A, and its continued presence in many ongoing trials for a variety of tumour types and combinations (van der Graaf *et al.*; Sternberg *et al.*, 2010).

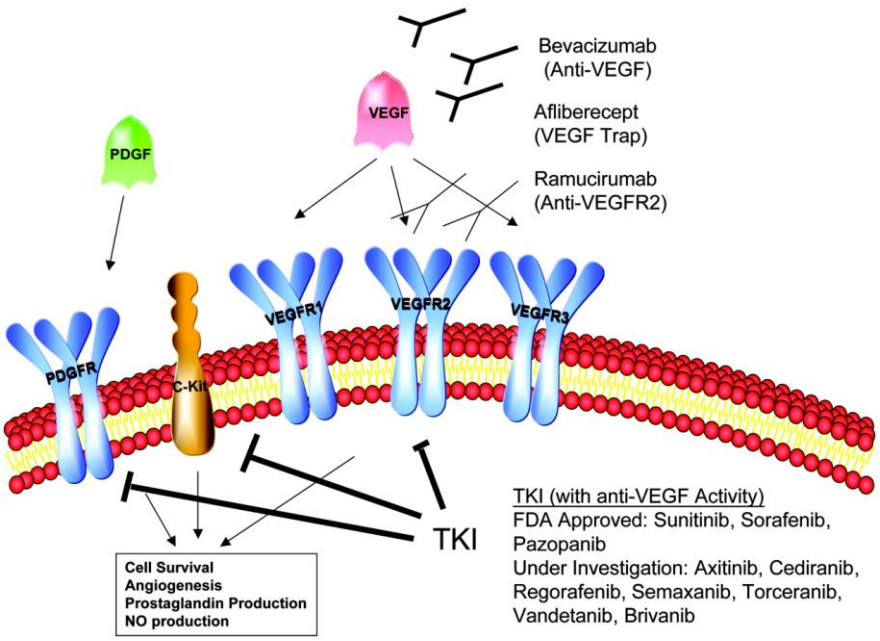

Figure 1.19 The VEGF Receptor and Ligand System and Current Anti-angiogenic Therapeutic Strategies.

Tyrosine kinase inhibitor (TKI). Platelet derived growth factor and receptor (PDGF and PDGFR). Adapted from (Nazer et al., 2011).

Pazopanib has also recently been identified as having potential clinical value in the treatment of melanoma, evidenced by observations of inhibited tumour cell growth *in vitro* in addition to its anti-angiogenic effect (Gril *et al.*, 2011).

An unbiased combinational drug screen of 108 targeted therapies on 36 melanoma cell lines identified specific synergy between VEGFR2/PDGFR inhibitors and B-RAF/MEK inhibitors in B-RAF mutated cell lines (Friedman *et al.*, 2015). This study demonstrated increased sensitivity of PLX4270 (a B-RAF-V600E inhibitor) intrinsically resistant melanoma cell lines to combined treatment with cediranib (VEGFR2/PDGFR inhibitor) and PLX4720, an effect that was replicated with MEK inhibition both *in vitro* and *in vivo* (Friedman *et al.*, 2015). This study also reported the elevated expression of VEGFR2 as a predictive marker of poor B-RAF/MEK inhibitor response, highlighting possible crosstalk between MAPK cell signalling and VEGFR2 expression. Additionally other, studies have demonstrated that cutaneous melanoma cell lines bearing a B-RAF mutation are more sensitive than N-Ras mutated melanoma cells to treatment with pazopanib, with mutational status correlating with anti-angiogenic activity (Gril *et al.*, 2011). Moreover, both studies suggest that B-RAF status may affect pazopanib sensitivity in melanoma, further highlighting the association between MAPK signalling and angiogenesis. Given the cross talk of both VEGF and MAPK signalling pathways in survival, growth, angiogenesis and chemotaxis of melanoma, it therefore maybe more applicable to modulate VEGF receptor activity with pazopanib in concert with targeting other VEGF promoting pathways such as the MAPK signalling by use of the MEK inhibitor trametinib, as a more efficient means through which to hamper cutaneous and uveal melanoma at both primary and metastatic sites. Nevertheless, such approaches may also require the investigation of crosstalk with other cell signalling mechanisms that may impair the efficacy of such combined approaches, including autophagy, which is well documented to be induced by many drugs to counteract apoptotic signalling.

1.7 Autophagy

Autophagy, derived from the greek words ‘auto’ and ‘phagy’ meaning to eat one’s self, refers to the lysosomal mediated catabolic process for the degradation and re cycling of cellular debris (Boya *et al.*, 2013). Activated in times of nutrient/oxidative stress or starvation, autophagy participates in many normal physiological processes to maintain cellular energy and homeostasis including its pivotal role in cellular differentiation, innate and adaptive

immunity and aging (Lum *et al.*, 2005; Martinez-Vicente and Cuervo, 2007; Beth Levine and Kroemer., 2008; Deretic and Levine, 2009).

Autophagy levels within cells are under tight control by signalling pathways that work in concert with environmental cues (Levine and Kroemer, 2008). Key signalling regulators of autophagy include AKT/mTOR and class I and class III phosphoinositide 3-kinase (PI3K) (Jung *et al.*, 2010), and more than 20 evolutionarily conserved genes known as autophagy regulatory genes (ATG) that code for Atg proteins (autophagy related proteins).

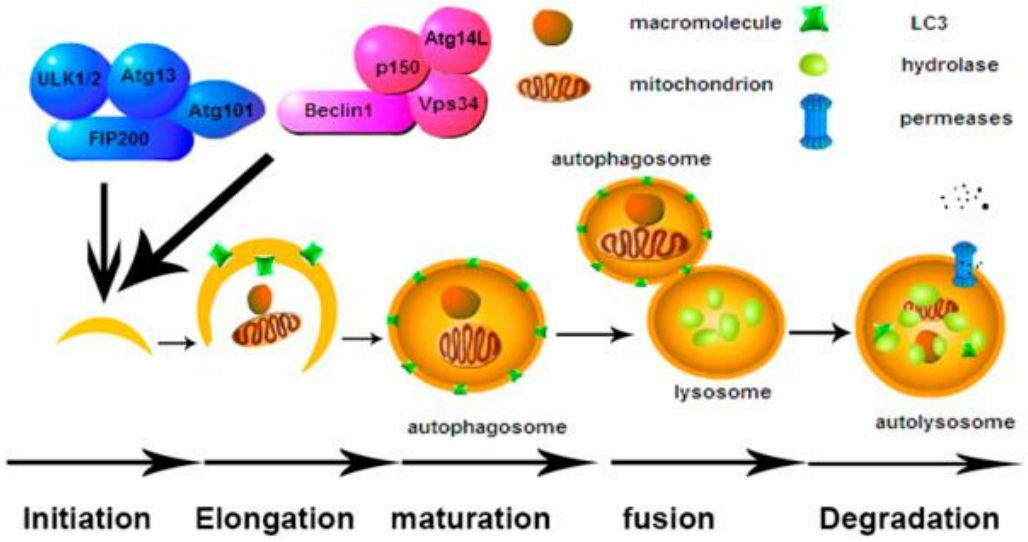

Figure 1.20 The Process of Autophagy
Autophagy involves 5 component steps, including, initiation, nucleation/elongation, maturation, fusion and finally degradation. An ULK complex including ULK1/2, Atg13, FIP200 and Atg101 is key to the initiation phase of autophagy forming the pre-autophagosomal structure (PAS). Next, during the nucleation phase a complex of Beclin1, Vsp34, Atg14L and p150 is recruited to the PAS for autophagosomal construction. The subsequent elongation phase then enables the formation of a double membrane vesicle in which cytoplasmic contents are sequestered, the closing of which results in the completed autophagosome. The autophagosome then fuses with a lysosome where cytoplasmic contents are degraded by lysosomal hydrolase to generate free amino acids, fatty acids and unconjugated LC3-I, that can be recycled in a cell-autonomous fashion back to the cytoplasm by lysosomal permease, or delivered to distinct sites to sustain cellular energy and homeostasis. Taken from (Sun et al., 2013).

The process of autophagy consists of five essential component steps (Figure 1.20) the first of which is called the 'initiation phase'. During autophagy initiation, a stable complex consisting of ULK1, Atg13, and FIP200 integrates stress signals and initiates the autophagy process. mTOR next, disassociates from ULK1 allowing it to phosphorylate Atg13 and FIP200 which results in the localisation of ATG proteins and active ULK1 complex to a specialised site termed the pre-autophagosomal structure (PAS) (Mizushima, 2010; Mizushima *et al.*, 2011).The second

'nucleation' event recruits Atg proteins, in particular Beclin1, Vps34, Ambra1 and UVRAG as well as lipids to the PAS for autophagosome construction (Mizushima et al., 2011). This process is followed by the 'elongation' phase when the autophagosome forms *de novo*, in which cytoplasmic contents are sequestered. This process requires two unique protein conjugation systems, the first resulting in the formation of an Atg5–Atg12 conjugate, in a noncovalent complex with Atg16 (Orsi et al., 2010; Roy and Debnath, 2010), and the second, in which the C-terminally lipid-conjugated LC3 (Atg8) is conjugated to phosphatidyl-ethanolamine (PE), recruiting cytosolic proteins and organelles to the phagopore (Fujita et al., 2008; Simonsen and Tooze, 2009; Roy S and Debnath J, 2010). Additionally, LC3 binds cargo adaptor proteins such as p62 which bind ubiquinated cargo from the cytoplasm (Ichimura and Komatsu, 2010), enabling the fusion of the autophagosome with the lysosome to form an autolysosome marking 'maturation'. The cytosolic contents along with p62 are finally degraded by lysosomal hydrolases, and the resulting products recycled and released back into the cytosol through permeases completing the process (Orsi et al., 2010).

1.7.1 Autophagy Deregulation and Influence in Melanoma Therapy

Although principally a cellular survival process, autophagy in cancer is complex, with both tumour suppressing and tumour promoting roles often referred to as the 'autophagy paradox' (Figure 1.21). While the maintenance of autophagy prevents the build-up of cytotoxic waste, suppressing tumourigenesis (Degenhardt K *et al.*, 2006; Mathew *et al.*, 2007), its re activation in later stages of tumourigenesis promotes tumour growth and survival in a nutrient deprived hypoxic environment, such as that of melanoma (Sivridis *et al.*, 2011).

As with many cancers autophagy is thus deregulated in melanoma (Corazzari et al., 2013). Immunohistochemical studies of Beclin1 expression in cutaneous melanoma, have reported its down regulation, with strong expression being evident in benign naevi, while expression is decreased as melanoma develops, suggesting a reduction of autophagy in early stage disease (Miracco *et al.*, 2010). Electron microscopy studies have also demonstrated the increased presence of double membraned autophagosomes in advanced stage melanomas within hypoxic regions, that correlate with early disease mortality (Sivridis *et al.*, 2011). Further, Immunohistochemical studies of endogenous LC3-II have demonstrated weak expression in normal melanocytes and naevi compared to cutaneous malignant melanomas supporting the reinstatement of autophagy in more advanced tumour stages (Lazova et al., 2010; Miracco et

al., 2010), and further supported by recent studies of p62 expression, revealing biphasic expression, reflecting the paradoxical role of autophagy in cancer (Ellis *et al.*, 2014a) (Figure 1.21). Further, in cutaneous melanoma autophagy maybe influenced by B-RAF mutational status, as basal autophagy is enhanced in B-RAF ^{V600E} melanomas as a consequence of chronic induction of ER stress, promoting chemo resistance (Armstrong *et al.*, 2011; Corazzari *et al.*, 2015).

Dysregulation of autophagy in uveal melanomas is only an emerging concept, with few publications dedicated to the expression of markers of autophagy in primary tumours. However consistent with studies in cutaneous melanoma, a immunohistochemical study of 99 primary uveal melanomas, demonstrated endogenous LC3 and beclin1 expression in 50% of patients was associated with intense pigmentation and tumour hypoxia, consistent with autophagy activation as a survival response to low oxygenation (Giatromanolaki *et al.*, 2011). In contrast, loss of Beclin1 expression by uveal melanomas has also been associated with worse prognosis (Ho and Ganesan, 2011).

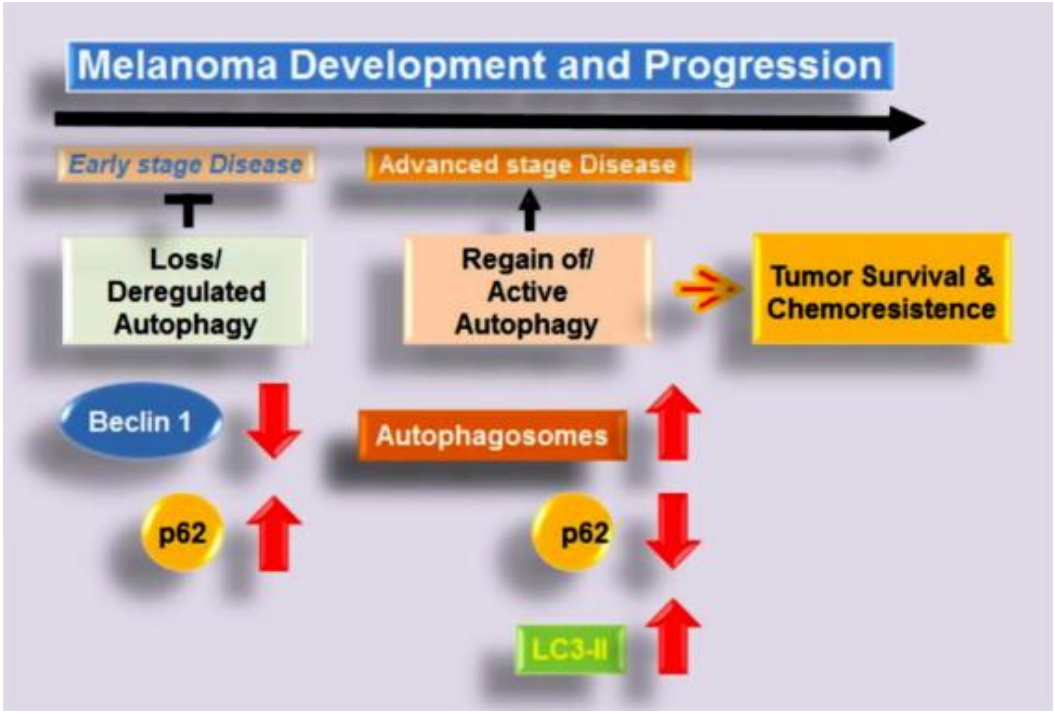

Figure 1.21 The Autophagy Paradox
In early stage disease autophagy may be lost or deregulated detected by loss of Beclin1 expression and a concomitant increase in p62 expression. However, in advanced melanoma autophagy maybe regained detected by an increase in autophagosomes and LC3-II expression and a decrease in p62, promoting the tumour survival and chemo resistance. Taken from (Corazzari et al., 2013).

In addition to its deregulation in melanoma, autophagy may also play a detrimental role in the response to cytotoxic therapy, with many examples, not only in melanoma therapy, whereby autophagy is induced by traditional chemotherapeutics as well as by novel targeted agents to counteract apoptotic signalling, and as such is key to the development of the process of acquired drug resistance (Apel et al., 2008; Vazquez-Martin et al., 2009; Claerhout et al., 2010; Marino et al., 2010; Ren et al., 2010; Guo et al., 2012). Supporting this concept in a phase III clinical trial of temozolomide and sorafenib, the development of secondary tumours in 12 AJCC stage IV melanoma patients, was associated with the increased presence of autophagosomes suggestive of high autophagy levels (Ma et al., 2011b). Further, in B-RAF inhibitor resistant xenograft models of cutaneous melanoma, combined B-RAF V600E and autophagy inhibition promotes tumour regression (Ma *et al.*, 2014). Similarly, it has been recently demonstrated, that MEK and autophagy inhibition increases cell death of B-RAF inhibitor-induced resistant metastatic melanoma cell lines, identifying autophagy as a key promoter of B-RAF inhibitor resistance (Martin et al., 2015).

Given other observations of drug-induced autophagy in melanoma therapy (Mariño *et al.*, 2007; Hammerová *et al.*, 2012; Xie *et al.*, 2013), clinical trials have now been initiated incorporating the lysosomal inhibitor hydroxychloroquine as monotherapy (NCT01273805) or in combination with current treatment regimens (NCT01128296), (NCT00909831) and (NCT00714181). Nevertheless, given blockade of autophagy may drive secondary tumourigeneis, other strategies to counteract the pro-survival effects of autophagy in advanced stage melanoma are under investigation, including the use of specific cannabinoids to drive its exacerbation and promote autophagic cell death (Armstrong *et al*., 2015). Modulation of autophagy in uveal melanoma for therapeutic benefit has largely remained unexplored, however one study of uveal melanoma cell lines harbouring GNAQ mutations revealed the induction of autophagy, in response to combined MEK and AKT inhibition, but which resulted in enhanced cell apoptosis, and inhibition of tumour growth in a xenograft mouse model (Ambrosini *et al.*, 2013b). Debate therefore remains as to whether autophagy modulation in uveal melanoma is a viable therapeutic approach together with the optimal means though which to harness modulation for optimal therapeutic benefit.

1.8 Aims and Objectives

Chemotaxis, MAPK and VEGF signalling are key to melanoma development, migration and metastasis but their interplay remains largely undefined in the context of both cutaneous and uveal melanomas. Understanding the role and cross talk between key mediators of these 3 signalling pathways, namely CXCR4, CXCL12 and VEGFR2 will inform on the development of more efficacious strategies to prevent melanoma migration and metastasis, the central aim to the current thesis.

To this the specific objectives were to:

- To define the prognostic significance of CXCR4, CXCR7 and CXCL12 expression in uveal and cutaneous melanoma, and the potential for autocrine signalling and impact on MAPK activation.
- To investigate the potential crosstalk between CXCR4-CXCL12 mediated chemotaxis and MAPK signalling and other pro-survival signalling mechanisms (e.g. autophagy) in cutaneous and uveal melanoma.
- To define the prognostic significance of VEGFR2 expression in relation to CXCR4 expression in cutaneous and uveal melanoma, and the potential efficacy of VEGFR2 blockade to inhibit cell viability and CXCR4-CXCL12-mediated-chemotaxis.
- To explore the potential cross talk between VEGFR2 and CXCR4-CXCL12 signalling in both cutaneous and uveal melanoma and the potential for the combined inhibition of both pathways as a strategy to inhibit tumour migration and promote cell death.

Derived results will thus inform on novel combinational and more efficacious targeted approaches through which to prevent melanoma, survival, migration and metastasis.

Chapter 2

Materials and Methods

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2.1 Growth and Maintenance of Human Cutaneous and Uveal Metastatic Melanoma, Hela, EA.hy.926, CXCR7 Overexpressing Chinese Hamster Ovary and Human Umbilical Vein Cell (HUVEC) Lines

Human metastatic cutaneous cell lines CHL-1 (B-RAF wild-type), A375, WM-164 (B-RAF V600E mutated), WM-1361 (N-Ras mutated), human endothelial cell line EA.hy926 or CXCR7 overexpressing Chinese hamster ovary (CHO) cells (a kind gift from Professor Simi Ali, Newcastle University) were grown and maintained in Dulbecco's modified Eagles Medium (DMEM, Lonza, Vervies, Belgium) supplemented with 10% fetal calf serum (FCS, Sigma, St Louis, U.S.A) and 5% penicillin streptomycin (P/S, Lonza, Vervies, Belgium). All cell lines were obtained from American Type Culture Collection (ATCC, LGC Standards, Middlesex, U.K) with authenticity validated by commercial single nucleotide polymorphism (SNP) genotyping assays for the presence or absence of mutant B-RAF V600E, B-RAF V600D, N-Ras G61L or N-Ras G61A as previously described (Hiscutt *et al.*, 2010).

Human metastatic uveal melanoma cell lines OM413, MEL290, C918 (GNAQ/GNA11 wild-type), 92.1, MEL270, OMM2.3 (GNAQ mutated) and OMM1 (GNA11 mutated), (all a kind gift from Professor Richard Marais, Manchester CRUK) were grown and maintained in RPMI-1640 medium (Sigma, St Louis, USA) supplemented with 10% FCS (Sigma) and 5% P/S (Lonza). UPMM1, UPMM3 (GNAQ mutated), UPMD1 and UPMD2 (GNA11 mutated), human metastatic melanoma cell lines (also kindly supplied by Professor Marais) were grown and maintained in Nutrient Mixture F-12 Ham (Sigma, St Louis, USA) supplemented with 10% FCS (Sigma) and 5% P/S (Lonza). Validation of common GNAQ and GNA11 mutations (GNAQ Q209, GNAQ R183, GNA11 Q209, GNA11 R183) of all uveal melanoma cells was performed by sequencing of PCR amplified DNA as detailed in section 2.8 (Appendix 1). Melanocytic lineage of all uveal melanoma cells was confirmed by detection of melan-A by reverse transcription PCR or immunofluorescence as detailed in section 2.7 and 2.11 (Appendix 1).

Human Umbilical Vein Endothelial (HUVEC, PCS100010, ATCC) cells were grown and maintained in 0.2% gelatin (Sigma) coated tissue culture flasks with endothelial cell basal medium (Lonza, Vervies, Belgium) supplemented with Endothelial Growth Medium Bullet Supplement (Lonza, Vervies, Belgium), 10% FCS (Sigma), 1µg/ml hydrocortisone acetate

(Sigma), and 50nM N-6,2'-O-dibutyryl-adenosine 3', 5'-cyclic monophosphate (Sigma) and 5% P/S (Lonza).

All cell lines were stored long term as early passage numbers in 90% FCS (Sigma) and 10% Dimethyl sulfoxide (DMSO) (Fisher Scientific, Leicestershire, UK) in liquid nitrogen. Cells were grown to 70% confluence before passaging, by washing in sterile PBS (Lonza) and detaching cells using trypsin EDTA (Lonza, Vervies, Belgium). Once cells were detached, complete medium containing FCS was added to stop cell surface protein cleavage, and the cells were transferred to a new culture flask (Corning, New York, U.S.A). All cell lines were maintained at 37 ^{o}C in a humidified atmosphere of 5% $C0_2$ in air, up to a maximum of 50 passages and used for all experiments at approximately 70% confluence.

2.2 Growth and Maintenance of Human Primary Dermal Fibroblasts, Keratinocytes, and Melanocytes

Primary human epidermal keratinocytes, dermal fibroblasts and melanocytes were isolated from redundant foreskin, breast or abdominal skin as previously described (Todd and Reynolds, 1998). Full ethical permission was obtained for the use of redundant normally disposed tissue, (NRES reference 08/H0906/95+5) with all studies performed in accordance with the Declaration of Helsinki Principles.

Isolated primary keratinocytes were expanded in human keratinocyte growth supplement MCDB medium (Sigma, St Louis, U.S.A), supplemented with histidine 37.3 mg/L, Isoleucine 98.4 mg/L, methionine 13.4 mg/L, phenylalanine 14.4 mg/L, tryptophan 9.2 mg/L, tyrosine 19.55 mg/L, ethanolamine 6.1µg/L, phosporylethanolamine 14.1 mg/L, Calcium 70µM, sodium bicarbonate 1.76 mg/L adjusted to pH 7.4 (all supplementary chemicals obtained from Sigma, St Louis, U.S.A). Dermal fibroblasts were expanded and maintained in Dulbecco's Modified Eagles Medium (Lonza), supplemented with 10% FCS (Sigma) and 5% P/S (Lonza). Primary melanocytes were expanded and maintained in melanocyte 254 medium (Life Technologies, Paisley, U.K), supplemented with human melanocyte growth supplement (Life Technologies, Paisley, U.K) comprising of bovine pituitary extract (BPE) (0.2% v/v), fetal bovine serum (0.5% v/v), recombinant human insulin-like growth factor-I (1 µg/ml), bovine transferrin (5 µg/ml) basic fibroblast growth factor (3 ng/ml), Hydrocortisone (0.18 µg/ml), Heparin (3 µg/ml) Phorbol 12-myristate 13-acetate (PMA) (10 ng/ml).

2.3 Primary Cohorts of Cutaneous and Uveal Melanomas, and Cutaneous Melanoma Lymph Node Metastases

Formalin-fixed paraffin-embedded (FFPE) tissue was obtained from a retrospective cohort of 77 primary melanomas comprising 13 benign melanocytic naevi and 64 primary American Joint Committee on Cancer (AJCC) stage I, II or III melanomas at the time of diagnosis, following excision at the Royal Victoria Infirmary, Newcastle-Upon-Tyne between January 2003 and May 2005.

For studies in primary uveal melanoma, a cohort of 13 formalin-fixed paraffin-embedded (FFPE) primary uveal melanomas (comprising of 5 with disomy and 8 monosomy of chromosome 3, with variable spindle, epithelial, or mixed tumour cell morphology) was obtained from Dr Lucianne Irion, (Department of Hisopathology, Clinical Sciences, Central Manchester and Manchester Children's University Hospitals NHS Trust, Manchester).

An additional cohort of 9 formalin-fixed paraffin-embedded (FFPE) primary cutaneous melanomas and patient matched FFPE metastatic lymph nodes (14 in total, 5 un-matched with primary cutaneous melanoma), or 2 normal lymph nodes (as controls) was obtained from Mr Jing Kim, and Dr Paul Barrett (Plastic Surgery and Department of Pathology, Durham University, Durham, Newcastle upon Tyne).

Full ethical permission for all studies with primary cutaneous, uveal melanomas, or metastatic lymph nodes was obtained, NRES (Ref; 08/H0906/95+5).

2.4 Chemical and Drug Treatment of Cells *in Vitro*

Trametinib (GSK 112021B) a specific ATP allosteric inhibitor of MEK 1 and MEK 2, was dissolved in DMSO at a concentration of 10mM and stored in aliquots at -80°C, and added to cell culture medium at final concentrations of 0.1, 0.5, 1, 2.5, 5,10, and 32nM.

Pazopanib (GSK GW786034), a selective multi-targeted tyrosine kinase inhibitor with primary targets of VEGFR2, VEGFR1 and VEGFR3 receptor tyrosine kinases (lesser biological action on Fibroblast growth factor receptor-1, Platelet derive growth factor receptor beta, KIT proto-oncogene tyrosine kinase receptor and Colony stimulating factor 1) was dissolved in DMSO at a concentration of 10mM, and after gentle warming in a 37°C water bath, aliquots were stored at -80°C, and added to cell culture medium at final concentrations of 0.1, 0.17, 0.5, 0.85, 1, 1.71, 4.28, 5, 8.56, 10, 17.12, 25µM.

Chloroquine (CQ, Sigma-Aldrich) a lysosomal inhibitor, was dissolved in sterile nanopure water at a concentration of 10mM, and stored at room temperature, protected from light, with final concentrations of 10μM added to cell cultures for 72 hours continuously or the final 2 hours of experiments if assaying LC3 I-II autophagic flux by western blotting.

Temozolomide (Sigma, T2577) an alkylating chemotherapeutic agent, was dissolved in DMSO at a concentration of 100mM, aliquoted and stored at -20°C, before addition to cell cultures at a final concentration of 50μM for 4 or 72 hours.

Recombinant CXCL12 (SDF-1α, CN-11, Almac, Craigavon, UK) was reconstituted in sterile PBS (LONZA) containing 0.1% bovine serum albumin (BSA, Alpha Diagnostics, San Antonio, U.S.A) at a concentration of 10μM, aliquoted and stored at -20°C, before addition to cell cultures at a final concentration of 10nM for 16 hours.

Recombinant human VEGF 165 protein (293-VE, R&D systems, Abingdon, U.K) was reconstituted in 0.1% BSA in PBS at a concentration of 100μg/ml, aliquoted and stored at -20°C, before addition to cell cultures at final concentrations of 10ng/ml for 16 hours.

2.5 Transient Transfection of B-RAF^{WT} and B-RAF^{V600E} in Human Metastatic Melanoma Cells and Analysis of CXCR4 Subcellular Localisation

CHL-1 wild-type metastatic melanoma cells were seeded in 6 well tissue culture plates (Corning Incorporated, New York, U.S.A) at a density of 4x10^{5}cells/well in a volume of 3ml complete culture medium and allowed to adhere overnight.

Pre-transfection mixtures (per well) were prepared containing 100μl OPTIMEM transfection medium (Life Technologies; Invitrogen) to which 1μg of pEFm-B-RAF^{WT} or pEFm-B-RAF^{V600E} (Davies *et al.*, 2002b; Flockhart *et al.*, 2009) (a kind gift from Professor Richard Marais, Cancer Research UK Manchester Institute, The University of Manchester) and lipofectamine 2000 was added at a ratio of 1:3 DNA to Lipofectamine 2000 (DNA:Lipofectamine 2000). pEFm-B-RAF^{WT} or pEFm-B-RAF^{V600E} pre-mixtures are then added to lipofectamine mixtures and left at room temperature for 20 minutes before being added to cells dropwise, and incubation for 24 hours. Cells are then re-seeded at a density of 2x10^5 cells/well for a further 48 hours. Cells subjected to Lipofectamine treatment in absence of cDNA were used as a control.

B-RAF over-expression and downstream activation of ERK was verified by western blot analysis of P-ERK, total ERK and GAPDH loading control, at 72 hours post transfection as described in section 2.14.

CXCR4 subcellular localisation was determined either by immunofluorescence (as described section 2.11) or by western blot analysis for CXCR4 expression (section 2.14), following cell subcellular fractionation into cytoplasmic and nuclear fractions (Pierce, Thermo Fisher Scientific, Illinois, U.S.A) performed in accordance to manufacturer's instructions. Successful cell fractionation was confirmed by western blotting for loading controls MEK 1/2 (cytoplasmic fraction) or Lamin A/C (nuclear fraction) as described section 2.14.

2.6 siRNA Knockdown of VEGFR2 in Human Metastatic Melanoma Cell Lines

CHL-1 or WM-164 cells were seeded at a density of $1.5x10^5$ cells/well, in 6 well culture plates in 3ml of complete culture medium and allowed to attach overnight by incubation at 37^{o}C. For the transient transfection of a single well, 2.5µl of lipofectamine RNAiMax reagent (Invitrogen, Carlsbad, California, U.S.A) was added to 125µl Opti-MEM1x reduced serum medium (life technologies, New York, U.S.A) before addition to respective pre-siRNA mixtures consisting of 2.5µl (for CHL-1 transfection) or 5µl (for WM-164 transfection) of ON-TARGET plus KDR (VEGFR2) siRNA (SMART pool siRNA, Dharmacon, GE lifesciences, Lafayette, Colorado, U.S.A), or Stealth RNAi™ siRNA Negative Control (none target) (Invitrogen, Carlsbad, California, U.S.A) in 125µl Opti-MEM 1x reduced serum medium (Life technologies, U.S.A), and incubated at room temperature for 15 minutes. Cells were then washed in PBS before addition of 1ml Opti-MEM 1x reduced serum medium (Life technologies, U.S.A) and the subsequent addition of 250µl of each siRNA and lipofectamine mixture dropwise and corresponding to final concentrations of 40nM or 80nM siRNA. After continued culture for 6 hours, the transfection solution was removed from cells and replaced with complete culture medium containing FCS. To assess the effect of VEGFR2 knockdown on cell chemotaxis and the effect of pazopanib, cells were used in chemotaxis assays as described in section 2.16, 24 hours following transfection completion with the experiment ending 72 hours post transfection. mRNA levels of VEGFR2 were determined by qPCR as described in section 2.9.

2.7 Reverse Transcription Polymerase Chain Reaction (RT-PCR) for Melan-A Expression in Uveal Melanoma Cell Lines

To detect the presence of Melan-A mRNA in uveal melanoma cell lines, RNA was extracted from C918, OM413, MEL290, 92.1, MEL270, OMM2.3, UPMM1, UPMM3, UPMD1, UPMD2 and OMM1 uveal melanoma cell lines using an RNeasy Mini Kit (Qiagen, Hilden, Germany) as per manufacturer's instructions. The integrity of isolated RNA was revealed by electrophoresis through 1% agarose gels and for each sample, with isolated RNA reverse transcribed to cDNA using a High Capacity cDNA reverse transcription kit (Applied Biosystems, Vilnius, Lithuania), and amplified by PCR using a GeneAmp RNA PCR kit (Applied Biosystems, Roche, New Jersey, USA), again according to the manufactures instructions. PCR of cDNA was performed with specific primers to Melan-A and GAPDH loading control (Invitrogen, Life Technologies, California, USA, and designed in collaboration with Dr Asif Tulah, ICM, Newcastle University) using oligonucleotides as described in Table 2.1. For each sample, a 20µl PCR reaction consisted of 2µl DNA templates mixed with AmpliTaq PCR master mix (Applied Biosystems, California, U.S.A) for each primer as detailed in Tables 2.2.

	Primer Oligonucleotides 5'to 3'	**PCR Product Molecular Weight (base pairs)**
Melan-A	Forward – ACT CTT ACA CCA CGG CTG AA	254
	Reverse – GTG AAT AAG GTG GTG GTG ACT G	
GAPDH	Forward – GGT GAA GGT CGG AGT CAA CGG A	496
	Reverse – GAG GGA TCT CGC TCC TGG AAG A	

Table 2.1 Oligonucleotides for Determination of Melan-A and GAPDH Expression in Uveal Melanoma Cell Lines

PCR for Determination of Melan-A or GAPDH Expression	
Master mix	2µl
De-ionised water	11.08µl
Gold Buffer	2µl
Magnesium (1.5mM)	1.2µl
dNTP	0.8µl
AmpliTaq Gold DNA Polymerase	0.12µl
Forward and Reverse Primers (10mM)	0.4µl each
DNA	2µl

Table 2.2 Constituents of 20µl PCR Reactions for Determination of Melan-A or GAPDH Expression

PCR conditions were: initial denaturation at 95°C for 10 minutes, followed by 28 cycles of denaturation of 4°C for 30 seconds, annealing at 57°C for Melan-A or 55°C for GAPDH for 30 seconds, extension at 72°C for 1 minute, and a final extension at 72°C for 7 minutes, using a Gene Amp PCR system 9700 thermocycler (Applied Biosystems, California, USA). PCR products were then separated by electrophoresis through 2% agarose electrophoresis gels before bands were visualising using a FlourChem UV imager (Alpha Innotech, California, USA), to confirm the presence of Melan-A mRNA expression.

2.8 Reverse Transcription Polymerase Chain Reaction (RT-PCR) and Sequence Analysis to Verify Activating Mutations in Uveal Melanoma Cell Lines

To confirm the GNAQ/GNA11 mutational status of uveal melanoma cell lines, DNA was extracted from C918, OM413, MEL290, 92.1, MEL270, OMM2.3, UPMM1, UPMM3, UPMD1, UPMD2 and OMM1 uveal melanoma cell lines using a QIAmp DNA mini Kit (Qiagen, Hilden, Germany) as per manufacturer's instructions. PCR was performed with specific primers (Invitrogen, Life Technologies, California, USA) to amplify areas of commonly mutated genes

GNAQ Q209, GNAQ R183, GNA11 Q209 and GNA11 R183 obtained from published literature (Van Raamsdonk *et al.*, 2010; Laviv Y *et al.*, 2012), and using oligonucleotides as described in Table 2.3. A 20µl PCR reaction for each replicate sample analysed comprised 2 or 4µl DNA templates mixed with AmpliTaq PCR master mix (Applied Biosystems, California, U.S.A) for each primer as detailed in Table 2.4.

Mutation	**Primer Oligonucleotides 5'to 3'**	**PCR Product Molecular Weight (base pairs)**
GNAQ Q209	Forward - TTT TCC CTA AGT TTG TAA GTA GTG C	222
	Reverse - CCT CAT TGT CTG ACT CCA CG	
GNAQ R183	Forward - TGG TGT GAT GGT GTC ACT GAC ATT CTC AT	183
	Reverse - AGC TGG GAA ATA GGT TTC ATG GAC TCA GT	
GNA11 Q209	Forward - CGC TGT GTC CTT TCA GGA TG	150
	Reverse - CCA CCT CGT TGT CCG ACT	
GNA11 R183	Forward - GTG CTG TGT CCC TGT CCT G	249
	Reverse - GGC AAA TGA GCC TCT CAG TG	

Table 2.3 Primer Oligonucleotides for Determination of GNAQ/GNA11 Mutations in Uveal Melanoma Cell Lines

PCR Reaction Mixture for Determination of Uveal Melanoma Mutations	
Master mix	2µl
De-ionised water	11.08µl or 13.08µl
Gold Buffer	2µl
Magnesium (1.5mM)	1.2µl
dNTP	0.8µl
AmpliTaq Gold DNA Polymerase	0.12µl
Forward and Reverse Primers (10mM)	0.4µl each
DNA	2µl or 4µl

Table 2.4 Constituents of 20µl PCR Reactions for Determination of Uveal Melanoma Mutations

PCR conditions were: initial denaturation at 95°C for 10 minutes, followed by 28 cycles of denaturation of 4°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 1 minute, a final extension at 72°C for 7 minutes. Each complete PCR cycle was repeated twice using a Gene Amp PCR system 9700 thermocycler (Applied Biosystems, California, USA). PCR products were then subjected to electrophoresis through 2% agarose gel, visualised using a FlourChem UV imager (Alpha Innotech, California, USA) and the correct molecular weight DNA product cut out from the gel and purified using a GeneJET Gel Extraction Kit (Thermo Scientific, Lithuania). Products were then sequenced by Source BioScience (Nottingham, UK) with sequencing chromatograms analysed using Chromas 2.4 soft-wear (Technelysium Pty Ltd, South Brisbane, Australia).

2.9 Real Time Reverse Transcriptase Polymerase Chain Reaction (qPCR)

qPCR was used to quantify VEGFR2 mRNA expression in HUVEC cells, human metastatic cutaneous melanoma cell lines CHL-1, WM-164, WM-1361, or human metastatic uveal melanoma cells OM413, MEL270, OMM2.3 and UPMD2. qPCR was also used to verify siRNA

knockdown of VEGFR2 in CHL-1 and WM-164 human metastatic cutaneous melanoma cell lines. All cell lines were seeded at $2x10^5$ cells/well in a volume of 3ml/well of their respective cell culture medium, in 6 well flat bottom cell culture plates (Corning Inc) for 24 hours, prior to washing with ice cold PBS, and the extraction of RNA using a ReliaPrep™ RNA Cell Miniprep System (Promega, Madison, Wisconsin, U.S.A) including DNase digestion as per manufacturer's instructions. RNA was stored at -80^oC, prior to conversion to single-stranded cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Vilnius, Lithuania) as per manufacturer's instructions. 20µl PCR reverse transcription reactions were prepared for each sample as detailed in Table 2.5 and with subsequent PCR performed using a Gene Amp PCR system 9700 thermocycler (Applied Biosystems, California, USA) with thermocycler conditions of 10 minutes 25 ^oC, 120 minutes 37 ^oC, 5 minutes 85 ^oC then 4 ^oC ∞. cDNA was stored at 4 ^oC, or -20 ^oC for long term storage.

High Capacity Reverse Transcription	
10X RT Buffer	2µl
25X dNTP Mix (100mM)	0.8 µl
10X RT Random Primers	2 µl
Multiscribe Reverse Transcriptase	0.5 µl
RNase Inhibitor	0.2 µl
Nuclease-free Water	4.5 µl
RNA (same concentration in every reaction)	10 µl

Table 2.5 Constituents of a Single Reverse Transcription Reaction

qPCR was subsequently used to determine the relative expression levels of VEGFR2 mRNA, relative to the house keeping control of 18s mRNA. Master reaction mixes were prepared for each gene probe as described in Tables 2.7 and 2.8, using TaqMan Universal PCR Master Mix (Applied Biosystems, Warrington, UK), and pre-designed Gene Expression Assays for VEGFR2 (KDR human Hs00911700_m1, Applied Biosystems, California, U.S.A) or 18s as a housekeeping gene (Integrated DNA Technologies, Leuven, Belgium), (Table 2.6). 8µl of master mix and 2µl

cDNA were combined in each well of a 96 well MicroAmp Fast Optical Reaction Plate (Applied Biosystems, California, U.S.A). Duplicate samples for each gene samples were prepared incorporating both a non-template control (H_2O only) and positive control of HUVEC cell cDNA, before plates were sealed with optical film, briefly centrifuged at 1200 RPM for 30 seconds and samples subjected to qPCR analysis using a StepOne Plus Real-Time PCR instrument (Applied Biosytems, California U.S.A), with 40 cycles of: 2 minutes at 50^{o}C hold, 10 minutes at 95^{o}C hold, 15 seconds at 95^{o}C denature, and 1 minute 60^{o}C anneal/extend. Real-time mRNA expression analysis was determined using StepOne Soft wear V2.3 (Applied Biosystems, California, U.S.A). An average of VEGFR2 Cycle threashold (Ct) values for each sample was normalised to 18s and the fold change in VEGFR2 gene expression calculated using the comparative 2–[delta][delta]Ct method, where [delta][delta]Ct = [delta]Ct,VEGFR2 - [delta]Ct,18s, as previously described (Livak and Schmittgen, 2001).

18s TaqMan® Gene Expression (FAM reporter -TAMRA quencher) Probe 5'-3'	FAM TCCT TTG GTC GCT CGC TCC TCT
18s Forward Primer Oligonucleotides 5'-3'	CGA ATG CCT CAT TAA ATC AGT TAT GG
18s Reverse Primer Oligonucleotides 5'-3'	TAT TAG CTC TAG AAT TAC CAC AGT TAT

Table 2.6 Probe and Oligonucleotides for 18s Housekeeper Gene

KDR (VEGFR2) TaqMan® Gene Expression	
Master mix (x2)	5µl
Nuclease free Water	2.5µl
KDR TaqMan® Gene Expression Assay (FAM reporter-NFQ quencher probe)	0.5µl
cDNA	2µl

Table 2.7 Constituents of a Single PCR Reaction using KDR (VEGFR2) TaqMan Gene Expression Assay

18s TaqMan® Gene Expression	
Master mix (x2)	5µl
Nuclease free Water	2.35µl
18s Forward & Reverse Primers	0.2µl
18s FAM-TAMRA Probe	0.25 µl
cDNA	2µl

Table 2.8 Constituents of a Single PCR Reaction Using 18s Primer and Probe

2.10 Cell Viability Assay

Melanoma cells were seeded at $5x10^3$ (A375, WM-1361, WM-164, OM413, MEL-270, UPMD2) or $2.5x10^3$ (CHL-1) in 100µl/well of 96 flat well tissue culture plates (Corning Incorporated, New York, U.S.A). After 16, or 72 hours the cell culture medium was replaced with 100µl of fresh media or media in the presence or absence of a dose range of pazopanib or trametinib ranging from 0.5-75µM and 0.1-50nM respectively or with a fixed dose of both drugs at a ratio of 1: 1712.5 trametinib : pazopanib (nM). Cell viability was determined by addition of 20µl of Aqueous Non-Radioactive Cell Proliferation Assay (CellTitre-96, Promega, Southampton, UK) to each well (including blank wells containing just media) for 4 hours and absorbance measured at 490 nm using a Spectra Max 250 plate reader (Molecular Devices).

Cell viability data were normalized to control cells and expressed as a mean of individual experiments. Variation in cell viability between treatment conditions was analyzed by One-Way analysis of variance (ANOVA) with Dunnett's post hoc correction test or using Kruskal-Wallis analysis with Dunn's post hoc test.

2.11 Immunofluorescence for the Detection and Quantification of CXCR4, CXCR7, CXCL12, Melan-A and VEGFR2 in Human Melanoma or Endothelial Cell Lines

Human metastatic uveal and cutaneous melanoma cells, HUVEC cells (positive control for VEGFR2), EA.hy926 or CXCR7 overexpressing Chinese hamster ovary (CHO) cells (positive control for CXCR7) primary dermal fibroblasts, keratinocytes or melanocytes, were seeded on

to cover slips at a density of $2.5x10^5$ cells /well in 3mls of respective culture medium in 6 well tissue culture plates (TPP, Sigma, Poole, U.K) for 24 hours.

For detection of CXCR4 expression, cells were serum starved in 0.2% BSA in DMEM (Lonza), RPMI-1640 (Sigma) or Nutrient Mixture F-12 Ham (Sigma) for 16 hours prior to cell fixation.

For all analyses, cell culture medium was removed and cells washed with PBS before fixing in ice cold 4% paraformaldehyde (Sigma, Saint Louis, U.S.A) in PBS for 15 minutes at room temperature. After a further 3 washes with PBS, cells were permeabilized in 0.2% Triton X-100 (Thermo Fisher Scientific, Illinois, U.S.A) in PBS for 10 minutes at room temperature. A blocking step was performed to block non-specific primary antibody binding, by incubating slides in 20% swine serum (Serotec, Oxford, UK) in PBS for 30 minutes at room temperature.

Coverslips were then transferred to glass slides, with the coverslip/cell area marked around with a hydrophobic pen, before incubation with 100ul of primary antibody; anti-human CXCR4 monoclonal antibody (Clone 44716) (R&D systems, Abingdon, U.K) at a concentration of 2.5 ug/ml, anti-human CXCR7 monoclonal antibody (11G8, R&D systems, Abingdon, U.K) at a concentration of 10µg/ml, anti-human CXCL12 (ab9797, Abcam, Cambridge, UK) diluted 1:500, anti-human Melan-A monoclonal antibody diluted 1:250 (ab731-500 Abcam, Cambridge, UK), Mel-5 (Anti-TRP1 antibody, Abcam, Cambridge, UK) diluted 1:250, anti-human VEGFR2 (FLK-1 SC-6251, Santa Cruz, Heidelberg, Germany) diluted 1:50 in 20% swine serum (Serotec, Kidlington, U.K) or 20% FCS (Sigma) in PBS ,or as a negative control, 20% swine serum (Serotec) or 20% FCS (Sigma) in PBS, overnight at 4^oC.

Cells were then washed in PBS thrice before incubation with a secondary fluorescent antibody, Alexa Fluor® 488 Goat Anti-Mouse IgG (Life Technologies, Eugene, Oregon USA) or Alexa Fluor® 488 Goat Anti-Rabbit IgG (Life Technologies, Eugene, Oregon USA) diluted 1:250 with diamidino-2-phenylindole dye (DAPI, Thermo Fisher Scientific, Illinois, U.S.A) diluted 1:1000 in 20% swine serum (Serotec) or FCS (Sigma) in PBS for 1 hour at room temperature. After final washing 3 times in PBS for 5 minutes, cover slips were mounted onto fresh glass slides using hardest fluorescence mounting medium (Vector labs, Burlingame, California, U.S.A) and stored at room temperature prior to analysis. Fluorescence was visualised and images captured with a Leica TCS SP2 UV confocal microscope and analysed using LC2 2.61 software (Leica Microsystems GmbH Heidelberg).

CXCL12, CXCR7 or VEGFR2 fluorescence per cell (relative to DAPI) was quantified above background fluorescence from 4 representative images per cell line respectively using ImageJ software (Rasband, W.S., ImageJ, Maryland, USA).

2.12 Immunofluorescence for the Detection and Quantification of CXCR4, CXCL12 and Melan-A in Formalin Fixed Paraffin Embedded (FFPE) Primary Cutaneous and Uveal Melanomas

5µm FFPE tissue sections derived from benign naevi or primary melanomas or human tonsil as a positive control (for CXCR4) were de-paraffinised in xylene (Thermo Fisher Scientific, Illinois, U.S.A) for 10 minutes at room temperature then dehydrated in 100% and 95% ethanol, each for 5 minutes (Barbro Ehlin-Henriksson *et al.*, 2006). Sections were rinsed in de-ionised water and washed in Tris buffered saline pH 7.6 (TBS) for 1 minute before antigen retrieval was performed by heating slides in 10mM Tris hydrochloric acid (pH 7.4) for CXCR4 or in 10mM Sodium Citrate (pH 6) to reveal Melan-A and CXCL12, for 1 minute in a pressure cooker. Sections were rapidly cooled in cold de-ionised water and washed for 20 minutes in TBS. Cells were permeabilised in 0.2% Triton X-100 (Thermo Fisher Scientific, Illinois, U.S.A) in TBS for 10 minutes at room temperature. After blocking for non-specific primary antibody binding with 20% (v/v) swine serum (Serotec) or 20% FCS (Sigma) in TBS for 1 hour at room temperature, sections were marked with a hydrophobic pen, and incubated with primary mouse anti-human CXCR4 monoclonal antibody (Clone 44716) (R&D systems, Abingdon,UK), diluted to a final concentration of 10µg/ml in TBS containing 20% (v/v) FCS, primary rabbit anti-human melan-A (ab731-500 Abcam, Cambridge, UK) diluted 1/250 or primary mouse anti-human CXCL12 (ab9797, Abcam, Cambridge, UK) 1/500 in TBS containing 20% (v/v) FCS, at 4°C overnight. An additional slide of each section was used as a negative control, omitting incubation with primary anti-CXCR4, melan-A or CXCL12 antibody or with the addition of an isotype control primary antibody anti-Rabbit IgG polyclonal Isotype control antibody (ab171870, Abcam, Cambridge, UK) or anti-mouse IgG polyclonal Isotype control antibody (ab37355, Abcam, Cambridge, UK) at the same concentrations as respective primary antibodies in TBS containing 20% (v/v) FCS. Sections were subsequently washed thrice for 5 minutes each in TBS, before incubation with secondary antibody tetramethylrhodamine isothiocyanate (TRITC) conjugated anti-Mouse IgG (Sigma-Aldrich, Gillingham, United Kingdom), Alexa fluor Goat anti-rabbit IgG 568 (a11036, Thermo Fisher Scientific, Illinois, U.S.A), or Alexa fluor Goat anti-mouse IgG 488 (a11001, Thermo Fisher Scientific, Illinois, U.S.A) diluted 1:250 in TBS containing 20% (v/v) FCS

for 1 hour at room temperature. All subsequent procedures were performed shielding the sections from light to prevent degradation of the secondary antibody fluorophore. Following incubation with diamidino-2-phenylindole dye (DAPI, Thermo Fisher Scientific, Illinois, U.S.A) diluted 1:1000 in TBS, for 10 minutes to reveal nuclear staining, sections were mounted using fluorescence mounting medium (DAKO, California, U.S.A). Auto fluorescence was blocked using Sudan Black (Sigma-Aldrich, Gillingham, United Kingdom) 0.3% (w v^{-1}) in 100% ethanol for 20 minutes, and slides finally stored at 4°C prior to image analysis. Images were captured with a Leica TCS SP2 UV confocal microscope and analysed using LC2 2.61 software (Leica Microsystems GmbH Heidelberg). CXCL12 fluorescence per tumour cell (relative to melan A) or within the epidermis of melanoma tissue sections (relative to DAPI) was quantified above background fluorescence from 4 or 2 representative images per tumour respectively using ImageJ soft wear (Rasband, W.S., ImageJ, Maryland, USA).

2.13 Immunohistochemistry for the Detection and Quantification of CXCR4, CXCR7, VEGFR2, Melan-A, Beclin-1 and P62 in Formalin Fixed Paraffin Embedded Primary Uveal Melanomas or Primary Cutaneous Melanomas and Patient Matched Metastatic Lymph Nodes

5µm formalin fixed and paraffin embedded tissue sections derived from benign naevi or primary melanomas, tonsil (CXCR4 positive control), HUVEC cells (VEGFR2 positive control), or CHL-1 and A375 metastatic melanoma cells with known p62 and Beclin-1 expression (both markers of autophagy and used as positive controls) were de-paraffinised through immersion in xylene (Thermo Fisher Scientific, Illinois, U.S.A) for 10 minutes at room temperature before being dehydrated in 100%, followed by 95% ethanol each for 5 minutes (Barbro Ehlin-Henriksson *et al.*, 2006). Sections were then rinsed in de-ionised water before heating in antigen retrieval buffer; 10mM Tris hydrochloric acid (pH 7.4) for CXCR4, Melan-A and p62 detection, 10mM Tris hydrochloric acid (pH 9) for CXCR7 detection, or 10mM Sodium Citrate (pH6) for Beclin-1 and VEGFR2, for 1 minute in a pressure cooker to unmask antigenic sites. Sections were then rapidly cooled in cold de-ionised water and washed for 20 minutes in TBS before sections were marked with a hydrophobic pen and then cells permeabilised in 0.2% Triton X-100 (Thermo Fisher Scientific, Illinois, U.S.A) in TBS for 10 minutes at room temperature. Endogenous peroxides were then blocked by incubation in 3% hydrogen peroxide (Sigma, Poole, U.K) in water for 10 minutes at room temperature. Sections were then

washed 3 times in TBS prior to incubation with avidin solution (Avidin/Biotin blocking kit, Vector laboratories, Burlingane, USA) for 15 minutes to block avidin binding sites. Sections were then again washed with TBS and subsequently incubated for a further 15 minutes with biotin solution (Avidin/Biotin blocking kit, Vector laboratories, Burlingane, USA) to block endogenous biotin receptors. Sections were then washed in TBS prior to a protein blocking step by incubation in 20% swine serum (AbD Serotec, Oxford, UK) in TBS or 20% FCS (Sigma) in TBS, for 1 hour at room temperature, washing in TBS and incubation with primary anti-human CXCR4 monoclonal antibody (Clone 44716) (R&D systems) at a concentration of 2.5 µg/ml, anti-human Melan-A monoclonal antibody (ab731-500 Abcam, Cambridge, UK) diluted 1:500, anti-beclin-1 monoclonal antibody (Beclin-1 612113, BD Biosciences, UK) diluted 1:100 or anti-p62 monoclonal antibody (SQSTM1 Sc28359, Santa Cruz Biotechnology, Heidelberg, Germany) diluted 1:50, anti-human CXCR7 monoclonal antibody (11G8, R&D systems, Abingdon, U.K) at a concentration of 5µg/ml, or anti-human VEGFR2 (FLK-1 SC-6251, Santa Cruz, Heidelberg, Germany) diluted 1:500 in 20% swine serum (AbD Serotec) in TBS overnight at 4°C, or for 1 hour at room temperature in the case of Beclin-1, p62 and Melan-A. An additional slide of each section was also included as a negative control, omitting incubation with primary antibody. After 3 washes with TBS, primary antibody binding was detected with biotinylated horse anti-mouse IgG secondary antibody (Vectastain Elite ABC Kit (Mouse IgG) or anti-rabbit IgG secondary antibody (Vectastain Elite ABC Kit (Rabbit IgG), Vector laboratories, Burlingane, U.S.A) diluted 1:200 in 20% swine serum (AbD Serotec) in TBS or 20% FCS (Sigma) in TBS for 30 minutes at room temperature. Sections were further washed thrice in TBS before staining with ABC Reagent from a Vectastain Elite kit (Vector laboratories) for 30 minutes at room temperature. Following 3 more washes in TBS sections were incubated with VIP solution (Vector VIP Peroxidase Substrate Kit, Vector laboratories, Burlingane, USA), for 10 minutes according to the manufacturers specifications. Slides were next rinsed in tap water for 5 minutes and sections counterstained with hematoxylin solution (Harris Modified, Sigma Aldrich diagnostics, St Louis, U.S.A) for 2 minutes. Following washing in tap water with multiple changes for 10 minutes, sections were dehydrated through 75% and 100% ethanol for 5 seconds each, cleared in histoclear (Sigma, Poole, UK) for 2 minutes before being left to air dry. Finally, sections were mounted with a cover using DPX mountant (Thermo Fisher Scientific, UK) and visualised by light microscopy. Images were captured using the Zeiss Axio Imager microscope (Carl Zeiss Microscopy New York, U.S.A).

2.14 Western Blotting

Human metastatic cutaneous melanoma cell lines CHL-1, WM-164, WM-1361, or human metastatic uveal melanoma cells OM413, MEL270, OMM2.3 and UPMD2 were seeded at $2x10^5$ cells/well in a volume of 3ml/well of their respective cell culture medium, in 6 well flat bottom cell culture plates (Corning Incorporated, New York, U.S.A) and allowed to attach overnight. For experiments analysing cells viability and autophagic flux in response to trametinib treatment, the medium in each well was replaced with 3mls of fresh media or media containing trametinib (2.5, 5, 32nM) or temozolomide (50µM) for 4 or 72 hours in the presence or absence of Chloroquine (10µM) for the duration of the experiment or the final 2 hours of incubation before cells were subsequently harvested for western blot analysis. Experiments investigating the potential for autocrine CXCR4-CXCL12 cell signalling in WM-164 metastatic melanoma cell line, cells were seeded at $1.5x10^5$ cells/well in 6 well tissue culture plates 24 hours prior to the addition of 1µg/ml anti-CXCL12 neutralising antibody (Abcam Cambridge, UK, clone 9797) or IgG isotype control antibody (Abcam Cambridge, UK, clone 171870) for 5-30 minutes before harvesting cell lysates for western blotting.

Protein from human metastatic cutaneous and uveal melanoma cells in the presence or absence of treatment with Trametinib, Chloroquine or temozolomide was obtained after washing cells with PBS, and lysis on ice with 50µl of cell lysis buffer (0.1 M Tris-HCl pH 7.4, 25mM NaF, 0.1M NaCl, 2mM EDTA (pH 8), 1mM benzamidine, 0.1mM sodium orthovanadate, 0.1% Triton-X100) containing 150 µl/ml protease inhibitor cocktail (Promega, Southampton, UK). The adherent cells were then scraped in lysis buffer from 6 well plates into eppendorf micro centrifuge tubes.

Protein was extracted by sonication, using a probe sonicator (Soniprep 150, MSE, UK) with 2 pulses for 5 seconds at an amplitude of 7 microns and protein concentration determined by Bradford protein quantification assays (Pierce Biotech, Rockford, USA) according to the manufacturer's specifications, with protein absorbance measured using SpectraMAX 250 plate reader (Molecular Devices Ltd. UK). 10 µg of protein lysates were diluted 1:3 in 4x sample buffer (250 mM Tris HCL (pH 8), 8% sodium dodecyl sulphate (SDS), 40% glycerol, 10% β-mercaptoethanol and bromophenol blue) prior to denaturation at 95^oC for 5 minutes on a heat block. Proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) including a Bench Mark^{TM} Prestained Protein Ladder (Invitrogen,

Paisley, UK; 10748-010), through 4-20% tris-glycine gels (Bio-Rad, UK) immersed in a SDS running buffer (25 mM Tris base, 190 mM glycine 0.1% SDS). Proteins were subsequently transferred from the gel onto a Trans-Blot Turbo PVDF membrane (Bio-rad, UK) using a Bio-rad Turbo-blotter (1.3A, 25V; Bio-Rad, UK) for 7 minutes before membranes were blocked for non-specific primary antibody binding with 5% non-fat milk (OXOID ltd, Basingstoke, UK) diluted in TBS for 1 hour at room temperature. Membranes were then incubated over night at 4^{o}C with primary antibodies; anti-human MEK1/2 (Rabbit monoclonal antibody, diluted 1:8000, #9122 Cell Signalling, Life Technologies, Thermo Fisher Scientific, USA), anti-human ERK1/2 (Rabbit monoclonal antibody, diluted 1:5000, p44/p42 MAPK, #9102, Cell Signalling, Life Technologies, Thermo Fisher Scientific, USA), anti-human Phospho–ERK1/2 (Rabbit monoclonal antibody, diluted 1:5000, Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (197G2) #4377, Cell Signalling, Life Technologies, Thermo Fisher Scientific, USA) anti-human LC3 I-II (Rabbit monoclonal antibody, diluted 1:2000, #2775, Cell Signalling, Life Technologies, Thermo Fisher Scientific, USA), anti-human CXCR4 (Rabbit polyclonal antibody, diluted to 0.1ug/ml, (ab2074), Abcam, Cambridge, UK), anti-human Cleaved Caspase 3 (Monoclonal rabbit antibody, diluted 1:1000, #9664, Cell Signalling, Life Technologies, Thermo Fisher Scientific, USA), anti-human B-RAF (Mouse monoclonal antibody, diluted 1:40,000 (sc-5284, Santa Cruz, Heidelberg, Germany), anti-human Lamin A/C (Mouse monoclonal antibody, diluted 1:1000, (4C11) # 4777, Cell Signalling, Life Technologies, Thermo Fisher Scientific, USA) or for 1 hour at room temperature in the case of anti-human GAPDH loading control (Rabbit monoclonal antibody, diluted 1:5000, #2118s, Cell signalling, Life Technologies, Thermo Fisher Scientific, USA) diluted in 5% Bovine Serum Albumin (Alpha Diagnostics, Texas, USA) 0.2% Tween, Tris Buffered saline (TBS/T). Membranes were then washed thrice for 15 minutes in TBS/T before 1 hour incubation with peroxidase labelled anti-rabbit IgG or anti-mouse IgG secondary antibodies (at dilutions of 1:2000 (for the detection of Cleaved Caspase 3), 1:5000 or 1:10,000 (for detection of GAPDH), Vector Laboratories, Burlingame, USA) diluted in TBS/T containing 5% non-fat milk. Membranes were then washed again thrice for 15 minutes in TBS/T, before proteins were visualised using ECL-plus system (Clarity Western ECL-Substrate, Bio-Rad, USA) according to the manufacturer's specifications. Band density quantification was determined using Image J soft-wear (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2015) or Odyssey FC Image studio software (Li-cor Biosciences, Lincoln, Nebraska, U.S.A).

2.15 Enzyme-Linked Immunosorbent Assay for the Detection of CXCL12

Cutaneous and uveal metastatic melanoma cell lines, EA.hy.926 endothelial cells, primary keratinocytes, dermal fibroblasts or melanocytes were seeded in 1ml culture medium at $5x10^4$ cells/well in 24 well tissue culture plates (TPP, Sigma, Poole, U.K) and incubated overnight at 37^{o}C. Media was replaced with 300µl of DMEM/10% FCS (Sigma Chemical Co), 5% P/S (Lonza) and incubation continued for 72 hours before supernatant was removed, centrifuged and stored at -20^{o}C. Human biliary epithelial cell supernatants were provided by Dr Isabella Swidenbank, (Newcastle University) and also stored at -20^{o}C. A commercial sandwich enzyme-linked immunosorbent assay (ELISA), Human CXCL12/SDF-1 alpha Quantikine ELISA Kit (R&D, Minneapolis, Minnesota, U.S.A) was used to detect CXCL12 secretion as per manufacturer's specifications. Absorbance was measured at 492 nm using a Spectra Max 250 plate reader (Molecular Devices, Workingham, UK). CXCL12 concentration in pg/ml was determined relative to a standard curve of known concentrations of CXCL12.

In vitro CXCL12 secretion by cell lines and primary cells was expressed as a mean of individual experiments for cell lines or 4 independent tissue donors for primary cells, and analysed by Mann-Whitney U test.

2.16 Transwell Chemotaxis Assays

24 hours prior to assay, the cell culture media of each cutaneous or uveal melanoma cell line was replaced with serum free DMEM (CHL-1 and WM-164 cells), or RPMI (OM413 cells) media supplemented with 0.2% BSA and 5% P/S. The undersides of inserts (8µm pore size, BD Falcon, Oxford, UK) were coated with 30µl of 2.5µg/ml fibronectin (Sigma Aldrich, St Louis, U.S.A) diluted in DMEM, or RPMI media supplemented with 0.2% BSA 5% P/S for 1 hour at room temperature. Excess fibronectin was removed and inserts air dried for 1 hour. The 24 well companion plate (Falcon, Becton Dickinson Labwear, Franklin Lakes, New Jersey, U.S.A) was blocked with 1ml PBS +1% BSA for an hour, before addition of 800µl/well of human CXCL12, Stromal Cell Derived Factor 1 chemokine (Almac, Edinburgh, U.K) at concentrations of 1, 10 or 50nM diluted in DMEM, or RPMI media +0.1% BSA, or supernatant derived from primary dermal fibroblasts, to wells with their respective tissue culture media as a control. For experiments evaluating the migratory effect of recombinant CXCL12, rabbit anti-human CXCL12 (ab9797, Abcam, Cambridge, UK) or rabbit anti-IgG isotype control (ab171870, Abcam,

Cambridge, UK) antibodies, were added to the well media or dermal fibroblast supernatants at a concentration of 1µg/ml. Melanoma cells were applied to the top of inserts at a concentration of $2x10^5$ cells in 500µl of DMEM, or RPMI media + 0.2% BSA after detachment with 2mM Ethylenediamine tetra-acetic acid (VWR, Leicestershire, UK) in PBS. For experiments evaluating the potential inhibitory effects of trametinib or pazopanib alone or in combination, cells were treated for the duration of the experiment in the presence of 2.5, 5 or 32nM trametinib or 1, 5 or 25µM pazopanib alone or in combination at a fixed dose ratio of 1:1712.5 (trametinib nM: pazopanib nM). For experiments evaluating the effect of knockdown of VEGFR2 on cell chemotaxis, wild-type or B-RAF mutated melanoma cell lines subjected to transfection with a control scrambled oligonucleotide (none target siRNA) or following transient knockdown with a pool of siRNA targeting VEGFR2 were applied to the insert in the presence or absence of 10ng/ml recombinant human VEGF 165 protein (293-VE, R&D, Minneapolis, Minnesota, U.S.A) for the duration of the experiment. After incubation at 37^{o}C for 16 hours, un-migrated cells were gently swabbed from within the insert, before migrated cells were fixed in ice cold methanol overnight, and before immersion in Mayer's haematoxylin solution (Sigma, Aldrich, St Louis, Missouri, U.S.A) for 20 minutes, Scott's tap water for 10 minutes, and dehydration with 50, 75, 90 and 100% ethanol and final mounting on coverslips with DPX (Raymond A Lamb, Life Technologies, Thermo Fisher Scientific, USA). Total numbers of migrated cells were recorded in 9 representative fields of vision (as shown in figure 2.1) of each filter, with 3 filters used per treatment condition.

Cell migration/chemotaxis data were recorded as cell migration per high powered field (HPF), and normalised to control cells, and expressed as mean of individual experiments. Data were analysed using one-way ANOVA with Dunnett's post hoc correction or by Kruskal-Wallis with Dunn's post hoc test. The effect of individual drug treatments and combined drug treatments on cell chemotaxis was assessed using a one-way analysis of variance with Bonferroni's post hoc correction.

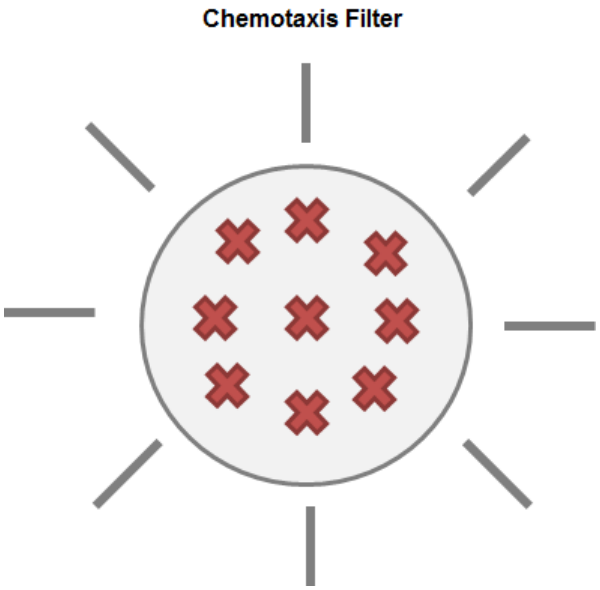


Figure 2.1 Chemotaxis Filter and Areas of High Powered Field of Vision Analysed
Example of a chemotaxis filter, the areas to be analysed for migrated cells are depicted by the red cross.

2.17 Biomarker Analysis

The mean percentage of CXCR4 or VEGFR2 positively stained cells in each tumour section was derived as a mean of 10 representative images per tumour section (example selection of areas to image Figure 2.2), captured using Zeiss Axio Imager microscope (Carl Zeiss Microscopy New York, U.S.A) and expressed as a mean percentage of cells expressing total CXCR4 (nuclear and cytoplasmic expression) or VEGFR2 using Leica QWin V3 software (Leica Microsystems, Newcastle upon Tyne). Nuclear and cytoplasmic CXCR4 staining were graded in each image by eye using the following criteria: 1= no staining, 2= under 50% of cells positively stained, 3= 50-75% of cells positively stained, 4= over 75% of cells positively stained. Inter-assay variability was assessed by repeated staining of the same strongly positive or negative tumour sections in each assay with reproducible percentage staining positivity confirmed.

Mean CXCL12 fluorescence per tumour cell (relative to melan-A) or within the epidermis of melanoma tissue sections (relative to DAPI) was quantified above background fluorescence from 4 or 2 representative images per tumour respectively, using ImageJ soft wear.

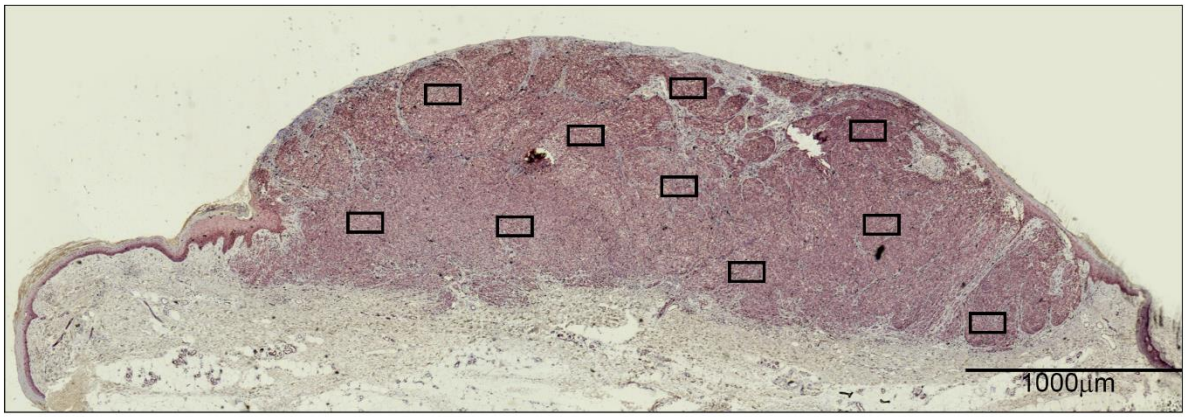


Figure 2.2 Example Image of VEGFR2 Expression in a Primary Cutaneous Melanoma, with Areas Highlighted for Expression Analysis

Example image of VEGFR2 expression in AJCC stage II primary cutaneous melanoma. The square boxes represent areas selected for 20x magnification high powered field examination. Image taken at 2.5x magnification. Scale bar -1mm.

For biomarker studies of CXCR4, CXCL12 or VEGFR2 data were correlated with clinical outcome after a minimum of seven years' follow-up, allowing the correlation of CXCR4, CXCL12 and VEGFR2 expression in the primary tissue sample with the time to development of first metastasis (disease free survival; DFS). Disease recurrence was defined by the time to first radiological or tissue diagnosis of metastatic disease (nodal or systemic) from the point of initial primary tumour excision, or time to death from melanoma (melanoma specific mortality; MSM). Reporting of the data was performed in line with the REMARK guidelines for tumour marker prognostic studies (McShane *et al.*, 2005).

Mean percentage total CXCR4 expression or mean nuclear CXCR4 staining score was compared with AJCC disease stage by One-way Analysis of Variance (ANOVA). Independent group analysis between localised and metastatic disease (eventual AJCC stage I/II versus eventual AJCC stage III/IV disease) was determined by Mann-Whitney *U*. The Difference between "high" and "low" CXCR4 expression levels for survival curve analysis was analysed by Wilcoxon Signed Rank Test, highlighting that 50% CXCR4 expression as an appropriate cut off point, as this expression level differed significantly in both localised and metastatic disease. Univariate and subsequent Log-rank (Mantel-Cox) survival analysis was undertaken using R 2.15.0 (R Foundation for Statistical Computing). Comparison of nuclear CXCR4 expression in localised and metastatic tumours (eventual AJCC stage I/II versus eventual AJCC stage III/IV disease), and comparison between wild-type and B-RAF/N-Ras mutated tumours was performed using an Unpaired T test.

In vivo tumoural CXCL12 Immunofluorescence data were expressed as mean fluorescence per cell and comparison made between AJCC disease stage by Kruskal-Wallis test with post hoc Dunn's multiple comparison test. Mann-Whitney *U* was used for analysis of CXCL12 fluorescence between localised and metastatic disease (eventual AJCC stage I/II versus eventual AJCC stage III/IV disease) or B-RAF mutated and wild-type melanomas, while Spearman's Rank Correlation was used to correlate CXCL12 expression with time to metastasis and CXCR4 expression. Pearson Product Moment Correlation analysis was used to correlate CXCL12 expression with nuclear CXCR4 expression.

In vivo CXCL12 Immunofluorescence within the epidermis beside the tumour bulk or epidermis overlying the tumour, was expressed as the mean expression per cell and comparison analysed using the Wilcoxon Signed Rank test. Correlation of CXCL12 expression in the epidermis beside the tumour with time to metastasis or tumoural cytoplasmic CXCR4 expression was analysed using Pearson product moment correlation.

In vivo uveal melanoma CXCL12 immunofluorescence data were expressed as the total mean fluorescence per cell. Mann-Whitney U was used for comparison of cutaneous and uveal melanomas, and unpaired T- test for analysis of uveal melanomas and differing chromosome 3 status.

Mean percentage VEGFR2 tumoural expression and mean percentage epidermis VEGFR2 expression was compared with AJCC disease stage by Kruskal Wallis analysis. Independent group analysis of VEGFR2 expression % (tumour) between localised and metastatic disease (eventual AJCC stage I/II versus eventual AJCC stage III/IV disease) was determined by Mann-Whitney *U*. Comparison between wild-type and B-RAF/N-Ras mutated tumours was performed using Mann-Whitney *U*. Correlation between Mean percentage VEGFR2 expression and mean percentage total CXCR4 expression was determined by Spearman's r correlation. Comparison between mean percentage tumour and epidermis VEGFR2 expression was assessed by Kruskal-Wallis test, and correlation determined by Spearman's r correlation.

2.18 Statistics

Throughout this thesis statistical analyses were performed using IMB SPSS statistic Soft-wear (version 22 for windows, IBM soft-wear Group, Chicago, Illinois, U.S.A) and GraphPad Prism (version 5.00 for windows, GraphPad Software, San Diego California USA). For all experiments

throughout data were assessed for normality by Shapiro-Wilk Test. Where there was not enough data point to analyse data by Shapiro-Wilk Test, normality was not assumed and non-parametric tests were applied. Normally distributed data were further assessed for homogeneity of variance by Levene Test, or sphericity by Mauchly's Test, before using the appropriate statistical test having satisfied statistical assumptions. Statistical significance is indicated by P values of $P< 0.05$ *, $P< 0.01$ **, $P< 0.001$ ***, throughout the thesis with values $P > 0.05$ deemed non-significant (ns).

In vitro basal VEGFR2 mRNA expression in cell lines and primary cells was determined by qPCR with an average of VEGFR2 Cycle threshold (Ct) values for each sample normalised to 18s mRNA Ct values. Normalised VEGFR2 mRNA expression between all cell lines and primary cells was analysed by Kruskal-Wallis test with Dunn's post hoc correction. Normalised VEGFR2 mRNA expression between VEGFR2 siRNA and non-target siRNA was compared by Students T-Test.

In vitro immunofluorescent expression of CXCR7 fluorescence per cell in EA.hy.926 and CXCR7 overexpressing Chinese Hamster Ovary cells was compared to null primary control cells by Students-T test. CXCL12 or VEGFR2 immunofluorescent expression by cell lines and primary cells was expressed as mean fluorescence per cell (relative to DAPI) and compared using Mann-Whitney *U* test.

ImageJ or Odyssey FC Image studio soft-wear (Li-cor) was used to determine band intensity of western blots. For each experiment protein intensity was normalised to GAPDH (loading control) intensity for each treatment condition and expressed relative to control of each individual experiment. Differing protein expression was analysed by One-way analysis of variance (ANOVA) with Dunnett's post hoc correction.

Analysis of the effect of drug combinations of trametinib and pazopanib on cell viability or cell chemotaxis compared with either drug given alone, was assessed using a one-way analysis of variance with Bonferroni's post hoc correction.

Chapter 3

CXCR4, CXCR7, and CXCL12 Expression and Signalling in Cutaneous and Uveal Melanoma

Chapter 3 CXCR4, CXCR7 and CXCL12 Expression and Signalling in Cutaneous and Uveal Melanoma

3.1 Introduction

The growing interest in the important role of the tumour microenvironment in cancer has accelerated the understanding of cancer biology. The CXCR4-CXCL12 chemokine axis provides the crosstalk between cancer cells and their microenvironment and hence is the target of much research and therapeutic interventions.

The CXCR4-CXCL12 chemokine axis plays a diverse role in directing cellular migration, as early as embryogenesis in humans where it regulates the migration and positioning of embryonic stem cells (Domanska *et al.*, 2013). The *de novo* synthesis of the chemokine CXCL12 throughout our body allows establishment of chemokine gradients where cells expressing the cognate receptor CXCR4 can be directed to locations in the body of high chemokine concentration, down 'cellular highways' (Zlotnik *et al.*, 2011). In adults the CXCR4-CXCL12 axis also has an important role in immune responses directing CXCR4 expressing leukocytes along gradients of CXCL12 present at the site of inflammation, and in neovascularisation, recruiting progenitor cells from the bone marrow to the site of new vessel formation (Loetscher P *et al.*, 2000; Roeliene C. Kruizinga *et al.*, 2009). Unsurprisingly in line with the Darwinian evolution of cancer, cancer cells may hijack the chemokine axis, upregulate chemokine receptors or secrete chemokine ligands which have diverse effects on cancer cell behaviour, the tumour microenvironment and establishment of metastasis.

Over a decade ago Muller and colleagues in a seminal paper, demonstrated the overexpression of CXCR4 in primary and metastatic breast tumours (Muller *et al.*, 2001b). We now know CXCR4 is the most widely over expressed chemokine receptor on malignant cells including melanoma, where aberrant expression results from oncogenic mutations, inactivation of tumour suppressor genes and hypoxia (Schioppa *et al.*, 2003; Guerra *et al.*, 2007; Pivarcsi *et al.*, 2007). The gain of CXCR4 expression in the context of melanoma determines destination, allowing tumour cells to behave like leukocytes and home towards the source of CXCL12 secreted at common sites of metastasis (Cardones *et al.*, 2003). The expression of chemokine receptors by tumour cells not only permits melanoma metastasis, but also promotes tumour cell survival and proliferation by activation of the MAP/ERK pathway (Robledo *et al.*, 2001). The significance of CXCR4 protein expression has also been demonstrated in both cutaneous and uveal melanomas. In uveal melanoma Scala *et al* correlated CXCR4 tumour expression with an epithelioid cell phenotype, conferring a worse

prognosis, while subsequent studies have shown increased CXCR4 expression correlates with the increased likelihood of hepatic metastasis (Scala *et al.*, 2007; Dobner *et al.*, 2012). In terms of cutaneous melanoma, elevated levels of CXCR4 expression have been linked to tumour ulceration, thickness as well as the development of metastasis (Longo-Imedio *et al.*, 2005; Scala *et al.*, 2005; Tucci *et al.*, 2007; Toyozawa *et al.*, 2012). However, to date, the significance of CXCR4 as a prognostic biomarker in melanoma has remained undefined.

Strikingly, the mechanisms mediating the regulation and expression of CXCL12 within primary tumours, and the microenvironment is also lacking. CXCL12 is widely expressed by many different tumour types and commonly induced by hypoxia or as a result of hormone triggered pathways where it exerts a wide variety of effects including, promoting tumour cell proliferation via the activation of AKT or ERK cell signalling or survival through induction of NFκB signalling (Wang CY *et al.*, 1996; Sonoda *et al.*, 2001; Bachelder *et al.*, 2002; Kryczek *et al.*, 2005). CXCL12 also has potent angiogenic activity and through synergistic action with VEGF induces endothelial cell proliferation, and recruitment of pro-angiogenic plasmacytoid dendritic cells (Curiel, 2004; Kryczek *et al.*, 2005). In metastasis, CXCL12 aids tumour dissemination by the induction of metalloproteinases that break down the extracellular matrix, and through up regulation of integrins that can promote tumour cell adhesion (Burger *et al.*, 2003; Singh *et al.*, 2004). The appropriate trafficking of immune cells and their retention in a tumour is important for immune detection and eradication, however CXCL12 can generate an immunosuppressive tumour microenvironment through recruitment of CD4+ T regulatory cells and plasmacytoid dendritic cells that induce immunosuppressive IL-10 secretion from T cells (Zou *et al.*, 2001; Zou *et al.*, 2004a). However, although many *in vivo* studies describe the diverse effects of CXCL12 in the tumour microenvironment, studies in melanoma are limited mainly to *in vitro* studies demonstrating the migration of tumour cells towards recombinant CXCL12 or enriched supernatants derived from liver cell types (O'Boyle *et al.*, 2013). Only one *in vivo* study in cutaneous melanoma, correlated absence of CXCL12 expression with poor prognosis (Robledo *et al.*, 2001; Di Cesare *et al.*, 2007a; Mitchell *et al.*, 2014). The role of CXCL12 in tumour progression particularly in primary melanoma is hence largely undefined.

To add complexity to the CXCR4-CXCL12 signalling axis, recent studies have identified CXCR7 as a chemokine receptor that binds to CXCL12 with 10 times the affinity of CXCR4 (Balabanian *et al.*, 2005). However, despite its classical features as a chemokine receptor, CXCR7 is in general, regarded as a ‘decoy’ receptor that ‘scavenges’ and hence removes monomeric

CXCL12 to dampen or inhibit CXCR4-CXCL12 signalling (Naumann *et al.*, 2010). Supporting these observations, studies in CXCR7 knockout mice or in the presence of CXCR7 inhibitors *in vivo*, have been shown to increase CXCL12 levels by up to 5 fold (Berahovich *et al.*, 2014). The scavenging of CXCL12 allows CXCR7 the ability to modulate circulating chemokine levels possibly priming a chemokine gradient for effective cell chemotaxis. Furthermore, the modulation of circulating CXCL12 levels by CXCR7 has also been shown to effect leucocyte CXCL12 homing, and hence may indirectly modulate tumour infiltration (Berahovich *et al.*, 2014). CXCR7 also has the ability to heterodimerise with CXCR4, which is thought to affect CXCR4 G-protein signalling, therefore CXCR7 has the potential to modulate CXCR4 downstream signalling including proliferation, survival and cell migration (Décaillot *et al.*, 2011b). Indeed the up regulation of CXCR7 has been shown to promote proliferation in breast cancer, tumour growth in lung cancer and migration in colon cancer (Décaillot *et al.*, 2011b; Hernandez *et al.*, 2011; Xu *et al.*, 2011). However, to date there have been no reports of CXCR7 expression on melanoma tumour cells, although notably one study demonstrates high expression by tumour associated blood vessels (Sánchez-Martín *et al.*, 2011b). The possibility of a role for CXCR7 in tumour angiogenesis is also postulated given it's known up regulation by hypoxia and VEGF, and ability to compromise vascular permeability when expressed on endothelial cells (Maksym *et al.*, 2009a; Totonchy *et al.*, 2014).

There is strong evidence of the importance of the CXCR4-CXCL12 axis in the migration of cancer cells to metastatic sites however the role of CXCR4, and CXCL12 within the primary tumour and microenvironment remains poorly defined. The possible interplay with CXCR7 in CXCR4-CXCL12 exclusive events and the urgent need for novel prognostic biomarkers in melanoma therefore leads to the aims of the current chapter; to define the expression of CXCR4, CXCR7 and CXCL12 in uveal and cutaneous metastatic melanoma cell lines and *in vivo* in a cohort of primary tumours as well as to define the interplay between components of the CXCR4/CXCR7-CXCL12 axis.

3.2 Results

3.2.1 CXCR4 is a Putative Prognostic Biomarker for AJCC Stage II Cutaneous Melanomas

A pre-optimised immunohistochemical assay (A.McConnell Mres 2013) was used to quantify CXCR4 expression in a cohort of 64 melanocytic naevi and primary mealnomas of differeing AJCC stage. The mean percentage positively stained cells was calculated from 10 representative 20X high powered fields using Leica QWin software. Results demonstrated variable CXCR4 expression in both the cytoplasm and the nucleus of both benign naevi and all AJCC stage primary melanomas (Figure 3.1).

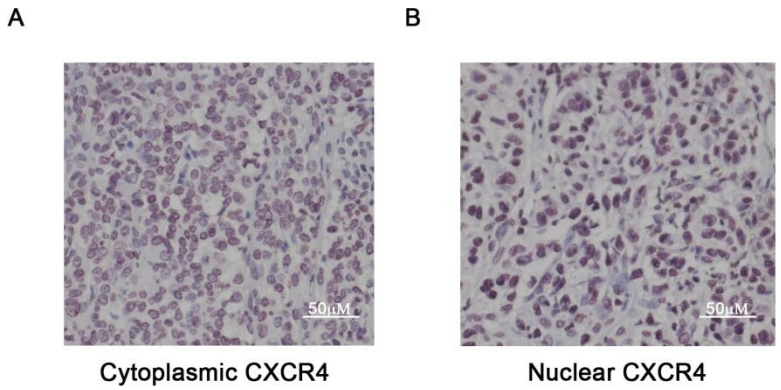


Figure 3.1 Cytoplasmic and Nuclear CXCR4 Epression in Primary Cutaneous Melanomas
Example pictomicrographs of Cytoplasmic CXCR4 expression A.) or Nuclear CXCR4 expresion B.) in primary cutanous melanomas. Images were acquired at x20 magnification. Scale bar represents 50 µm.

Correlation of total CXCR4 expression with eventual AJCC disease stage revealed a general decrease in total CXCR4 expression from naevi to AJCC stage II melanomas with a significant increase in CXCR4 expression from stage II melanomas to stage III/IV melanomas (One-way ANOVA, *P=0.0296, Figure 3.2 A). Furthermore, correlation of total CXCR4 expression in localised and metastatic disease revealed a significant increase in mean percentage CXCR4 expression from 37.48% in localised melanomas to 56.08% in tumours which metastasised (Mann-Whitney *U*, *P=0.0369, Figure 3.2 B). These results thus highlight up regulation of CXCR4 expression associated with the development of metastatic disease, consistent with the reported role of CXCR4 in cancer cell migration (Kim *et al.*, 2006).

The significant difference in CXCR4 expression between localised and metastatic tumours allowed a differential cut off point of 50% CXCR4 expression that differed significantly between localised and metastatic tumours, calculated using a Wilcoxon signed-rank test.

Therefore 50% CXCR4 expression was considered an appropriate cut off point, for further analysis. Univariate analysis of mean percentage total CXCR4 expression in all AJCC stage melanomas revealed a non-significant decrease in disease free survival from 65.5% for individuals expressing low CXCR4 (<50%), to 47.6% in individuals expressing high CXCR4 (>50%) over seven years (Log-rank (Mantel-Cox) test HR = 1.99 (95% CI 0.81 – 4.91) P=0.13, Figure 3.2 C). However, stratification according to AJCC stage II melanomas at diagnosis, revealed high CXCR4 expression (>50%) was associated with significantly decreased disease free survival to 30.77%, compared to 73.68% of individuals with low CXCR4 expression (<50%) and who remained disease free over 7 years (Log-rank (Mantel-Cox) test HR = 3.24 (95% CI 1.08 – 9.73) *P=0.036) (Figure 3.2 D). Collectively, these results therefore highlight a 3-fold risk of disease reoccurrence for individuals with high total CXCR4 expression (>50%), further suggesting high CXCR4 expression (>50%) may be a useful prognostic biomarker for disease progression in AJCC stage II melanomas.

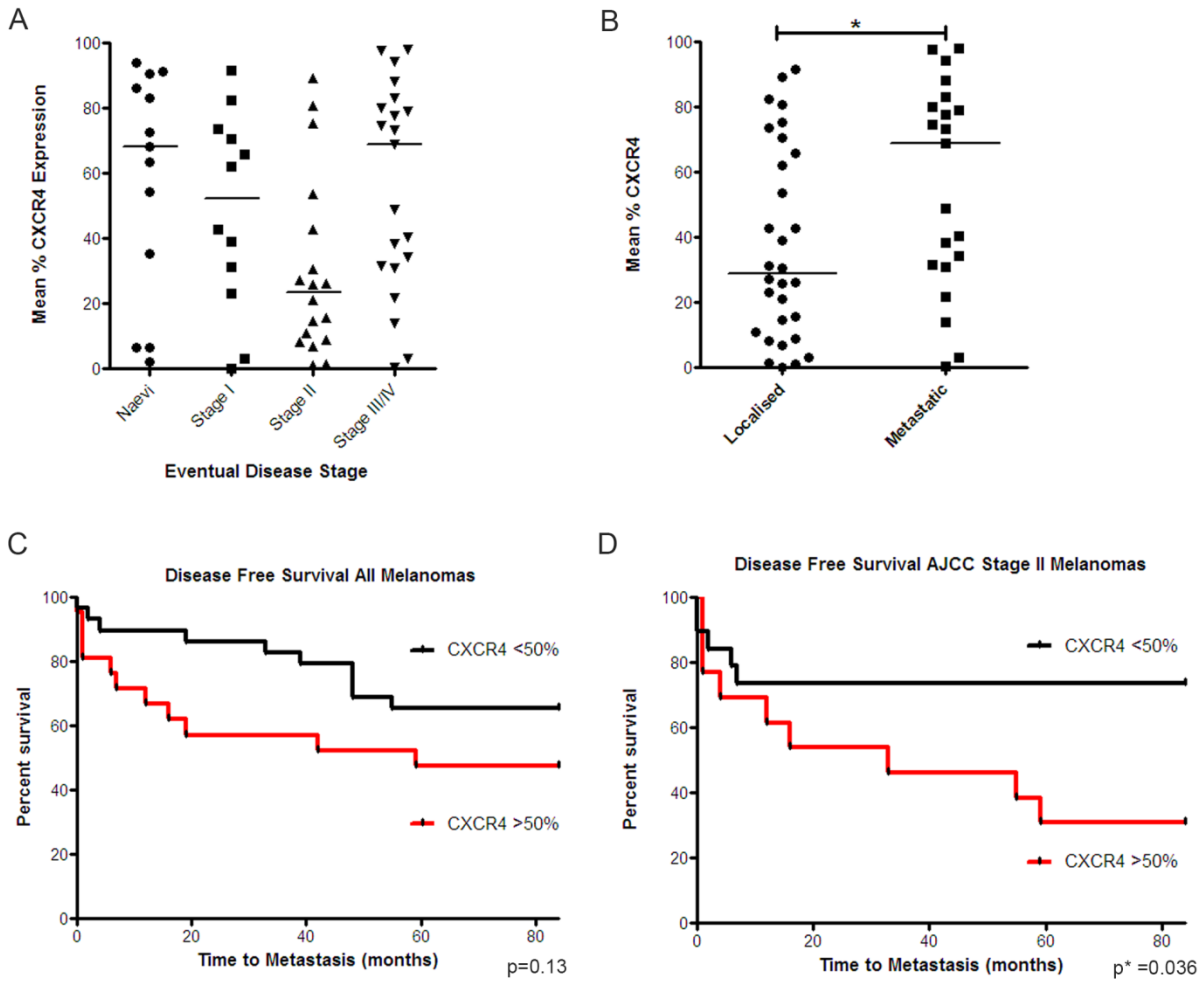


Figure 3.2 CXCR4 Expression in Primary Melanomas.
*A.) Scatter graph representing the mean (mean of 10 HPF) % positive cells for CXCR4 expression in eventual stage melanocytic naevi or AJCC stage I, II or III melanomas. The horizontal bar represents the median % staining for each group. B.) Scatter graph representing the mean % positive CXCR4 staining cells in localised (eventual AJCC stage of disease I/II) and metastatic melanomas (eventual AJCC stage III/IV) after 7 years follow up. Horizontal bars represent median staining percentage, (Mann-Whitney U P *= 0.0369) C.) Kaplan-Meier survival curve showing univariate analysis of % CXCR4 expression and disease free survival (7 years) in all stage melanomas. Vertical lines represent individual patients developing a metastasis. Log-rank (Mantel-Cox) test HR = 1.99 (95% CI 0.81 – 4.91) P = 0.13.D.) Kaplan-Meier survival curve showing Univariate analysis of % CXCR4 expression and disease free survival (7 years) in stage II melanomas. Vertical lines represent individual patients developing a metastasis. Log-rank (Mantel-Cox) test HR = 3.24 (95% CI 1.08 – 9.73) *P = 0.036.*

Studies in breast, renal and lung cancer demonstrate the expression of nuclear CXCR4 correlates with the increased risk of metastasis, however in cutaneous melanoma this has remained undefined (Na *et al.*, 2008; Woo *et al.*, 2008; Linhui Wang *et al.*, 2009). Further analysis of nuclear CXCR4 expression in all AJCC stage melanomas revealed a stepwise but non-significant increase in nuclear CXCR4 expression with disease progression (One-way ANOVA, P=0.78, Figure 3.3 A). However, when comparing nuclear CXCR4 expression in localised and metastatic disease, results revealed a significant increase in nuclear CXCR4

expression in metastatic disease (Unpaired t-test *P=0.027, Figure 3.3 B). In addition, correlation of nuclear CXCR4 expression in all AJCC stage wild-type with B-RAF/N-Ras mutated melanomas, revealed a significant increase in nuclear CXCR4 expression in those tumours bearing an activating mutation in either B-RAF or N-Ras (Unpaired t-test ***P = 0.0004, Figure 3.3 C). Collectively these data suggest an association of nuclear CXCR4 expression with oncogenic N-Ras/B-RAF signalling and the evolution of a more aggressive tumour phenotype.

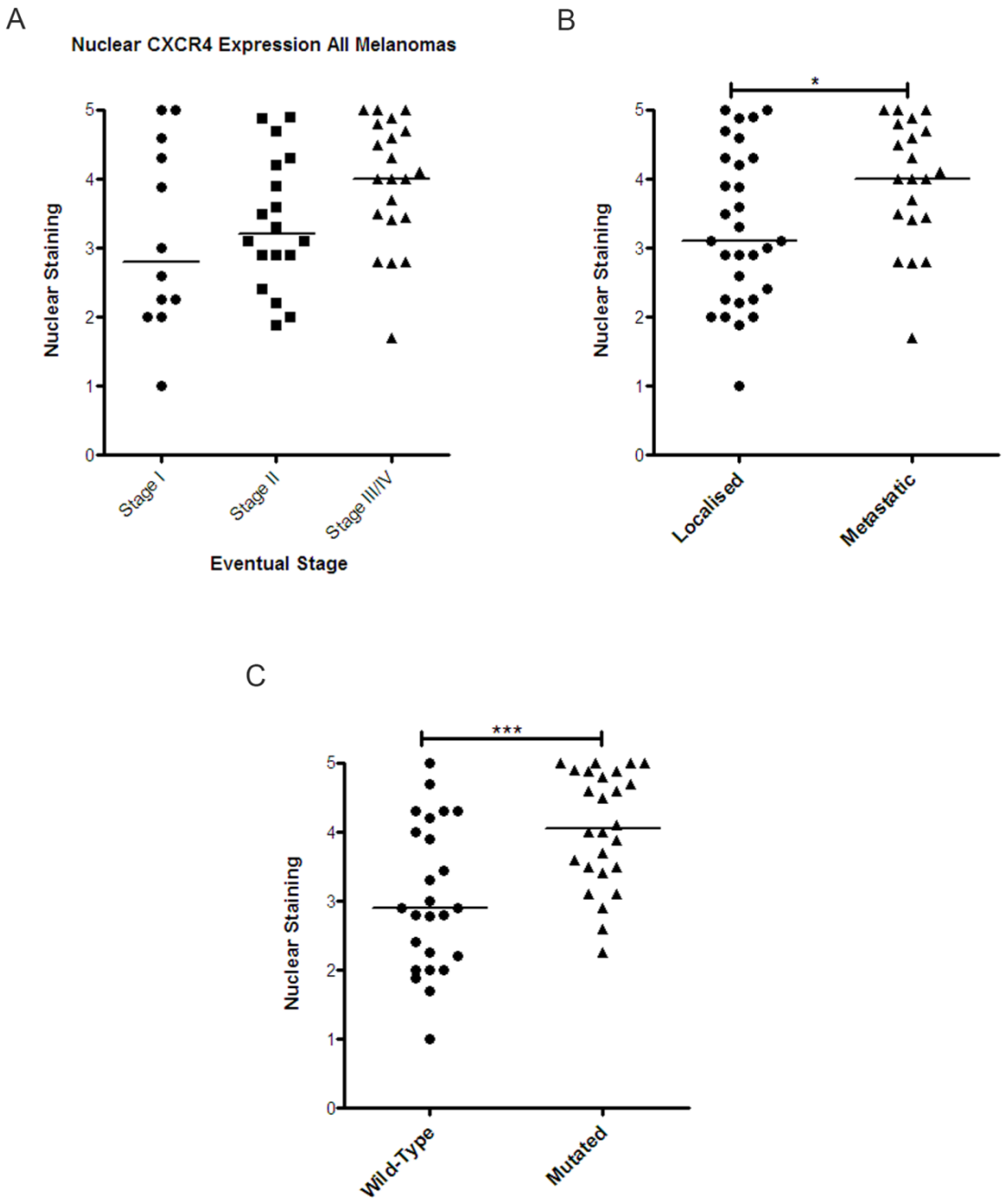


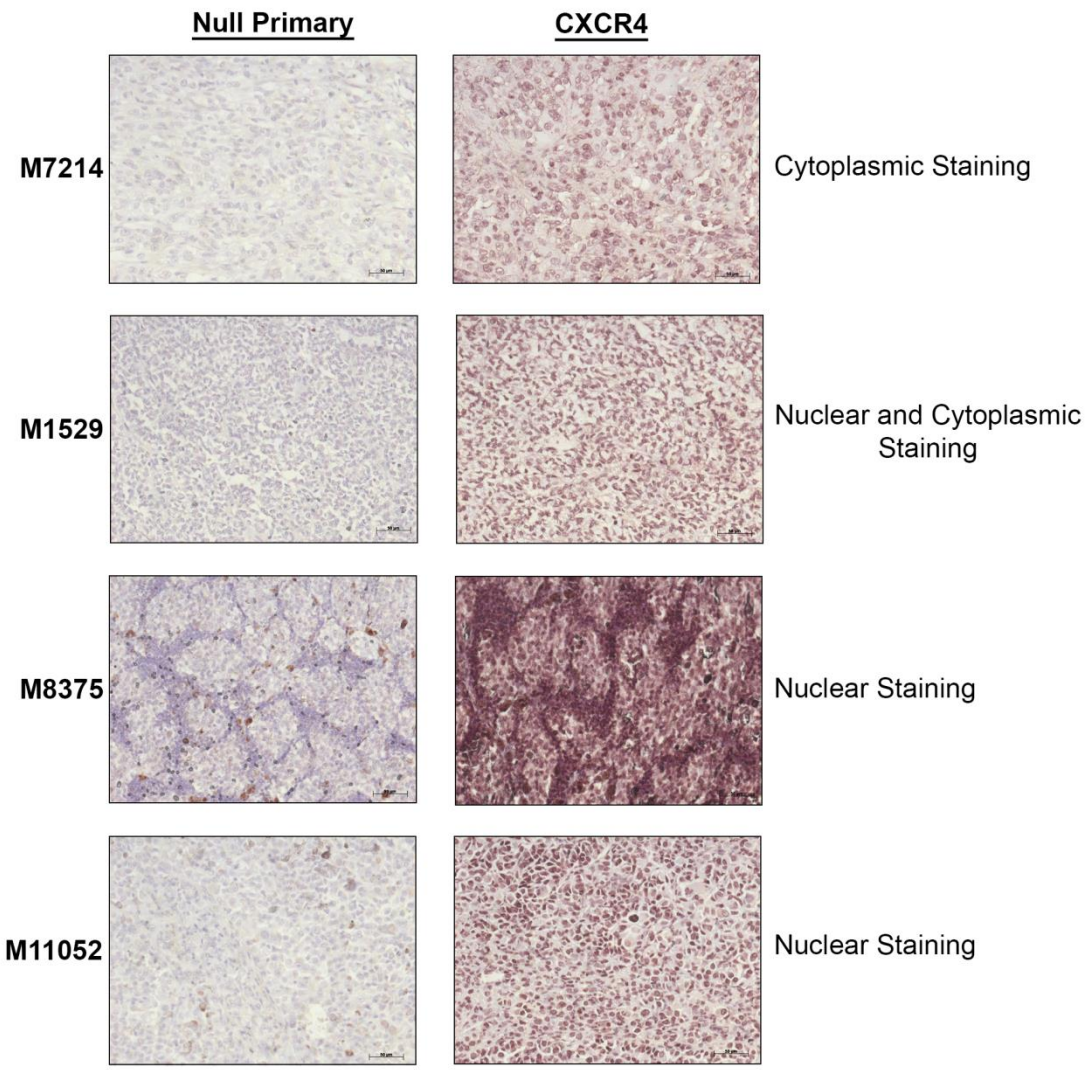
Figure 3.3 Nuclear CXCR4 Expression in Primary Melanomas

*A.)Scatter graph representing the mean (mean of 10 HPF) nuclear CXCR4 score in eventual stage melanocytic naevi or AJCC stage I, II or III melanomas. B.) Scatter graph representing the mean nuclear CXCR4 score in localised (eventual AJCC stage of disease I/II) and metastatic melanomas (AJCC stage III/IV) after 7 years follow up (Unpaired t-test *P = 0.027) C.) Scatter graph representing the mean nuclear CXCR4 score in B-RAF/N-Ras wild-type and B-RAF/N-Ras mutated melanomas all AJCC stages (Unpaired t-test *** P = 0.0004). The horizontal bar represents the mean nuclear CXCR4 staining score for each group.*

3.2.2 CXCR4 is Strongly Expressed by Primary Uveal Melanomas

Using the same optimised immunohistochemistry protocol as performed in Section 3.2.1, CXCR4 expression was determined in a small cohort of 12 primary uveal melanoma tumours. Results demonstrated CXCR4 expression in 83% of tumours, where expression, be it nuclear or cytoplasmic, was observed in all tumour cells (Figure 3.4), consistent with results obtained from a study of 70 uveal melanomas which demonstrated total CXCR4 expression in 98.6% of tumours, with expression levels of 75-100% (Dobner *et al.*, 2012). In the current primary uveal melanoma cohort however, both nuclear and cytoplasmic CXCR4 expression were observed, although in general results revealed expression as either cytoplasmic or nuclear, with dual CXCR4 expression observed in 2 tumours (Figure 3.4) and with no apparent correlation between subcellular localisation or monosomy or disomy of chromosome 3 (Figure 3.4 B).

A



B

Primary Uveal Tumours	Chromosome 3 Status	CXCR4 Staining	CXCR4 Subcellular Localisation
M13705/12	Monosomy	Negative	
M7871/13	Monosomy	Negative	
M12068	Monosomy	100%	Cytoplasmic
M8375	Monosomy	100%	Nuclear
M7214	Monosomy	100%	Cytoplasmic
M1529	Monosomy	100%	Cytoplasmic and Nuclear
M11052(A6)	Monosomy	100%	Nuclear
M11052(A7)	Monosomy	100%	Nuclear
M07885/12	Disomy	100%	Nuclear
M9219/12	Disomy	Undetermined due to melanin staining	
M1135	Disomy	100%	Cytoplasmic
M13759	Disomy	100%	Nuclear
M16827	Disomy	100%	Nuclear, some cytoplasmic

Figure 3.4 Immunohistochemistry of CXCR4 Expression in Primary Uveal melanomas
A.) Representative images of CXCR4 immunohistochemical expression in a cohort of primary uveal melanomas. Images were acquired at x20 magnification. Scale bar represents 50µm. B.) Summary table of cohort features and CXCR4 immunohistochemical expression.

3.2.3 CXCR4 is Expressed by Cutaneous and Uveal Metastatic Melanoma Cell Lines and Primary Melanocytes

To further explore any impact of hyper-activating mutations in MAPK signalling on nuclear and cytoplasmic expression of CXCR4, both total and nuclear/cytoplasmic CXCR4 expression were evaluated in a panel of B-RAF/N-Ras wild-type or mutated cutaneous or wild-type and GNAQ/GNA11 mutant uveal melanoma cell lines, and compared with expression in normal cutaneous melanocytes.

Western blot analysis of total CXCR4 expression revealed variable but consistent expression of CXCR4 in primary melanocytes as well as in cutaneous CHL-1 (B-RAF/N-Ras wild-type), WM-164 (B-RAF^{V600E} mutated) and WM-1361 (N-Ras mutated) metastatic melanoma cell lines, and in OM413 (GNAQ/GNA11 wild-type), MEL270, OMM2.3 (GNAQ mutated) and UPMD2 (GNA11 mutated) uveal melanoma cell lines, which in all melanoma cell lines was enhanced compared to expression in primary melanocytes (Figure 3.5).

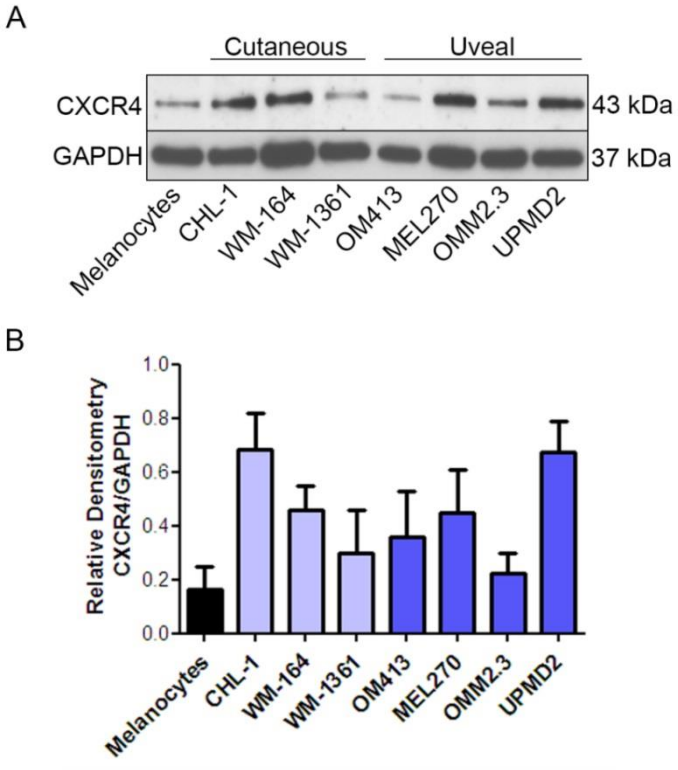


Figure 3.5 Total CXCR4 Expression in Human Uveal Melanoma Cell Lines.
Representative western blot of CXCR4 and GAPDH (loading control) expression in A.) CHL-1 (wild-type) WM-164 (V600E B-RAF mutated), or WM-1361 (N-Ras mutated) human metastatic cutaneous melanoma cells or OM413 (GNAQ/GNA11 wild-type), MEL270, OMM2.3, (GNAQ mutated) and UPMD2 (GNA11 mutated) human uveal melanoma cell lines. B.) Each bar represents the mean of 4 replicates of CXCR4 band intensity normalised to GAPDH band intensity (CXCR4/GAPDH) for each cell line, and expressed relative to the mean of each individual experiment (mean ± SD, N = 4).

Immunofluorescence analysis for subcellular localisation of CXCR4 revealed expression mainly in the cytoplasm of primary melanocytes, (Figure 3.6), whereas in a select panel of both cutaneous and uveal metastatic melanoma cell lines (Figure 3.7) consistent expression of both nuclear and cytoplasmic CXCR4 was observed. Interestingly and consistent with findings of increased nuclear CXCR4 in primary cutaneous metastatic melanomas (Figure 3.3 B), results demonstrated increased nuclear CXCR4 expression in metastatic OMM2.3 uveal melanoma cells compared to expression in MEL270, the primary uveal melanoma cell line derived from the same patient (derived from the liver metastasis).

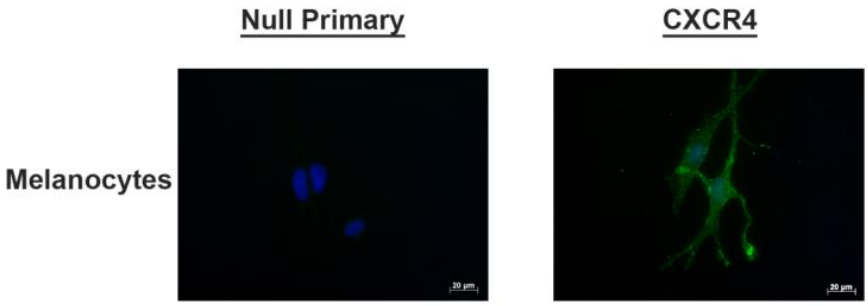


Figure 3.6 Immunofluorescent Analysis of CXCR4 Expression in Primary Melanocytes.
Representative image of CXCR4 or null primary antibody control expression in primary melanocytes. Green depicts CXCR4 positivity and blue DAPI nuclear staining. Images taken by confocal microscopy with a magnification 20x scale bar = 50µm.

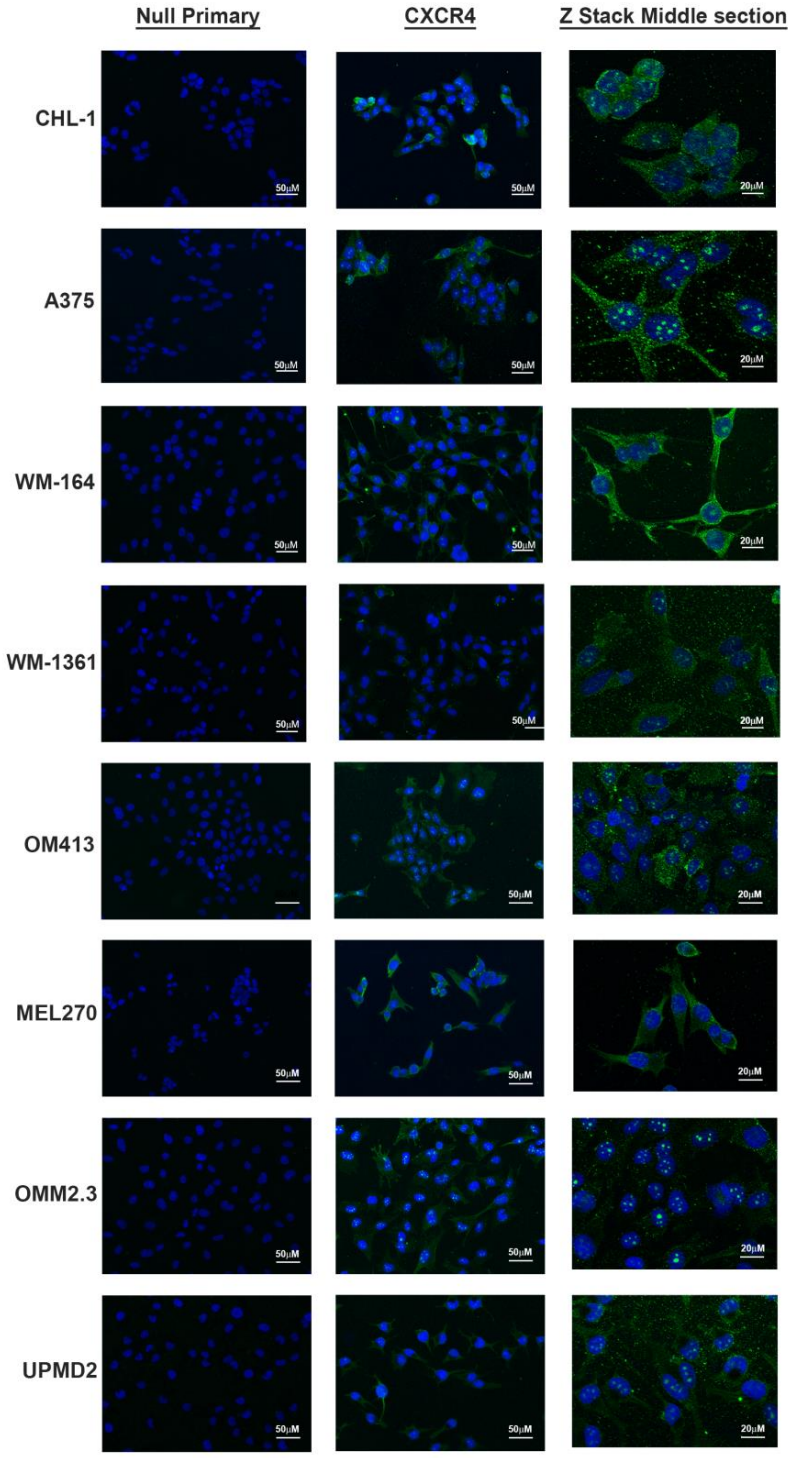


Figure 3.7 Immunofluorescence for the Expression of CXCR4 in Cutaneous and Uveal Metastatic Melanoma Cell Lines.

Representative images from 3 replicate experiments for the immunofluorescent expression of cutaneous metastatic melanoma cell lines CHL-1, A375, WM-164 and WM-1361 or null primary control or uveal melanoma cell lines OM413, MEL270, OMM2.3 and UPMD2 or null primary. Green depicts CXCR4 positivity and blue DAPI nuclear staining. Images were acquired by confocal microscopy with a magnification 20x scale bar = 50µm or Z stacks of cells taken and the middle cross section of the cell shown, with a magnification of 40x, scale bar 20µm.

3.2.4 Oncogenic B-RAF Does Not Enhance Nuclear Localisation of CXCR4

To test the hypothesis that activated MAPK in cutaneous melanoma cells signalling mediated by B-RAF/N-Ras (or GNAQ/GNA11) mutation may result in the translocation of CXCR4 expression to the nucleus as suggested by *in vivo* data demonstrating the significant increase in nuclear CXCR4 expression in tumours bearing an activating mutation in either B-RAF or N-Ras (Unpaired T-test ***P=0.0004, Figure 3.3 C), B-RAF^{WT} or B-RAF^{V600E} were transiently transfected into B-RAF^{WT} CHL-1 cells prior to determining CXCR4 subcellular localisation by western blotting and immunofluorescence. Expression of B-RAF^{WT} or B-RAF^{V600E} was confirmed by a significant increase in total B-RAF expression in cells transfected with both B-RAF^{WT} or B-RAF^{V600E} compared to un transfected cells (One way Analysis of variance with Dunnett's Post Hoc correction *P<0.05, **P<0.005 for B-RAF^{WT} or B-RAF^{V600E} transfected cell respectively , Figure 3.8 A and B), with activation of oncogenic B-RAF signalling confirmed by a significant increase in the phosphorylation of ERK (One way analysis of variance with Dunnett's Post Hoc correction ***P<0.001) (Figure 3.8 A and C). Western blotting of cytoplasmic and nuclear cell components following cell fractionation revealed consistently and significantly lower expression of CXCR4 in the cytoplasm compared to nuclear subcellular location in all conditions (Paired T test ***P<0.0001). However, there was no significant change in CXCR4 expression observed in either subcellular locations of cells overexpressing B-RAF^{WT} or B-RAF^{V600E} compared to un transfected cells (One-way analysis of variance P>0.05 ns) (Figure 3.8 D and E). Immunofluorescence analysis of CXCR4 expression in both cytoplasmic and nuclear fractions of CHL-1 cells over expressing B-RAF^{WT} or B-RAF^{V600E} also revealed no significant changes in the subcellular CXCR4 location compared to un-transfected cells, (Figure 3.9), collectively suggesting that, at least, *in vitro* oncogenic B-RAF^{V600E} does not confer enhanced nuclear localisation of CXCR4.

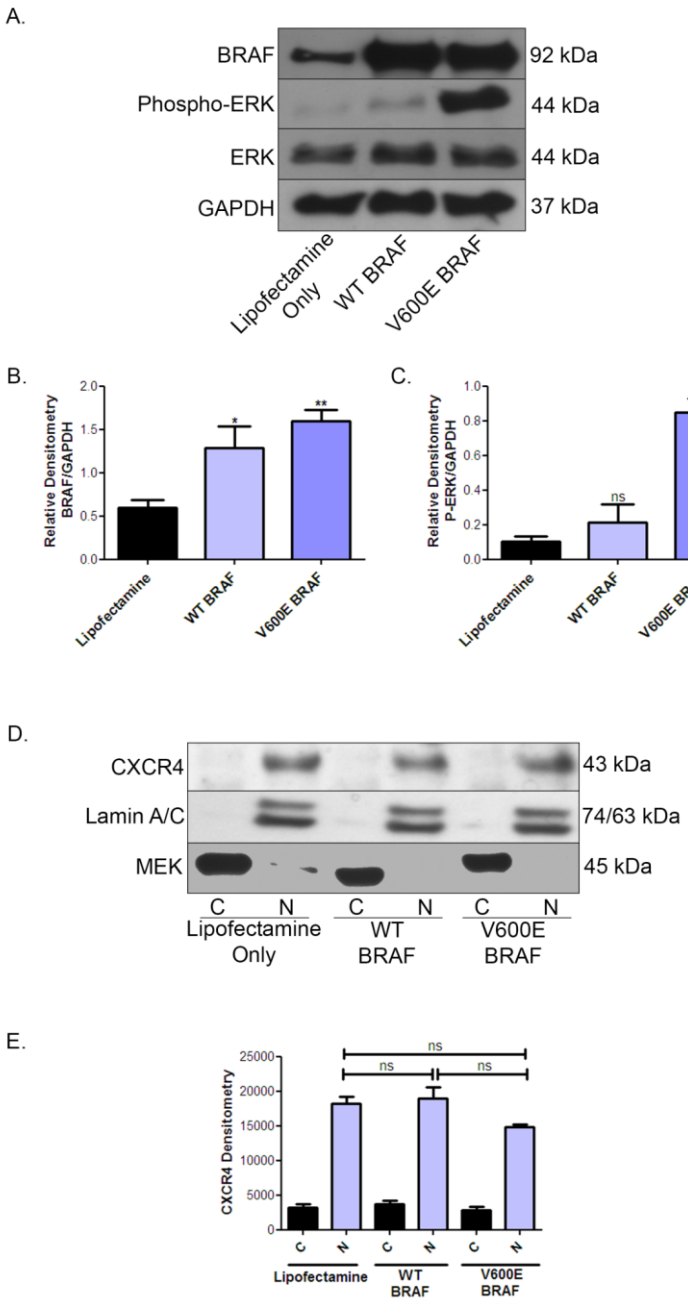


Figure 3.8 Oncogenic B-RAF Does Not Enhance Nuclear Localisation of CXCR4

*A.) Western blot for the expression of total B-RAF, Phospho-ERK, ERK and GAPDH loading control in CHL-1 cells (lipofectamine only) or in CHL-1 cells transiently transfected for 72 hours with B-RAF^{WT} (WT B-RAF) or B-RAF^{V600E} (V600E B-RAF) B and C) Relative expression of B-RAF or pERK in CHL-1 cells (lipofectamine) or CHL-1 cells transiently transfected for 72 hours with WT B-RAF or V600E B-RAF. Each bar is the mean of 3 replicate experiments normalised to GAPDH band intensity, and expressed relative to the mean of each individual experiment (mean ± SD N=3), statistics acquired by One-way analysis of variance, P=0.0143 with Dunnett post- hoc test, *P<0.05, **P<0.01. ***P<0.0001. D.) Western blot for the expression of CXCR4, Lamin A/C (Nuclear loading control) and MEK (Cytoplasmic loading control) in cytoplasmic and nuclear subcellular fractions of CHL-1 cells (lipofectamine only) or CHL-1 cells transiently transfected for 72 hours with WT B-RAF or V600E B-RAF. E.) Relative expression of CXCR4 in CHL-1 cells (lipofectamine) or CHL-1 cells transiently transfected for 72 hours with WT B-RAF or V600E B-RAF Each bar represents the mean of 3 replicate experiments expressed relative to the mean of each individual experiment (mean ± SD N=3), statistics acquired by One-way analysis of variance with Dunnett's post-hoc test, P>0.05 ns.*

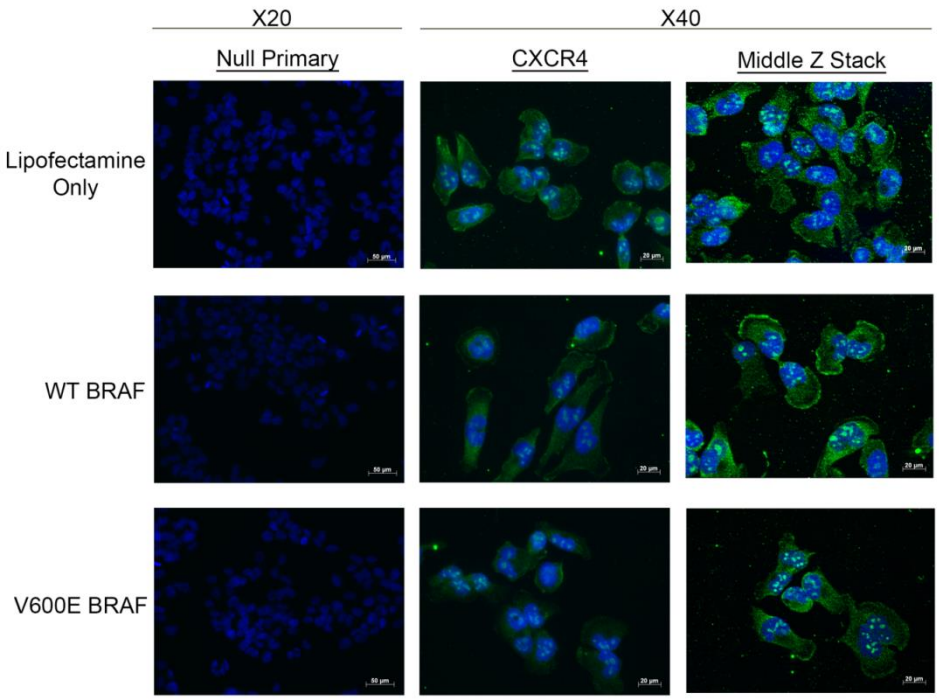


Figure 3.9 Oncogenic B-RAF Does Not Enhance Nuclear Localisation of CXCR4
Representative images for the immunofluorescent expression of CXCR4 in 3 replicate experiments of CHL-1 cells (lipofectamine only) or CHL-1 cells transiently transfected for 72 hours with B-RAF^{WT} (WT B-RAF) or B-RAF^{V600E} (V600E B-RAF) or null primary antibody control. Green depicts CXCR4 positivity and blue DAPI nuclear staining. Images taken by confocal microscopy with a magnification 20x scale bar = 50µm, 40x scale bar = 20µm or Z stacks of cells taken and the middle cross section of the cell shown, with a magnification of 40x, scale bar 20 µm (N=3).

3.2.5 CXCR7 is Not Expressed by Cutaneous and Uveal Metastatic Melanoma Cell Lines

CXCR7 has recently been described as an additional binding partner to CXCL12 and although originally thought to 'scavenge' CXCL12 to dampen, inhibit or regulate CXCR4-CXCL12 signalling, CXCR7 has also been shown to induce cell proliferation and migration in some cancer cell types (Naumann *et al.*, 2010; Décaillot *et al.*, 2011b; Singh and Lokeshwar, 2011; Xu *et al.*, 2011; Yates *et al.*, 2013). Furthermore, its reported expression on tumour associated endothelial cells suggests a contribution to tumour angiogenesis (Wang *et al.*, 2008b). Given the unclear role of CXCR7 in melanoma, its potential contribution to the CXCL12-CXCR7 axis and to processes that were previously thought to be monogamously CXCL12-CXCR4 driven, the expression and role of CXCR7 in melanomas was therefore further evaluated. Immunofluorescence analysis for the expression of CXCR7 was performed in primary cutaneous metastatic CHL-1, WM-164, metastatic uveal melanoma cell lines OM413 and UPMD2 (Figure 3.10) or primary melanocytes (Figure 3.11) and compared with expression in known control expressing cell lines; HeLa, EA.hy926 or Chinese hamster Ovary (CHO) cells

stably over expressing CXCR7 (a kind gift from Professor Simi Ali, Newcastle University, Figure 3.10).

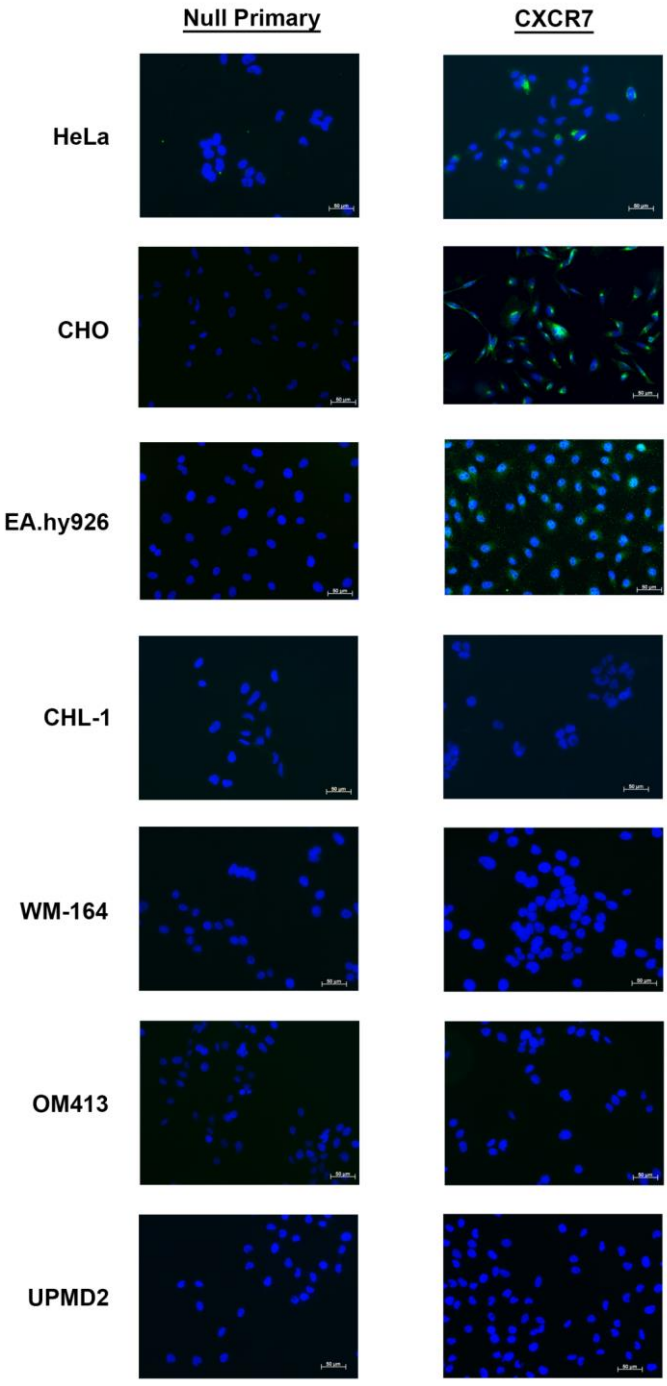


Figure 3.10 Immunofluorescence for the Expression of CXCR7 in Cutaneous and Uveal Metastatic Melanoma Cell Lines.

Representative images from 3 replicate experiments for the immunofluorescent expression of CXCR7 in Hela, Chinese Ovary cells stably expressing CXCR7 (CHO), EA.hy926 cells (positive controls), or cutaneous metastatic melanoma cell lines CHL-1, and WM-164 or metastatic uveal melanoma cell lines OM413 and UPMD2 or null primary antibody control. Green depicts CXCR7 positivity and blue DAPI nuclear staining. Images taken by confocal microscopy with a magnification 20x scale bar = 50µm.

Results demonstrated cytoplasmic CXCR7 was expressed by Hela cells, EA.hy926 and CHO cell stably expressing CXCR7 (Figure 3.10). However, there was no apparent expression of CXCR7 by either the cutaneous or uveal melanoma cell lines (Figure 3.10) or in primary melanocytes (Figure 3.11).

Further quantification of CXCR7 expression also confirmed the significant expression of CXCR7 on EA.hy926 endothelial cells as well as a positive control CHO cells over expressing CXCR7 (Unpaired T test **P= 0.0016 and **P=0.0018 respectively, Figure 3.12).

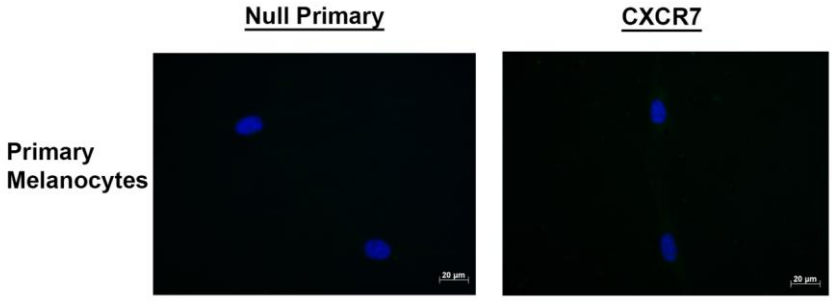


Figure 3.11 CXCR7 is not Expressed by Primary Melanocytes
Representative images for the immunofluorescent expression of CXCR7 in primary melanocytes. Green depicts CXCR7 positivity and blue DAPI nuclear staining. Images taken by confocal microscopy with a magnification 20x scale bar = 50µm (N=3).

Collectively these data therefore suggest CXCR7 expression is negligible on cutaneous and uveal melanoma cells but is expressed by endothelial cell types, consistent with previous studies *in vivo* demonstrating expression of CXCR7 within the tumour microenvironment of cutaneous melanoma and specifically expression by the endothelial cells of tumour associated blood vessels (Sánchez-Martín *et al.*, 2011b).

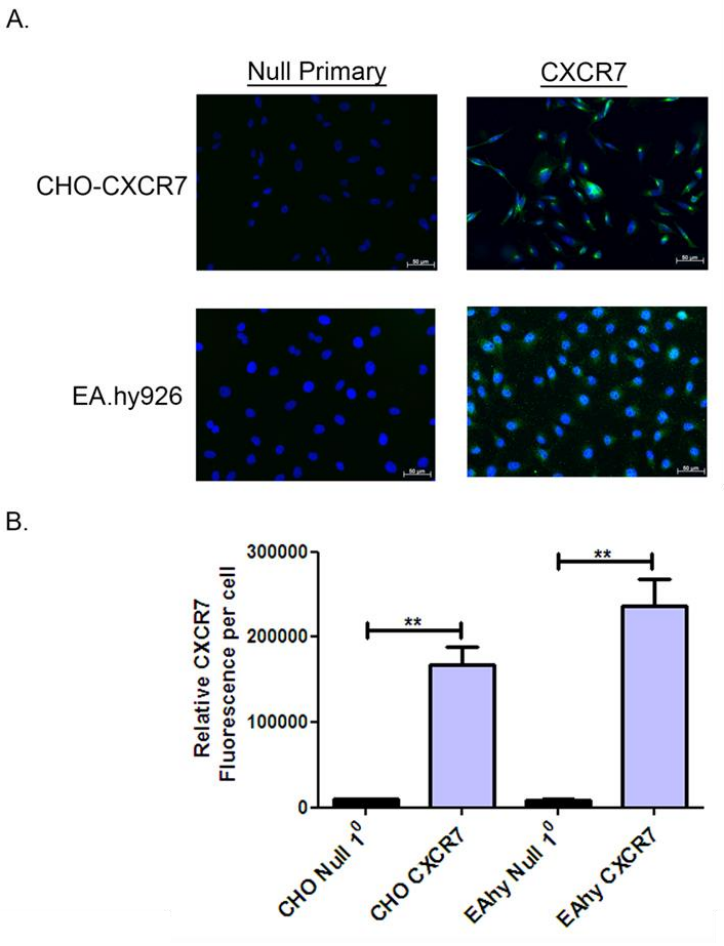


Figure 3.12 Immunofluorescence for the Expression of CXCR7 in Chinese Hamster Ovary Cells Overexpressing CXCR7 and EA.hy926 Umbilical Vein Endothelial Cells

*A.) Representative images from 3 replicate experiments of CXCR7 expression in Chinese hamster ovary cells (CHO-CXCR7, positive control), or EA.hy926 endothelial cells or following staining with a null primary antibody control. Green fluorescence represents CXCR7 expression and cell nuclei are represented by blue DAPI staining. Images were acquired by confocal microscopy with a magnification 20x scale bar = 50µm (N=3). B) Relative CXCR7 fluorescence/expression in CHO-CXCR7 or EA.hy.926 cells compared to expression in corresponding cells stained with null primary antibody control. Statistics acquired by unpaired T test **P<0.01. Each bar is the mean +/- SEM (N=3).*

3.2.6 CXCR7 is Not Expressed by Melanocytic Naevi or Primary Cutaneous Melanomas but is Expressed Within the Tumour Microenvironment

To confirm previous reports of CXCR7 expression within the tumour microenvironment of cutaneous melanoma *in vivo* and to further assess any potential expression by melanocytes or melanoma cells, the expression of CXCR7 was determined in cohort of 24 primary FFPE cutaneous melanomas of differing disease stage or melanocytic naevi using a previously optimised immunohistochemistry assay (optimised in collaboration with Barnaby Pathy, undergraduate 2015), and optimised using Hela FFPE cell pellets (Figure 3.13) (Sánchez-Martín *et al.*, 2011b).

Results confirmed *in vitro* studies, with no apparent expression of CXCR7 expression detected in any of the 24 analysed cutaneous melanomas or benign nevi. However, notably, in 7 tumours (30%) expression of CXCR7 was clearly detectable within the tumour microenvironment of the dermis seemingly on cells lining the inner lumen of blood vessels and capillaries, postulated to be endothelial cells (Figure 3.13). In 1 advanced stage melanoma there was also strong CXCR7 expression in the immune infiltrate of the tumour, possibly on T or B lymphocytes, tumour associated macrophages or dendritic cells known to express CXCR7 (Sánchez-Martín *et al.*, 2013). Nevertheless, the expression of CXCR7 within the tumour microenvironment was varied and could not be correlated to any particular disease stage or with B-RAF/N-Ras mutational status. Thus, data confirm the absence of CXCR7 on melanoma tumour cells, but with expression evident within the tumour microenvironment of a proportion of melanomas.

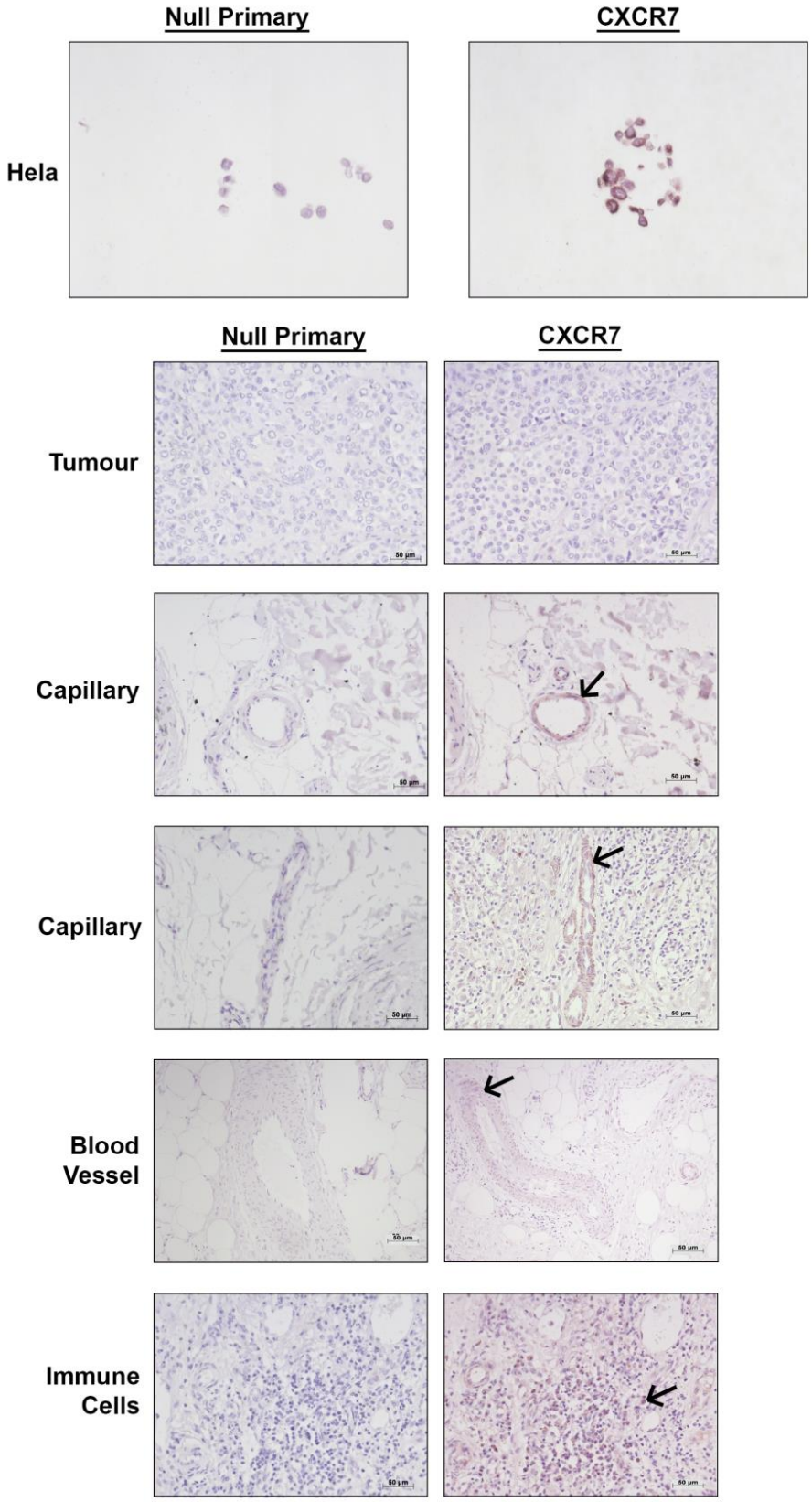


Figure 3.13 Immunohistochemical Expression of CXCR7 in Primary Cutaneous Melanomas.
Representative images of CXCR7 immunohistochemical expression in FFPE Hela cells (positive control) or a cohort of primary cutaneous melanomas or following staining with a null primary antibody control. Images were acquired at x20 magnification. Arrow depicts positive CXCR7 staining in capillaries, blood vessels and immune cells.Scale bar = 50µM.

3.2.7 CXCR7 is Not Expressed by Primary Uveal Melanomas

The immunohistochemical expression of CXCR7 was also determined within a small cohort of 7 FFPE primary uveal melanomas (Figure 3.14) with results again confirming *in vitro* studies in uveal melanoma cell lines (section 3.2.5), with no detectable expression of CXCR7 on primary uveal melanoma tumour cells (Figure 3.14 iii), or cells within the normal retina or choroid of the eye (Figure 3.14 iv).

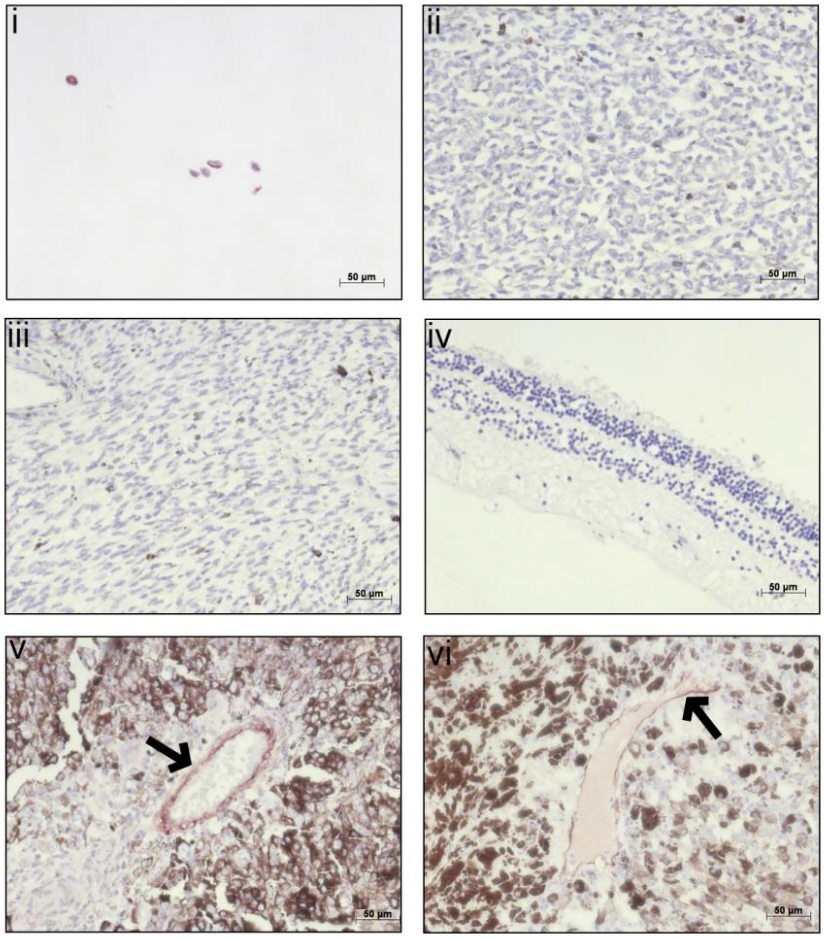


Figure 3.14 Immunohistochemical Expression of CXCR7 in Primary Uveal Melanomas
Representative images of CXCR7 expression on Hela cells (positive control, (i) or Primary Uveal melanomas (ii- vi) or following staining with null primary antibody control. Images depict Primary Uveal Melanoma tumour cells (iii), the choroid and retina of the eye (iv) or capillaries within primary uveal melanomas (v and vi) Black arrow depicts positive CXCR7 expression. Images were acquired at x40 magnification. Scale bar = 50µm.

Similarly, to observations in cutaneous melanoma (section 3.2.6), CXCR7 expression was detected on cells lining capillaries within the bulk of 3 tumours (42%) (Figure 3.14 v and vi), suggesting as with cutaneous melanoma, CXCR7 expression is present within the

microenvironment of some primary uveal melanomas and specifically expressed by endothelial cell types.

3.2.8 Autocrine CXCR4-CXCL12 Cell Signalling Activates the Pro-survival MAPK Signalling in Cutaneous Metastatic Melanoma Cell Lines

In melanoma, the CXCR4-CXCL12 axis is well known to promote the specific homing of CXCR4 positive tumour cells to regions with high levels of CXCL12 found at the secondary sites of metastasis. In addition to promoting organ specific migration, CXCL12 can also promote cell proliferation, survival, angiogenesis and immunosuppression, all factors that can have a pro-tumorigenic effect on the primary tumour and its microenvironment. However, the effect of CXCL12 and its presence in the primary melanoma tumour microenvironment remains poorly understood, with the few studies performed, reporting non-significant and contradictory results. To assess the role of CXCL12 in the primary tumour environment of both cutaneous and uveal melanomas, initial immunofluorescence studies for expression of CXCL12 were therefore performed in cutaneous and uveal metastatic melanoma cell lines, as well as in EAhy.926 endothelial cells and primary cells known to be present within the local tumour microenvironment including, primary melanocytes, primary keratinocytes, and primary dermal fibroblasts. CXCL12 fluorescence was quantified using ImageJ software, where thresholds were applied to limit fluorescence intensity for green (CXCL12) and blue (cell nuclei) with a standardised threshold limit of 8 and 11 respectively. CXCL12 fluorescence per cell was determined by division of total CXCL12 fluorescence per image by number of DAPI pixels per image (Figure 3.15).

Interestingly results revealed variable expression of cytoplasmic CXCL12 by all cell lines and primary cells, indicating that under normal and unstimulated cell culture conditions, CXCL12 is likely produced *in situ* (Figure 3.15 A and B). Results also revealed increased CXCL12 expression in primary melanocytes compared to expression in primary keratinocytes or dermal fibroblasts and moreover, compared to expression in all melanoma cell lines, suggesting a trend for CXCL12 down regulation by melanomas (Figure 3.15 C). Expression of CXCL12 between differing cutaneous and uveal melanoma cell lines however, did not significantly vary (Figure 3.15 C). Quantitative and statistical analysis of CXCL12 expression in primary melanocytes, keratinocytes and dermal fibroblasts also revealed significantly less expression in primary keratinocytes compared to both primary dermal fibroblasts and melanocytes (Man Whitney *U* * P=0.0117 and **P= 0.0047 respectively, Figure 3.15 C).

To investigate whether CXCL12 is secreted from each cell type, CXCL12 was quantified in cell culture supernatants derived from confluent cutaneous and uveal melanoma cells, primary melanocytes, keratinocytes and fibroblasts, the control medium of each cell type as well as in supernatants derived from primary biliary epithelial cells obtained from a liver resection using a commercial ELISA assay (Figure 3.16).

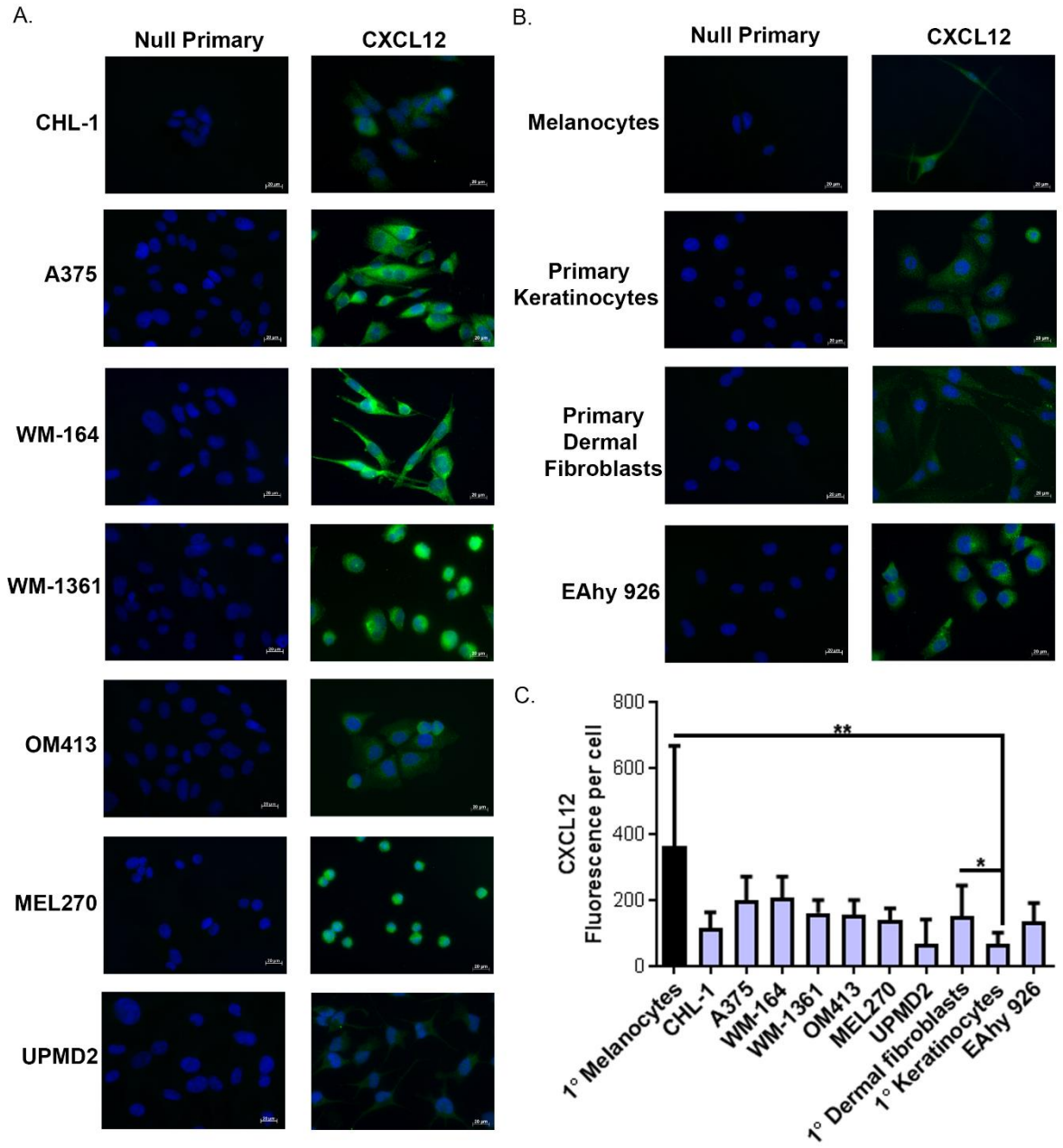


Figure 3.15 Immunofluorescence Analysis of the Expression of CXCL12 in Cutaneous and Uveal Metastatic Melanoma Cell Lines and Cells within the Tumour Microenvironment

*A.) Representative image from 3 replicate experiments for the immunofluorescent expression of CXCL12 or following staining with null primary antibody negative control in cutaneous metastatic melanoma cell lines CHL-1, A375, WM-164, WM-1361 or metastatic uveal melanoma cell lines OM413, MEL270 and UPMD2 or B.) in known primary cells in the tumour microenvironment; primary melanocytes, primary keratinocytes, primary dermal fibroblasts (all primary cells are representative images of 4 individual donors) or in the endothelial cell line EAhy.926. Green depicts CXCL12 positivity and blue, DAPI nuclear staining. Images taken by confocal microscopy, magnification 40x. Scale bar = 20µm. C. Mean CXCL12 fluorescence per cell within cutaneous and uveal metastatic melanoma cell lines, primary dermal fibroblasts, keratinocytes, or EAhy926 cells. Each bar is the mean CXCL12 fluorescence per cell of >3 replicates (mean ± SD N=3, n=6). Statistics acquired by Mann Whitney U *P<0.05, **P<0.01.*

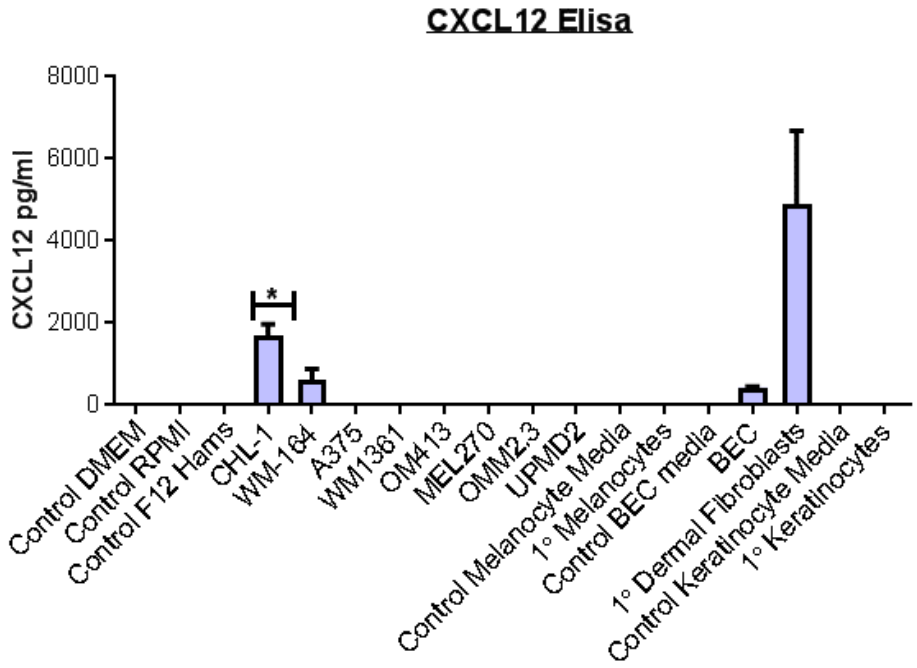


Figure 3.16 Secretion of CXCL12 by Cutaneous Metastatic Cell Lines, Cells within the Tumour Microenvironment and Billary Epithelial Cells.

*CXCL12 (pg/ml) secretion by human metastatic cutaneous melanoma cell lines CHL-1, WM-164, A375, WM-1361, human metastatic uveal melanoma cell lines OM413, MEL270, OMM2.3, UPMD2, Primary (1°) melanocytes, dermal fibroblasts, keratinocytes and primary biliary epithelial cells (BEC), or control DMEM, RPMI, F12 Hams, melanocyte, BEC or keratinocyte specific growth medias. Each bar represents the mean of 3 independent experiments of cell lines or 4 separate donors for primary cells +/- SD. Statistics acquired by Mann Whitney U *P=>0.01 (N>3).*

Surprisingly although all cells tested previously displayed cytoplasmic expression of CXCL12, only CHL-1 (B-RAF/N-Ras wild-type) and WM-164 (B-RAF^{V600E} mutated) cutaneous metastatic melanoma cell lines, primary biliary epithelial cells and dermal fibroblasts secreted detectable levels of CXCL12 (Figure 3.16). Significantly higher secretion of CXCL12 was detected in B-RAF/N-Ras wild-type cutaneous metastatic melanoma cells CHL-1 compared to WM-164 B-RAF^{V600E} mutated cells (Mann Whitney *U* *P=0.0286) with no observed secretion by B-RAF/N-Ras mutated A375 and WM-1361 suggesting that CXCL12 secretion maybe down regulated as a result of hyper activating mutations in MAPK signalling. The observed secretion of CXCL12 from cutaneous metastatic melanoma cell lines, previously shown to express CXCR4 (section 3.2.3) thus uncovers the interesting possibility that these cell lines may display autocrine CXCL12 signalling, as previously reported in head and neck cancer cells (Wang *et al.*, 2008a). However, there was no detectable secretion of CXCL12 in either wild-type, or GNAQ/GNA11 mutant metastatic uveal melanoma cell lines, suggesting in line with previous reports in the literature that uveal melanoma cell lines do not secrete CXCL12 (Di Cesare *et al.*, 2007b).

Although CXCL12 expression was evident within the cytoplasm of primary melanocytes, there was however, no detectable secretion of CXCL12.

The observed consistently high levels of CXCL12 secreted by primary dermal fibroblasts also suggests that these cells may act as the main secretory source of CXCL12 in normal skin or within the local primary tumour microenvironment while the observed lower secretion of CXCL12 from primary biliary epithelial cells further confirmed the liver as a CXCL12 secreting source with the potential to attract CXCR4 positive melanoma cells as widely described (Wald *et al.*, 2004; Hong *et al.*, 2009; Saiman *et al.*, 2015).

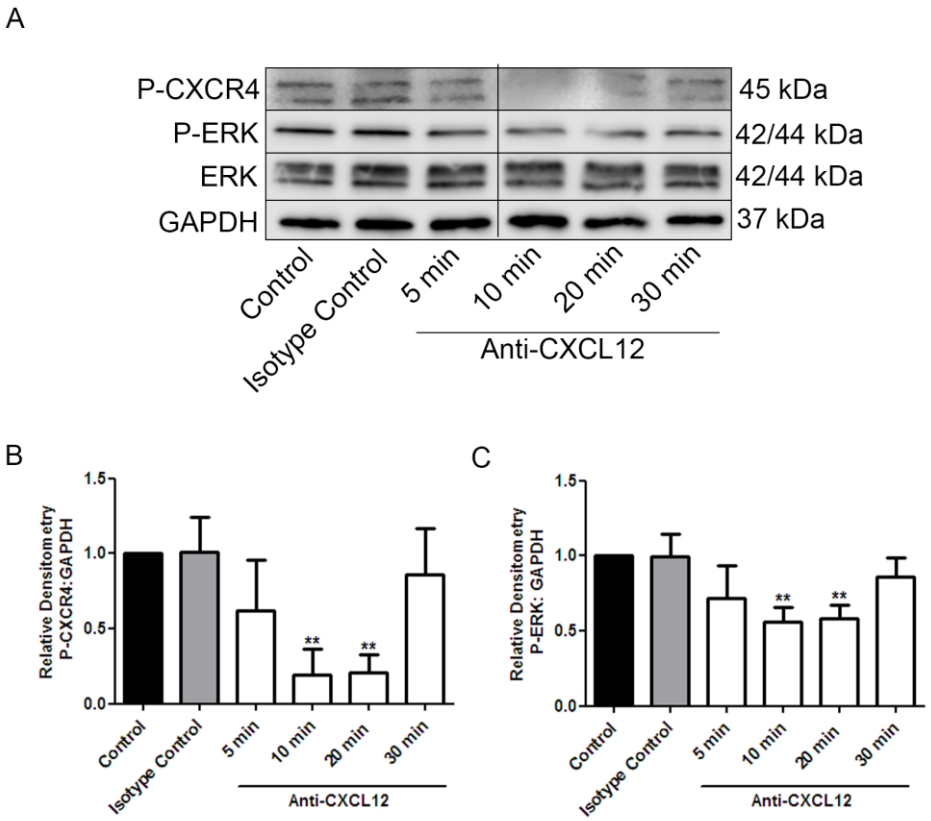


Figure 3.17 Autocrine CXCR4-CXCL12 Cell Signalling in Cutaneous Melanoma

*A.) Representative Western blot of Phospho-CXCR4 (P-CXCR4), Phospho-ERK (P-ERK), total ERK and GAPDH loading control expression in WM-164 (B-RAF^{V600E} mutated) metastatic melanoma cells treated with Anti-CXCL12 neutralising antibody or IgG isotype control for 5, 10, 20, 30 minutes. N=3. B.) Densitometric analysis of P-CXCR4 relative to GAPDH expression. Each bar represents the mean of 3 replicates of P-CXCR4 band intensity normalised to GAPDH band intensity (P-CXCR4/GAPDH), and expressed relative to the mean of each individual experiment (mean ± SD, N = 3). Statistics acquired by one-way ANOVA with Dunnett's post hoc correction **P= 0.01. C.) Densitometric analysis of P-ERK relative to GAPDH expression. Each bar represents the mean of 3 replicates of P-ERK band intensity normalised to GAPDH band intensity (P-ERK/GAPDH), and expressed relative to the mean of each individual experiment (mean ± SD, N = 3). Statistics acquired by one-way ANOVA with Dunnett's post hoc correction **P= 0.003.*

To investigate the possibility of CXCR4-CXCL12 autocrine cell signalling and its impact on MAPK cell signalling, a neutralising antibody to CXCL12 or isotype control antibody was incubated with B-RAF mutated WM-164 metastatic melanoma cells that secrete CXCL12. Western blotting revealed a significant reduction of phospho-CXCR4 expression after treatment with CXCL12 neutralising antibody for 10 or 20 minutes (one-way ANOVA with Dunnett's post hoc correction **P= 0.001, Figure 3.17 A). This was accompanied by concurrent significant inhibition of MAPK cell signalling and a concomitant reduction in phospho-ERK expression (one-way ANOVA with Dunnett's post hoc correction **P=0.003, Figure 3.17 B and C). Together these data suggest that endogenous CXCL12 secreted form metastatic melanoma cells may act in an autocrine manner activating CXCR4 receptors on melanoma cells to promote pro-survival MAPK cell signalling.

3.2.9 Epidermal CXCL12 Expression Prevents Melanoma Metastasis

To investigate further the possibility of CXCL12 autocrine signalling in cutaneous melanomas at the primary site and determine the expression of CXCL12 in the tumour microenvironment, immunofluorescence for the expression of CXCL12 was performed in a FFPE cohort of 24 melanocytic naevi or primary melanomas of differeing AJCC stage as well as in 2 FFPE samples of normal human skin, with double staining for Melan-A, as a melanocytic marker. CXCL12 fluorescence intensity was quantified using ImageJ softwear, with thresholds applied to limit fluorescence intensity for green (Melan-A) and red (CXCL12) at a standardised threshold limit of 8 and 6 respectively. CXCL12 fluorescence per cell was acquired by division of total CXCL12 fluorescence per image by the number of Melan-A pixels per image.

Results demonstrated the expression of Melan-A by melanocytes at the stratum basale of the epidermis in normal skin as expected (Figure 3.18) with CXCL12 expression detected, as previously documented in both the sweat glands and capillaries (Pablos *et al.*, 1999; Avniel *et al.*, 2005; Mitchell *et al.*, 2014). In line with *in vitro* observations (section 3.2.8) and previous literature, CXCL12 expression was also observed within the dermis, located around dermal fibroblasts (Figure 3.18 A. B) (Pablos *et al.*, 1999; Avniel *et al.*, 2005; Mitchell *et al.*, 2014). However, contradictory to findings from the present *in vitro* studies (section 3.2.8) and previous reports, CXCL12 expression was also observed in the epidermis located around keratinocytes, with increased intensity towards the stratum corneum, and suggesting the

possibility that CXCL12 expression in the epidermis increases in line with increasing epidermal differentiation (Figure 3.18) (Avniel *et al.*, 2005).

Studies in all AJCC stage primary cutaneous melanomas, confirmed Melan-A expression by all tumour cells which co-localised to varying degrees with tumoural CXCL12 expression (Figure 3.19 Ai, Bi). Interestingly in all primary melanomas studied, CXCL12 was also expressed in the same pattern as that observed in normal skin, with expression observed within the epidermis adjacent to the tumours (Figure 3.19 A iii, B ii). However, CXCL12 expression in the epidermis overlying primary melanomas appeared to be reduced (Figure 3.19 A iv).

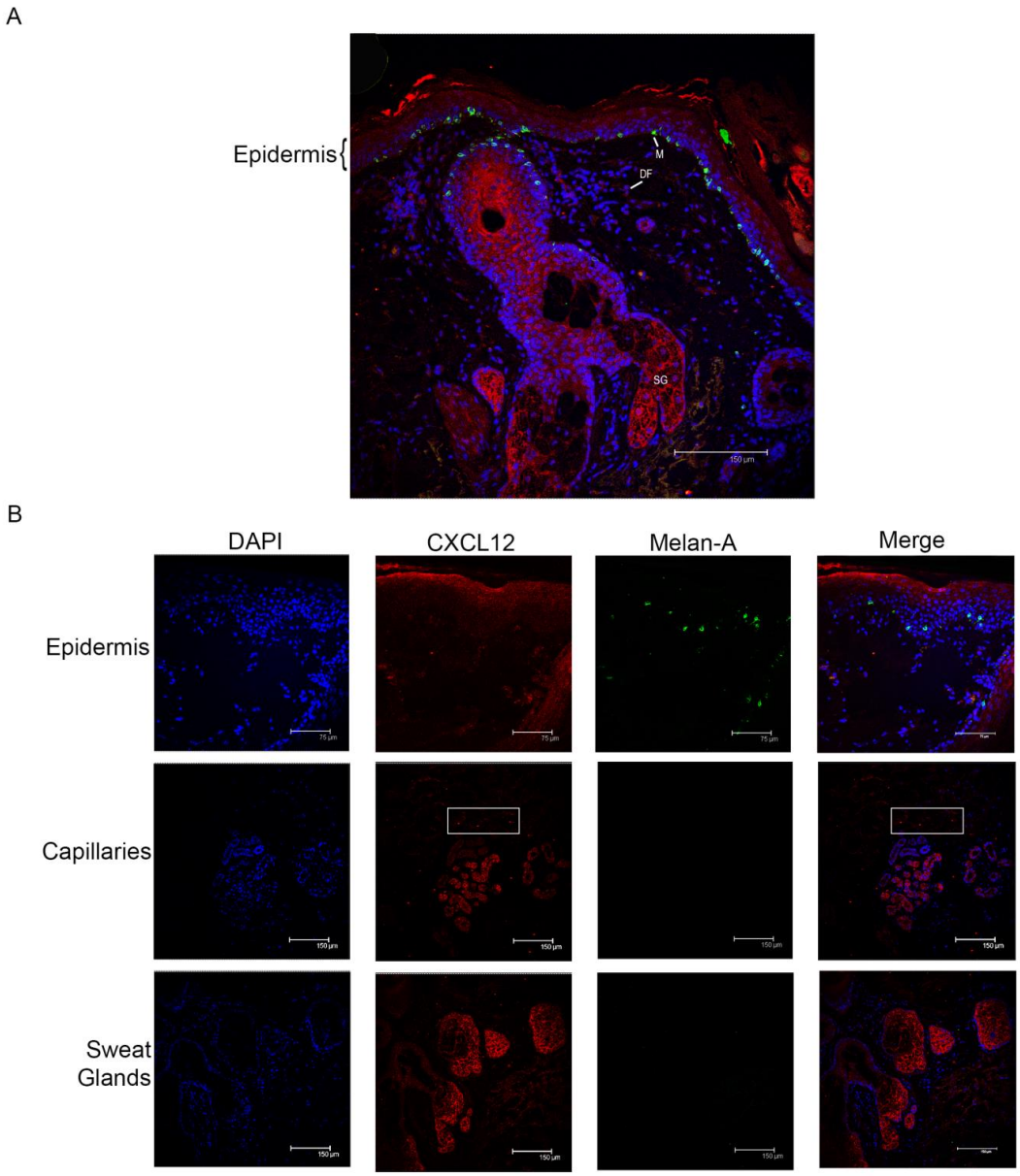


Figure 3.18 Immunofluorescence analysis of Melan-A and CXCL12 in Normal Skin
A.) Representative images of Melan-A (green), CXCL12 (red) expression or DAPI nuclear staining (blue) in FFPE sections in the epidermis, capillaries or sweat glands of normal human skin. White box highlights dermal fibroblasts. Images were acquired by confocal microscopy with a magnification 20x. Scale bar = 150 or 50µm.

Analysis of CXCL12 within primary melanomas revealed consistent expression of CXCL12 by tumour cells albeit it at low levels, which did not vary significantly between AJCC disease stage (Figure 3.20 A) (Kruskal-Wallis test P=0.2488 ns) or between localised or metastatic melanomas (Figure 3.20 B.) (Mann Whitney U P=0.1138 ns). Furthermore, there was no significant correlation between CXCL12 expression and time to metastasis in advanced tumours (Spearman's rank correlation P=0.3680 ns), collectively suggesting that although CXCL12 is present or possibly secreted by tumour cells in an autocrine manner; this does not

apparently influence disease progression. Remarkably, however, comparison of CXCL12 expression with B-RAF/N-Ras mutational status revealed significantly reduced CXCL12 expression in B-RAF/N-Ras mutant tumours compared to expression in wild-type tumours (Figure 3.20 D.) (Students T test *P=0.0323), consistent with the observations seen *in vitro* (section 3.2.8) showing B-RAF mutated cell lines secrete significantly less CXCL12 compared to B-RAF wild-type melanoma cell lines, and further supporting the hypothesis that the down-regulation of CXCL12 secretion is mediated by activation of MAPK signalling.

Given the association between high nuclear CXCR4 expression and the presence of a B-RAF/N-Ras mutation in primary melanomas observed in section 3.2.1, the expression of CXCL12 with either total, nuclear or cytoplasmic CXCR4 was therefore compared in the same combined cohort of melanocytic nevi and primary melanomas of varying AJCC disease stage (Figure 3.20 E and F). Results however, demonstrated there was no apparent correlation between total CXCR4 and CXCL12 expression (Spearman's rank correlation P=0.5301, Figure 3.20 E) or indeed between cytoplasmic CXCR4 and CXCL12 expression (data not shown), supporting data from a previous study in cutaneous melanoma where similarly no relationship between CXCR4 and CXCL12 expression was observed (Toyozawa *et al.*, 2012). Nevertheless, the comparison of nuclear CXCR4 with mean CXCL12 expression across the whole cohort revealed a trend, albeit insignificant in the current cohort size, for high nuclear CXCR4 expression with low CXCL12 expression (Spearman's rank correlation P=0.057 ns).

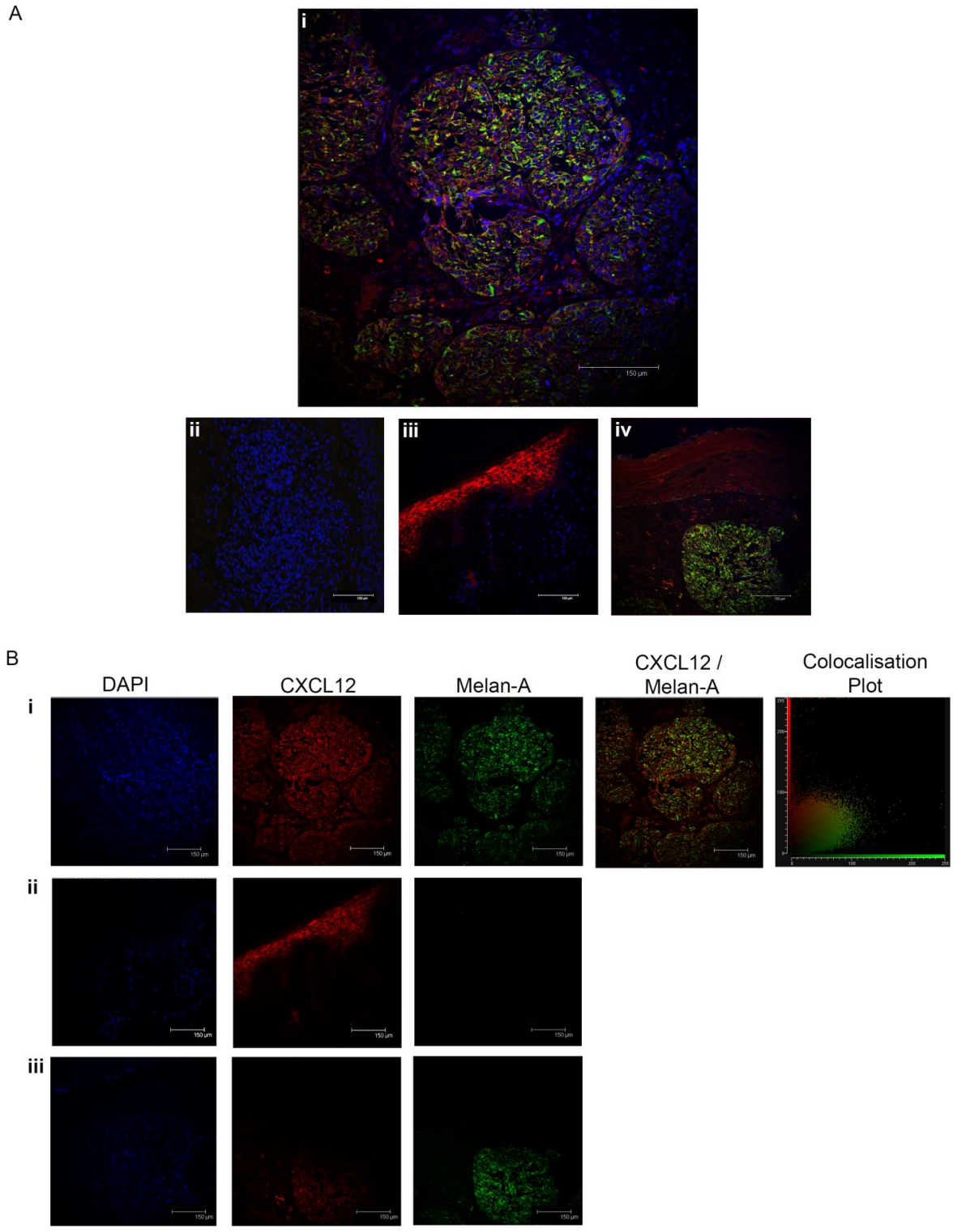


Figure 3.19 Immunofluorescence for the Expression of CXCL12 in Primary Cutaneous Melanomas

A.i) Representative image of Melan-A (green), CXCL12 (red) expression or nuclear DAPI (blue) staining in a primary cutaneous melanoma AJCC stage III. ii.) Nuclear DAPI staining only following treatment with null primary antibody control. iii) CXCL12 expression depicted in the epidermis beside or iii) above the primary tumour. B.) Representative images of blue DAPI staining or Melan-A (green) and CXCL12 (red) expression in a primary melanoma AJCC stage III depicted i.) in the tumour bulk, including co-localisation plot of Melan-A and CXCL12 expression or ii.) in the epidermis beside or iii) above the tumour. Images taken by confocal microscopy with a magnification 20x scale bar = 150µm.

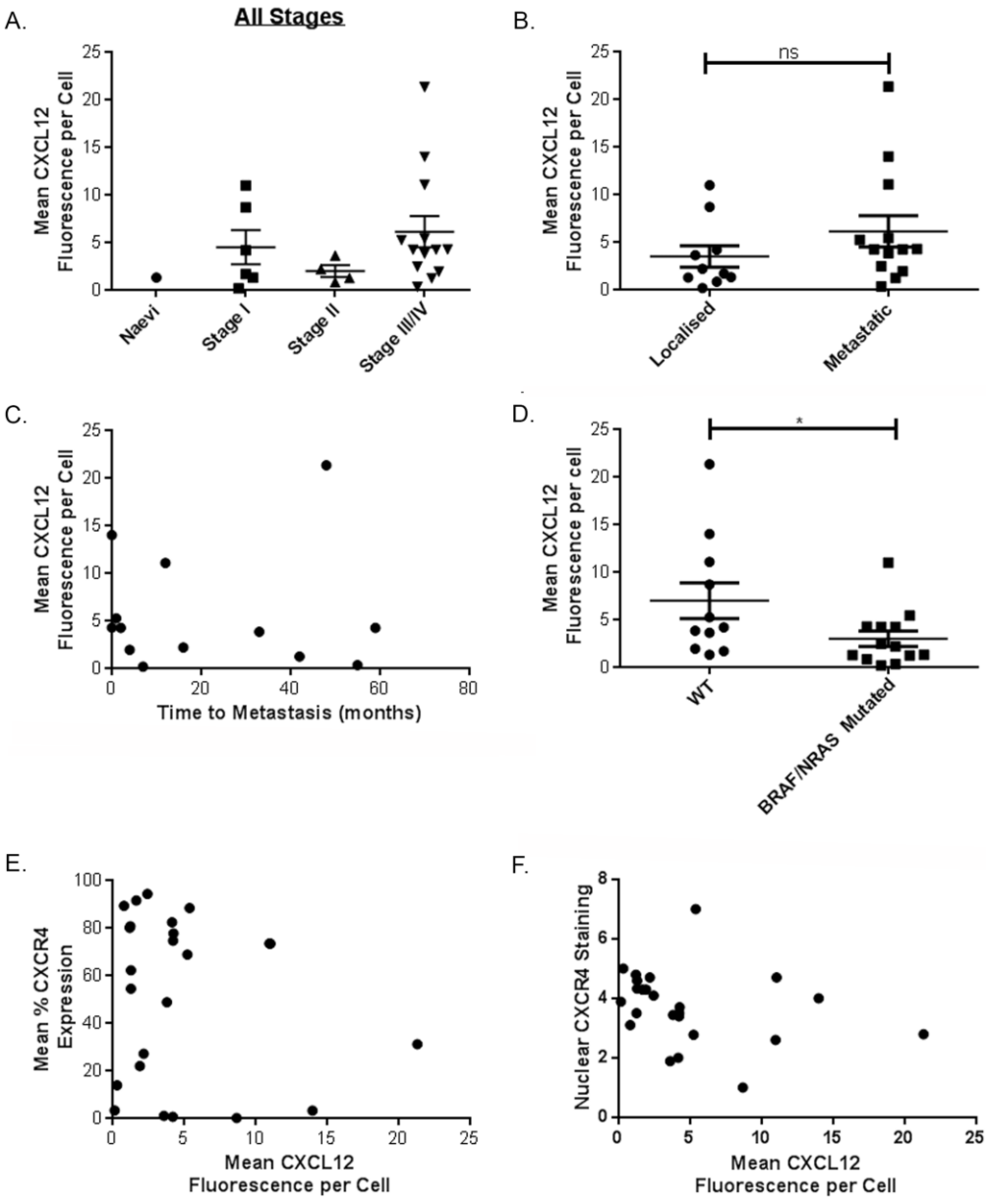


Figure 3.20 Expression of CXCL12 and Comparison with CXCR4 expression in a Cohort of Melanocytic Naevi and Primary Melanomas of Differeing AJCC Stage

*A.) Scatter graph representing the mean (mean of 4 high powered fields of view (HPF) +/- SD) CXCL12 fluorescence/expression per cell in eventual stage melanocytic naevi or AJCC stage I, II or III melanomas. B.) Scatter graph representing mean CXCL12 fluorescence/expression per melanoma cell in localised (eventual AJCC stage of disease I/II) or metastatic melanomas (eventual AJCC stage III/IV) after 7 years follow up. Horizontal bars represent mean CXCL12 fluorescence per cell (mean of 4 HPF +/- SD). C.) Scatter graph representing mean CXCL12 fluorescence/expression per cell (mean of 4 HPF +/- SD) in metastatic melanomas (eventual AJCC stage III/IV) in relation with time to metastasis (months) after a 7 year follow up. D.) Scatter graph representing the mean CXCL12 fluorescence per cell in all AJCC stage B-RAF/N-Ras wild-type or mutant melanomas. Horizontal bars represent mean CXCL12 fluorescence per cell (mean of 4 HPF +/- SD). Statistics acquired by students T test *P<0.05. E.) Scatter graph representing mean CXCL12 fluorescence per cell (mean of 4 HPF in eventual stage melanocytic naevi or AJCC stage I, II or III melanomas in relation to mean total % CXCR4 expression (mean of 10 HPF). F.) Scatter graph representing mean CXCL12 fluorescence per cell (mean of 4 HPF) in eventual stage melanocytic naevi or AJCC stage I, II or III melanomas in relation to mean total % nuclear CXCR4 (mean of 10 HPF).*

To further explore observations of CXCL12 expression in normal and peri-tumoural epidermis, CXCL12 fluorescence was additionally quantified in 2 samples of normal skin, and the peri-tumoural or epidermis overlying primary melanomas within the same patient cohort. Threshold limits for CXCL12 and Melan-A fluorescence of 11 and 6 were applied respectively, with mean CXCL12 fluorescence derived by the division of total CXCL12 fluorescence per image by the number of DAPI pixels. Results revealed a trend for increased CXCL12 expression in the epidermis of normal skin compared to the epidermis adjacent or overlying primary melanomas (Figure 3.21 A), although a comparison of CXCL12 levels in the adjacent epidermis or the epidermis overlying primary melanomas demonstrated this effect was insignificant (Wilcoxon signed-rank test P=0.0710 ns, Figure 3.21 B). Comparative analysis of CXCL12 expression in the adjacent epidermis of all AJCC stage melanomas with tumour progression however, revealed increased CXCL12 expression correlated significantly with increased time to metastasis (Figure 3.21 C) (Pearson's correlation *P=0.0136), suggesting CXCL12 in the immediate tumour microenvironment may limit disease progression. Furthermore, a significant correlation of adjacent epidermal CXCL12 expression with tumoural expression of cytoplasmic CXCR4 expression was also observed (Pearson's correlation **P=0.0098, Figure 3.21 B.), highlighting the possible relationship of CXCL12 expression in the microenvironment with chemokine receptor localisation in the tumour, and proposing a previously un-described intimate relationship between micro environmental CXCL12, melanoma phenotype and patient outcome.

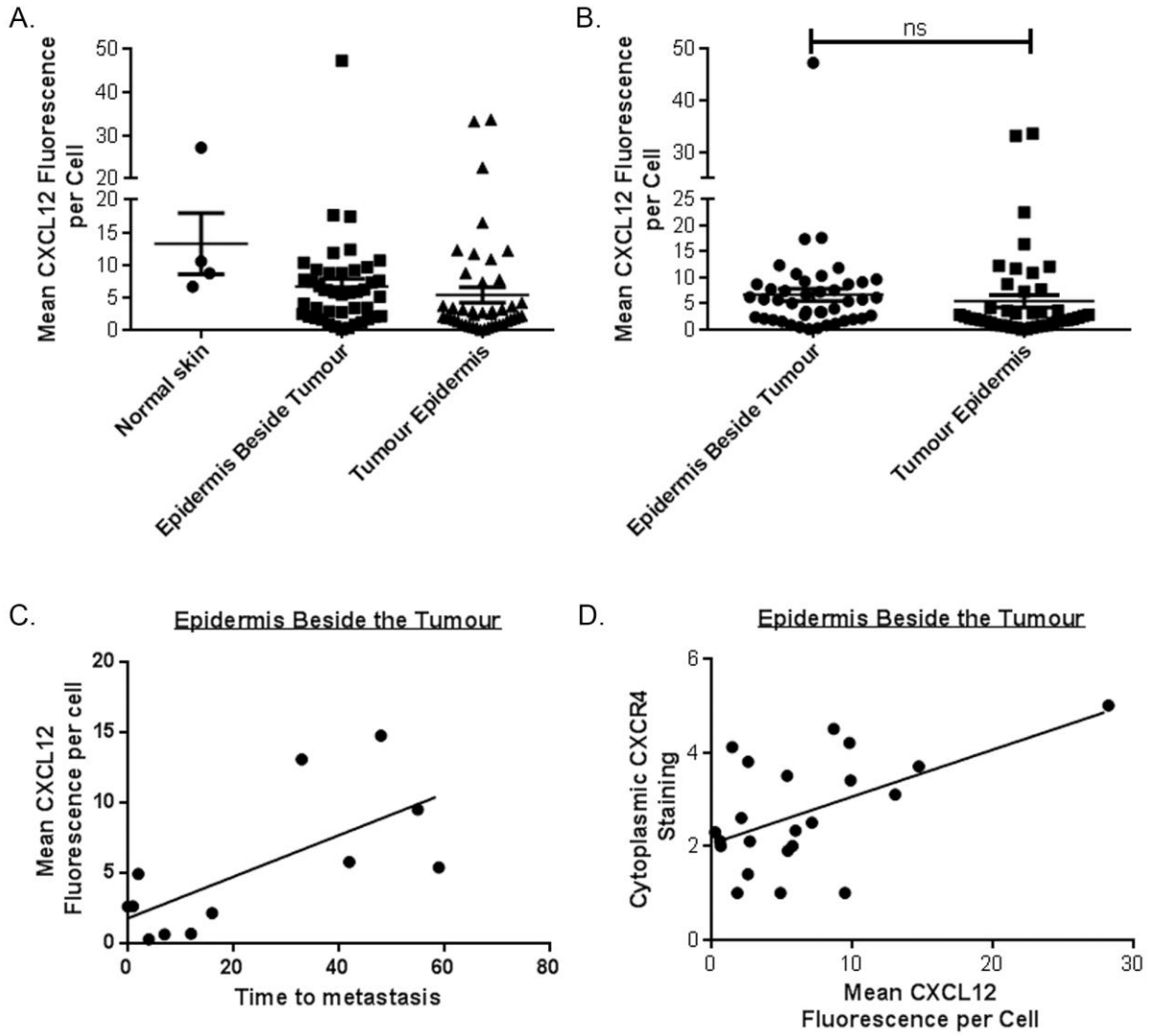


Figure 3.21 Immunofluorescence for the Expression of CXCL12 in the Epidermis of Normal Skin, Tumoural Epidermis and Epidermis in the Melanoma Microenvironment.

*A.) Scatter plot representing CXCL12 fluorescence per cell in each high powered view (HPV) in the epidermis of normal skin, adjacent (beside) or overlying (above) all AJCC stage melanomas. Horizontal bars represent mean CXCL12 fluorescence per cell +/- SD. B.) Scatter plot representing mean CXCL12 fluorescence per cell (Mean of 2 HPV) in the epidermis beside and above all AJCC stage melanomas. Statistics acquired by Wilcoxon signed-rank test, P>0.05 ns. C.) Scatter graph representing mean CXCL12 fluorescence per cell (mean of 2 HPF) in the epidermis beside eventual AJCC stage III/IV melanomas in relation to time to metastasis (months) after 7 year follow up. Statistics acquired by Pearson's correlation *P<0.05. D.) Scatter graph representing mean CXCL12 fluorescence per cell (mean of 2 HPF) in the epidermis beside all AJCC stage melanomas in relation to relative tumoural cytoplasmic CXCR4 expression. Statistics acquired by Pearson's correlation **P<0.01.*

3.2.10 Downregulation of CXCL12 is Associated with Monosomy of Chromosome 3 in Primary Uveal Melanomas

CXCL12 has been defined as a mediator of choroidal neovascularisation and vasculogenesis, recruiting bone marrow derived vascular progenitors and endothelial precursors to the developing or injured eye, likely regulated by hypoxia and VEGF (Sengupta *et al.*, 2005; Jin *et al.*, 2006; Ruiz de Almodovar *et al.*, 2006). However, the expression of CXCL12 in the normal eye and in uveal melanoma is currently poorly defined. Given the observations of strongly positive CXCR4 expression observed in primary uveal melanomas (section 3.2.2) and the unknown role of CXCL12 at the primary tumour site, expression of CXCL12 and Melan-A were evaluated by immunofluorescence under the same experimental and analysis conditions described in section 3.2.9 in a cohort of 7 primary uveal melanomas (Figures 3.22 and 3.23).

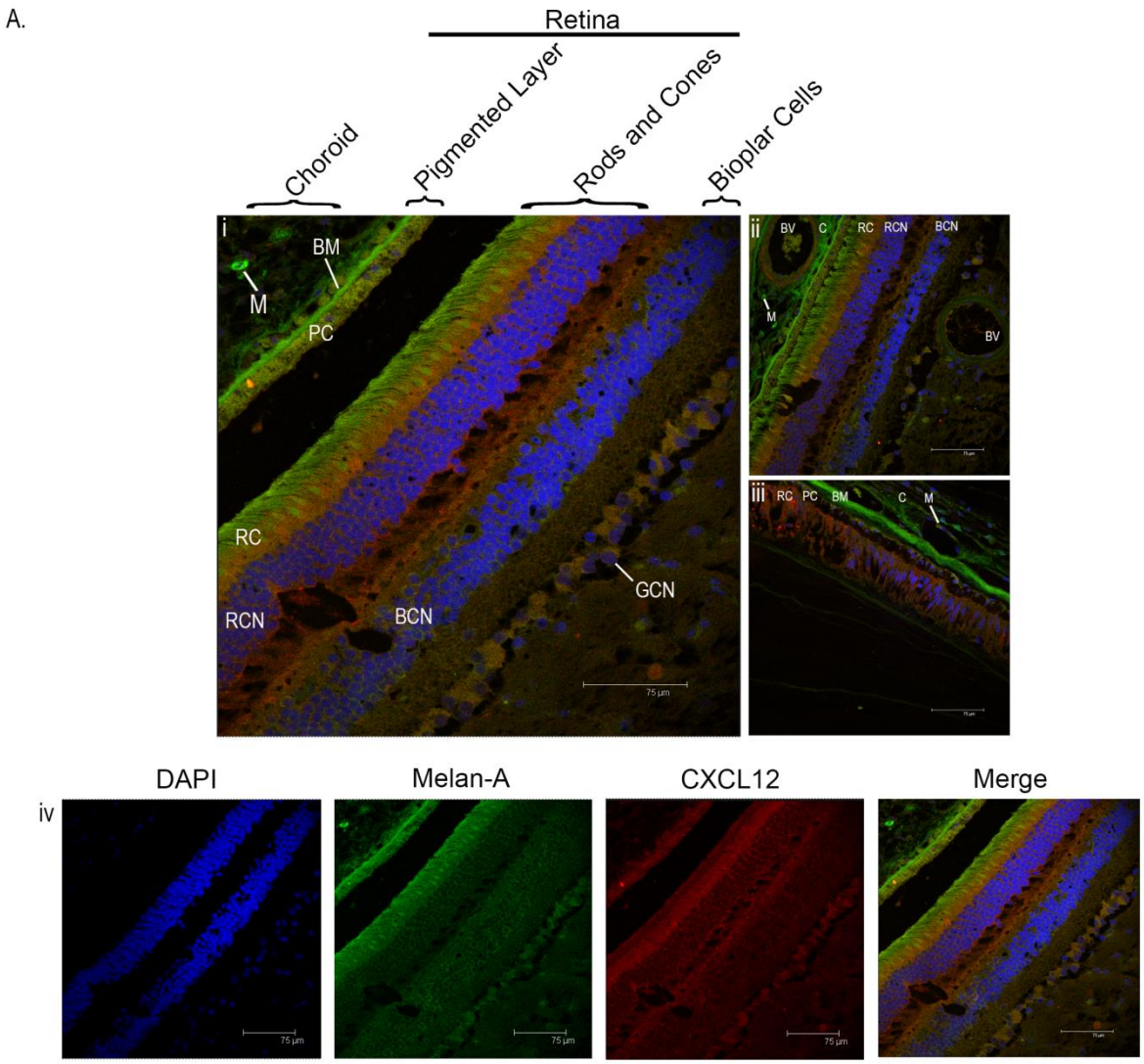


Figure 3.22 Immunofluorescence for the Expression of CXCL12 and Melan-A in the Normal Choroid and Retina of Primary Uveal Melanomas

Representative images of CXCL12 (red) or Melan-A (green) expression, or DAPI nuclear staining (Blue) or merged expression in the normal choroid and retina of primary uveal melanomas. Images taken by confocal microscopy with a magnification 20x, scale bar = 75µm. M= Melanocytes, C= choroid, BM= Bruch's membrane, PC= pigmented cells, RC= rods and cones, RCN=rods and cones nuclei, BCN=bipolar cell nuclei, GCN= ganglion cell nuclei, BV= blood vessels.

In the normal choroid and retina of all primary uveal melanomas (area far from tumour site), results demonstrated strong melan-A expression in melanocytes of the choroid, and pigmented cells of Bruch's membrane and outward protrusions of rods and cones of the retina, with weak expression in ganglion cells facing the posterior cavity of the eye (Figure 3.22). CXCL12 immunoreactivity was generally intense, strongest in the retina of the eye, with particular expression seen in the retinal pigment epithelium, ganglion cells and within blood vessels of the choroid co-localised with Melan-A and consistent with previous reports, proposing the retina and choroid as CXCL12 rich sources within the eye (Crane *et al.*, 2000; Hasegawa *et al.*, 2008; Otsuka *et al.*, 2010).

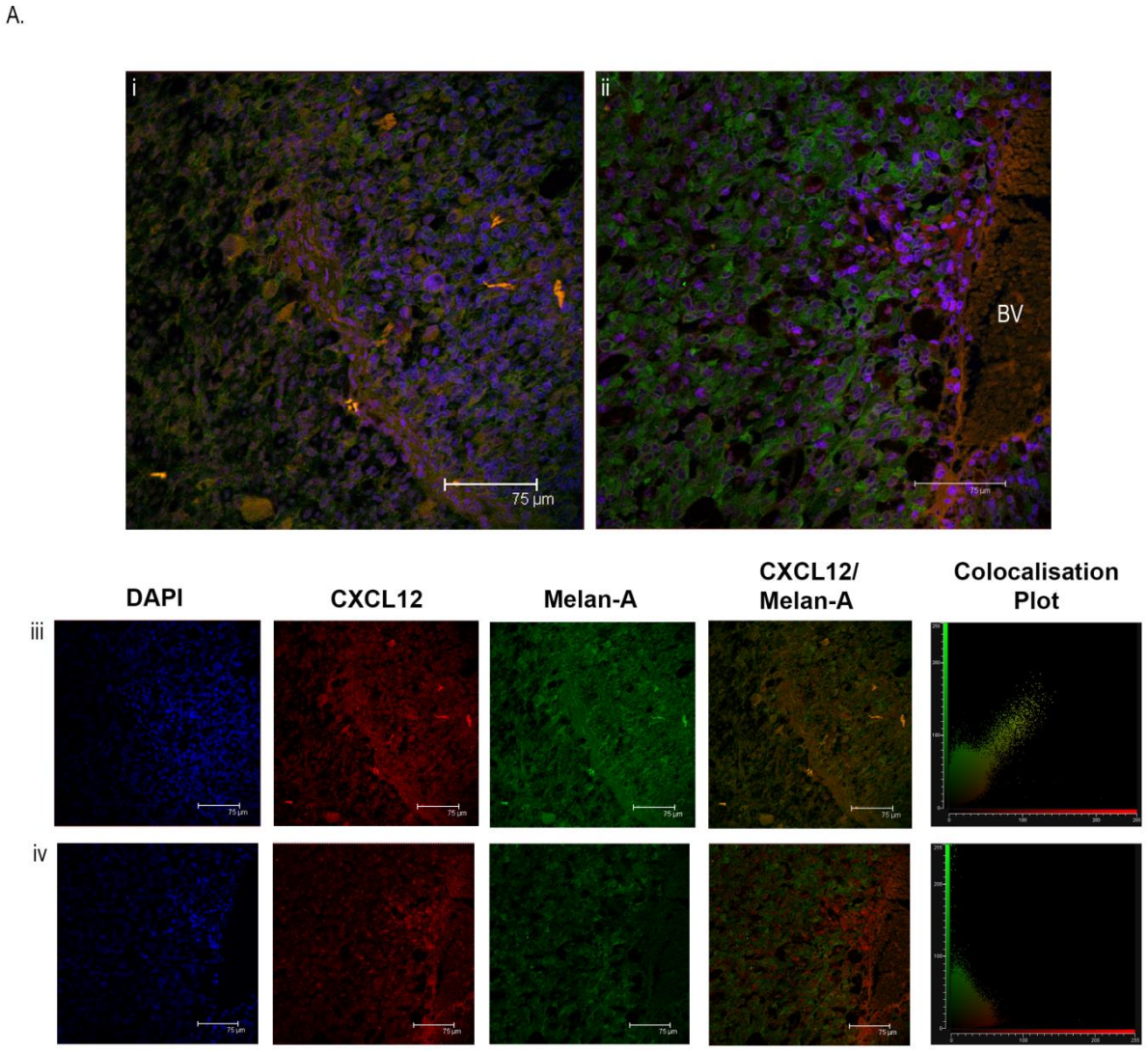


Figure 3.23 Immunofluorescence for the Expression of CXCL12 in Primary Uveal Melanomas
A.) i,ii,iii,iv Representative images of melan-A (green) and CXCL12 (red) expression in primary uveal melanomas tumour cells with melan-A/ CXCL12 co-localisation plot, blue depicts nuclear staining. BV=blood vessel. Images taken by confocal microscopy with a magnification 20x scale bar = 75µm.

CXCL12 expression also varied between primary uveal melanoma tumours but co-localised with Melan-A, suggesting the potential for autocrine CXCL12 secretion (Figure 3.23 i). Notably, high levels of CXCL12 immunoreactivity were observed within and around blood vessels inside the tumour bulk, although this observation was not apparent in all tumours (Figure 3.23 ii, iv). Nevertheless, these observations suggest blood vessels may serve as another source of CXCL12 in primary uveal tumours if the tumour is vascularised. A comparison of CXCL12 fluorescence levels in uveal compared to levels expressed by primary cutaneous melanomas, further revealed significantly increased CXCL12 fluorescence per cell in primary uveal melanomas (Mann Whitney *U* **P=0.0274, Figure 3.24 Aii), indicating a reliance on CXCL12 signalling at the primary site. Interestingly similar to the observed lower levels of CXCL12 expression seen in B-RAF/N-Ras mutated cutaneous melanomas (section 3.2.9), uveal melanomas with monosomy of chromosome 3 (strongest prognostic indicator of metastasis) also displayed significantly lower CXCL12 expression compared to those tumours with disomy of chromosome 3 (Students T test **P=0.0099, Figure 3.23 A iii). Collectively these data thus suggest the down regulation of CXCL12 at the primary site, is associated with the metastatic potential of uveal melanoma and that reduced CXCL12 expression by both cutaneous and uveal melanomas is associated with tumour progression, while conversely, higher CXCL12 levels in the local tumour environment may limit disease spread.

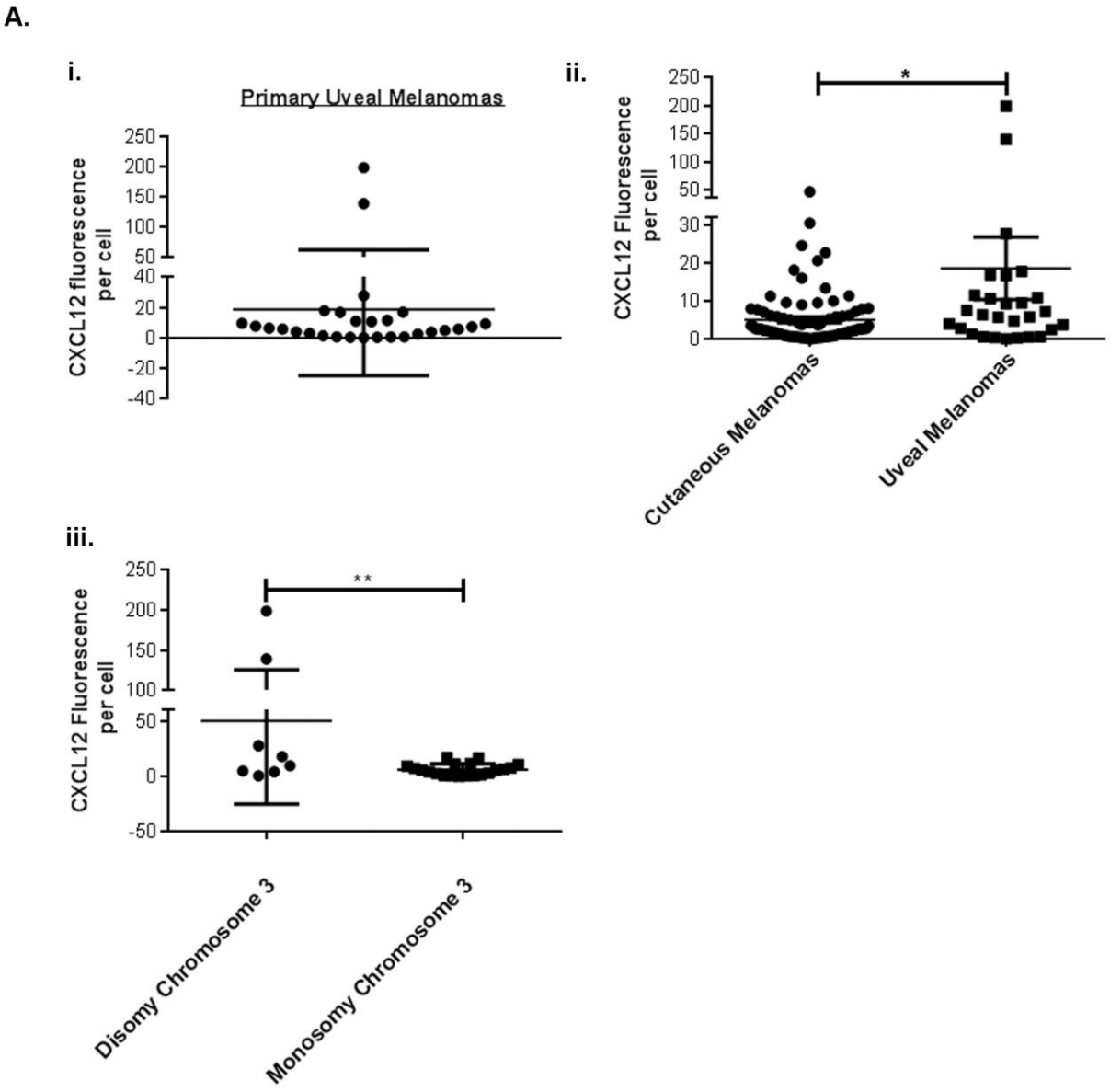


Figure 3.24 CXCL12 Expression in Primary Uveal and Cutaneous Melanoma Tumours

*A.) i Scatter plot representing CXCL12 fluorescence per cell for each high powered view (HPV) of primary uveal melanomas. Horizontal bar represents mean CXCL12 fluorescence per cell +/- SD. ii.) Scatter plot representing CXCL12 fluorescence per cell for each HPV in primary cutaneous melanomas and primary uveal melanomas. Horizontal bar represents mean CXCL12 fluorescence per cell +/- SD. Statistics acquired by Mann Whitney U *P<0.05. iii.) Scatter plot representing CXCL12 fluorescence per cell for each HPV in primary uveal melanomas with disomy or monosomy of chromosome 3. Horizontal bar represents mean CXCL12 fluorescence per cell +/- SD. Statistics acquired by student's T test **P<0.01. N=7, n=28.*

3.3 Discussion

3.3.1 CXCR4 Expression in Cutaneous and Uveal Melanoma

Tumour thickness and ulceration are currently the two components of the AJCC melanoma staging system that provide the most important prognostic information for patients diagnosed with primary cutaneous melanomas (Balch *et al.*, 1980; Balch *et al.*, 2009a). However, neither tumour thickness or ulceration or any other prognostic indicators are able to reliably identify 'high risk' patients, whose tumours will eventually progress, hence emphasising the acute demand for novel biomarkers with predictive accuracy of metastatic progression. Current evidence suggests chemokines and their receptors play a critical role in melanoma metastasis with the CXCR4–CXCL12 axis involved in many key aspects of tumour progression, including cell proliferation, survival, immune evasion, invasion, angiogenesis and cell chemotaxis (Bachelder *et al.*, 2002; Hall and Korach, 2003; Murakami *et al.*, 2003; Balkwill, 2004; O'Boyle *et al.*, 2013). CXCR4 expression is frequently up regulated in both cutaneous and uveal melanomas, where in cutaneous melanoma increased expression has been linked with increased Breslow thickness, ulceration, metastasis as well as poor patient outcome (Longo-Imedio *et al.*, 2005; Scala *et al.*, 2005; Tucci *et al.*, 2007; Li *et al.*, 2009; Toyozawa *et al.*, 2012). High CXCR4 expression in uveal melanoma on the other hand is associated with the increased likelihood of liver metastasis (Li *et al.*, 2009). Results from the present study, highlight the reduction in total CXCR4 expression in AJCC stage I/II cutaneous melanomas relative to expression detected in benign naevi, a pattern which may reflect the importance of CXCR4-CXCL12 autocrine signalling in early stage disease which in turn may promote cell survival and proliferation through activation of MAPK and PI3K signalling, downstream of CXCR4 (Murakami *et al.*, 2003; Balkwill, 2004; Balkwill, 2012). Conversely, the significant increase in total CXCR4 expression demonstrated in metastatic compared to localised melanomas as well as the significant increase in CXCR4 expression from AJCC stage II to III/VI disease reinforces the pivotal role of CXCR4 expression in metastatic spread. In this respect the up regulation of CXCR4 expression in locally invasive melanomas likely enables tumour exit from the primary site, migration down chemokine gradients and subsequent metastasis to organ specific locations that secrete CXCL12 including the liver, lungs, bone and brain, hence providing the rationale for CXCR4 as a therapeutic target (O'Boyle *et al.*, 2013). Consistent with the association of high CXCR4 expression and the increased risk of metastasis, results from the present study also showed individuals bearing AJCC stage II cutaneous melanomas with high

CXCR4 expression (>50%) were over 3 times more likely to develop metastasis compared to individuals with lower CXCR4 expression (<50%). Collectively these data thus show, in addition to being a potential therapeutic target, CXCR4 expression defines a high risk melanoma AJCC stage II sub group, and hence may be a valuable putative clinical prognostic biomarker.

In uveal melanoma the strongest prognostic indicators for poor outcome are monosomy of chromosome 3, cell phenotype (epithelioid) and tumour diameter (>1.5cm). However, these factors again do not allow for the reliable identification of high risk patients. Current data supports previous observations of increased expression of CXCR4 in primary uveal melanomas with CXCR4 expression detected in 100% of tumour cells within 83% of primary uveal melanomas evaluated (Scala *et al.*, 2007; Li *et al.*, 2009; Franco *et al.*, 2010; Dobner *et al.*, 2012). However, the lack of any expression in the remaining uveal melanoma cohort suggests CXCR4 expression may be an 'all or nothing' scenario. Previous studies have correlated CXCR4 expression within an epithelioid tumour cell type demonstrating expression in this context increases the risk of metastasis with reduced time to disease progression (Scala *et al.*, 2007; Franco *et al.*, 2010). Although the cohort of uveal melanomas in the present study was of too small to demonstrate any association with tumour phenotype or chromosome 3 status, the increased expression of CXCR4 observed nevertheless suggests an important role for this chemokine receptor in uveal melanoma pathogenesis.

Interestingly immunohistochemical analysis demonstrated differential expression of nuclear and cytoplasmic CXCR4 expression in primary cutaneous and uveal melanomas. Previous studies have reported CXCR4 cytoplasmic expression in both cutaneous and uveal melanoma, while in many other cancers, nuclear CXCR4 expression is well documented, and commonly associated with the development of metastasis and a worse prognosis (Oda *et al.*, 2007; Akashi *et al.*, 2008; Na *et al.*, 2008; Woo *et al.*, 2008; Yoshitake *et al.*, 2008; Frank M. Speetjens *et al.*, 2009; Linhui Wang *et al.*, 2009; Dobner *et al.*, 2012; Toyozawa *et al.*, 2012). CXCR4 is a G-protein coupled cell receptor which unstimulated exists on the cell cytoplasmic membrane (Nguyen and Taub, 2002; Wang *et al.*, 2006). Stimulation of the receptor with its cognate ligand CXCL12 causes receptor dimerisation and internalisation to augment cell signalling before being recycled back to the cell surface by endocytosis or degradation by lysosomes. However, recent reports describe the identification of a latent receptor response where long term stimulation of CXCR4 with ligand results in its nuclear translocation (Linhui Wang *et al.*, 2009). Nuclear CXCR4 expression detected in the current cohort of cutaneous melanomas may

therefore reflect long term receptor stimulation, and may explain the observed trend of increased nuclear CXCR4 expression with disease progression. Interestingly results from the present study also demonstrated significantly increased nuclear CXCR4 expression in metastatic compared to localised disease, suggesting a possible role of nuclear CXCR4 in the evolution of an aggressive phenotype in cutaneous melanoma. Moreover, this observation was also reflected by studies of CXCR4 subcellular localisation *in vitro*, where both nuclear and cytoplasmic CXCR4 expression were detected in all cutaneous and uveal metastatic melanoma cell lines with the exception of studies in the primary uveal melanoma cell line, MEL270 (all other cell lines were derived from a tumour metastasis) in which nuclear CXCR4 expression was undetectable. In further support of the association of nuclear CXCR4 expression with metastasis, functional and ligand responsive CXCR4 in the nucleus of metastatic cells, has also been shown to promote tumour invasion (Linhui Wang *et al.*, 2009; Don-Salu-Hewage *et al.*, 2013). Although a mechanism by which nuclear CXCR4 promotes tumourigenesis has yet to be defined, the fact that CXCR4 is not recycled back to the cell membrane or degraded, steps important for signal termination, and is able to participate in intra-nuclear signalling, suggests that nuclear CXCR4 may ensure or intensify cell signalling, promoting the evolution of a metastatic phenotype (Pelchen-Matthews A *et al.*, 1999; Don-Salu-Hewage *et al.*, 2013).

Strikingly, the immunohistochemical analysis of the present cutaneous melanoma cohort revealed significantly increased nuclear CXCR4 expression in B-RAF mutant compared to wild-type tumours. Although a study in papillary thyroid carcinoma demonstrated CXCR4 up regulation by B-RAF activation, and total CXCR4 up regulation has been described in cutaneous melanoma, there have been no previous reports of any specific association of CXCR4 expression and B-RAF/N-Ras mutational status in cutaneous melanoma (Castellone *et al.*, 2004; Borrello *et al.*, 2005; Torregrossa *et al.*, 2012; Mitchell *et al.*, 2014). Since B-RAF is a bonafide oncogene in cutaneous melanoma and oncogenic signalling may up regulate CXCR4, this may account for the increased expression of nuclear CXCR4 seen in the present cohort of B-RAF mutant tumours, collectively suggesting that oncogenic B-RAF signalling and nuclear CXCR4 cooperatively commit the cell to an aggressive phenotype (Castellone *et al.*, 2004; Toyozawa *et al.*, 2012). However, although CXCR4 was observed in the cytoplasm and to a greater extent in the nucleus of B-RAF wild-type CHL-1 cells, transfection of wild-type or B-RAF^{V600E} did not significantly alter total CXCR4 levels or subcellular localisation. Discordance in results with *in vivo* observations may thus reflect the myriad of other unknown influences on

CXCR4 expression in an *in vivo* setting, including for example exposure to CXCL12. Given that nuclear CXCR4 expression increases with disease progression, results from the present study suggest perhaps that the acquisition of nuclear expression may occur over a prolonged time period, and thus explain the lack of such observation by transient transfection mutant B-RAF over 72 hours.

3.3.2 CXCR7: A Likely Scavenger of CXCL12 in the Cutaneous and Uveal Microenvironment

CXCL12 engages with both chemokine receptors CXCR4 and CXCR7 to regulate homeostatic and pathological processes. While expressed on immune cells, endothelial cells and some tumour cell types, the presence of CXCR7 on melanoma cells or any association with tumour progression remains undefined. Contrary to reports of CXCR7 in breast cancer , results from the present study however, clearly showed there was no expression of CXCR7 on melanoma cells *in vitro or in vivo*, reaffirming CXCR4 as the predominantly expressed chemokine receptor, and the likely primary driver of CXCL12 chemotaxis (Salazar *et al.*, 2014). Nevertheless, and consistent with previous reports of CXCR7 expression within the cancer microenvironment and specifically on endothelial cells of tumour associated vasculature, results confirmed the expression of CXCR7 on EA.hy926 and Hela endothelial cells *in vitro* as well as on endothelial cell types of capillaries and blood vessels within the microenvironment of 30-40% of primary cutaneous and uveal melanomas (Sánchez-Martín *et al.*, 2011b).

CXCR7 expression on endothelial cells is often associated with periods of accelerated growth and vascularisation, and given the known up regulation of CXCR7 in hypoxic regions of a tumour and by pro-angiogenic secreted factors such as VEGF and CXCL8, CXCR7 maybe a surrogate marker of tumour angiogenesis (Hattermann and Mentlein; Maksym *et al.*, 2009a). Although there were no obvious associations of CXCR7 expression with prognostic features in the current cutaneous and uveal melanoma cohort, CXCR7 may be playing some pro-tumorigenic role. CXCR7 has enhanced affinity over CXCR4 for CXCL12, and its ability to scavenge CXCL12, means that CXCR7 has the ability to modulate circulating chemokine levels. When CXCR7 is absent or inhibited, serum CXCL12 levels can increase 5 fold, demonstrating the potentially large modulatory role of CXCR7 (Jin *et al.*, 2013). The scavenging of CXCL12 by CXCR7 receptors on vascular endothelium sharpens the extracellular chemokine gradient of CXCL12 facilitating the chemotaxis of cells in and out the blood vessels. This could be particularly important within the tumour microenvironment as it may provide a precise

chemokine gradient assisting melanoma intravasation from CXCL12 present in the tumour into the blood. Conversely the sharpened chemokine gradient may also facilitate the extravasation of cells from the blood into tissues, which would allow CXCR7 to modulate leukocyte CXCL12 homing and may affect tumour infiltration (Jin *et al.*, 2013). While not as clear as the role for CXCR4 in melanoma pathogenesis, CXCR7 may nevertheless therefore support melanoma progression by fine tuning chemotactic gradients thereby providing optimal migratory conditions for tumour chemotaxis.

3.3.3 CXCL12: Implications of Expression and Signalling in Cutaneous and Uveal Melanoma

CXCL12 plays a critical role in driving melanomas chemotaxis, organ specific metastasis, cell proliferation and survival, as well as influencing angiogenesis and tumour immune infiltration demonstrating a regulatory capacity in both temporal and spatial cancer pathogenesis (Balkwill, 2004). However, studies of CXCL12 expression and distribution with the melanoma microenvironment are less well defined. In the present study the skin and retina of the eye were identified as rich sources of CXCL12 expression *in vivo. In vitro* studies also highlighted primary dermal fibroblasts and biliary epithelial cells as prominent secretors of CXCL12 suggesting these may be the dominant sources of CXCL12 chemokine gradients for CXCR4 expressing melanomas *in vivo*. Data from the present study in both the skin and eye suggests the establishment of a CXCL12 chemokine gradient whereby melanocytes at the epidermal/dermal junction sit above strong sources of chemokine secretors within the dermis of the skin or the retina of the eye, making it unsurprising that melanoma cells evolve to take advantage of such a chemokine source to invade and metastasise.

Studies of CXCL12 expression identified widespread cytoplasmic expression of CXCL12 by all cutaneous and uveal melanoma cell lines. Of note primary melanocytes expressed CXCL12 most strongly suggesting CXCL12 expression may be down regulated by melanoma cells. CXCL12 was also detected within the supernatants of CXCR4 positive cutaneous metastatic melanoma cell lines CHL-1 and WM-164 suggesting that CXCL12 is secreted from melanoma cells potentially resulting in autocrine chemokine signalling. The ability of a cancer cell to secrete chemokines together with the expression of the cognate receptor allows the cell to self-perpetuate advantageous cell signalling indefinitely. Autocrine chemokine-receptor loops are thought to give cancer cells a survival advantage and in glioma and head and neck cancer have been shown to promote cell survival and resistance to chemotherapy (Wang *et al.*,

2008a; Hattermann *et al.*, 2010). Probably the best example of such signalling in melanoma is the CXCR2-CXCL8 chemokine axis where by CXCL8 acts as an autocrine growth factor, where blocking its interaction with CXCR2 inhibits melanoma cell growth *in vitro* (Norgauer J *et al.*, 1996; Homey *et al.*, 2002). Neutralisation of CXCL12 caused deactivation of CXCR4 and downregulation of MAPK cell signalling in WM-164 metastatic melanoma cell line, defining an autocrine signalling mechanism whereby tumoural secretion of CXCL12 is able to promote activation of pro-survival MAPK cell signalling. Previous studies have illustrated CXCL12-induced activation of MAPK signalling and melanoma cell growth, suggesting CXCL12 may contribute to melanomagenesis (Robledo *et al.*, 2001; Murakami *et al.*, 2002); however the present study is the first to demonstrate that endogenous CXCL12 drives MAPK cell signalling in melanoma. However, given that none of the other melanoma cell lines or primary melanocytes secreted CXCL12, it may be that CXCL12 secretion is cell line specific. Nevertheless, as CXCL12 was expressed within the cytoplasm of all melanoma cell lines to some extent, these data question whether there may be a mechanism of chemokine retention in play. Supporting this hypothesis, studies have demonstrated that chemokines may possess a endoplasmic reticulum retention signal that may prevent receptor cell surface expression (Yang *et al.*, 1997; X Baia *et al.*, 1998). Collectively these data therefore suggest early interaction of chemokines and chemokine receptors within the cell cytoplasm may prevent normal chemokine receptor trafficking to the cell surface which may account for accumulation of CXCL12 and CXCR4 within the cytoplasm of melanoma in absence of any CXCL12 secretion.

Expression of CXCL12 was detected in the current cohort of primary cutaneous melanomas at low levels, however, whether or not it is secreted in an autocrine manner or if perhaps originated from an alternative source within the tumour microenvironment could not be differentiated. As CXCL12 expression did not differ significantly between naevi and all AJCC stage melanomas, localised and metastatic tumours or correlate with time to metastasis, this suggests that although present, CXCL12 does not play a prominent role in disease progression or metastasis, results that are corroborated by previous studies (Toyozawa *et al.*, 2012). However, given its expression in all disease stages including naevi, and the fact that *in vitro* experiments suggest a mechanism of CXCR4-CXCL12 autocrine cell signalling, a general role for CXCL12 in primary tumours is postulated, where the tumour cells uptake endogenously derived CXCL12, driving pro-survival MAPK cell signalling allowing sustained tumour maintenance and survival. The low levels of CXCL12 expressed within the primary tumour and

lack of association with disease progression are perhaps not surprising given that abundant endogenous CXCL12 within the primary site would logically act to retain CXCR4 positive tumour cells, rather than facilitate their migration towards CXCL12 at distant sites. In support of this hypothesis, studies have shown trends for increased CXCR4 expression with the absence of CXCL12 expression in melanomas as well as the enhanced metastatic potential of CXCR4 positive cancer cells following the epigenetic silencing of CXCL12 (Wendt *et al.*, 2006; Mitchell *et al.*, 2014). Alternatively, secreted CXCL12 may be degraded by tumour proteases including MMPs, cathepsin G and elastase known to be upregulated in the tumour microenvironment of nearly all cancer types (Delgado *et al.*, 2001; McQuibban *et al.*, 2001; Valenzuela-Fernández *et al.*, 2002). Although significantly higher than that detected in primary cutaneous melanomas, CXCL12 was also detected albeit again at low levels in all primary uveal melanomas. The role of CXCL12 in uveal melanoma is uncertain with only 2 studies documenting expression of CXCL12 in 27% and 41% of primary uveal tumours respectively (Franco *et al.*, 2010; Dobner *et al.*, 2012). In one study CXCL12 was associated with increased tumour diameter and an epithelioid cell type, implying that unlike in cutaneous melanoma, CXCL12 within primary uveal melanomas may exert a pro-tumourigenic effect (Franco *et al.*, 2010; Dobner *et al.*, 2012). In both these studies CXCL12 expression was also reported to display low immunoreactivity, much lower than other chemokines such as CCL19 and CCL21, alluding to the possibility that CXCL12 is not of paramount importance within primary tumours and that its effects are more likely relevant once tumour cells have evaded the primary site.

To date only one study has evaluated the relationship between CXCL12 expression and B-RAF/N-Ras mutational status in primary cutaneous melanomas, however researchers were unable to find any association (Mitchell *et al.*, 2014). An interesting outcome from the present study was the observation of significantly reduced CXCL12 expression in B-RAF/N-Ras mutated compared to wild-type cutaneous melanomas, indicating for the first time, that oncogenic B-RAF signalling may promote down regulation of CXCL12 expression, or CXCL12 secretion from primary tumour cells. This trend was also recognised in primary uveal melanoma where down regulation of CXCL12 was associated with monosomy of chromosome 3, the strongest prognostic indicator of metastasis. Together these data thus support a pattern of CXCL12 down regulation in the primary tumour which one could hypothesise, may assist the emergence of an aggressive phenotype enabling CXCR4 positive tumour cells to escape from the primary site and encouraging metastasis towards a distant CXCL12 secreting site. This

trend is further supported by literature reporting the increased ratio of CXCL12:CXCR4 mRNA in thin compared to thick cutaneous melanomas (Monteagudo *et al.*, 2012). Given the fact B-RAF^{V600E} mutated melanoma cell lines express higher levels of cytokines including MMP-1 compared to wild-type cutaneous melanoma cell lines this raises the possibility that CXCL12 down regulation is a reflection of enhanced degradation by MMP-1 secreted by B-RAF mutated melanomas (Whipple and Brinckerhoff, 2014). Nevertheless, in general, low CXCL12 expression in primary tumours appears to be an advantageous quality although the mechanism and impact of this observation remains to be determined.

Similarly to other reports, studies of CXCR4 and CXCL12 expression in the present cohort of primary cutaneous revealed no significant correlation between total CXCR4 and CXCL12 expression, perhaps surprising given the close relationship between CXCR4 and CXCL12 but which may reflect the low expression levels of CXCL12 observed, or the unimportance of CXCR4-CXCL12 signalling in the primary tumour (Toyozawa *et al.*, 2012; Mitchell *et al.*, 2014). Nevertheless, data from the current study demonstrated the association of B-RAF/N-Ras mutant cutaneous melanomas expressing increased levels of nuclear CXCR4 expression with low levels of CXCL12 expression leading to the hypothesis that oncogenic B-RAF signalling promotes both nuclear CXCR4 translocation and down regulation of CXCL12 expression which may be key to the emergence of an aggressive cutaneous melanoma phenotype.

Specific studies of CXCL12 in cancer have focused mainly on tumour expression, with very little research into the function of CXCL12 within the tumour microenvironment or its interaction with the primary tumour, and even less so in the context of studies of melanoma. The observation of increased epidermal CXCL12 expression adjacent to some primary cutaneous melanomas compared to expression in the epidermis overlying the tumour prompted further investigation. Although epidermal CXCL12 expression did not significantly differ between the adjacent and overlying tumoural epidermis, the presence of increased levels of CXCL12 in the adjacent epidermis did however, correlate with a reduced time to tumour metastasis, there by suggesting the presence of CXCL12 in the adjacent epidermis limits tumour progression. Although the precise mechanisms mediating this affect are unclear it is possible that the high CXCL12 gradient within the adjacent epidermis may act to retain CXCR4 positive tumour cells at the epidermal/dermal junction thereby promoting radial growth and delaying melanoma vertical invasion into the dermis with subsequent metastasis. Adjacent epidermal CXCL12 expression was also associated with cytoplasmic CXCR4 expression by melanoma cells. These

data are coherent since they suggest tumoural CXCR4 is chronically activated by microenvironmental CXCL12 and therefore internalised from the melanoma cell membrane. Importantly, chronic CXCR4 signalling by melanoma is supported by our *in vitro* observation of basal pCXCR4 levels; phosphorylation of CXCR4 being characterised by ligand occupancy and a pre-requisite of receptor internalisation (Marchese, 2014). Given the association between CXCR4 nuclear expression with B-RAF/N-Ras mutated tumours and the increased likelihood of the development of metastatic disease, it may be postulated that B-RAF/N-Ras wild-type cutaneous melanomas expressing increased levels cytoplasmic CXCR4 with relatively low levels of nuclear CXCR4 expression are more likely to remain localised. Therefore, the association of epidermal CXCL12 expression adjacent to the primary tumour with tumoural cytoplasmic CXCR4 expression may thus reflect a subset of tumours more likely to remain localised, in contrast cutaneous B-RAF/N-Ras mutated tumours with high levels of nuclear CXCR4 expression which unresponsive to epidermal CXCL12 are more likely to metastasise.

In conclusion, increased CXCR4 expression within primary cutaneous and uveal melanoma is highlighted as a putative biomarker for metastasis and disease progression. The pro-tumourigenic role of CXCR4 is likely further aided by CXCL12 autocrine signalling promoting enhanced MAPK activation within tumour cells, and when secreted from distant sites promoting tumour cell mobilisation and migration from the primary tumour. Although present within primary melanomas, the most influential role played by CXCL12 maybe within the primary tumour microenvironment where CXCL12 chemokine gradients are in part modulated by CXCR7 sequestration, may promote retention of CXCR4 positive cells within the primary location. Data thus reinforces the intricate and intercalating roles of the CXCR4/CXCR7-CXCL12 chemokine axis within primary melanomas, the immediate tumour microenvironment and sites of distant metastasis, including the liver.

3.4 Summary

- High CXCR4 expression (>50%) is a prognostic biomarker for AJCC stage II cutaneous melanoma
- Nuclear CXCR4 expression is associated with B-RAF/N-Ras mutant cutaneous melanomas and an aggressive metastatic phenotype.
- CXCR4 is strongly expressed by primary uveal melanomas
- CXCR7 is not expressed by cutaneous or uveal melanomas *in vitro* or *in vivo* but is present on vascular endothelium within the tumour microenvironment *in vivo.*
- CXCL12 is expressed by primary melanocytes, keratinocytes, and dermal fibroblasts within normal skin
- CXCL12 is expressed by both cutaneous and uveal melanoma cell lines and primary cutaneous and uveal melanomas
- CXCL12 is secreted by primary dermal fibroblasts and biliary epithelial cells, and select cutaneous melanoma cell lines.
- Secretion of CXCL12 by WM-164 cutaneous melanoma cell line acts in an autocrine manor activating CXCR4 receptors and MAPK cell signalling.
- Activating mutations in B-RAF/N-Ras in cutaneous melanoma or monosomy of chromosome 3 in uveal melanoma are associated with decreased tumoural expression of CXCL12
- High levels of CXCL12 expression in the adjacent epidermis to cutaneous melanomas are associated with tumoural cytoplasmic expression of CXCR4 and increased time to disease progression

Chapter 4

Crosstalk Between CXCR4-CXCL12 and MAPK Cell Signalling

Chapter 4 Crosstalk Between CXCR4-CXCL12 and MAPK Cell Signalling

4.1 Introduction

In cancer, chemokines maybe present within tumours themselves, within the stroma of the tumour microenvironment or enriched at specific metastatic sites. It is often the balance of chemokines within the tumour or microenvironment and summation of their divergent functions that impacts tumour growth and metastasis. The CXCR4-CXCL12 chemokine axis has an important regulatory role in many aspects of tumorigenic processes including shaping leukocyte recruitment or tumour stromal content, angiogenesis, tumour cell proliferation, invasion and survival (as previously discussed section 1.5.). However, the overriding detrimental role of the CXCR4-CXCL12 axis in malignant processes is the promotion of tumour cell migration, that leads to enhanced invasive capabilities and eventual organ specific metastasis of tumour cells.

A number of studies in different cancers have correlated CXCR4 and CXCL12 expression with tumour metastasis and worse prognosis (Li *et al.*, 2004; Kang *et al.*, 2005; Scala *et al.*, 2005; Toyozawa *et al.*, 2012), with additional evidence demonstrating the inhibition of this axis reduces metastatic ability with improved patient outcome (Liang *et al.*, 2004b; Mori *et al.*, 2004; Sun *et al.*, 2005). Indeed results from chapter 3 demonstrate the upregulation of CXCR4 in AJCC stage II primary cutaneous melanomas, is predictive of metastasis and worse prognosis, emphasising the potential for targeting CXCR4 to prevent melanoma metastasis (O'Boyle *et al.*, 2013).

It has long been recognised that cancer cells metastasise to their own favoured sites in the body (Paget, 1889); favoured sites that naturally express chemokine ligands, and with cancer cells expressing the cognate chemokine receptor, circulating passively within our vasculature sensing a local chemokine source, then extravasating into the secreting tissue. Further, it is well established that in melanoma the CXCR4-CXCL12 chemokine axis promotes tumour cell dissemination to high CXCL12 secreting distant sites such as the lymph nodes, liver, and lungs (Ben-Baruch, 2008; O'Boyle *et al.*, 2013). The source of CXCL12 at these sites is generally assumed to be secreted primarily from stromal fibroblasts (Micke and Östman, 2005; Orimo *et al.*, 2005), with results derived from chapter 3 additionally highlighting CXCL12 expression by keratinocytes within the epidermis as protective of tumour metastasis, the chemoattractant stimulus for which is postulated to be mediated by a haptotaxic mechanism. However, findings from the present study also suggest that CXCL12 downregulation in

melanomas is associated with more aggressive B-RAF/N-Ras mutant cutaneous melanomas or uveal melanomas with monosomy of chromosome 3. Collectively, these data suggest that tumoural secretion of CXCL12 may be reduced to provide a localised decline in CXCL12 chemokine concentration, an effect that CXCR7 expression by skin vasculature could also potentiate, allowing melanoma cell migration towards higher CXCL12 concentrations. Given that 36% of melanoma patients develop recurrence, with local recurrence seen in 63-87% of these patients (Balch *et al.*, 2009a), the noted potent secretion of CXCL12 by primary dermal fibroblasts and their location, it is logical to question whether melanoma cells, firstly migrate towards primary dermal fibroblasts, and secondly if this is mediated by CXCL12-specific secretion.

Given the important role that CXCR4-CXCL12 cell signalling has in cancer metastasis, it is perhaps unsurprising that this axis has been exploited therapeutically. Targeting has mainly focused on CXCR4 where small molecule inhibitors, peptide inhibitors and antibodies have been trialled (Tamamura *et al.*, 2003; Cashen AF, 2009; Kuhne *et al.*, 2013). Although many of the agents are currently in clinical trials, only one agent has been approved for clinical use; Plerixafor (Genzyme, previously called AMD3100), for use pre-bone marrow transplantation as an immune-stimulant in lymphoma and myeloma. Plerixafor has shown some promise in melanoma, inhibiting CXCL12 induced chemotaxis *in vitro* and reducing lung metastases *in vivo* (D'Alterio *et al.*, 2012). Other CXCR4 inhibitors have shown similar blockade of CXCR4-CXCL12 chemotaxis in melanoma *in vitro* (Liang *et al.*, 2012; O'Boyle *et al.*, 2013). Although the concept of inhibiting CXCR4-CXCL12 cell signalling to prevent metastasis has been proven there are however, some potential pit falls in targeting chemokine receptors in this manner. Firstly, rarely do cancer cells only express one chemokine receptor. Blocking one chemokine axis may mediate cancer metastasis to differing sites under the influence of a different chemokine axis. Secondly, chemokine axes are involved in many aspects of immunity and developmental processes (Mithal *et al.*, 2012), and hence their inhibition may impair potential anti-tumour chemokine immune responses, or establish other unwanted side effects related to immune system dysfunction (Weitzenfeld and Ben-Baruch, 2014). In particular CXCR4-CXCL12 is crucial for haematopoiesis, and blocking this axis may result in cytopenia and mobilization of haematopoietic stem cells to blood (Weitzenfeld and Ben-Baruch, 2014).

Such potential serious implications of targeting the chemokine system specifically, thus questions whether there may be a better way to target melanoma migration and metastasis.

In the context of CXCR4-CXCL12 mediated chemotaxis, migration is mitigated through CXCL12 binding and activating CXCR4, a heterotrimeric G protein coupled receptor. Activation of the receptor leads to a cascade of downstream signalling pathways including the PKC, PI3K and MAPK pathways that enable intracellular calcium mobilisation and cell migration (Robledo *et al.*, 2001). Previous studies have illustrated an association between CXCR4-CXCL12 activation and MAPK cell signalling (Robledo *et al.*, 2001; Murakami *et al.*, 2002). Furthermore, recent studies from the Lovat lab have demonstrated a link between hyper-activation of MAPK and melanoma cell migration, illustrated by transfection of CHL-1 wild-type melanoma cells with mutant B-RAF and subsequent increased CXCR4-CXCL12 cell migration (O'Boyle *et al.*, 2013). Results from the previous chapter further illustrated autocrine secretion of CXCL12 can promote activation of pro-survival MAPK cell signalling in the cutaneous melanoma cell line WM-164. Collectively these data provide a potential rational for preventing CXCR4-CXCL12 mediated cell migration by inhibiting the MAPK pathway, and leads to the initial aim of the current chapter to test whether MEK inhibition with the MEK 1/2 specific clinical inhibitor trametinib prevents melanoma CXCR4-CXCL12 chemotaxis.

Although targeted therapies such as B-RAF/MEK inhibition have revolutionised melanoma treatment in recent years, researchers are now faced with the almost inevitable emergence of drug resistance and the task of identifying strategies to overcome drug-induced resistance. Autophagy has been identified as a key player in the acquisition of drug resistance to many different chemotherapeutic regimens (Amaravadi RK and Thompson CB, 2007). This self-cannibalisation process, primarily activated as a housekeeping stress response to adverse cellular challenges including hypoxia and nutrient deprivation, is also activated by many anti-cancer therapies to counter act drug-induced cytotoxicity (Degenhardt K *et al.*, 2006). Targeting autophagy however, has become somewhat of a controversial issue within the literature, stemming from autophagy's contradictory roles in tumour progression deemed the 'autophagy paradox', where defective autophagy can drive neoplastic formation, whereas efficient autophagy may promote tumour survival (Mathew *et al.*, 2009; Roy S and Debnath J, 2010; Ellis *et al.*, 2014b). There is evidence that targeting cyto-protective autophagy maybe beneficial and sensitise cells to many different anti-cancer regimes, including traditional chemotherapies and radiotherapy, hormonal treatments and newer targeted agents such as B-RAF and MEK inhibitors (Carew JS *et al.*, 2007; Apel *et al.*, 2008; Qadir *et al.*, 2008; Del Bello *et al.*, 2013; Yao *et al.*, 2015; Goulielmaki *et al.*, 2016). Conversely exacerbation of autophagy

may lead to cell death (type II) or 'autophagic cell death', mediated by large scale autophagic vacuolization and extensive degradation of intracellular contents that leads to cell death (Liu and Levine, 2015). Therefore the induction of autophagy to promote autophagic cell death in some cancers has emerged as a therapeutic strategy including melanoma (Ambrosini *et al.*, 2013b; Xie *et al.*, 2013; Amirouchene-Angelozzi *et al.*, 2014). It is clear that both inhibiting and inducing autophagy can harness this process for therapeutic benefit, however in which cancer, what drug, regime, or timeframe, remains enigmatic. Hence, in the current chapter the specific effect of trametinib-induced MEK inhibition on autophagy in uveal and cutaneous melanoma was investigated, together with potential strategies through which to harness autophagy to enhance the efficacy of MEK specific targeted therapy.

Collectively the specific aims of this chapter were to investigate whether CXCR4-CXCL12 mediated chemotaxis of cutaneous melanoma cell lines (both B-RAF/N-Ras mutant and wild-type) within the cutaneous microenvironment may be mediated by CXCL12 secreted by dermal fibroblasts, and to question if this and CXCR4-CXCL12 mediated chemotaxis of uveal melanomas may additionally be prevented by treatment with the MEK specific inhibitor, trametinib and any potential impact of this strategy on autophagy.

4.2 Results

4.2.1 CXCR4 Expressing Cutaneous and Uveal Metastatic Melanoma Cells Migrate Towards Human Recombinant CXCL12 and CXCL12 Rich Supernatant Derived from Primary Cutaneous Dermal Fibroblasts

Studies in chapter 3 demonstrate CXCL12 in the immediate melanoma microenvironment of the adjacent epidermis is protective of metastasis, however high levels of CXCR4 expression correlate with the increased risk of metastasis in AJCC stage II melanomas, suggesting the CXCR4-CXCL12 axis likely influences primary tumour escape to distant sites. This coupled with the downregulation of CXCL12 by more aggressive B-RAF/N-Ras mutant melanomas suggests directional migration sensing, leading to the hypothesis that an alternative CXCL12 source, likely derived from primary cutaneous dermal fibroblasts may influence CXCR4 positive tumour cell escape, and provide a chemotactic gradient to potentiate deeper invasion within the dermis and subsequent migration to distant sites. To investigate this possibility a series of conventional transwell CXCR4-CXCL12 chemotaxis experiments were performed to confirm the migration of CXCR4 expressing melanoma cells towards both human recombinant CXCL12 (rCXCL12) and supernatants derived from primary dermal fibroblasts as well as the potential to inhibit such chemotaxis within the cutaneous environment by MEK inhibition with trametinib.

Results demonstarted both CXCR4 expressing cutaneous CHL-1 (B-RAF/N-Ras wild-type) and B-RAF mutant WM-164 cells as well as OM413 (GNAQ/GNA11 wild-type) uveal melanoma cells significantly migrated towards 10nM rCXCL12 compared to no chemokine control (One-way ANOVA, with Bonferroni's multiple comparison post hoc correction, or Kruskal-Wallis test with Dunn's multiple comparison post hoc correction, P***< 0.001), (Figure 4.1). However, this effect was significantly abrogated by co-treatment with a neutralising anti-CXCL12 antibody (One-way ANOVA with Bonferroni's multiple comparison post hoc correction, or Kruskal-Wallis test with Dunn's multiple comparison post hoc correction, P***<0.001), while there was no observed migration in the presence of an isotype control antibody, (One-way ANOVA, with Bonferroni's multiple comparison post hoc correction, or Kruskal-Wallis test with Dunn's multiple comparison post hoc correction, ns P>0.05) (Figure 4.1).

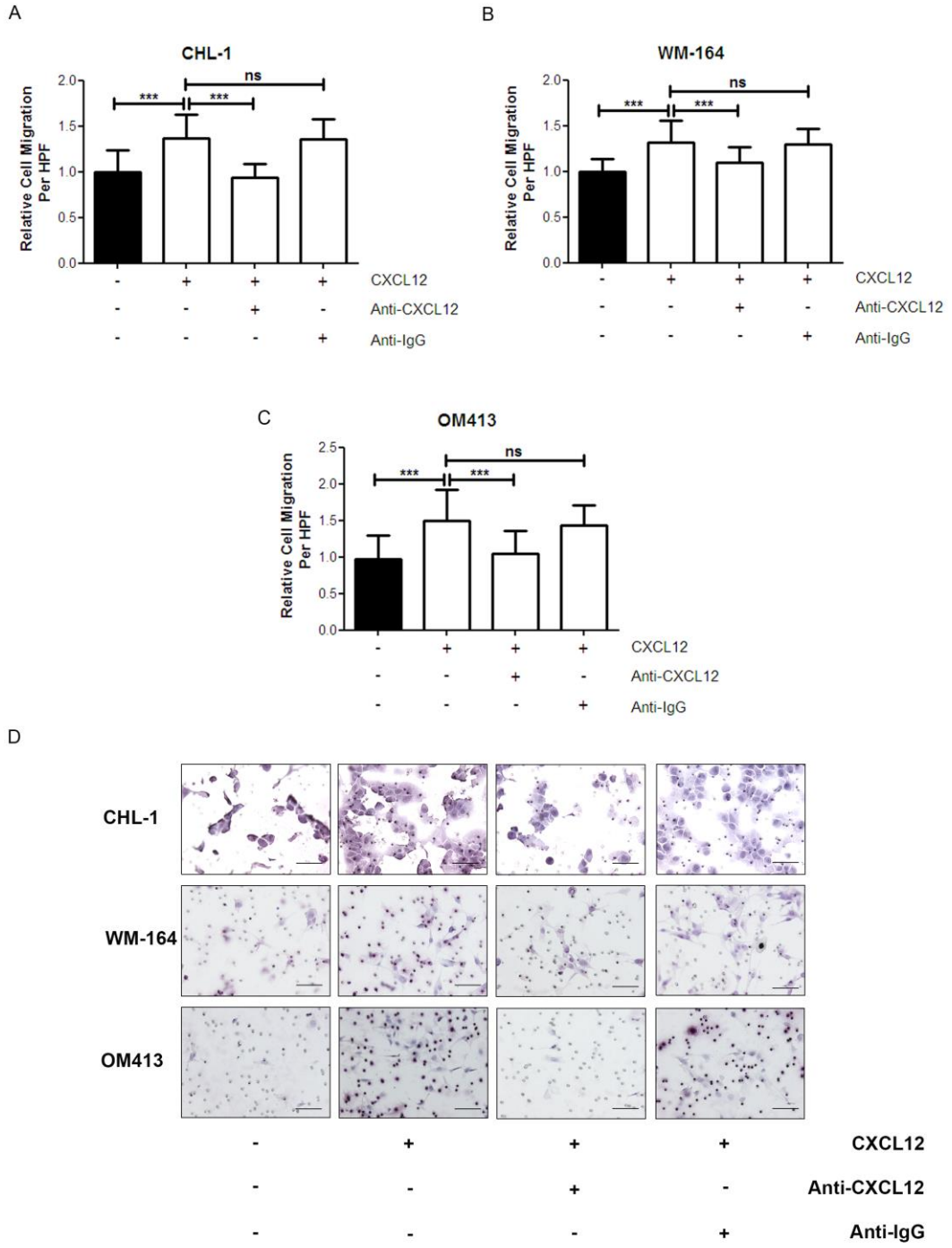


Figure 4.1 Cutaneous and Uveal Metastatic Melanoma Cell Lines Migrate Towards Human Recombinant CXCL12

*Relative cell migration per high field vision (HPF) of A.) CHL-1 or B.) WM-164 cutaneous metastatic melanoma cells or C.) OM413 metastatic uveal melanoma cells over 16 hrs towards 10nM human recombinant CXCL12 (CXCL12) in the presence or absence of anti-CXCL12 neutralising antibody, or Isotype control anti-IgG antibody, expressed relative to migration towards control media without chemokine. Each bar represents the mean of 9 replicate filters from 3 independent experiments + SD. Statistics were acquired by one-way analysis of variance, with Bonferroni's multiple comparison post hoc correction, or Kruskal-Wallis test with Dunn's multiple comparison post hoc correction, where ns = P>0.05, ***P<0.001. D.) Representative photomicrographs from one high powered field (HPF) of migrated CHL-1, WM-164 or OM413 metastatic melanoma cells in the presence or absence of CXCL12, anti-CXCL12 or anti-IgG. Images were acquired at X20 magnification; scale bar represents 100µm.*

4.2.2 CXCR4 Expressing Cutaneous Metastatic Melanoma Cells Migrate Towards CXCL12 in Supernatants Derived from Primary Dermal Fibroblasts

To question whether cutaneous melanoma cells also migrate towards CXCL12 secreted from primary dermal fibroblasts, supernatants collected from primary dermal fibroblasts following 72 hours of culture were incorporated as the chemoattractant in transwell chemotaxis assays with CHL-1 or WM-164 cutaneous melanoma cells, in the presence or absence of CXCL12 neutralising antibody. Results demonstrated a significant increase in cell migration of both metastatic melanoma cell lines towards primary dermal fibroblast supernatant compared to control (DMEM +10% FCS) (Kruskal-Wallis test with Dunn's multiple comparison post hoc correction, ***P< 0.001, Figure 4.2), an effect that was particularly evident in WM-164 cells (Figure 4.2 B). The migration of melanoma cells was significantly blocked by the CXCL12 neutralising antibody (Kruskal-Wallis test with Dunn's multiple comparison post hoc correction ***P< 0.001, Figure 4.2), however no significant effect was seen with the isotype control antibody (Kruskal-Wallis test with Dunn's multiple comparison post hoc correction, ns P>0.05, Figure 4.2).

Collectively these data therefore confirmed CXCR4-CXCL12 chemotaxis of CXCR4 expressing cutaneous and uveal metastatic melanoma cell lines towards rCXCL12, with no apparent effect of the presence of an activating MAPK mutation, as well as CXCR4-CXCL12-mediated chemotaxis of cutaneous melanoma cells towards dermal fibroblasts.

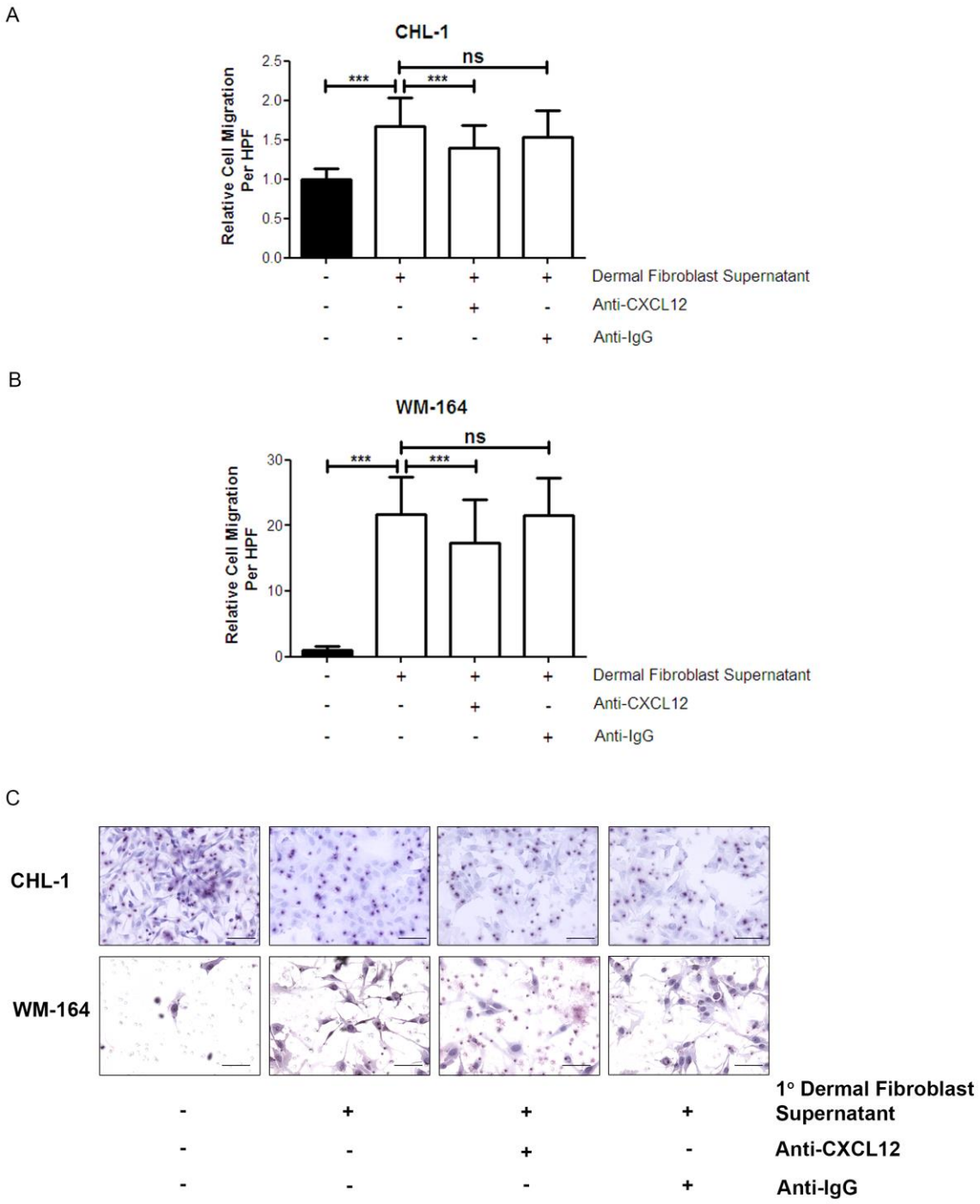


Figure 4.2 Cutaneous Melanoma Cell Lines Migrate Towards CXCL12 Secreted by Primary Dermal Fibroblasts

*Relative cell migration per high field vision (HPF) of A.) CHL-1 or B.) WM-164 cutaneous metastatic melanoma cells towards CXCL12 rich supernatants derived from primary dermal fibroblasts over 16hs, in the presence or absence of anti-CXCL12 neutralising antibody, or Isotype control anti-IgG antibody, expressed relative to migration towards control complete culture media. Each bar represents the mean of 9 replicate filters from 3 independent experiments each with a different donor of primary dermal fibroblast supernatants + SD. Statistics were acquired by Kruskal-Wallis test with Dunn's multiple comparison post hoc correction, where ns = P>0.05, ***P<0.001. D.) Representative photomicrographs from one high powered field (HPF) of migrated CHL-1, or WM-164 metastatic melanoma cells in the presence or absence of dermal fibroblast supernatants, anti-CXCL12 antibody or anti-IgG antibody. Images were acquired at X20 magnification; scale bar represents 100µm.*

4.2.3 MEK Inhibition Prevents CXCR4-CXCL12 Chemotaxis of Cutaneous and Uveal Melanoma Cells towards Human Recombinant CXCL12

To determine if MEK inhibition could inhibit CXCR4-CXCL12 melanoma chemotaxis, additional chemotaxis assays of cutaneous and uveal melanoma cells towards recombinant CXCL12 were performed in the presence of trametinib. Initial dose response assays of trametinib-induced inhibition of cell viability were first performed in order to optimise assay time frames and to rule out any effects of inhibited cell migration due to reduction of cell viability. MTS cell viability assays were performed with cutaneous and uveal melanoma cell lines after 16 hours' incubation with a dose escalating range of trametinib around the clinically achievable dose of 32nM.

Results revealed a dose dependant decrease in cell viability in all cell lines with the exception of uveal OM413 cells in which there was no significant inhibition of cell viability at any dose of trametinib at this time point (One-way analysis of variance with Dunnett's multiple comparison post hoc correction P=0.4215 Figure 4.3 C). As expected B-RAF mutant cutaneous WM-164 or GNA11 mutated uveal UPMD2 cells (Figure 4.3 B and D) were more sensitive to trametinib than wild-type cell lines CHL-1 and OM413 (Figure 4.3 A and C), with significant inhibition of cell viability observed even with 5nM trametinib (Kruskal Wallis test with Dunn's multiple comparison post hoc correction, **P<0.01, ***P<0.001, Figure 4.3 B and D). Trametinib concentrations of 2.5nM, 5nM and the maximum clinically achievable concentration of 32nM trametinib were therefore selected for use in all subsequent chemotaxis assays. UPMD2 GNA11 mutated metastatic uveal melanoma cell line, although migrated towards rCXCL12, the basal level of migration was particularly low and hence once inhibitors were added to chemotaxis assays it was impossible to determine a dose response, so this cell line was omitted from assays.

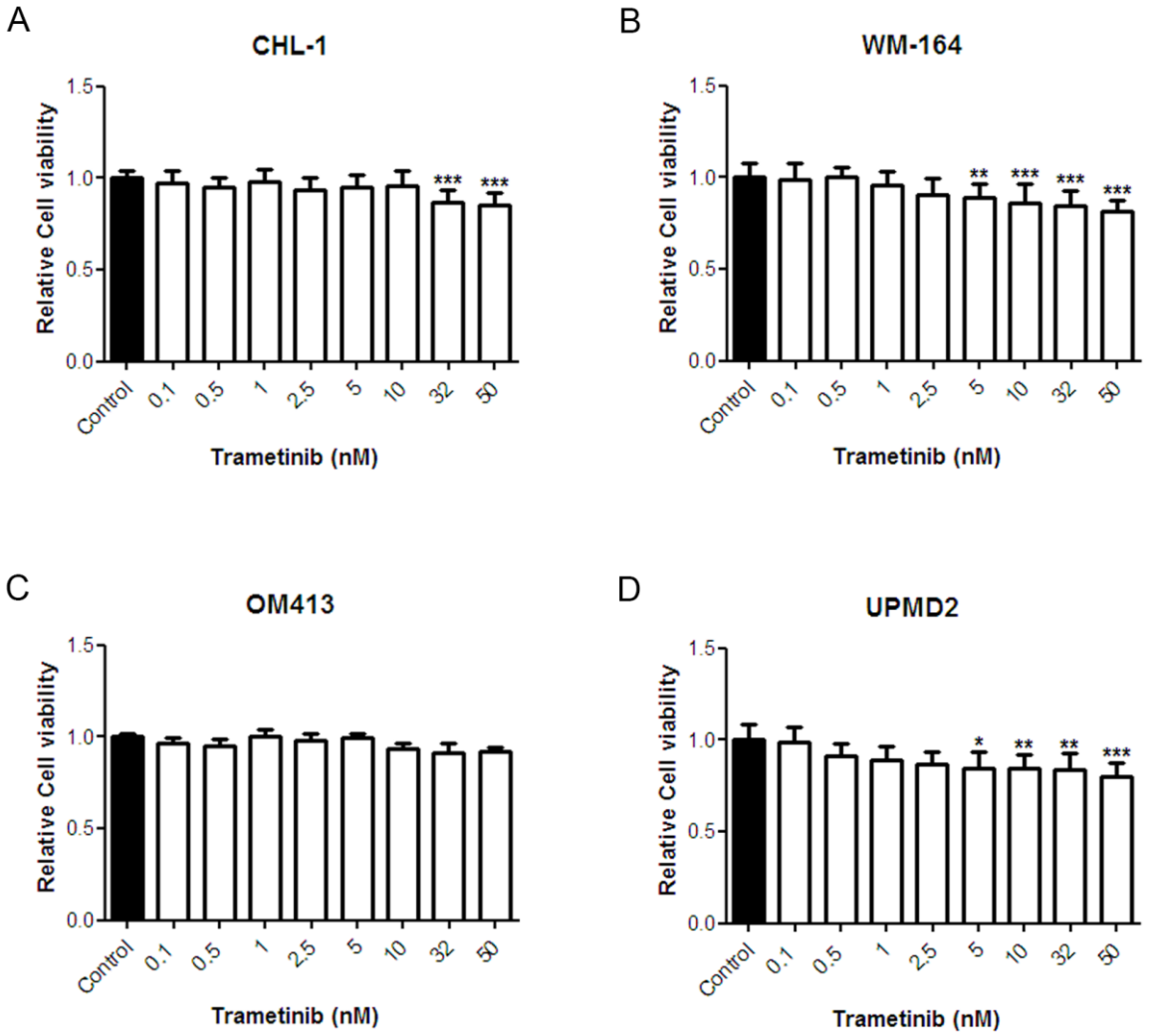


Figure 4.3 Trametinib-induced Inhibition of Cell Viability of Cutaneous and Uveal Metastatic Melanoma Cell Lines

*Cell viability of A.) CHL-1, B.) WM-164 cutaneous metastatic melanoma cell lines or C.) OM413 or, D.) UPMD2 uveal metastatic melanoma cell lines in the presence of 0.1-50nM trametinib for 16 hours, relative to control untreated cells. Each bar represents mean cell viability of 12 replicates from 3 independent experiments + SD. Statistics acquired by one-way analysis of variance with Dunnett's multiple comparison post hoc correction or Kruskal-Wallis test with Dunn's multiple comparison post hoc correction, where *P<0.05, **P<0.01, ***P<0.001.*

To confirm the ability of trametinib to inhibit MAPK signalling in cutaneous and uveal melanoma cells CHL-1, WM-164, OM413 and UPMD2 cells were treated in the presence of 2.5, 5 or 32nM trametinib for 16 hours prior to western blot analysis of phospho-ERK, total ERK and GAPDH (loading control) expression. Results revealed increased basal phospho-ERK expression in B-RAF mutant WM-164, compared to B-RAF/N-Ras wild-type CHL-1 cells (Figure 4.4 A), however increased expression of phospho-ERK was also apparent in both OM413 GNAQ/II wild-type and GNA11 mutated UPMD2 cells (Figure 4.4 B). Although OM413 cells do not harbour a GNAQ or GNA11 activating mutation, literature reports the presence of a B-RAF

mutation in these cells which therefore likely contributes to the observed increase in phospho-ERK expression (Griewank *et al.*, 2012). Nevertheless, trametinib induced the dose dependent inhibition of phospho-ERK expression in all cell lines, collectively indicating its potent activity as a MAPK signalling inhibitor at all employed concentrations.

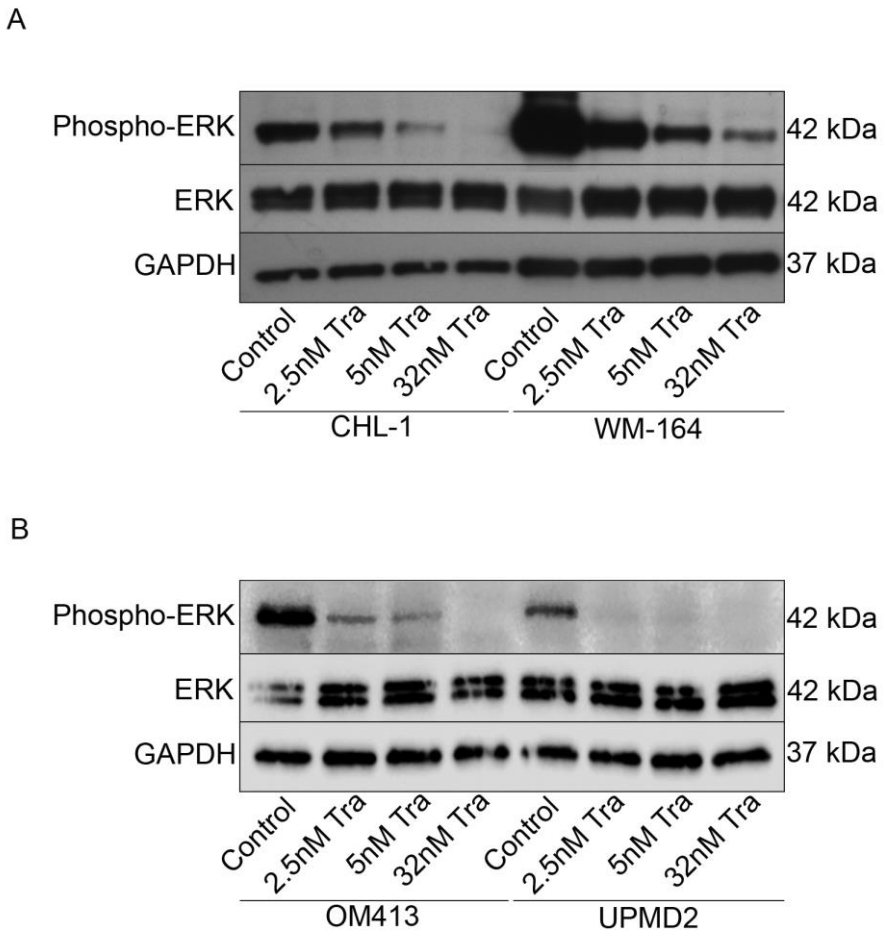


Figure 4.4 The Effect of Trametinib on MAPK Signalling in Cutaneous and Uveal Metastatic Melanoma Cell Lines

Representative western blots of, Phospho-ERK (42 kDa), total ERK (42 kDa), and GAPHD (loading control, 37 kDa) expression in A.) CHL-1 or WM-164 cutaneous metastatic melanoma cells or B.) OM413 or UPMD2 uveal metastatic melanoma cells after treatment with 2.5, 5 and 32nM trametinib (TRA) for 16 hours.

Having optimised trametinib doses, the potential for this MEK inhibitor to prevent CXCR4-CXCL12 chemotaxis was evaluated by co-treating CXCR4 positive CHL-1, WM-164 or OM413 cells for 16 hrs with 2.5, 5 or 32 nM trametinib when incorporated into transwell chemotaxis assays towards rCXCL12 (Figure 4.5).

Results demonstrated that trametinib at all concentrations induced the significant dose dependent inhibition of chemotaxis of all cells towards rCXCL12 compared to control, and importantly at concentrations of 2.5nM in WM-164 cells and 2.5 or 5nM in CHL-1 and OM413, doses at which there was no significant effect on cell viability (Kruskal-Wallis test with Dunn's post hoc multiple comparison test, *P<0.05, ***P<0.001, Figure 4.5).

Collectively these data therefore demonstrate the potent ability of the MEK specific inhibitor trametinib to inhibit CXCR4-CXCL12 mediated chemotaxis of both B-RAF mutant and wild-type cutaneous or GNAQ/GNA11 wild-type metastatic uveal melanoma cells.

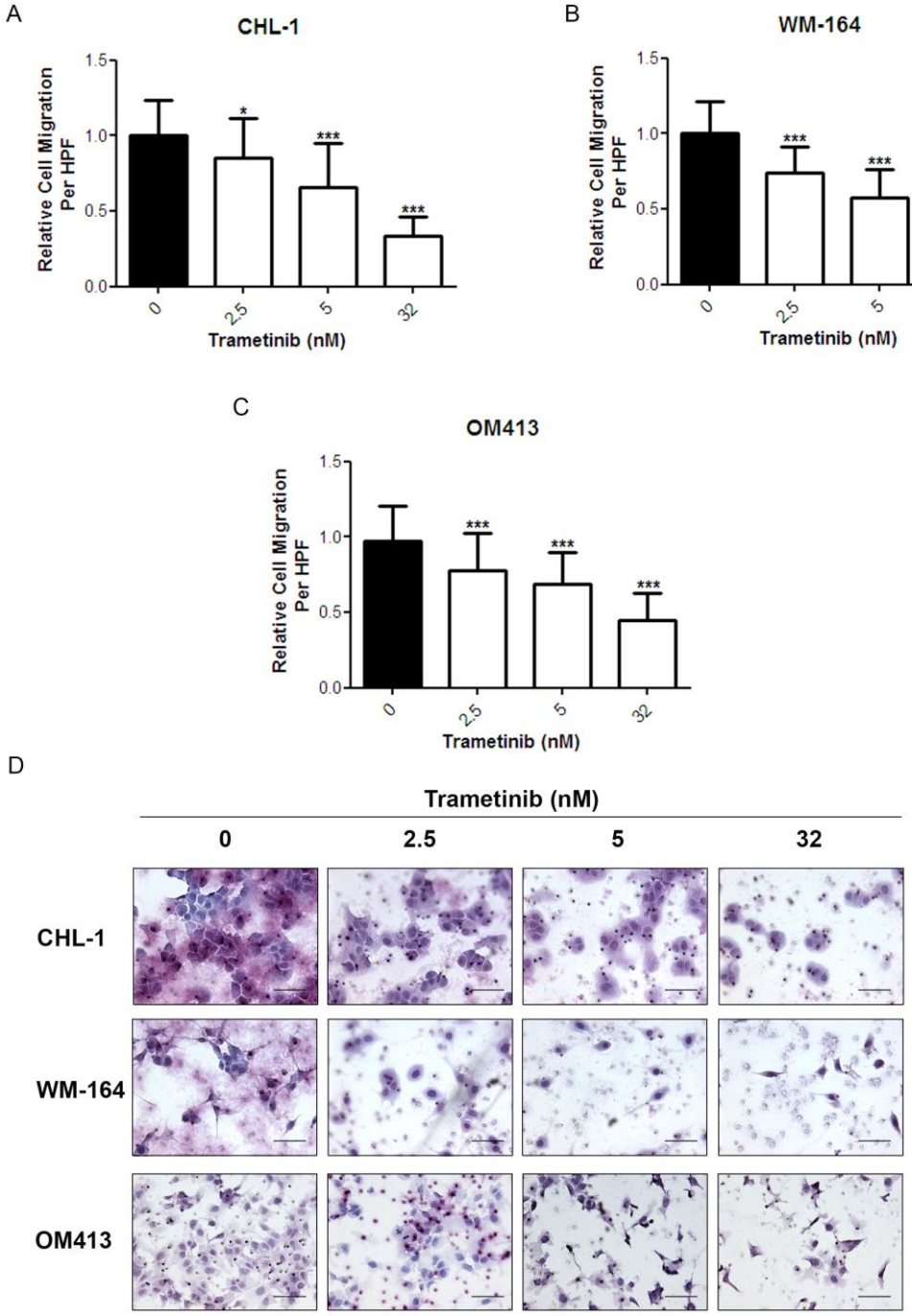


Figure 4.5 Trametinib Inhibits Cutaneous and Uveal Metastatic Melanoma Cell Line Migration towards Human Recombinant CXCL12

*Relative cell migration per high powered field of vision (HPF) of A.) CHL-1 or B.) WM-164 cutaneous metastatic melanoma cells or C.) OM413 metastatic uveal melanoma cells towards 10nM human recombinant CXCL12 following treatment for 16 hrs in the presence or absence of 2.5, 5 or 32nM trametinib and expressed relative to cell migration towards recombinant CXCL12 of untreated cells. Each bar represents the mean of 9 replicate filters from 3 independent experiments + SD. Statistics were acquired by Kruskal-Wallis test with Dunn's multiple comparison post hoc correction, where *P<0.05, ***P<0.001. D.) Representative photomicrographs from one high powered field (HPF) of migrated CHL-1, WM-164 or OM413 metastatic melanoma cells in the presence or absence of 2.5, 5, 32nM trametinib. Images were acquired at X20 magnification; scale bar represents 100µm.*

4.2.4 Trametinib Prevents CHL-1 Melanoma Cell Migration towards Supernatants Derived from Primary Dermal Fibroblasts

Next to determine the potential of trametinib to inhibit CXCR4-CXCL12 mediated chemotaxis of CXCR4 positive cutaneous melanoma cells towards CXCL12 rich dermal fibroblast supernatants, trametinib was titrated into chemotaxis assays for 16 hours with dermal fibroblast supernatants as the chemoattractant stimulus. As with experiments using recombinant CXCL12, results clearly demonstrated the dose dependent ability of trametinib to significantly inhibit CHL-1 cell migration towards primary dermal fibroblast supernatant (Kruskal-Wallis test with Dunn's multiple comparison post hoc correction, ***P<0.001) (Figure 4.6 A).

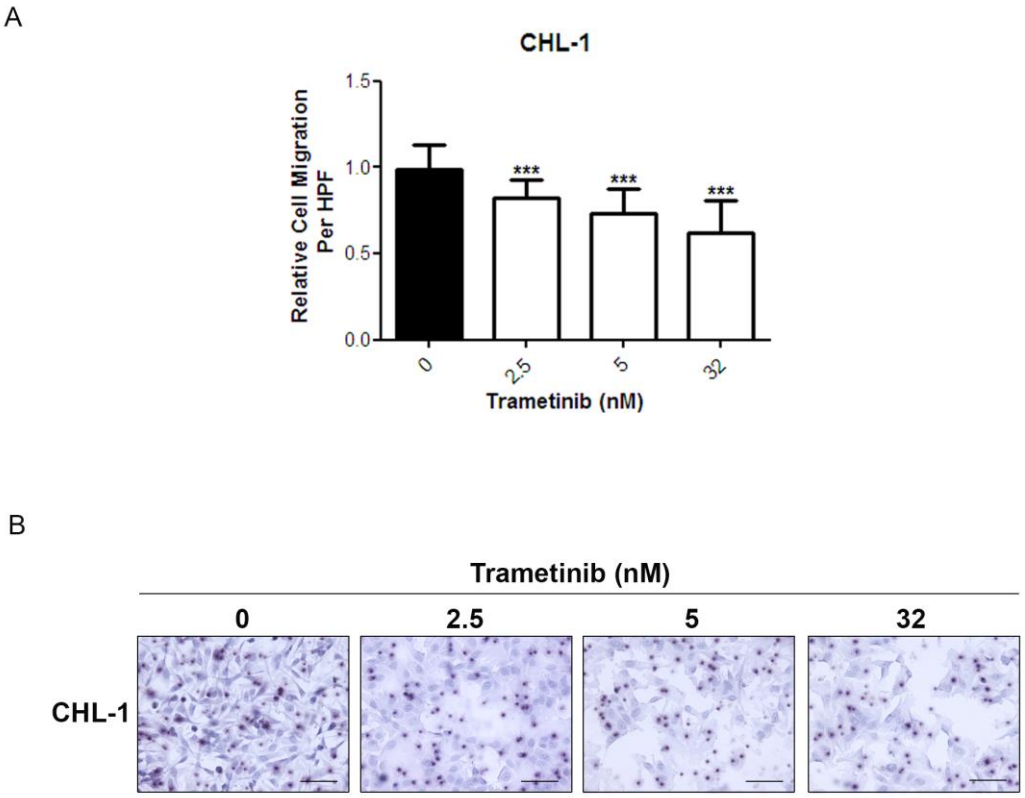


Figure 4.6 Trametinib Inhibits Cutaneous Melanoma Cell Line Migration towards Dermal Fibroblast Supernatants

*Relative cell migration per high powered field of vision (HPF) of A.) CHL-1 cutaneous metastatic melanoma cells towards supernatant derived from primary dermal fibroblasts in the presence of 2.5, 5 or 32nM trametinib, expressed relative to cell migration towards untreated cells. Each bar represents the mean of 9 replicate filters from 3 independent experiments each with a different donor of primary dermal fibroblast supernatants + SD. Statistics were acquired by Kruskal-Wallis test with Dunn's multiple comparison post hoc correction, where ***P<0.001. B.) Representative photomicrographs from one high powered field (HPF) of migrated CHL-1 metastatic melanoma cells towards primary dermal fibroblast supernatant in the presence or absence of 2.5, 5, 32nM trametinib. Images were acquired at X20 magnification; scale bar represents 100µm.*

4.2.5 Increased Basal Autophagy in Uveal Melanomas *in vivo* is Associated with Monosomy of Chromosome 3

In line with the paradoxical role of autophagy in cancer, studies have documented increased autophagic activity in metastatic cutaneous melanomas raising the possibility that active autophagy may serve to protect tumour cells from the cytotoxic effects of trametinib (Ma *et al.*, 2011a). Furthermore, several biomarker studies have confirmed the autophagy status of cutaneous melanomas with differing markers (Hersey and Zhang, 2008; Lazova *et al.*, 2012; Ellis *et al.*, 2014b), but to date only one study has reported the autophagy status of uveal melanomas (Giatromanolaki *et al.*, 2011). Specifically Giatromanolaki *et al* report increased Beclin-1 expression in uveal melanomas is associated with hypoxia and pigmentation and a poor clinical outcome, leading to their conclusion that increased autophagic activity in uveal melanomas is associated with a more aggressive phenotype (Giatromanolaki *et al.*, 2011).

Given Beclin-1 expression alone is not a reliable marker of autophagy (Grishchuk *et al.*, 2011) pre-optimised immunohistochemical assays for p62 as well as Beclin-1 expression and the expression of Melan-A as a melanocyte marker were therefore adopted in order to further confirm the potential status of autophagy in a cohort of 13 primary uveal melanomas with primary cutaneous melanomas of known Beclin-1 and p62 expression included as positive/negative controls (Table 4.1).

Primary Uveal Tumours	Chromosome 3 Status	Melan-A	Beclin-1	P62
M13705/12	Monosomy	++	Variable expression	Variable expression ++/+
M7871/13	Monosomy	++	Weak expression	++
M12068	Monosomy	+	Strong expression	++
M8375	Monosomy	+	Variable expression	Variable expression ++/+
M7214	Monosomy	+	Variable expression	Variable expression ++/+
M1529	Monosomy	+	Variable expression	Weak expression in tumour centre +, strong expression +++ around edges
M11052(A6)	Monosomy	++	Variable expression	Weak expression in tumour centre +, strong expression +++ around edges
M11052(A7)	Monosomy	++	Variable expression	Weak expression in tumour centre +, strong expression +++ around edges
M07885/12	Disomy	+	Weak expression	+++
M9219/12	Disomy	+	Weak expression	+++
M1135	Disomy	No staining	No staining	No staining
M13759	Disomy	No staining	No staining	No staining
M16827	Disomy	+++	Weak expression	Weak expression in tumour centre +, strong expression +++ around edges

Table 4.1 Immunohistochemical Expression Melan-A, Beclin-1 and P62 in Primary Uveal Melanomas

Visual analysis revealed differential expression of Melan-A, Beclin-1 and p62 among all tumours with no expression of any marker detected in 2 primary tumours with disomy for chromosome 3 (Table 4.1). Notably however, p62 expression in 4 tumours was weaker in the centre of the tumour compared with much stronger staining observed at the tumour edge, which was also associated with the maintained expression of beclin-1 (Table 4.1, Figure 4.7). In addition, reduced expression of p62 in the tumour core centre was also associated, in general, with tumours with monosomy of chromosome 3 (Table 4.1, Figure 4.8). Collectively, these data thereby suggest the presence of increased autophagic activity within the hypoxic environment of the tumour core of primary uveal melanomas bearing monosomy of chromosome 3, and association with a more aggressive phenotype. Studies in a larger cohort however, are required to verify these findings, and any correlation with GNAQ/GNA11 mutational status.

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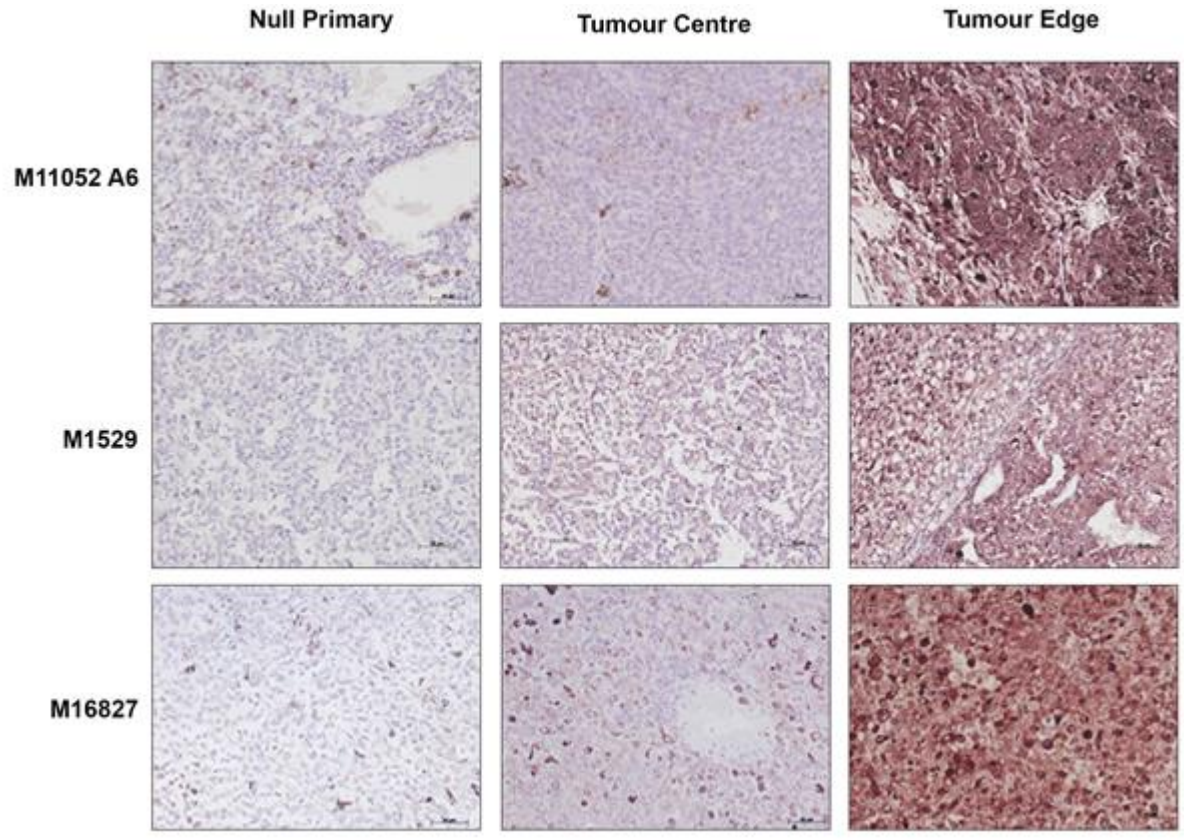

Figure 4.7 Autophagy is Increased within the Hypoxic Core of Metastatic Uveal Melanomas
Representative images for the immunohistochemical expression of p62 (or null primary negative) control in 3 primary uveal melanomas (M11052A6, M1529, M16827). Images represent p62 expression at the tumour edge or centre of the tumour bulk acquired by confocal microscopy with a magnification 20x scale bar = 50µm.

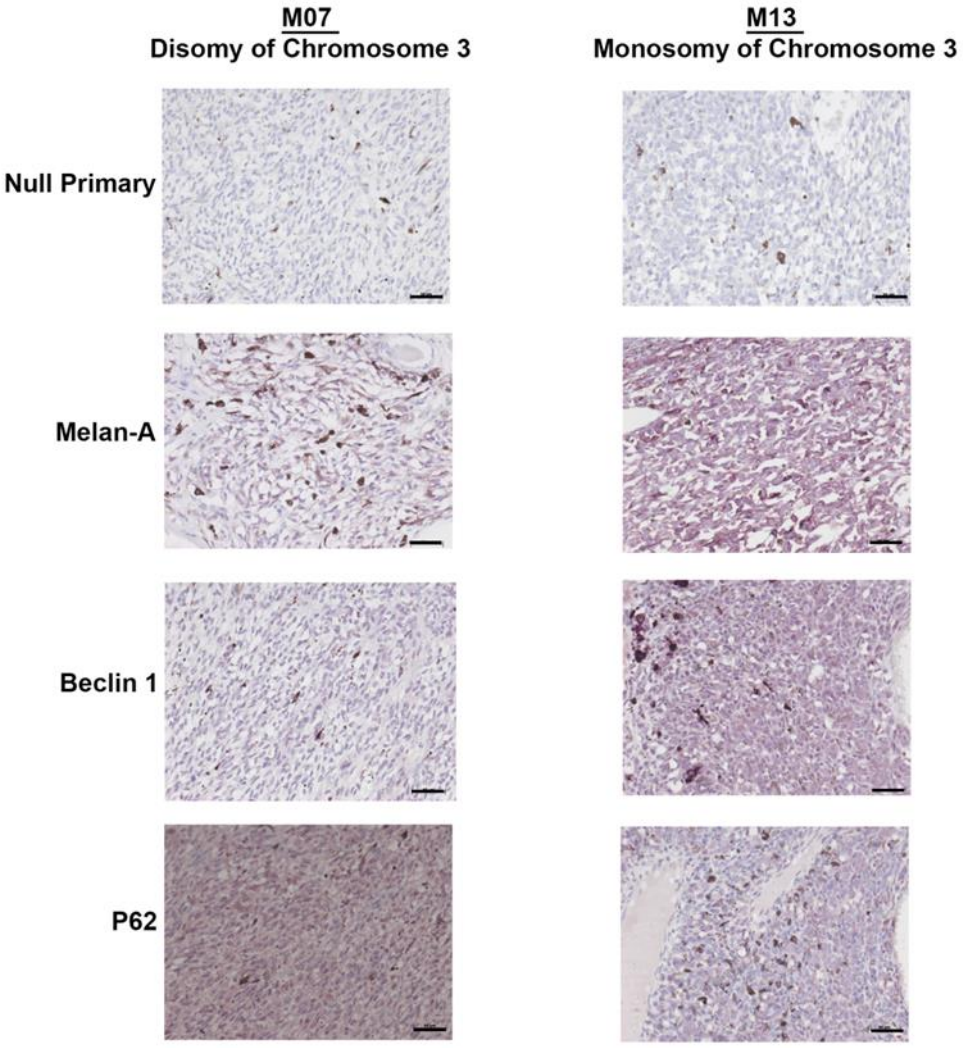

Figure 4.8 Increased Basal Autophagic Activity is Associated with Monosomy of Chromosome 3 in Metastatic Uveal Melanomas.

Representative images for the immunohistochemical expression of for Melan-A, Beclin1 and p62 (or null primary negative control) in two uveal melanomas; M07 with disomy of chromosome 3 and M13 monosomic for chromosome 3. Images were acquired by confocal microscopy with a magnification of 20X, scale bar = 50µm.

4.2.6 Combined Treatment with Trametinib and Chloroquine Potentiates Apoptosis of Uveal Melanoma *in Vitro*

Since studies *in vivo* confirmed an apparent increase in basal autophagy in metastatic uveal melanomas with an aggressive phenotype, western blotting for LC3 conversion in the presence or absence of the lysosomal inhibitor chloroquine was used to establish basal autophagy levels in metastatic uveal melanoma cell lines. Furthermore, it is also well documented that the efficacy of drug-induced apoptosis can be hindered by the capacity of many drugs, including MEK inhibitors, to induce pro-survival autophagy to counter act apoptotic effects (Martin *et al.*, 2015; Yao *et al.*, 2015). The potential for clinically achievable concentrations of trametinib to induce autophagy in uveal melanoma cell lines was also evaluated in comparison to the effects of treatment with the clinically approved chemotherapeutic agent temozolomide. Specifically, OM413 (GNAQ/GNA11 wild-type), MEL270 (GNAQ mutated), or UPMD2 (GNA11 mutated) uveal melanoma cell lines were treated with clinically achievable concentrations of trametinib or temozolomide, for 4 hours in the presence or absence chloroquine prior to western blotting for the expression of total or phosphorylated/active ERK 1/2 or LC3 I-II.

As shown by figure 4.9, trametinib induced the significant inhibition of pERK1/2 relative to total ERK1/2 in all cell lines, while no effect was observed in response to treatment with temozolomide or chloroquine alone (One-way ANOVA with Dunnett's post-hoc test ***P<0.001). However, treatment of all uveal melanoma cell lines resulted in increased basal autophagy as evidenced by the small but nevertheless increased expression of LC3-II (Figure 4.10). Furthermore, the co-treatment of all cell lines with either trametinib or temozomide and chloroquine blocked LC3 flux (Figure 4.10), as evidenced by maintained/increased levels of LC3-II expression. Collectively these data demonstrate trametinib induces potent inhibition of MAPK signalling in uveal melanoma cells, but is accompanied by a concomitant increase in autophagic activity.

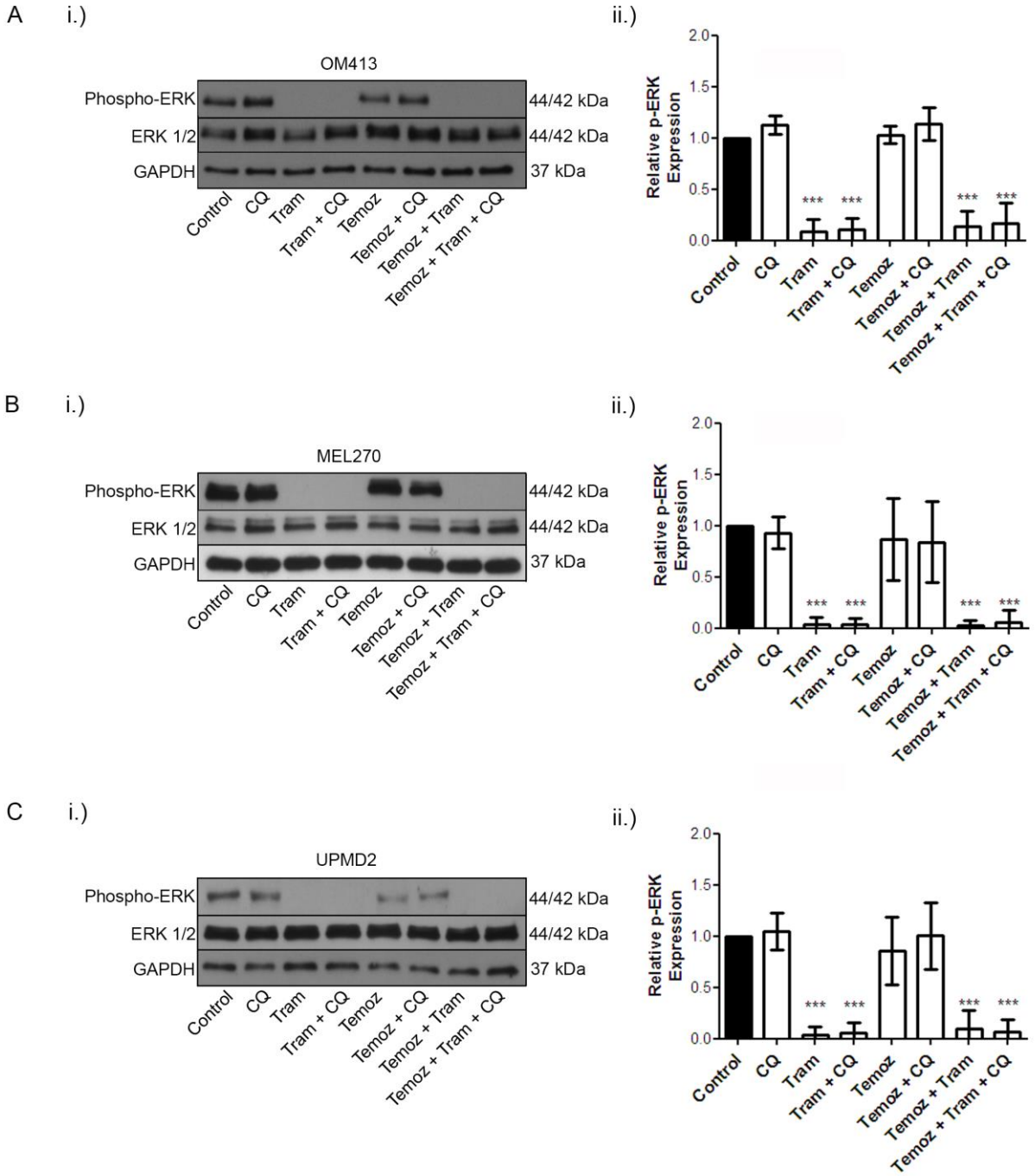

Figure 4.9 The Effect of MEK Inhibition on MAPK Signalling in Human Uveal Melanoma Cell Lines.

*Representative western blots for the expression of phospho- ERK1/2 (p-ERK) 44/42 kDa, total ERK1/2 44/42 kDa, and GAPDH (loading control) 37 kDa expression in Ai.) OM413 (GNAQ/GNA11 wild-type) Bi.) Mel270 (GNAQ mutated) and Ci.) UPMD2 (GNA11 mutated) uveal melanoma cells in the presence or absence or combined treatment for 4 hrs with of trametinib (32nM), temozolomide (50μM), alone or in combination with treatment with chloroquine (CQ, 10μM) for the final 2 hours' incubation. A, B, C ii.) Respective densitometric analysis representing p-ERK expression relative to GAPDH loading control, following the aforementioned treatment conditions. Each bar represents 3 replicates of p-ERK band intensity normalised to GAPDH band intensity (p-ERK/GAPDH) for each treatment condition, and expressed relative to control of each individual experiment (mean ± SD, N = 3). Statistics were acquired using one-way ANOVA with Dunnett's post-hoc test (***P<0.001).*

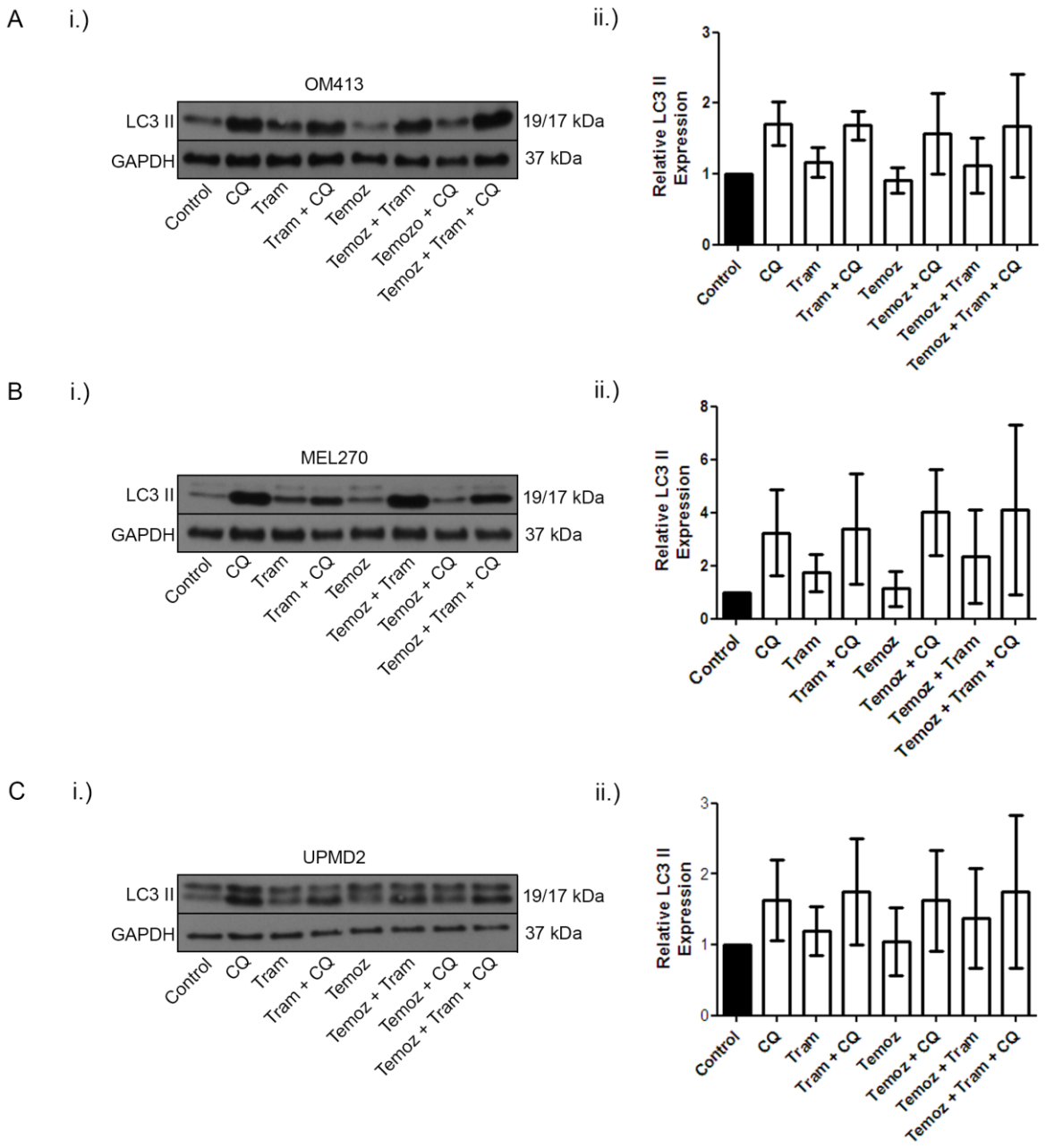

Figure 4.10 The Effect of MEK Inhibition on the Induction of Autophagy in Human Uveal Melanoma Cell Lines.

Representative western blots for the expression of LC3 I/II 19/17 kDa, and GAPHD (loading control) 37 kDa expression in Ai.) OM413 (GNAQ/GNA11 wild-type) Bi.) Mel270 (GNAQ mutated) and Ci.) UPMD2 (GNA11 mutated) uveal melanoma cell lines in the presence or absence or combined treatment for 4 hrs with trametinib (32nM), temozolomide (50μM), alone or in combination with treatment with chloroquine (CQ, 10μM) for the final 2 hours' incubation. A, B, C ii.) Respective densitometric analysis representing LC3 II expression relative to GAPDH loading control. Each bar represents 3 replicates of LC3 II band intensity normalised to GAPDH band intensity (LC3 II/GAPDH) for each treatment condition, and expressed relative to control of each individual experiment (mean ± SD, N = 3).

To test the hypothesis that trametinib-induced LC3 II is indicative of the induction of pro-survival autophagy, OM413, MEL270, and UPMD2 cells were subsequently treated with combined 32nM trametinib and 10μM chloroquine, for 72 hrs prior to analysing the effect on cell viability or apoptosis (as determined by western blotting for caspase 3 cleavage), in comparison with the effects derived by treatment of each cell line with combined temozolomide (50μM) and chloroquine. Results revealed no significant effect on the viability of any uveal melanoma cell line following treatment with chloroquine or temozolomide alone, however treatment of each cell line with trametinib resulted in a significant reduction in cell viability, particularly apparent in MEL270 and UPMD2 cells harbouring hyper-activating mutations in GNAQ/11 compared to effects observed in OM413 wild-type cell (One-way ANOVA with Dunnett's Post hoc correction, *P<0.05, **P<0.01, *** P<0.001, Figure 4.11 A-C i). Furthermore, co treatment of all cell lines with chloroquine and trametinib resulted in a trend for enhanced inhibition of cell viability compared to trametinib treatment alone (Figure 4.11, A-C, i). In contrast any effect of temozolomide on the inhibition of cell viability was only minimally potentiated by co-treatment with chloroquine and was insignificant when compared to control MEL270 and UPMD2 cells (Kruskal-Wallis test with Dunn's Multiple comparison post hoc test, ns P>0.05, Figure 4.11 B-Ci). Supporting these data, results also revealed little or no effect of treatment with temozolomide or chloroquine alone on the induction of cleaved caspase 3, while clear induction was observed in all cell lines under all trametinib treatment conditions (alone or in combination with chloroquine or temozolomide) (Figure 4.11 A-C ii, iii). Interestingly the most significant induction of cleaved caspase 3 was observed in OM413 and MEL270 cells following combined treatment with temozolomide, trametinib and chloroquine suggesting the combined treatment of trametinib and chloroquine may also increase the efficacy of temozolomide-induced apoptosis in some uveal melanomas.

Collectively these data suggest trametinib induces pro-survival autophagy in uveal melanoma cell lines and that the combined treatment with chloroquine may potentiate the efficacy of trametinib to induce apoptosis, thereby presenting a novel therapeutic strategy to enhance targeted MEK inhibition *in vivo.*

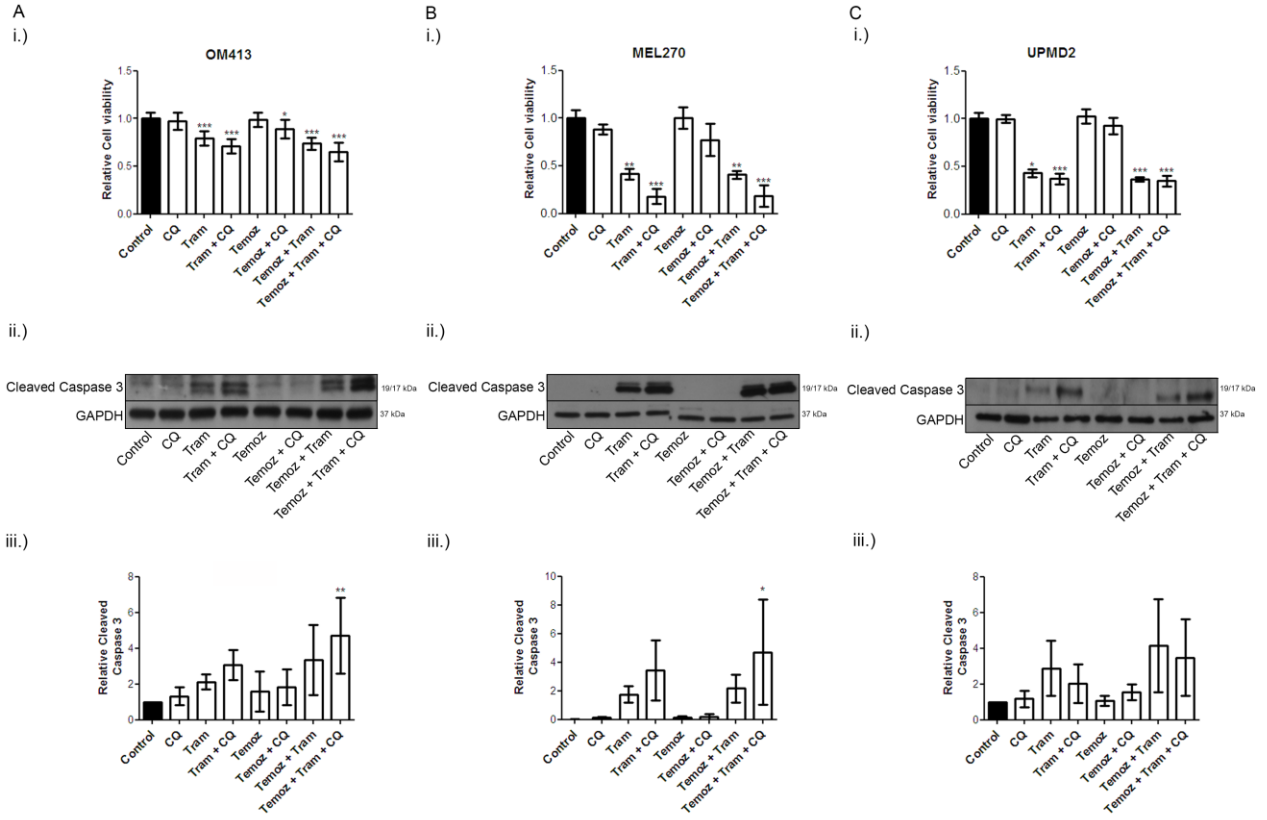

Figure 4.11 The Effect of MEK Inhibition on Survival in Human Uveal Melanoma Cell Lines.

*Relative cell viability of human uveal Ai.) OM413 (GNAQ/GNA11 wild-type), Bi.) Mel270 (GNAQ mutated) and Ci.) UPMD2 (GNA11 mutated) melanoma cell lines treated for 72 hrs with trametinib (32nM), temozolomide (50µM) or vehicle control, in the presence or absence chloroquine (CQ, 10µM). Each bar represents the mean of 12 replicates +/- SD from 3 independent experiments. Statistics acquired by One-way ANOVA with Dunnett's post hoc correction (*P<0.05, **P<0.01, ***P<0.001). Representative western blots for the expression of cleaved caspase 3 17/19 kDa, and GAPHD (loading control) 37 kDa in A ii.) OM413 B ii.) Mel270 and C ii.) UPMD2 uveal melanoma cells under the same experimental conditions or A, B, C iii.) Respective densitometric analysis representing caspase 3 cleavage expression relative to GAPDH loading control. Each bar represents 3 replicates of cleaved caspase 3 band intensity normalised to GAPDH band intensity (cleaved caspase 3/GAPDH) for each treatment condition, and expressed relative to vehicle control of each individual experiment (mean ± SD, N = 3). Statistics were acquired using one-way ANOVA with Dunnett's post-hoc test (*P<0.05, **P<0.01).*

4.2.7 Combined Treatment of Trametinib and Chloroquine Potentiates Apoptosis of Cutaneous Melanoma *In Vitro*

In line with the observed results in uveal melanoma cells (section 4.2.6) and data indicating MEK inhibition with trametinib results in concomitant up regulation of autophagy, similar studies were conducted in cutaneous melanoma cell lines to confirm increased basal autophagy (Armstrong *et al.*, 2011), the potential for trametinib-induced autophagy and to test the hypothesis that dual inhibition of MEK and autophagy may drive the efficacy of trametinib-induced cell death/apoptosis. To this aim, CHL-1 (wild-type), WM-164 (B-RAF mutated) and WM-1361 (N-Ras mutated) cutaneous melanoma cell lines were treated with clinically achievable concentrations of trametinib or temozolomide, for 4 hours in the presence or absence of chloroquine, before the effects on inhibition of MAPK signalling and induction of autophagic flux were determined by western blotting for phospho-ERK1/2 (p-ERK1/2), total ERK1/2 or LC3 I-II (Figures 4.12 and 4.13). As shown by figure 4.12, trametinib induced the significant inhibition of pERK1/2 relative to total ERK1/2 in all cell lines, while no effect was observed in response to treatment with temozolomide or chloroquine alone (One-way ANOVA with Dunnett post-hoc test, ***P<0.001). However, as in studies with uveal melanoma cell lines, trametinib appeared to increase basal autophagy in all cutaneous melanoma cell lines as evidenced by a small increase in expression of LC3-II (Figure 4.13), an effect also observed, albeit to a less extent, by treatment of all cell lines with temozolomide. As predicted, this effect was further potentiated by the blockade of autophagic flux with the addition of chloroquine, resulting in maintained accumulation of LC3-II expression. Collectively these data suggest similarly to observations in uveal melanoma that potent inhibition of MAPK signalling in cutaneous melanoma cells, by treatment with trametinib also results in a concomitant increase in autophagy.

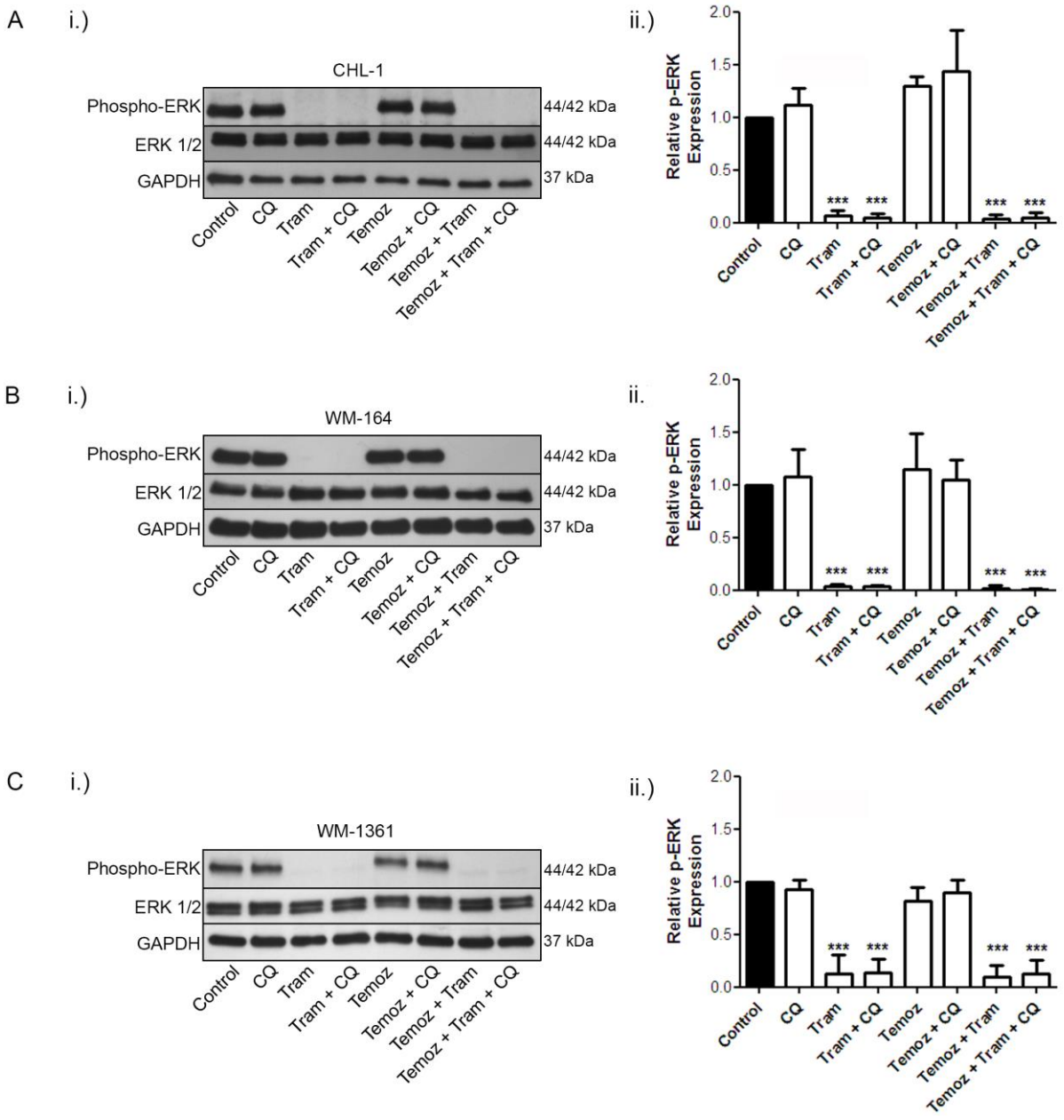

Figure 4.12 The Effect of MEK Inhibition on MAPK Signalling in Human Cutaneous Melanoma Cell Lines.

*Representative western blots for the expression of phospho-ERK1/2 (p-ERK) 44/42 kDa, total ERK1/2 44/42 kDa, and GAPDH (loading control) 37 kDa expression in Ai.) CHL-1(B-RAF/N-Ras wild-type) Bi.) WM-164 (B-RAF mutated) and Ci.) WM-1361 (N-Ras mutated) cutaneous melanoma cell lines in the presence or absence or combined treatment for 4 hrs with of trametinib (32nM), temozolomide (50μM), alone or in combination with treatment with chloroquine (CQ, 10μM) for the final 2 hour's incubation. A, B, C ii.) Densitometric analysis of p-ERK expression relative to GAPDH loading control, following treatments as outlined in A-C. Each bar represents 3 replicates of p-ERK band intensity normalised to GAPDH band intensity (p-ERK/GAPDH) for each treatment condition, and expressed relative to control of each individual experiment (mean ± SD, N = 3). Statistics were acquired using one-way ANOVA with Dunnett's post-hoc test (***P<0.001).*

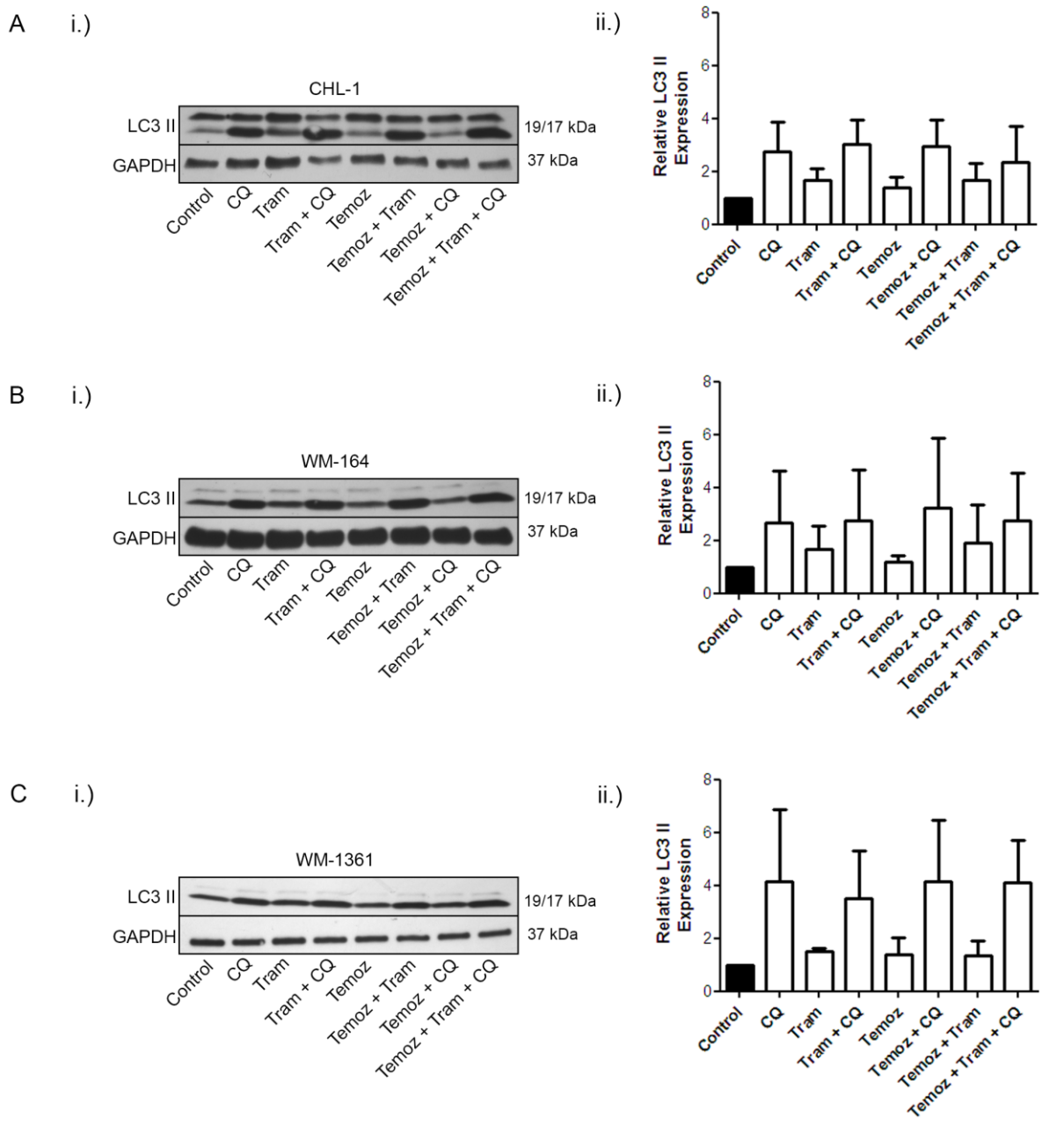

Figure 4.13 The Effect of MEK Inhibition on the Induction of Autophagy in Human Cutaneous Melanoma Cell Lines.

Representative western blots for the expression of LC3 I/II 19/17 kDa, and GAPDH (loading control) 37 kDa expression in Ai.) CHL-1 (B-RAF/N-Ras wild-type) Bi.) WM-164 (B-RAF mutated) and Ci.) WM-1361 (N-Ras mutated) cutaneous melanoma cell lines in the presence or absence or combined treatment for 4 hrs with of trametinib (32nM), temozolomide (50µM), alone or in combination with treatment with chloroquine (CQ 10µM) for the final 2 hours' incubation. A, B, C ii.) Densitometric analysis of LC3 II expression relative to GAPDH loading control. Each bar represents 3 replicates of LC3 II band intensity normalised to GAPDH band intensity (LC3 II/GAPDH) for each treatment condition, and expressed relative to control of each individual experiment (mean ± SD, N= 3).

Similarly, to studies in uveal melanoma cell lines, the effect of MEK inhibition with trametinib on cutaneous melanoma cell survival was then determined in the presence or absence of chloroquine. Results again revealed no significant effect on the inhibition of CHL-1, WM-1361 or WM-164 cell viability in the presence of chloroquine or temozolomide alone, however, trametinib induced a significant reduction in cell viability of WM-164 and WM-1361 cell lines bearing hyper-activating mutations in MAPK signalling compared to CHL-1 B-RAF/N-Ras wild-type cells (Kruskal-Wallis with Dunn's multiple comparison post hoc test, **P<0.01 and ***P<0.001, Figure 4.14 A-Ci). Furthermore, combined treatment of chloroquine and trametinib potentiated the effects of trametinib-induced cell death alone, (Kruskal-Wallis with Dunn's multiple comparison post hoc test, **P<0.01 and ***P<0.001, Figure 4.14 A-Bi), although this effect was not observed in WM-164 cells (Figure 4.14 Ci). Again, although clearly potentiating the effects of trametinib-induced cell death, the effect of chloroquine on the enhancement of temozolomide-induced inhibition of cell viability of all cutaneous melanoma cell lines, although evident, was less potent with only a significant effect seen in WM-164 cells (*P<0.05, Kruskal-Wallis with Dunn's multiple comparison post hoc test, Figure 4.14 Ci).

Supporting the enhanced inhibitory effects of combined treatment with trametinib and chloroquine on cell viability, western blotting for caspase 3 cleavage in WM-164 cells confirmed the mode of cell death as apoptosis as evidenced by the induction of cleaved caspase 3 following treatment of cells for 72 hours with trametinib +/- temozolomide +/- chloroquine treatment, with the greatest induction of apoptosis seen with a combination of all 3 drugs (Figure 4.14 C ii, iii). Collectively these results suggest as in studies in uveal melanoma cell lines, that trametinib treatment results in the induction of pro-survival autophagy to counter act apoptotic effects thereby providing further rationale for the dual inhibition of MEK and autophagy as a therapeutic strategy for cutaneous as well as metastatic uveal melanoma.

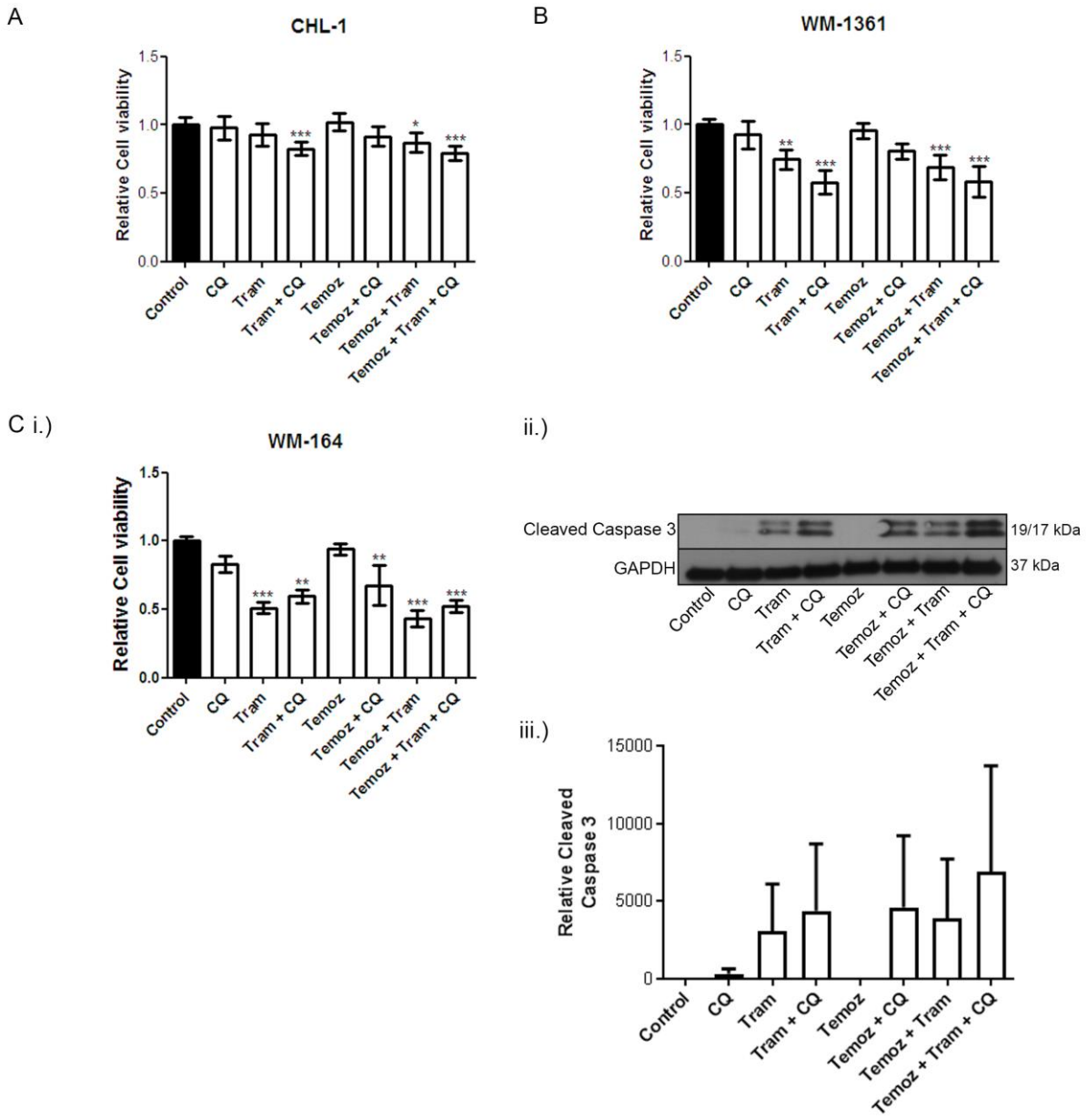

Figure 4.14 The Effect of MEK Inhibition on Survival in Human Cutaneous Melanoma Cell Lines.

*Relative cell viability of human cutaneous melanoma cells A.) CHL-1 (B-RAF/N-Ras wild-type) B.) WM-164 (B-RAF mutated) and C.) WM-1361 (N-Ras mutated) treated in the presence trametinib (32nM) or temozolomide (50μM), or vehicle control in the presence or absence chloroquine (CQ, 10μM) for 72 hours. Each bar represents the mean of a minimum of 12 replicates +/- SD from 3 independent experiments. Statistics acquired by One way ANOVA with Dunnetts post hoc correction (*P<0.05, **P<0.01,***P<0.001 C.ii) Representative western blots and iii) densitometric analysis for the expression of cleaved caspase-3 17/19 kDa, and GAPDH (loading control) 37 kDa, expression in WM-164 (B-RAF mutated) cutaneous melanoma cells under the same experimental conditions where each bar represents 3 replicates of cleaved caspase 3 band intensity normalised to GAPDH band intensity (cleaved caspase 3/GAPDH) for each treatment condition, and expressed relative to vehicle control of each individual experiment (mean ± SD, N = 3).*

4.3 Discussion

4.3.1 MEK Inhibition as a Strategy to Inhibit of CXCR4-CXCL12 Chemotaxis in Cutaneous and Uveal Melanoma

Results derived from this chapter reiterate the important role of CXCR4-CXCL12 cell signalling in melanoma cell migration and its possible role within the primary tumour microenvironment. Studies demonstrating the significant migration of CXCR4 positive cutaneous and uveal metastatic melanoma cells towards recombinant CXCL12, with reduced cell migration to an unstimulated level observed by inclusion of a neutralising CXCL12 antibody, underpin the potency of CXCL12 as a chemoattractant for melanoma cells. Although cutaneous melanoma cell migration towards recombinant CXCL12 has been explored comprehensively (O'Boyle *et al.*, 2013), the role of CXCR4-CXCL12 chemotaxis in uveal melanoma is an emerging topic of investigation in which the present results corroborate two previous studies reporting uveal melanoma migration towards recombinant CXCL12. However, unlike results derived from the present study, neither of these published studies were able to confirm migration was specifically mediated by CXCR4-CXCL12 chemotaxis, since in both cases the use of a neutralising CXCL12 blocking antibody was not included (Di Cesare *et al.*, 2007a; Chattopadhyay *et al.*, 2014).

CXCL12 in humans exists in 6 isoforms α, β, γ, ε, δ, and ϕ, generated from differential gene splicing events. Data from the present study report the effects of chemotaxis towards alpha recombinant CXCL12 ligand (CXCL12-α) and therefore can only be considered as CXCL12-α specific. Nevertheless, CXCL12-α and β are thought to be ubiquitously expressed, including within the skin (Cipriani *et al.*, 2006), while the -γ isoform is highly expressed within the brain and heart but only has low affinity for CXCR4. Other isoforms of CXCL12 are somewhat uncharacterised (Rueda *et al.*, 2008). Furthermore, it is likely that functional differences between differing CXCL12 isoforms exist, supported by studies demonstrating CXCL12-β but not CXCL12-α is able to modulate leukocyte infiltration in brain ischemia (Stumm *et al.*, 2002). Collectively these data further emphasise that the reported findings in the present study cannot be extrapolated to all CXCL12 isoforms and that future studies are required to specifically determine the potential contribution of other isoforms of CXCL12, and in particular CXCL12-β, to CXCR4-CXCL12 mediated chemotaxis of uveal or cutaneous melanoma.

The effect of MEK inhibition on uveal melanoma cell migration has also previously been explored, however, in contrast to results derived from the present study, results derived from a recent study by Chattopadhyay *et al* demonstrated MEK inhibition with MK-8033 had no effect on cell migration of wild-type or GNAQ/11 mutated cell lines, (Chattopadhyay *et al.*, 2014). Such discrepancy in results may reflect however, the lack of chemoattractant stimulus in the migration assay studies by Chattopadhyay *et al*, therefore only able to measure basal unstimulated migration, with cells presumably displaying lower levels of MAPK activation in the absence of a CXCL12 chemoattractant stimulus. In this context, results from the present study are the first to demonstrate the potential of MEK inhibition as a means to specifically prevent CXCR4-CXCL12 mediated chemotaxis in melanoma and the rationale for the use of trametinib to prevent CXCR4-CXCL12 mediated chemotaxis even in B-RAF/N-Ras or GNAQ/11 wild-type cutaneous or uveal metastatic melanomas. Since patients harbouring B-RAF/N-Ras wild-type cutaneous melanomas account for up to 26 % of all melanoma patients, and there are still no specific targeted therapies for this sub group, resulting in continued dismal survival rates (Dummer *et al.*, 2012; Hodis *et al.*, 2012a), the use of MEK specific inhibitors may therefore offer a novel targeted treatment approach. The confirmation of CXCR4 expression may be a companion predictive biomarker; an approach supported by results from the present and other studies demonstrating B-RAF/N-Ras wild-type cutaneous melanoma cell lines are as sensitive to MEK inhibition as mutated cell lines (Stones *et al.*, 2013; Ranzani *et al.*, 2015), and clinical trials demonstrating partial responses to trametinib in patients with B-RAF/N-Ras wild-type advanced melanomas (Falchook *et al.*, 2012).

Early stage melanomas classified as in radial growth phase have good prognosis following surgical excision, however melanomas that have transitioned to vertical growth phase with the presence of invasion into the dermis, display a much worse prognosis. The mechanism that promotes the transition from radial to vertical growth phase is unclear but is thought to involve the bidirectional interplay of tumour and stromal cells within the microenvironment (Villanueva J and Herlyn M, 2008). Supporting this hypothesis, results derived from studies in chapter 3, highlight the influence of epidermal CXCL12 expression on tumour progression. An interesting study by Whipple *et al* demonstrated cytokines secreted from melanoma cells (IL-1*β*, IL-6 and IL-8, and MMP-1) upregulate the secretion of CXCL12 from dermal fibroblasts facilitating tumorigenic behaviour (Whipple and Brinckerhoff, 2014). Having demonstrated the potent secretion of CXCL12-α from primary dermal fibroblasts, it was therefore

hypothesised that this may provide the chemoattractant gradient to facilitate the invasion and migration of tumour cells. In support of this concept, the present chemotaxis studies of both B-RAF/N-Ras mutated and wild-type metastatic cutaneous melanoma cell lines demonstrated significant tumour migration towards supernatants derived from primary dermal fibroblasts, and that CXCL12 within these supernatants specifically facilitates this migration. Furthermore, data from the present study demonstrate CXCR4-CXCL12 mediated chemotaxis of cutaneous melanoma cells (B-RAF/N-Ras wild-type or mutant) towards dermal fibroblast supernatant is inhibitable by treatment with trametinib, suggesting MEK inhibition may present a viable strategy through which inhibits vertical growth phase migration and cutaneous invasion. Nevertheless, it should be noted that the level trametinib inhibited chemotaxis of melanoma cells towards dermal fibroblast supernatant was less than the level of inhibition achieved towards rCXCL12, suggesting that although CXCL12 is clearly a chemoattractant secreted by dermal fibroblasts the concentration of CXCL12 in the supernatants is likely less than 10nM used in experiments employing recombinant CXCL12, or that other factors secreted by dermal fibroblasts may counteract the inhibitory effects of trametinib on chemotaxis. In this respect, the validation of results derived *in vitro,* should be further explored in an *in vivo* setting, since there is potential for CXCL12 to be attained within the primary melanoma microenvironment from sources other than dermal fibroblasts, with endothelial cells, immune cells (monocytic phagocytes) to name but a few potential sources, as well as CXCL12 within the circulation or bone marrow, likely to influence the CXCR4-CXCL12 chemotactic gradient at the primary tumour site (Sun *et al.*, 2010; Sánchez-Martín *et al.*, 2011a). Although dermal fibroblasts are likely a major secretor of CXCL12 within skin *in vivo,* extrapolation of *in vitro* results to a primary tumour setting are further complicated by the multitude of factors that may affect tumour cell migration, including the secretion of CXCL12 by the tumour itself, modulation of CXCL12 levels by CXCR7-mediated sequestration, CXCL12 cleavage by metalloproteinase, or the presence of other chemoattractant cytokines which may have a greater effect on cell migration. In addition, chemotaxis experiments in the present study were performed over a relatively short time period of 16 hours, in a controlled experimental setting, whereas *in vivo*, chronic chemokine stimulation and fluctuations in chemotactic gradients from several potential sources are likely to influence CXCR4-CXCL12 chemotactic responses. Moreover, although the transwell chemotaxis assay adopted in the present study is the most commonly used assay to assess cell migration *in vitro*, and allows great flexibility in the screening of multiple experimental conditions, it is nevertheless a 2D endpoint assay and as such more

complex assays may reveal greater insight. Such assays could include the use of the ACEA biosciences xCELLigence system, that integrates a traditional boyden chamber with electrodes, to provide a real-time assay of chemotaxis and cell kinetics, or the Ibidi µ Slide Chemotaxis assay, where chemotaxis can be observed using time lapse microscopy, allowing both efficiency of cell migration and directionality to be assessed. Limitations in the use of such assays still remain however, since neither mimics *in vivo* cell chemotaxis, as cells would have to migrate through the skins extracellular matrix. In this context the incorporation of a matrix (collagen/fibronectin) into 2D assays may be pertinent or the use of 3D chemotaxis assays, such as a spheroid migration assay, to assess single cell or collective cell movement in a physiologically relevant environment, with flexibility to incorporate other cell types into the assay and the assessment of the effect of cell-cell interactions on cell migration (Kramer *et al.*, 2013; Leight *et al.*, 2015).

4.3.2 Harnessing Autophagy Modulation to Promote the Cytotoxic Effects of MEK Inhibition

Although present results demonstrated effective dose-dependent inhibition of CXCR4-CXCL12 chemotaxis in B-RAF/N-Ras and GNAQ/11 mutated as well as wild-type cutaneous/uveal melanoma cells by trametinib, the effect of trametinib on the inhibition of cell viability, particularly in wild-type cell lines, was modest. Given the fact that many chemotherapeutic drugs activate autophagy as a compensatory survival mechanism to counteract pro-apoptotic signals (Wright *et al.*, 2013) and the inevitable emerging resistance of melanoma to MEK inhibition (Welsh *et al.*, 2016), this raises the possibility that the presence of a B-RAF or GNAQ/GNA11 activating mutations and/or treatment with trametinib leads to increased activation of pro-survival autophagy. Supporting previous studies, have highlighted the presence of increased autophagy in advanced cutaneous melanomas (Ellis *et al.*, 2014b), as well as a single study in primary uveal melanomas where increased autophagy was associated with earlier metastasis and worse prognosis (Giatromanolaki *et al.*, 2011). Results from this present study confirmed increased autophagic activity in primary uveal melanomas with an associated metastatic phenotype as evidenced in general, by the maintained presence of Beclin-1 and decreased p62 expression within the hypoxic core of poorly vascularised tumours with monosomy of chromosome 3 (Degenhardt K *et al.*, 2006; Giatromanolaki *et al.*, 2011). Interestingly, Giatromanolaki *et al* (Giatromanolaki *et al.*, 2011) found that depleted/reduced expression of Beclin-1 in primary uveal melanomas was associated with a better prognosis,

which may partially explain present observations in 2 uveal melanoma tumours with disomy of chromosome 3, which did not express any marker of autophagy including Beclin-1 and suggests that these tumours may display defective autophagy. Together these data thus highlight the potential for increased basal autophagy in advanced cutaneous or uveal melanoma (*in vivo*) regardless of the B-RAF or GNAQ/GNA11 mutational status.

Studies of basal autophagy in metastatic melanoma cell lines confirmed previous observations of increased basal LC3 II expression in cutaneous B-RAF mutant compared to B-RAF/N-Ras wild-type cells (Armstrong *et al.*, 2011), additionally revealing increased basal LC3 II expression in GNAQ/GNA11 mutant metastatic uveal melanoma cell lines as well as GNAQ/GNA11 wild-type OM413 cells, although in the latter case this was not surprising since OM413 cells bear an activating B-RAF mutation (Refaian *et al.*, 2015). Collectively therefore, increased basal autophagy in cutaneous and uveal melanoma appears to be associated with a metastatic phenotype with, in particular, the additional presence of an activating B-RAF or GNAQ/GNA11 mutation, which may explain in part, the inherent relative resistance of such tumours to the cytotoxic effects of trametinib. Results demonstrating further induction of LC3 II by trametinib in both cutaneous and uveal melanoma cells further supported the hypothesis of additionally increased drug-induced pro-survival autophagy and led to the question of whether dual inhibition of MEK and autophagy may offer a potential means through which to increase the efficacy and cytotoxic effects of trametinib in both uveal and cutaneous melanomas. Indeed, results demonstrated the co-treatment of all uveal melanoma, and cutaneous melanoma cell lines CHL-1 and WM-1361 with trametinib and chloroquine, led to increased inhibition of cell viability as well as increased expression of cleaved caspase 3, compared to cells treated with trametinib alone, collectively suggesting inhibiting basal/drug-induced autophagy with chloroquine enhances the pro-apoptotic effects of trametinib. Interestingly this effect was not B-RAF/N-Ras or GNAQ/11 mutation dependant as the response of cutaneous/uveal wild-type CHL-1 and OM413 cells to dual MEK and autophagy inhibition also resulted in increased apoptosis.

Hydroxychloroquine (HCQ, the clinically used formulation of Chloroquine), has been shown to improve the efficacy of many anti-cancer agents and in pre-clinical models augments cell death (Amaravadi *et al.*, 2007; Degtyarev *et al.*, 2008; Maclean *et al.*, 2008; Qadir *et al.*, 2008; Carew *et al.*, 2010; Fan and Weiss, 2011; Guo *et al.*, 2011b; Tang *et al.*, 2011; Yang *et al.*, 2011; Xie *et al.*, 2013). Furthermore Phase I studies of cutaneous metastatic melanoma (and

including other solid tumours) demonstrate in addition to its favourable safety profile, HCQ combined with temsirolimus or temozolomide, results in favourable anti-tumour activity and reduced autophagic activity (Rangwala *et al.*, 2014a; Rangwala *et al.*, 2014b). Coupled with the current use of HCQ in over 50 trials for various cancer types, including 4 specifically in melanoma (ClinicalTrials.Gov), and an open phase I/II trial of HCQ and trametinib for advanced B-RAF melanoma (NCT02257424), results from the present study may also support the use of HCQ to increase the efficacy and cytotoxic effects of MEK inhibition as a therapeutic strategy for metastatic uveal melanoma. In this context, it will be important to evaluate the efficacy of trametinib and HCQ in pre-clinical models of uveal melanoma and to address this potential I have successfully obtained additional supplementary funding from the MRC to initiate these studies in collaboration with Professor Marais, Cancer Research UK.

Nevertheless, although autophagy inhibition by HCQ at high micro-molar concentrations has a favourable safety profile, it is not a specific autophagy inhibitor but rather an inhibitor of lysosomal function, specifically blocking autophagosome lysosome fusion by increasing lysosomal PH and leading to cell cycle arrest. The use of more specific autophagy inhibitors such as SBI-0206965, a selective inhibitor of the serine/threonine autophagy-initiating kinase ULK1, or Spautin-1 that promotes the degradation of the Vps34-PI3 kinase complex, maybe thus a more beneficial approach to autophagy inhibition (Solomon and Lee, 2009; Jiang et al., 2010). An additional caveat to the use of such selective inhibitors or HCQ is their capacity to inhibit autophagy not only in tumour cells but also their potential to inhibit autophagy in normal cells where autophagy plays a homeostatic role. The kidneys are particularly sensitive to chemotherapy, and recent studies have shown autophagy to be protective of acute renal injury in response to cisplatin treatment (Takahashi *et al.*, 2012). Further, chronic use of HCQ has been linked with increased risk of retinopathies (Leung *et al.*, 2015). Moreover, the inhibition of autophagy maybe detrimental to immune responses, as autophagy promotes T cell survival (Kovacs *et al.*, 2012), dendritic cell MHC class II antigen presentation (Hayward and Dinesh-Kumar, 2010) and is a regulator of dendritic cell and T-cell immunological synapse formation important for adaptive immune responses (Wildenberg *et al.*, 2012). Additionally inhibition of autophagy may also affect immunogenic cell death mechanisms induced by chemotherapeutics, as autophagy is required for the immunogenic release of ATP from dying cancer cells, essential for immune effector cell recruitment to tumours (Michaud *et al.*, 2011; Martins *et al.*, 2012). With most pre-clinical models of autophagy inhibition performed in

xenograft models of immunocompromised animals, the effect of autophagy inhibition on the immune system has also not been fully considered.

Further, although inhibiting drug-induced autophagy or activated autophagy in advanced tumours seems attractive, autophagy nevertheless plays a paradoxical role in tumourigenesis; blocked or deregulated autophagy in early stages promotes genomic instability driving tumorigenesis while reactivation in response to increased metabolic stress in advanced tumours promotes tumour survival (Mathew *et al.*, 2009; White *et al.*, 2010). Inhibition of autophagy in advanced melanomas in this context may promote the emergence of secondary tumour development and hence driving autophagy exacerbation may be a more viable approach, further discussed below.

The role of autophagy in cell migration is controversial and remains relatively undefined. Three studies have indicated that autophagy inhibition (mediated by knock down of autophagy dependant regulatory Atg genes) increases cell migration of mouse embryonic fibroblasts (Tuloup-Minguez *et al.*, 2013; Qiang *et al.*, 2014; Yoshida *et al.*, 2016). Supporting these observations overexpression beclin-1 in tongue squamous cell carcinoma also inhibits cell migration (Weng *et al.*, 2014). Conversely however, one study in lung cancer demonstrated chemical inhibition of autophagy with 3-MA or knock down of atg5 inhibited TLR dependent migration suggesting TLR-induced autophagy is required for cancer cell migration (Zhan et al., 2014). Although controversial, these studies thus pose a further interesting caveat to results derived from the present study in that although dual MEK and autophagy inhibition promoted cutaneous and uveal melanoma cell death, the inhibition of autophagy in MEK resistant subpopulations may perhaps increase tumour migration and subsequent metastasis. It would therefore be interesting to incorporate trametinib and chloroquine into chemotaxis assays to assess the potential effect of autophagy inhibition on cell migration.

Taken on balance, although autophagy inhibition may potentiate the cytotoxic effects of trametinib, the possible role of autophagy inhibition in secondary tumour development, and implications on immune function and cell migration, may question whether in this context exacerbating autophagy induced by trametinib with drugs that use autophagy to exert their cytotoxic effects to promote autopahgic cell death may be more applicable. Metformin, terfenadine and Δ9-Tetrahydrocannabinol (THC) have demonstrated in melanoma to drive cell

death by induction of autophagy (Nicolau-Galmés *et al.*, 2011; Tomic *et al.*, 2011; Armstrong *et al.*, 2015). Interestingly in uveal melanoma Ambrosini *et al* also demonstrated the use of the MEK inhibitor selumetinib (AZD6244) combined with AKT inhibition (MK2206) induced synergistic cell death via the exacerbation of autophagy (Ambrosini *et al.*, 2013a). Therefore, strategies which alternatively harness autophagy induction by trametinib to promote apoptosis would be an interesting line of further investigation to pursue in uveal and cutaneous melanoma.

Collectively results derived from the present chapter highlight the ability of MEK inhibition with trametinib to prevent cutaneous/uveal CXCR4-CXCL12 chemotaxis, but reveal the induction of compensatory mechanisms in that trametinib-induced autophagy may limit the cytotoxic efficacy of this drug. Dual inhibition of MEK and autophagy may represent a novel therapeutic strategy through which to limit CXCR4-CXCL12 mediated chemotaxis as well as potentiate apoptosis of uveal and cutaneous melanomas.

4.4 Summary

- CXCR4 expressing cutaneous and uveal metastatic melanoma cells migrate towards human recombinant CXCL12 *in vitro*
- CXCR4 expressing cutaneous metastatic melanoma cells migrate towards CXCL12 rich supernatants derived from primary dermal fibroblasts *in vitro*
- MEK inhibition with trametinib inhibits CXCR4-CXCL12 chemotaxis of cutaneous and uveal melanoma cells towards human recombinant CXCL12 *in vitro*
- MEK inhibition with trametinib prevents the chemotaxis of cutaneous metastatic melanoma cells migration towards CXCL12 rich dermal fibroblast supernatant
- Increased autophagy in primary uveal melanomas *in vivo* is associated with an aggressive metastatic phenotype and monosomy of chromosome 3
- Increased basal autophagy in metastatic uveal or cutaneous melanoma cell lines *in vitro* is associated with the presence of an activating B-RAF or GNAQ/GNA11 mutation
- MEK inhibition with trametinib in uveal or cutaneous metastatic melanoma cell lines is associated with an increase in basal autophagy
- Concomitant inhibition of MEK (with trametinib) and autophagy (with chloroquine) increases apoptosis of uveal and cutaneous melanoma cell lines

Chapter 5

Crosstalk between VEGFR2 Cell Signalling and CXCR4-CXCL12 Chemotaxis in Cutaneous and Uveal Melanoma

Chapter 5 Crosstalk between VEGFR2 Cell Signalling and CXCR4-CXCL12 Chemotaxis in Cutaneous and Uveal Melanoma

5.1 Introduction

Angiogenesis and lymphangiogenesis are hallmarks of cancer, where the 'angiogenic switch' represents a rate limiting step in carcinogenesis (Hanahan and Folkman, 1996). The angiogenic switch is controlled by the balance between pro- and anti-angiogenic factors, expressed by tumour cells or within the tumour microenvironment. Of most importance in cancer is the principal pro-angiogenic factor, vascular endothelial growth factor A (VEGF-A) that binds to vascular endothelial growth factor receptor 2 (VEGFR2) on endothelial cells, inducing vascular permeability, disruption of adherent junctions, cell proliferation and migration required for new blood vessel formation. Lymphangiogenesis the formation of new lymphatic vessels, on the other hand is regulated by VEGF-C and VEGF-D that bind to VEGFR3 of lymphatic endothelial cells. However, proteolytic cleavage of VEGF-C, and VEGF-D can result in their enhanced binding to VEGFR2 on both lymphatic and blood vessel endothelium, making VEGFR2 an attractive target for both anti-angiogenesis and anti-lymphangiogenesis therapy (Hong *et al.*, 2004a; Hirakawa *et al.*, 2007).

Cutaneous melanoma is one of the most highly vascularised tumours, with tumour vascularity often surpassing Breslow depth as a determinant of patient survival (Ilmonen S *et al.*, 1999; Kashani-Sabet *et al.*, 2002). VEGF although absent from normal melanocytes, is often secreted by melanoma cells, correlating with angiogenesis and metastatic potential in tumour xenografts (Pötgens *et al.*, 1995; Claffey *et al.*, 1996). In cutaneous melanoma, increased VEGF expression has been associated with the transition from radial to vertical growth phase, and melanoma metastasis (Marcoval *et al.*, 1997; Vlaykova T *et al.*, 1999; Osella-Abate *et al.*, 2002). Similarly in uveal melanoma, increased aqueous concentrations of VEGF-A or tumoural expression is associated with larger tumours and correlated with distant metastasis particularly to the liver (Missotten *et al.*, 2006; Sahin *et al.*, 2007; Crosby *et al.*, 2011b). Although VEGF secretion from melanomas is thought to signal in a paracrine manner affecting local endothelial cells, identification of VEGF receptors on half of all melanoma cell lines, suggests that VEGF may also exert autocrine effects on tumour cells (Molhoek *et al.*, 2008). The presence of VEGF-VEGFR autocrine cell signalling loops has been demonstrated in many solid tumours and haematological malignancies, with disruption of the autocrine loop, reducing tumour growth, survival and migration (Kim *et al.*, 2005; Schoeffner *et al.*, 2005; Wey *et al.*, 2005; O'Donnell *et al.*, 2016). Autocrine VEGF cell signalling is particularly favourable to

cancer cells, as in some circumstances it may promote epithelial-mesenchymal transition leading to enhanced tumour invasion and survival (Goel and Mercurio, 2013). A probable reason for this phenotypic change is that VEGF receptors activate an abundance of downstream cell signalling pathways including phospholipase C, the MAP-kinase and phosphoinositide 3–Kinase/Akt, that results in calcium release, cell proliferation, migration and survival (Xia *et al.*; Wang *et al.*, 2008c). The expression of VEGF receptors has also been investigated in cutaneous melanomas, with evidence of all 3 receptors present to varying degrees in primary disease, however, VEGFR2 and VEGFR3 are thought to be most abundant, with VEGFR3 promoting tumour-associated lymphangiogenesis and VEGFR2 associated with angiogenesis and promotion of cell survival and migration (Mandriota *et al.*, 2001a; Straume and Akslen, 2001; Pisacane and Risio, 2005; Mehnert *et al.*, 2010). Specific studies of VEGFR2 in cutaneous melanomas reported VEGFR2 expression in 78%-89% of metastatic tumours, compared to expression in only 9% of primary melanocytic naevi, highlighting the potential therapeutic benefit of VEGF receptor targeted therapy (Mehnert *et al.*, 2010). Conversely, conflicting studies report, absence of VEGFR2 expression or only very low expression present in a mere 7% of metastatic melanomas (Molhoek *et al.*, 2011; Miettinen *et al.*, 2012). Such variable VEGFR2 expression may be attributable to the use of antibodies with differing epitope specificity, but caution the rationale for VEGFR2 specific targeted therapy. In uveal melanoma while, some VEGFR2 expression has been detected on uveal melanoma cell lines (Koch *et al.*, 2014a), there are to date, no documented studies of expression by primary uveal melanoma tumours.

Despite the assumed benefits of inhibiting angiogenesis in melanoma, no anti-angiogenic therapy has improved significantly overall patient survival, or has been approved for use in metastatic melanoma. With inconsistent data for VEGF receptor expression, the recent clinical approach to inhibiting angiogenesis in melanoma has been focussed towards inhibiting the ligand, with Bevacizumab (Avastin), a humanised monoclonal antibody against VEGF, preventing the interaction of VEGF ligand with cognate VEGF receptors. Bevacizumab has the additional potential to inhibit both paracrine and autocrine VEGF signalling in cutaneous melanoma. As a single agent, however, bevacizumab has displayed disappointing clinical activity in cutaneous melanoma (Varker *et al.*, 2007a), although in combination with other chemotherapies has been shown to improve the clinical outcome for various other cancers including breast and colon cancer (Miller *et al.*, 2007; Saltz *et al.*, 2008). In addition the clinical

efficacy of bevacizumab may limited, by inability to inhibit the interaction of VEGF with neuropilin receptors, known to promote growth, survival, invasion and tumour migration of cancer cells (Fukasawa M *et al.*, 2007). Nevertheless encouraging results with bevacizumab have been derived in pre-clinical models of uveal melanoma, where intraperitoneal injection of bevacizumab in an orthotopic model supressed primary tumour growth and the development of hepatic metastasis, suggesting this anti-angiogenic therapy may offer clinical benefit in uveal melanoma (Yang *et al.*, 2010). Over all, bevacizumab has had no effect on improved overall patient survival for melanoma (Corrie *et al.*, 2014), and hence clinical approaches to anti-angiogenic therapy have more recently turned to new generation tyrosine kinase inhibitors, with broader antiangiogenic profiles, able to target multiple signalling pathways and which through inhibition of intracrine VEGF signalling may offer improved anti-angiogenic therapy.

Pazopanib currently approved for treatment of advanced soft tissue sarcoma and advanced renal cell carcinoma (Sonpavde G and Hutson TE, 2007), is a multi-target tyrosine kinase inhibitor of vascular endothelial growth factor receptor-1, 2, and 3, platelet-derived growth factor alpha and beta, and cytokine receptor inhibitor (c-kit). The additional activity against c-kit may be of particular benefit, as up to 28% of melanomas harbour activating mutations or amplification of the receptor tyrosine kinase KIT (Becker *et al.*, 2007). Initial phase I trials have noted partial responses and disease stabilisation in advanced solid tumours including melanoma (Hurwitz *et al.*, 2009; Tan *et al.*, 2010; Burris *et al.*, 2012), and an interim analysis of a phase II trial demonstrated encouraging disease control rates of 80% with some complete and partial responses reported with combined use of pazopanib and paclitaxel as a first line therapy in unresectable melanoma (Fruehauf JP, 2012).

Cell migration is an important pre-requisite for angiogenesis and metastasis, highlighting possible crosstalk between CXCR4-CXCL12 and VEGF cell signalling in promotion of tumour progression. The crosstalk between these 2 important pathways has been eloquently demonstrated, in colon cancer where CXCR4-CXCL12 pathway activation induces VEGF production, an effect abrogated by the use of a CXCR4 inhibitor (Ottaiano *et al.*, 2006). In addition, breast cancer, osteosarcoma, glioma as well as renal cell carcinoma, VEGF signalling has been shown to upregulate CXCR4 expression, resulting in increased cell migration (Bachelder *et al.*, 2002; Zagzag *et al.*, 2005; Hong *et al.*, 2006; Oda *et al.*, 2006). Furthermore,

a correlation between CXCR4 and VEGF expression has recently been reported in a cohort of 53 patients with uveal melanoma (Franco *et al.*, 2010). CXCR4 and VEGFR2 expression as well as CXCL12 and VEGF secretion by tumour and endothelial cells, may be promoted by hypoxia-induced activation of the common regulator hypoxia inducible factor-1 (HIF-1), highlighting further potential crosstalk between VEGF and CXCR4-CXCL12 signalling (Ceradini *et al.*, 2004; Schutyser *et al.*, 2007; Adamcic *et al.*, 2012; Hu *et al.*, 2016). In addition recent studies of bevacizumab in a lung cancer model report the promotion of tumoural secretion of CXCL12 and the recruitment of CXCR4 positive fibrocyte cells, as a mechanism mediating drug resistance (Mitsuhashi *et al.*, 2015). Taken together these studies suggest that inhibition of both CXCR4-CXCL12 and VEGF-VEGFR2 signalling may be beneficial to prevent overlapping downstream migratory pathway activation but also resistance to anti-angiogenic therapy. Results from the previous chapter (4), demonstrated MEK inhibition provides a potential therapeutic strategy through which to prevent CXCR4-CXCL12 chemotaxis of melanoma cells. Given the know role of VEGF-VEGFR2 cell signalling in also mediating cell migration, dual inhibition of MEK and VEGFR2 cell signalling may hence represent a more potent means through which to prevent tumour migration and metastasis. In support of this hypothesis, dual inhibition of RAF (upstream of MEK) and VEGFR2 in a pre-clinical model of pancreatic cancer has been shown to result in reduced tumour growth and metastasis (Lang *et al.*, 2008). Further, the combination of a VEGFR2 inhibitor (cediranib) and a B-RAF inhibitor (PLX4720) has also been shown to promote the induction of apoptosis in melanoma cell lines and promote tumour regression in xenograft models, with the combination of these agents yielding greater effects than either drug alone (Friedman *et al.*, 2015).

To explore the potential cross talk between VEGFR2 and CXCR4-CXCL12 signalling in both cutaneous and uveal melanoma and the potential for the combined inhibition of both pathways as a strategy to inhibit tumour migration and promote cell death. The present chapter aimed to further characterise VEGFR2 expression in primary cutaneous and uveal melanomas in relation to CXCR4 expression and determine the potential efficacy of pazopanib alone and in combination with trametinib to inhibit cell viability and CXCR4-CXCL12 mediated chemotaxis.

5.2 Results

5.2.1 VEGFR2 is Expressed at Low Levels in Melanocytic Naevi and Primary Cutaneous Melanomas

To date there has been wide disparity with respect to melanoma expression of VEGFR2 (Mehnert *et al.*, 2010; Molhoek *et al.*, 2011). To further explore, the potential contribution of VEGFR2 to tumour progression, and possible correlation with CXCR4 expression and crosstalk with CXCR4-CXCL12 signalling, VEGFR2 expression was evaluated by semi quantitative immunohistochemical analysis in a FFPE cohort of 1 melanocytic naevi or 26 primary melanomas of differeing AJCC stage, with known CXCR4 expression. Immunohistochemical analysis was optimised using FFPE HUVEC endothelial cell pellets, well documented to express VEGFR2 (Zhang *et al.*, 2011), and used as a positive control in all subsequent immuohistochemistry experiments (Figure 5.1).

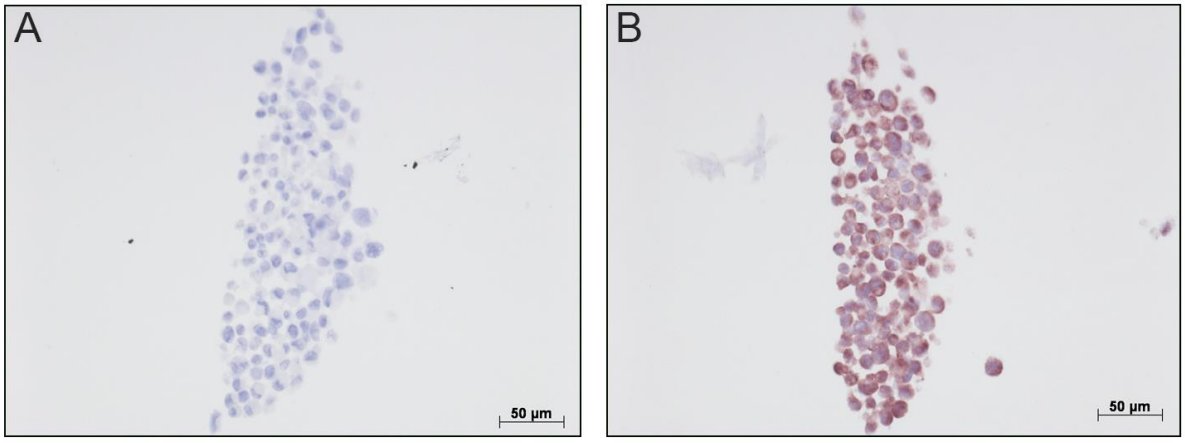

Figure 5.1 HUVEC Cells Express VEGFR2 Strongly
Representative images of VEGFR2 immunohistochemical expression in HUVEC cells, where A.) represents a null primary and B.) positive VEGFR2 staining. Images were acquired at x20 magnification. Scale bar represents 50µm.

A threshold for positive and negative tumoural VEGFR2 staining was set using images representative of strong VEGFR2 staining, the lowest threshold of positive staining and no VEGFR2 statining (Figure 5.2). Using Leica QWin software the mean percentage positively stained tumour cells, was derived from the analysis of up to 10 representative 20X high-powered fields.

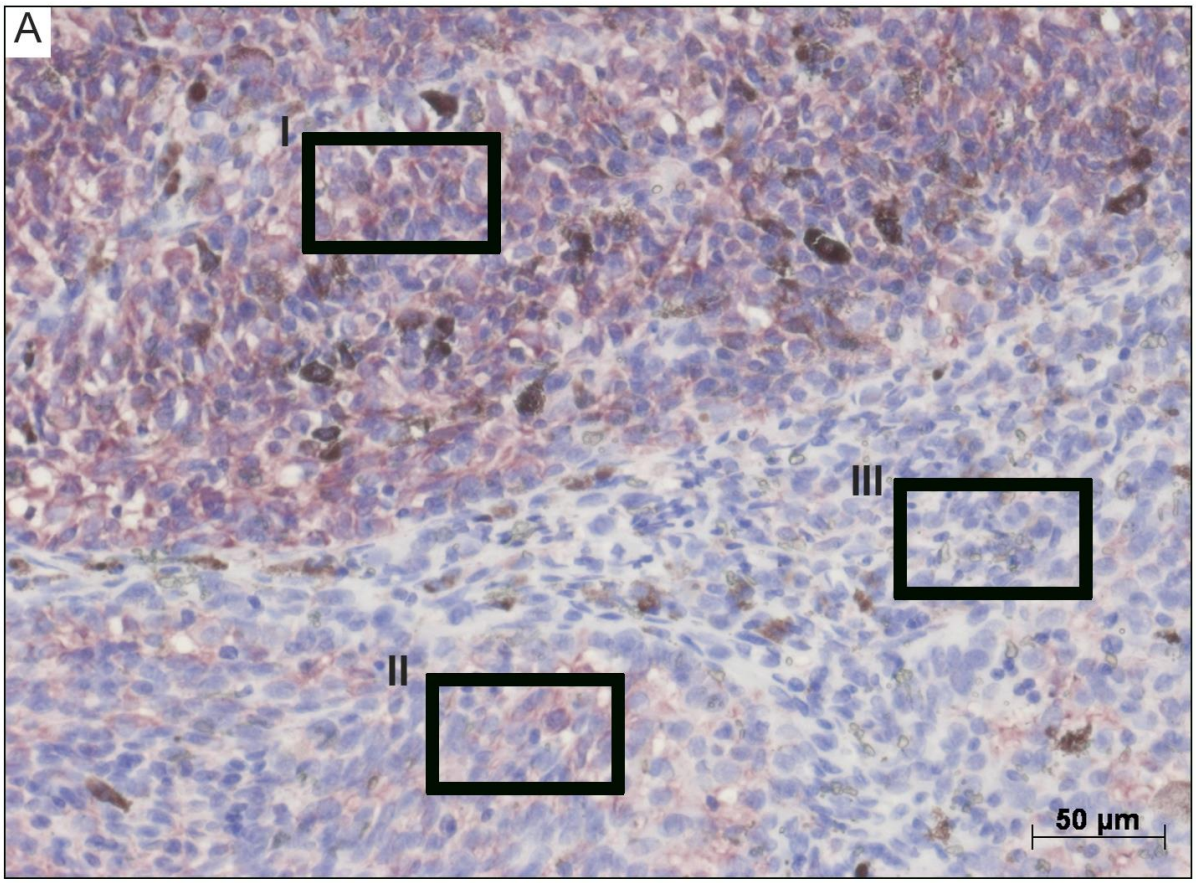

Figure 5.2 Threshold for Positive and Negative Tumoural VEGFR2 Staining
A.) Representative image of VEGFR2 immunohistochemical expression in an AJCC stage II primary cutaneous melanoma used to set thresholds of VEGFR2 expression detection where I.) Represents, positive VEGFR2 expression, II.) Lowest threshold of VEGFR2 positivity, or III.) Negative VEGFR2 expression. Images were acquired at x20 magnification. Scale bar represents 50µM.

Results revealed VEGFR2 was strongly expressed by endothelial cells within tumoural and dermal capillaries, sweat glands, sebaceous glands and hair follicles in all sections, and served as internal positive controls in each case (Figure 5.3 A-E). In addition the epidermal keratinocytes in some tumour sections displayed notable VEGFR2 expression (Figure 5.3 A, 5.4 E-F), so an additional representative images of epidermal VEGFR2 expression for each tumour was also acquired, with semi quantitative analysis perfomed by applying the same threshold as applied to quantify tumoural VEGFR2 expression.

Visual analysis of tumoural VEGFR2, revealed cytoplasmic expression, generally weak and heterogeneously expressed within melanomas (Figure 5.4). Semi quantitative analysis however, revealed 73% of all cutaneous melanomas across all AJCC disease stages displayed less than 1% mean VEGFR2 expression, with only one AJCC stage III melanoma expressing a mere 4.18% expression. Statistical analyses also confirmed a lack of any significant differences

in VEGFR2 expression between any AJCC disease stage, (Kruskal-Wallis test, P= 0.6726, Figure 5.5 A), localised and metastatic tumours (Mann Whitney U, P=0.5878, Figure 5.5 B), or between B-RAF/N-Ras wild-type or mutated tumours (Mann Whitney U, P= 0.2059, Figure 5.5 C).

Although the analysed cohort contained a broad range of mean % CXCR4 expressing melanomas, however results revealed there was no significant correlation between tumour VEGFR2 expression and CXCR4 expression (Spearman's r correlation, P=0.3248, Figure 5.5 D), or CXCL12 expression (Spearman's r correlation, P= 0.9892, data not shown). Collectively these data suggest that VEGFR2 is expressed heterogeneously at low levels within tumours and there is no association between CXCR4 and VEGFR2 expression by primary cutaneous melanomas.

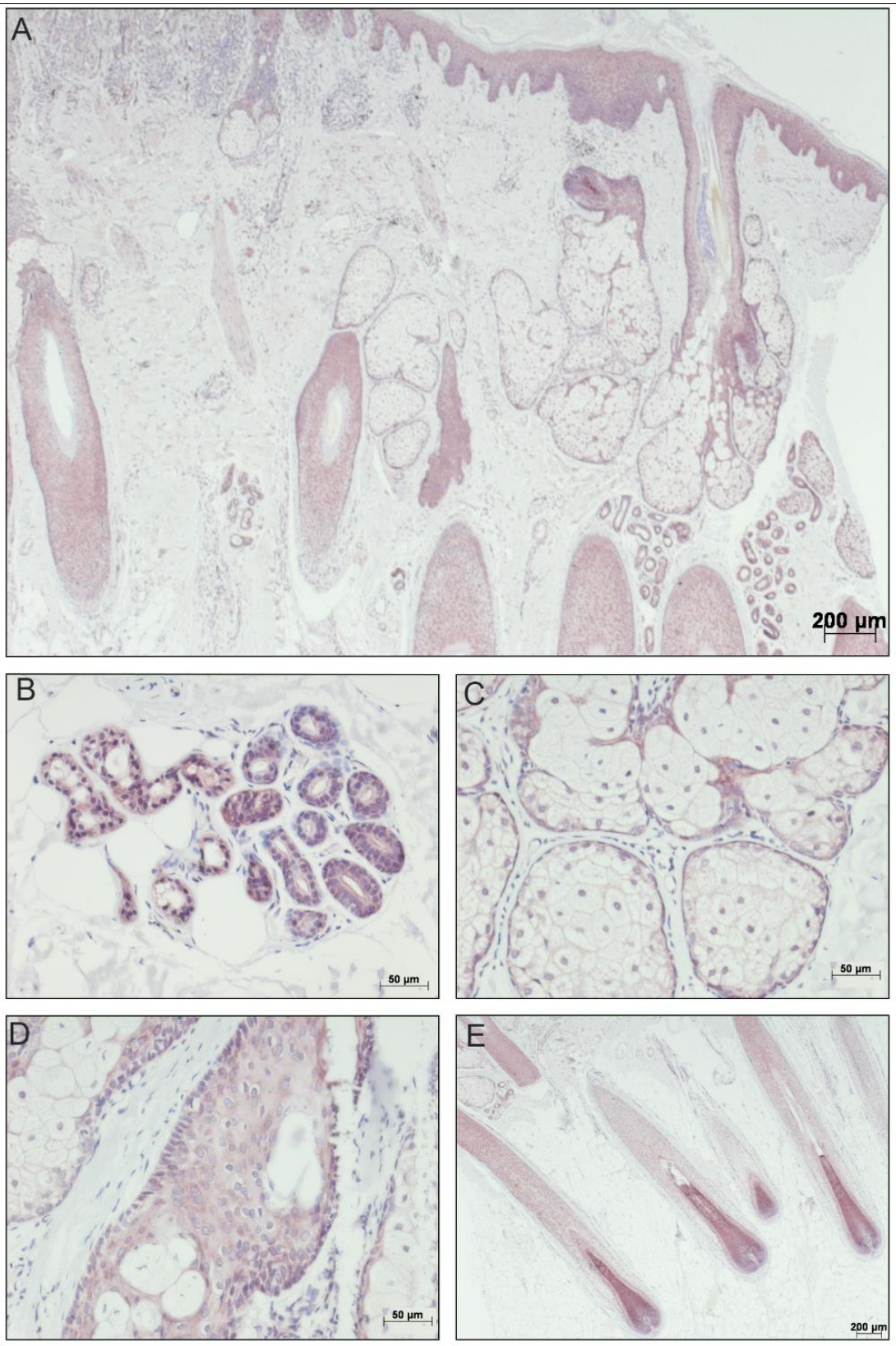

Figure 5.3 Immunohistochemical Expression of VEGFR2 in Skin.

A.) Representative image of VEGFR2 immunohistochemical expression in skin of a benign melanocytic naevus tumour. Images were acquired at x10 magnification. Scale bar represents 200µm. Representative images of VEGFR2 immunohistochemical expression in B.) Capillaries, C.) Sweat glands, D.) Sebaceous glands and E.) Hair follicles of a benign naevi tumour serving as internal positive control in each section. Images were acquired at x20 magnification. Scale bar represents 50µm.

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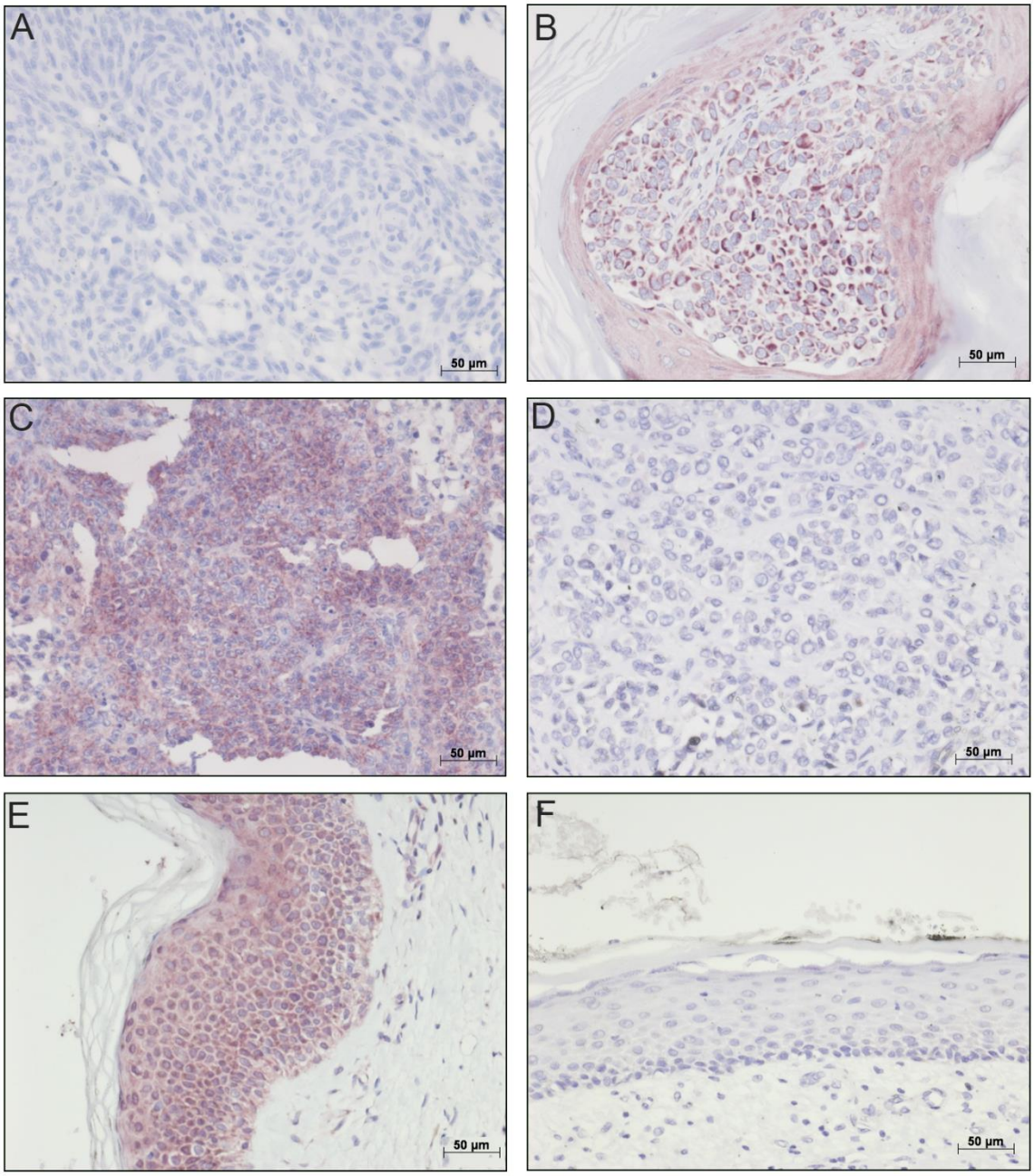

Figure 5.4 VEGFR2 is Expressed by Primary Cutaneous Melanomas at Low Levels
Representative images of VEGFR2 immunohistochemical expression in AJCC stage II primary cutaneous melanomas where A.) Represents a null primary, or B, and C.) Positive VEGFR2 Staining, D.) Negative VEGFR2 staining, E.) Positive staining of the epidermis or F.) Negative staining of the epidermis. Images were acquired at x20 magnification. Scale bar represents 50µm.

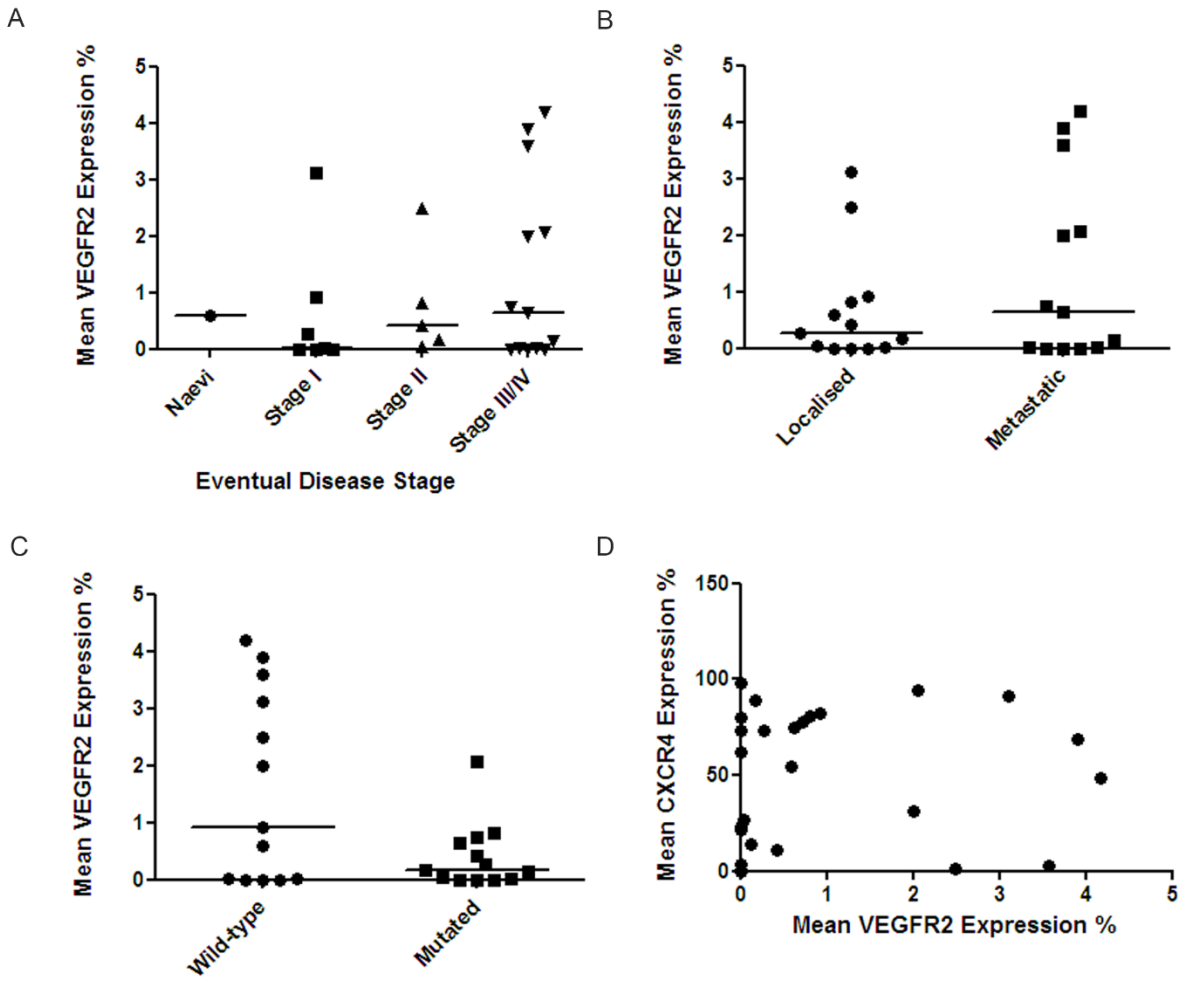

Figure 5.5 Primary Cutaneous Melanomas Express Low Levels of VEGFR2, with no Association with AJCC Stage, Localised or Metastatic Disease or CXCR4 Expression

A.) Scatter graph representing the mean VEGFR2 expression % in eventual stage melanocytic naevi or AJCC stage I, II or III melanomas or B.) localised (eventual AJCC stage of disease I/II) or metastatic melanomas (eventual AJCC stage III/IV) after 7 years follow up. C.) Scatter graph representing mean VEGFR2 expression % in all AJCC stage wild-type or B-RAF/N-Ras mutated primary melanomas. Each point is the mean of 10 high powered fields of view (HPF), horizontal bars represent median % VEGFR2 expression D.) Scatter graph representing mean VEGFR2 expression % in relation to mean CXCR4 expression % in all AJCC stage melanomas.

Similarly, to VEGFR2 expression within primary melanomas, mean VEGFR2 expression % within the overlying epidermis was also less than 1% in 80.7% of all tumours analysed, with levels of VEGFR2 expression >10% detected within the epidermis of just 3 tumours (2 AJCC stage II and 1 AJCC stage III) (Figure 5.6 A). There was no significant difference in epidermal VEGFR2 expression % between all AJCC stages (Kruskal-Wallis test, P=0.0935, Figure 5.6 A), or between tumour and epidermal VEGFR2 expression (P=0.2001 Mann Whitney U, Figure 5.6 B). However, there was a moderate positive correlation found between tumour and epidermal VEGFR2 expression % (Spearman's r= 0.4216, CI 95% 0.02-0.70, *P=0.0319, Figure 5.6 C). Collectively data suggests epidermal VEGFR2 expression is indicative of tumoural expression, and suggestive of a common upregulation of VEGFR2 within the skin as a whole.

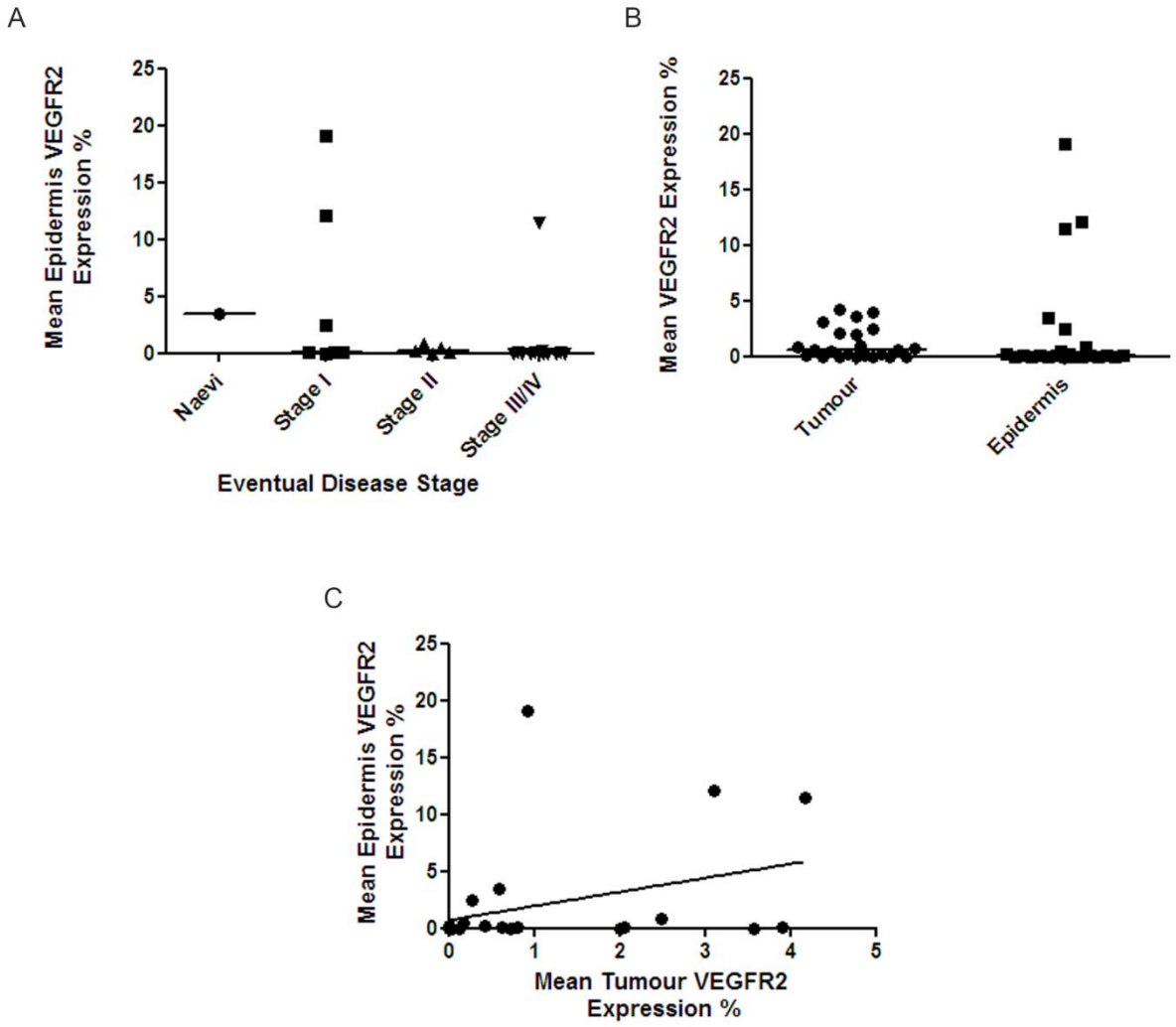

Figure 5.6 Potential Association of VEGFR2 Expression in Primary Cutaneous Melanomas and the Overlying Epidermis

A.) Scatter graph representing the mean epidermal VEGFR2 expression % (mean of 2 high powered fields HPF) in eventual stage melanocytic naevi or AJCC stage I, II or III melanomas or B.) mean VEGFR2 expression % in all AJCC stage primary melanoma tumours or overlying epidermis C.) mean VEGFR2 expression % in individual melanomas relative to mean VEGFR2 expression % in the corresponding epidermis. All horizontal bars represent median VEGFR2 expression %.

5.2.2 Association of VEGFR2 Expression in Primary Cuatneous Melanomas and Patient Matched Metastatic Lymph Nodes

The role and importance of VEGFR2 in promoting lymphangiogenesis is unclear, however the ability of VEGFR2 to bind mature VEGF-C and VEGF-D the primary mediators of lymphangiogenesis, questions whether VEGFR2 expression by melanoma cells in lymph node metastases may be integral to tumour metastasis to sentinel lymph nodes. To investigate this possibility, pre-optimised immunohistochemistry for VEGFR2 expression was performed in a FFPE cohort of 9 primary cutaneous melanomas and patient matched metastatic lymph nodes (Durham Cohort) and 2 normal lymph nodes, with FFPE HUVEC cell pellets used as a positive control and a previously VEGFR2 negative primary cutaneous melanoma (Newcastle Cohort) as a negative control.

Consistent with results derived above, HUVEC cells (positive control) stained strongly for VEGFR2 (data not shown), while little or no expression was detected in the primary cutaneous melanoma (negative control) derived from the Newcastle cohort (Figure 5.7 C). Visual analysis of VEGFR2 expression in the 2 normal lymph nodes (Figure 5.7 A and B) on the other hand revealed positive expression by immune cells. While analysis of 1 primary and patient matched metastatic lymph node metastases was not possible due to overwhelming melanin pigmentation. Interestingly, the immune infiltrate from lymph node metastases derived from some tumours also did not display any visual expression of VEGFR2 (Figure 5.7 G). Conversely, variable expression of VEGFR2 was detected in tumour cells in both primary cutaneous melanomas and patient matched metastatic lymph nodes, in general, revealing strong cytoplasmic expression throughout the tumour bulk (Figure 5.7 E-H).

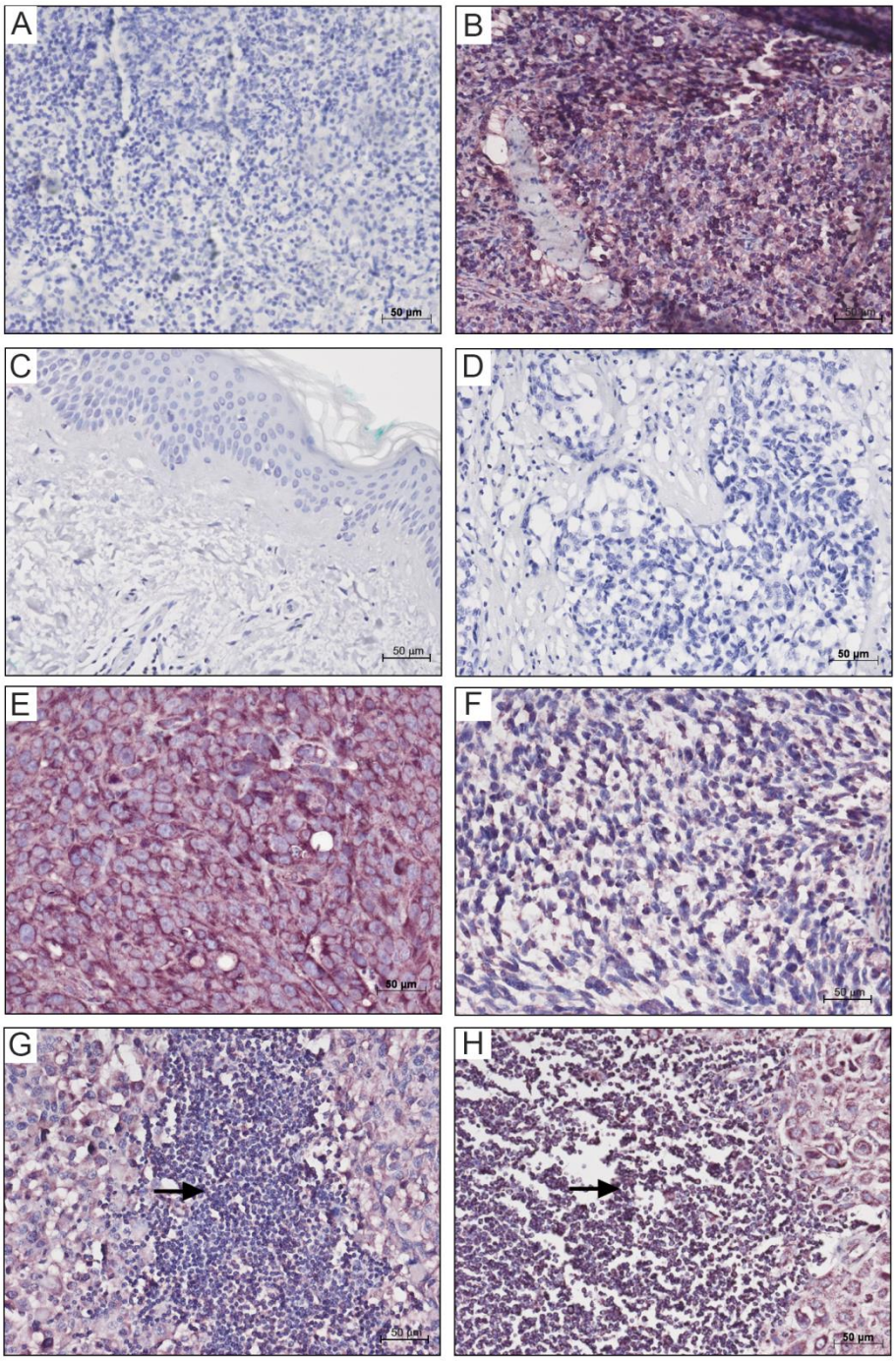

Figure 5.7 Immunohistochemistry for the Expression of VEGFR2 in a Cohort of Primary Cutaneous Melanomas with Patient Matched Metastatic Lymph Nodes

Immunohistochemistry for VEGFR2 expression in a cohort of primary cutaneous melanomas or patient matched metastatic lymph nodes, or normal lymph nodes derived from the Durham patient cohort where A.) Represents null primary antibody control staining in a normal lymph node, B.) Positive VEGFR2 staining in a normal lymph node C.) Negative VEGFR2 expression in a primary cutaneous melanoma from the Newcastle Cohort (negative control), D.) Null Primary antibody control staining in a primary cutaneous melanoma, E.) Positive VEGFR2 expression in a primary cutaneous melanoma, and F.) Weak VEGFR2 expression in a matched metastatic lymph node. G.) VEGFR2 expression in a metastatic lymph node with negative expression in the immune cells or H.) VEGFR2 expression in a metastatic lymph node with positive expression in the immune cells. Black arrows depict immune cells. Images were acquired at x20 magnification. Scale bar represents 50µm.

In comparison, there was no significant difference between VEGFR2 expression in primary cutaneous melanomas and expression in patient matched metastatic lymph nodes, suggesting if VEGFR2 expression is present in the primary tumour then expression is maintained within in the lymph node metastases (Paired T test, ns P=0.2086, Figure 5.8).

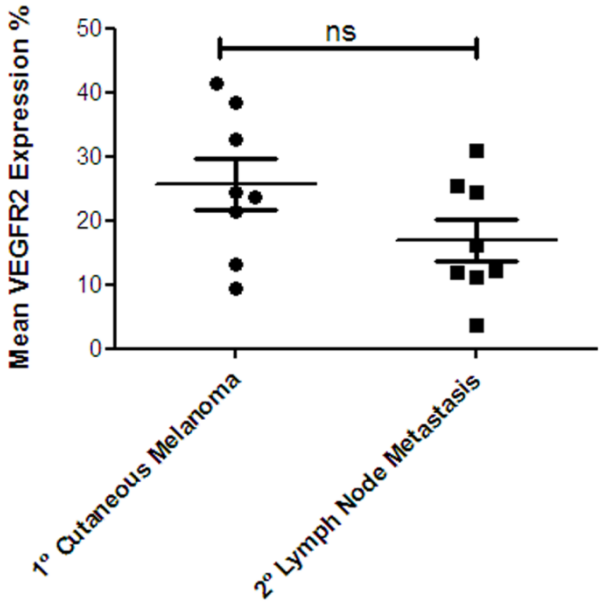

Figure 5.8 Association of VEGFR2 Expression in a Cohort of Primary Cutaneous Melanomas and Patient Matched Metastatic Lymph Nodes

A.) Scatter graph representing the mean (mean of 10 high powered fields of view (HPF)) VEGFR2 expression % in a cohort of primary cutaenous melanomas or secondary metastatic lymph nodes (Durham Cohort). Statistics Acquired by Paired T Test. Horizontal bars represent mean VEGFR2 expression % +/- SD.

Although the cohort of primary cutaneous melanomas and patient matched metastatic lymph nodes comprised patients with just eventual AJCC stage III/IV disease, and hence did not comprise a range of differing AJCC stage melanomas, when VEGFR2 expression was compared in tumours that remained localised (eventual AJCC stage I/II melanomas derived from the Newcastle cohort), with those that went on to develop metastases (eventual AJCC stage III/IV from the Durham and Newcastle patient cohort), a significant increase in VEGFR2 expression was observed in those tumours that subsequently developed metastasis (Mann Whitney U, ***P= 0.0004, Figure 5.9). Collectively these results imply VEGFR2 upregulation in primary cutaneous melanomas may be indicative of metastasis, in particular to lymph nodes, although a larger patient cohort would be required to validate this hypothesis.

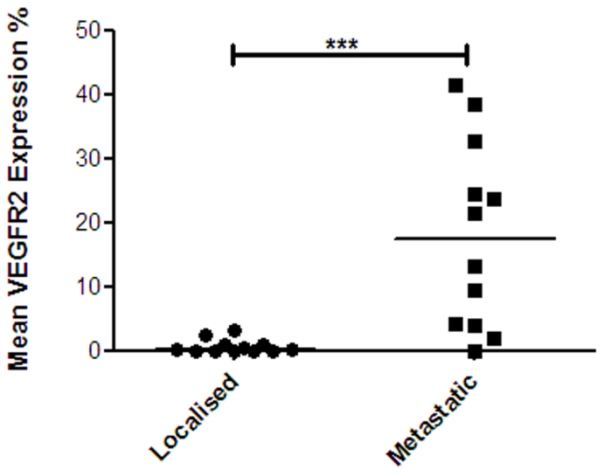

Figure 5.9 VEGFR2 Expression is Increased in Primary Tumours That Develop Metastatic Disease

*Scatter graph representing mean % (mean of 10 high powered fields of view (HPF)) VEGFR2 expression in localised (eventual AJCC stage I/II disease) or metastatic melanomas (eventual AJCC stage III/IV) after 7 years follow up in two primary melanoma patient cohorts derived from Newcastle or Durham. Horizontal bars represent median VEGFR2 expression %. Statistics acquired by Mann Whitney U ***P=0.0004.*

5.2.3 VEGFR2 Expression is Increased in Primary Uveal Melanomas

Using the same pre-optimised immunohistochemical assay used for the analysis of VEGFR2 in cutaneous melanoma, VEGFR2 expression was analysed in a cohort of 9 primary uveal melanomas with or without monosomy for chromosome 3 (Figure 5.10). Results revealed heterogeneous cytoplasmic expression of VEGFR2 in all uveal melanomas with the exception of one tumour with disomy of chromosome 3 (Figure 5.11 A). VEGFR2 was also detected within the retina and choroid of the eye, to varying degrees in all samples, and was most strongly expressed within micro vessels of the ganglion cell layer as well as rods and cones, as previously reported in normal eyes (Stitt et al., 1998; Kim et al., 1999).

However, there was no significant difference in VEGFR2 % expression between tumours bearing monosomy or disomy of chromosome 3 (Mann Whitney U, P=0.2619, Figure 5.11 B), although it should be noted that statistical analysis is limited in such a small sample size. Results demonstrate that on average 30% of uveal melanoma tumour cells express VEGFR2, however we detected expression up to 61.72% in a tumour with monosomy of chromosome 3. Together data suggests that VEGFR2 is a potential therapeutic target for uveal melanomas, in particular tumours with adverse prognostic features.

Furthermore, statistically, there was significantly greater VEGFR2 expression in uveal compared to cutaneous melanomas, (Mann Whitney U, ***P=0.0004, Figure 5.11 C) while interestingly, there was no significant correlation between VEGFR2 expression and the expression of either CXCR4, or CXCL12, (data not shown), perhaps also reflected by the small total cohort size of uveal melanomas employed by the present study.

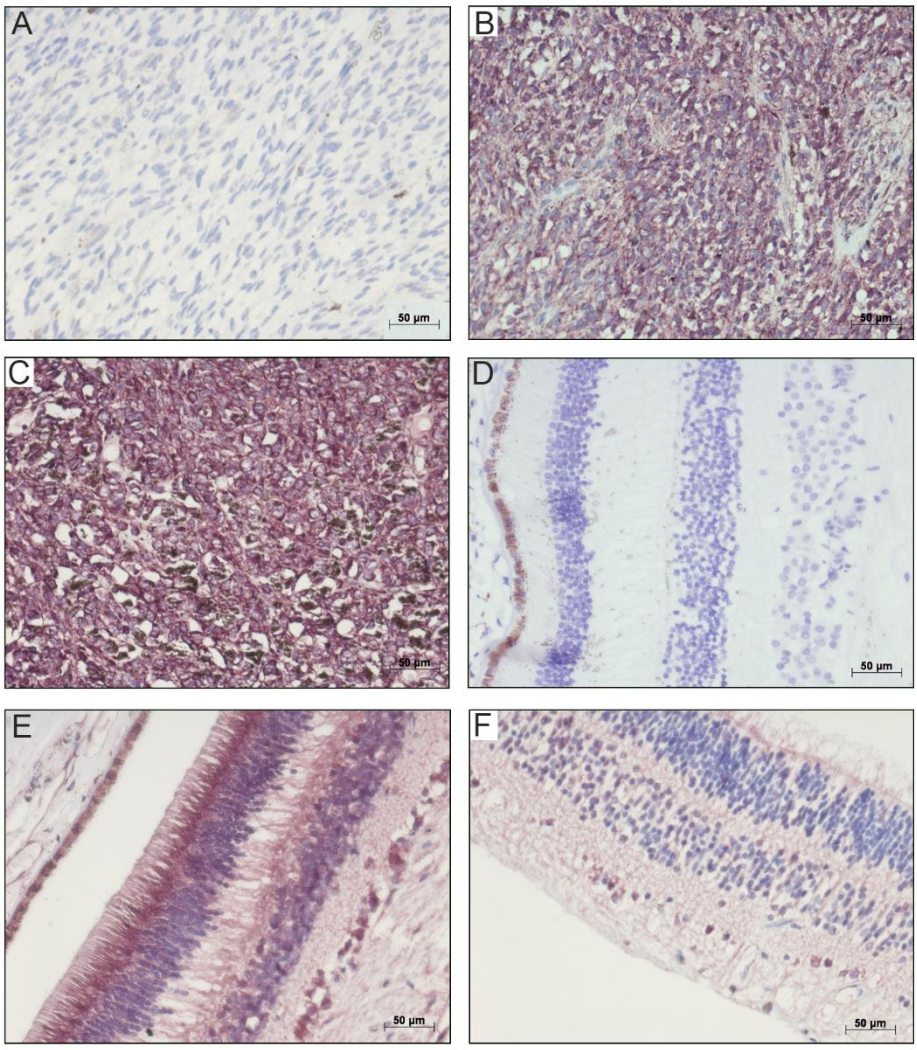

Figure 5.10 Immunohistochemistry for Expression of VEGFR2 in a Cohort of Primary Uveal Melanomas.

Immunohistochemistry for VEGFR2 expression in a cohort of primary uveal melanomas where A.) Represents a null primary control, B.) Positive VEGFR2 staining in a tumour with disomy of chromosome 3, C.) Positive VEGFR2 staining in a tumour with monosomy of chromosome 3, D.) Null Primary control of the retina and choroid of the eye E.) Positive VEGFR2 expression in the retina and choroid of the eye F.) Weak VEGFR2 expression in the retina and choroid of the eye. Images were acquired at x20 magnification. Scale bar represents 50µm.

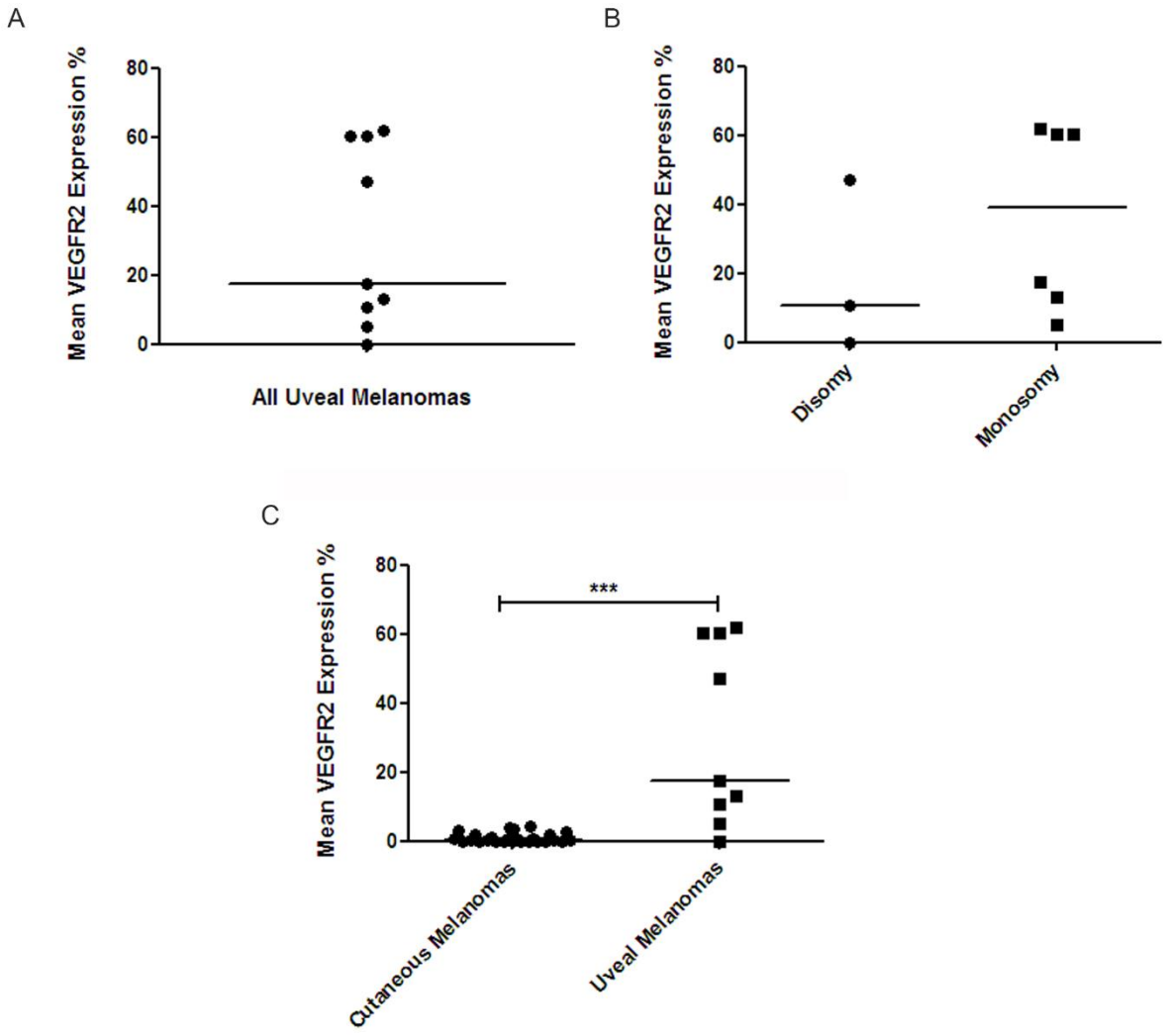

Figure 5.11 Quantification of Immunohistochemistry for the Expression of VEGFR2 in a Cohort of Primary Uveal Melanomas

*A.) Scatter graph representing the mean (mean of 10 high powered fields of view (HPF)) VEGFR2 expression % in a cohort of primary uveal melanomas. B.) Scatter graph representing mean VEGFR2 expression % in tumours that have disomy or monosomy of chromosome 3 C.) Scatter graph representing mean VEGFR2 expression % in primary cutaneous or uveal melanomas. Statistics acquired by Mann Whitney U Test ***P<0.001. All horizontal bars represent median VEGFR2 expression %.*

5.2.4 VEGFR2 is Expressed by Cutaneous and Uveal Melanoma *In Vitro*

Given the seemingly low expression of VEGFR2 in primary cutaneous melanomas, but which increased in tumours which subsequently metastasised to regional lymph nodes as well as seemingly increased expression by primary uveal melanomas, the immunofluorescent expression of VEGFR2 was also determined in a panel of cutaneous and uveal melanoma cell lines (Figure 5.12) as well as in keratinocytes and melanocytes (Figure 5.13) using HUVEC cells as a positive control (Slongo et al., 2007). In addition the immunofluorescent subcellular localistaion of VEGFR2 was determined by the acquisition of Z stacks of the middle section of fixed cells, since it has been suggested that a nuclear expression pattern is associated with benign naevi and microinvasive melanomas whereas a cytoplasmic and nuclear expression pattern are associated with a worse prognosis and advancement towards invasive melanomas (Figure 5.12 and 5.13) (Pisacane and Risio, 2005).

Results revealed differential expression of VEGFR2 among all cutaneous and uveal melanoma cell lines (Figure 5.12), with all cell lines expressing VEGFR2 but with weak expression displayed by the uveal metastatic melanoma cell line OM413. VEGFR2 expression was also evident in primary keratinocytes and melanocytes, (Figure 5.13). Intrestingly the subcellular localisation of VEGFR2 varied between cell lines with most displaying nuclear and cytoplasmic expression (CHL-1, A375, WM-1361, OM413, HUVEC, Figure. 5.12, and primary melanocyte cells Figure 5.13). Strikingly WM-164, OMM2.3 and primary keratinocytes expressed much greater nuclear VEGFR2 expression while in contrast, UPMD2 cells expressed VEGFR2 predominantly within the cytoplasm with only weak levels of VEGFR2 expression within the nucleus (Figure 5.12 and 5.13).

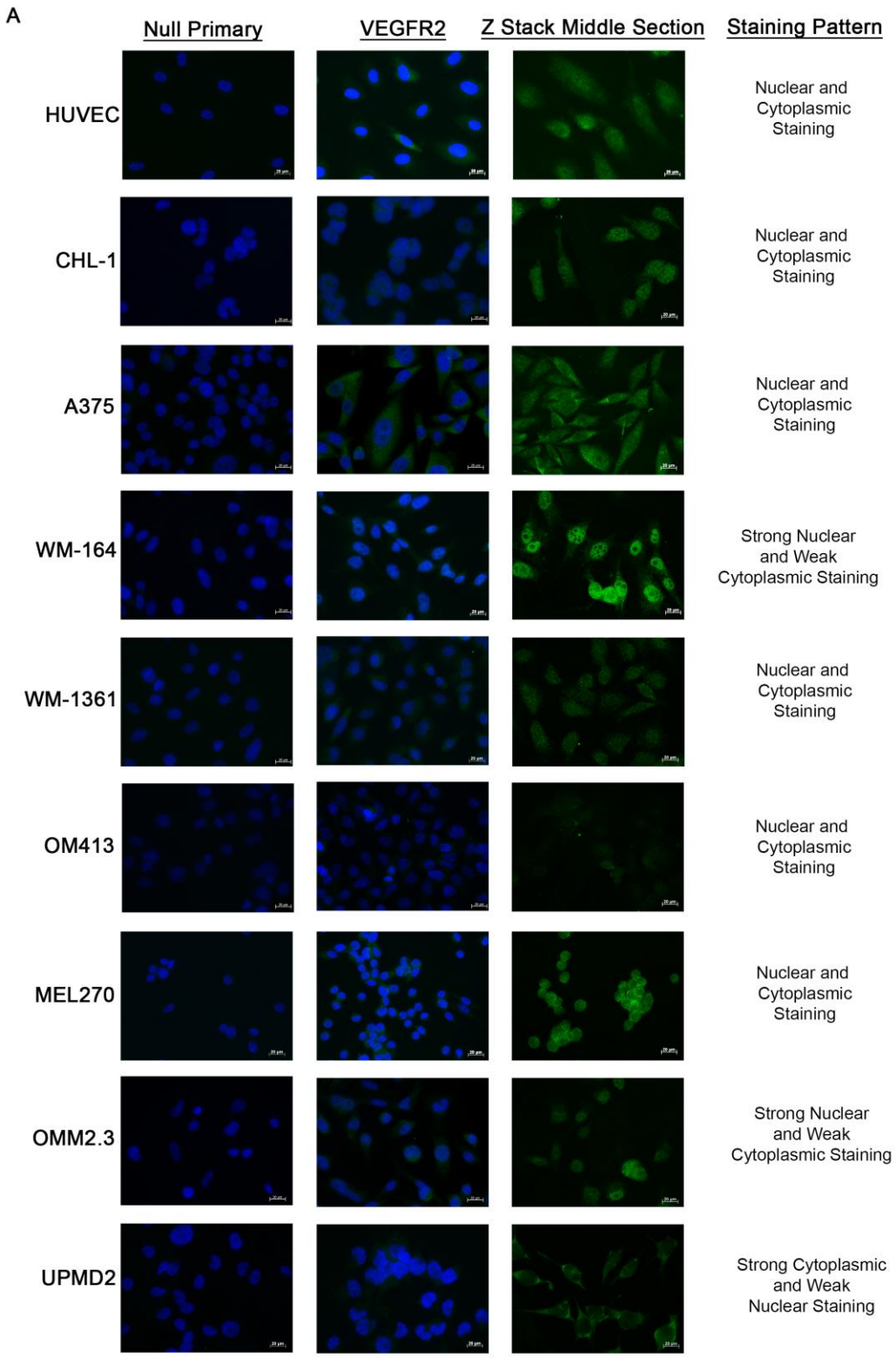

Figure 5.12 Immunofluorescent Expression of VEGFR2 in Cutaneous and Uveal Metastatic Melanoma Cell Lines

Representative images from 3 replicate experiments for the immunofluorescent expression of HUVEC cells (positive control), cutaneous metastatic melanoma cell lines CHL-1, A375, WM-164 and WM-1361, or uveal melanoma cell lines OM413, MEL270, OMM2.3 and UPMD2, or null primary control. Green depicts VEGFR2 positivity and blue DAPI nuclear staining. Images were acquired by confocal microscopy at 20x magnification, scale bar = 50µm or Z stacks of cells taken and the middle cross section of the cell shown, with magnification of 20x, scale bar 50µm.

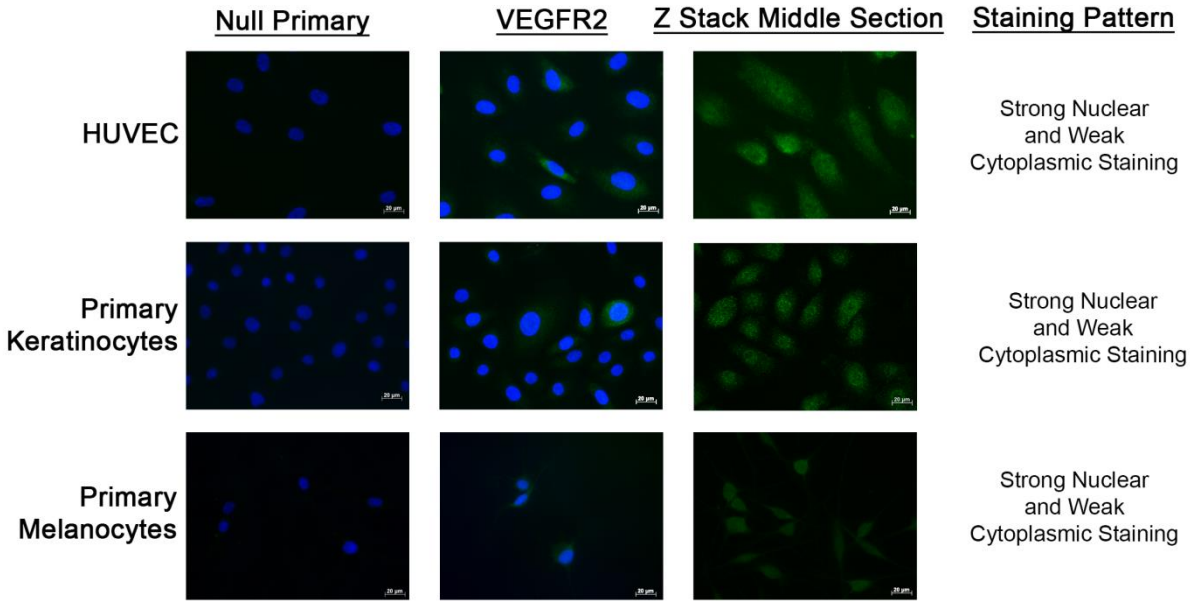

Figure 5.13 Immunofluorescent Expression of VEGFR2 in HUVEC cells, Primary Keratinocytes and Melanocytes

Representative images from 3 replicate experiments for the immunofluorescent expression of VEGFR2 in HUVEC cells, primary keratinocytes and primary melanocytes, or null primary control. Green depicts VEGFR2 positivity and blue DAPI nuclear staining. Images were acquired by confocal microscopy with magnification of 20x, scale bar = 50µm or Z stacks of cells taken and the middle cross section of the cell shown, with magnification of 20x, scale bar 50µm.

Given the large intra-variability of VEGFR2 expression detected by immunofluorescence (Figure 5.14 A), mRNA expression of VEGFR2 expression was also quantified in all cell lines and primary cells (Figure 5.14 B). Similarly, to immunofluorescent protein expression analysis, VEGFR2 mRNA expression was detectable in primary keratinocytes and melanocytes as well as in all cutaneous and uveal melanoma cell lines, with again very low level of expression in uveal OM413 cells (Figure 5.14 B). Interestingly while WM-164 cutaneous melanoma cells expressed high levels of VEGFR2 protein, these cells displayed very low expression of VEGFR2 mRNA (Figure 5.14). Differences in mRNA and protein expression are perhaps unsurprising given the myriad of post-transcriptional mechanisms, differences in protein half-life and experimental error or noise generated in both mRNA and immunofluorescent experiments. However overall, using mRNA and protein expression analysis results confirmed the expression of VEGFR2 by all cutaneous and uveal melanoma cell lines as well as in primary keratinocytes and melanocytes (Greenbaum *et al.*, 2003).

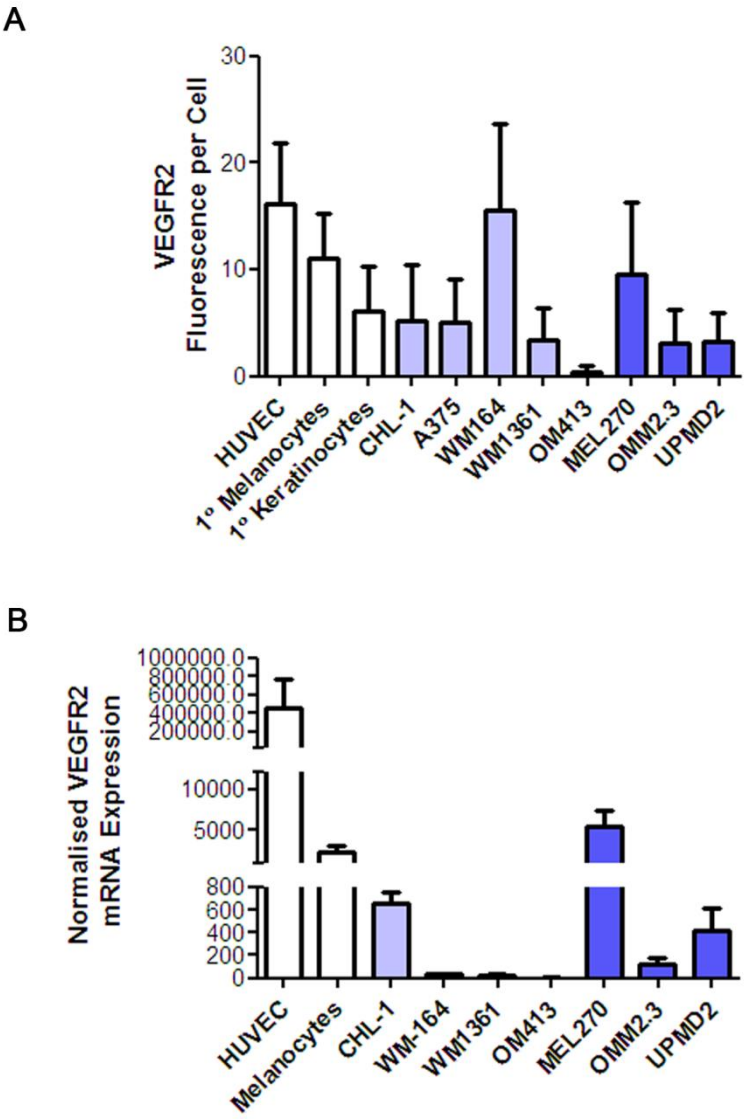

Figure 5.14 Differential Expression of VEGFR2 Protein and mRNA by Cutaneous and Uveal Metastatic Melanoma Cell Lines, HUVEC cells and Primary Keratinocytes and Melanocytes
A.) Mean VEGFR2 fluorescence per cell, within cutaneous and uveal metastatic melanoma cell lines, HUVEC cells (positive control) and primary melanocytes or keratinocytes. Each bar is the mean VEGFR2 fluorescence per cell derived from 4 representative images from 3 independent experiments (mean + SD, N=3). B.) Mean mRNA expression of VEGFR2 in cutaneous and uveal metastatic melanoma cell lines, HUVEC cells, and primary melanocytes or keratinocytes. Each bar is mean of VEGFR2 Cycle threshold for each sample normalised to 18s cycle threshold from 3 independent experiments (mean +SD N=3).

Results further revealed enhanced immunofluorescent expression of VEGFR2 by cutaneous and uveal melanoma cells grown in close proximity compared to cells cultured in monolayer at a lower density. The subcellular localisation of VEGFR2 also appeared to be more cytoplasmic within cells that had grown to larger cell clusters. The presence of a variable cluster size of cells with positive VEGFR2 expression led to the hypothesis that seeding density may affect expression and subcellular localisation. To test this hypothesis A375, WM-164 and

WM-1361 cells were seeded at variable densities for 24 hours, or CHL-1, WM-164 and WM-1361 cells were seeded at $1x10^4$ cells/well for 24-96 hours, prior to the analysis of the immunofluorescent/mRNA expression of VEGFR2 (Appendix 2). Results demonstrated that seeding density increased VEGFR2 expression and that VEGFR2 translocated from the nucleus to the cell cytoplasm at higher seeding density, which may therefore account for the variability in VEGFR2 expression and subcellular localisation observed in uveal/cutaneous melanoma cell lines (Appendix Figures 2.1-2.8). However, whether such an effect may be influenced by the secretion of VEGF from uveal or melanoma cell lines requires further evaluation.

5.2.5 Pazopanib Inhibits Cell Viability and CXCR4-CXCL12 Chemotaxis of Cutaneous and Uveal Melanoma Cell Lines

Given the expression of VEGFR2 by cutaneous and uveal melanoma cell lines *in vitro* (Section 5.2.4) as well as by primary cutaneous and uveal melanomas, and patient matched metastatic lymph nodes *in vivo* (Section, 5.2.1, 5.2.2 and 5.2.3), this provides a rationale for targeting VEGFR2 to prevent melanoma migration and metastasis especially in uveal melanoma. Previous clinical trials targeting the VEGF signalling pathways with the VEGF ligand trapping monoclonal antibody bevacizumab, have provided the proof of concept for use of angiogenesis inhibitors in cancer therapy (Varker *et al.*, 2007b). Furthermore bevacizumab prevents uveal melanoma cell migration *in vitro*, highlighting the role of VEGF signalling in melanoma cell migration (Logan *et al.*, 2013). Given that CXCR4-CXCL12 chemokine axis is the major mediator of melanoma cell migration, to investigate the potential of inhibiting VEGF cell signalling and CXCR4-CXCL12 mediated migration/chemotaxis by targeting the VEGFR2 receptor, experiments were initiated to test the efficacy of the pan VEGFR2 tyrosine kinase inhibitor, pazopanib in this capacity. Initially the effect of pazopanib on melanoma cell viability over a clinically achievable dose range was evaluated in both B-RAF/N-Ras wild-type and mutant cutaneous melanoma as well as GNAQ/GNA11 wild-type and mutant uveal melanoma cell lines (Figure 5.15).

Pazopanib induced a dose dependant decrease in cell viability of both cutaneous and uveal melanoma cell lines (Figure 5.15). WM-164 cutaneous melanoma cells were least sensitive to treatment with significant inhibition of cell viability seen only at the highest dose of 25µM pazopanib (Kruskal-Wallis test with Dunn's multiple comparison post hoc test, ***P<0.001, Figure 5.15 B). As there was no significant effect on cell viability with pazopanib in any cell line

at 1µM or in WM-164 and OM413 at 5µM, concentrations of 1, 5 and 25µM were selected to titrate in subsequent chemotaxis assays.

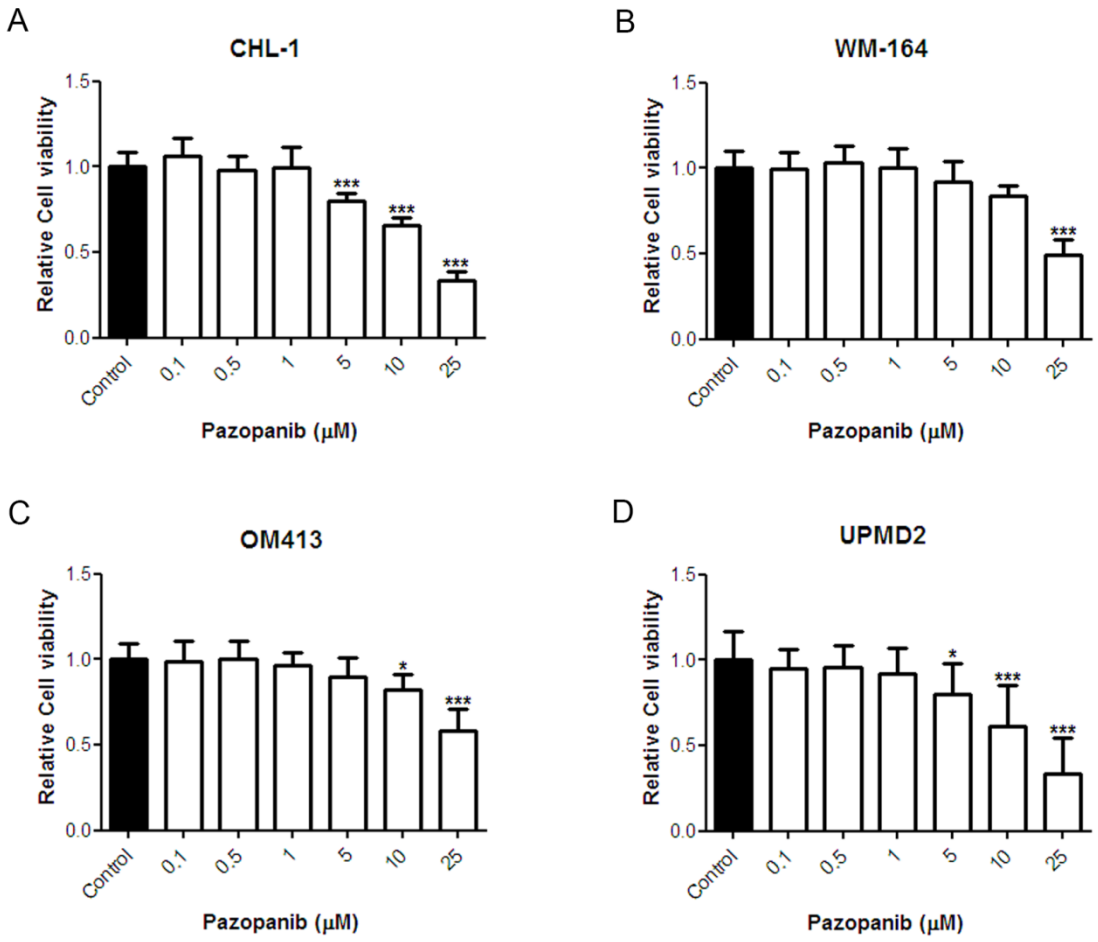

Figure 5.15 Pazopanib-induced Inhibition of Cell Viability of Cutaneous and Uveal Metastatic Melanoma Cell Lines

*Cell viability of A.) CHL-1, B.) WM-164, cutaneous metastatic melanoma cell lines or C.) OM413 or, D.) UPMD2 uveal metastatic melanoma cell lines in the presence or absence of 0.1-25µM pazopanib for 16 hours, relative to control untreated cells. Each bar represents mean cell viability of 12 replicates from 3 independent experiments +SD N=3. Statistics acquired by one-way analysis of variance with Dunnett's multiple comparison post hoc correction or Kruskal Wallis test with Dunn's multiple comparison post hoc correction, where *P<0.05, ***P<0.001.*

To determine the effect of pazopanib on CXCR4-CXCL12 cell chemotaxis of cutaneous and uveal metastatic melanoma cells, cells were treated with 1, 5, 25µM pazopanib prior to incorporation into transwell chemotaxis assays. Analysis of chemotaxis of CHL-1, WM-164 or OM413 cells revealed pazopanib (at all utilized concentrations) induced significant dose-dependent inhibition of chemotaxis towards rCXCL12 compared to control untreated cells (Kruskal-Wallis test with Dunn's multiple comparison post hoc test, *P<0.05, **P<0.01, ***P<0.001, Figure 5.16). However, although pazopanib induced a significant inhibition of

chemotaxis of CHL-1 cells at a concentration of 1μM pazopanib and for WM-164 and OM413 cells at concentrations of 5μM pazopanib (Kruskal-Wallis test with Dunn's multiple comparison post hoc test, ***$p<0.001$, Figure 5.16), there was no significant inhibition of cell viability observed at these concentrations (Figure 5.15). Collectively, these data suggest both trametinib and pazopanib display a more potent ability to inhibit cutaneous and uveal metastatic melanoma CXCR4-CXCL12 mediated chemotaxis compared to their effects on inhibition of cell viability, additionally further highlighting the apparent crosstalk between CXCR4-CXCL12 driven chemotaxis, MAPK and VEGFR2 cell signalling.

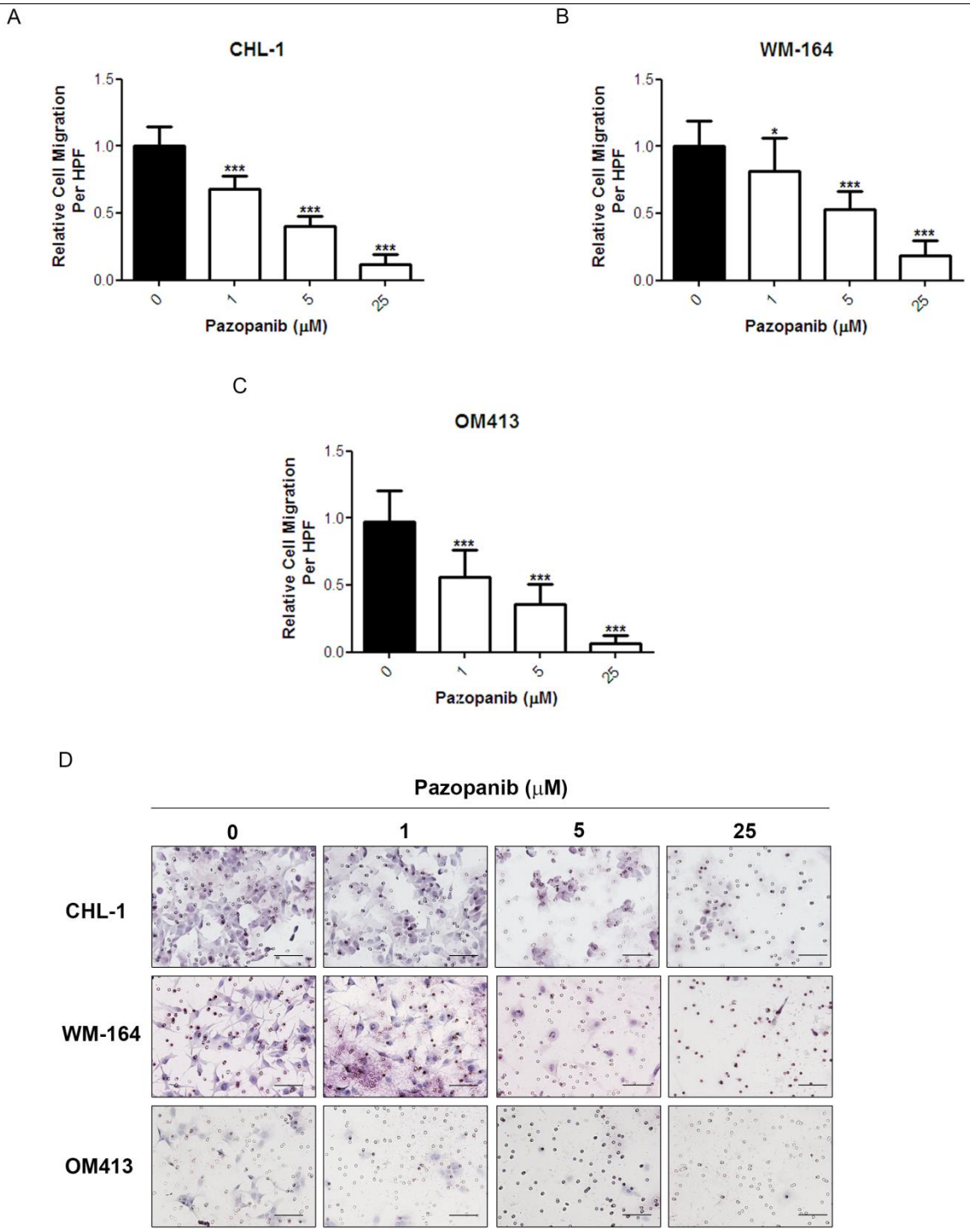

Figure 5.16 Pazopanib Inhibits Chemotaxis of Cutaneous and Uveal Metastatic Melanoma Cell Lines Towards rCXCL12

*Relative Migration (per high powered field) of A.) CHL-1, B.) WM-164 cutaneous metastatic melanoma cells or C.) OM413 metastatic uveal melanoma cells, towards 10nM recombinant CXCL12 in the presence of 1, 5 or 25μM pazopanib, expressed relative to migration towards recombinant CXCL12 of untreated cells. Each bar represents the mean of 9 replicate filters from 3 independent experiments + SD. Statistic acquired by Kruskal-Wallis test with Dunn's multiple comparison post hoc correction, where *P<0.05 and ***P<0.001. D.) Representative photomicrographs from one high powered field (HPF) of migrated CHL-1, WM-164 or OM413 metastatic melanoma cells towards rCXCL12 in the presence or absence of 1, 5, or 25μM pazopanib. Images acquired at X20 magnification, scale bar represents 100μm.*

Although clearly able to potently inhibit cutaneous and uveal melanoma cell chemotaxis, pazopanib is a multi-targeted tyrosine kinase inhibitor that primarily targets VEGFR2, but also has biological affinity for VEGFR1, VEGFR3 and to a lesser extent on fibroblast growth factor receptor-1, platelet derive growth factor receptor beta, KIT proto-oncogene tyrosine kinase receptor and colony stimulating factor receptor. It therefore remained unclear whether pazopanib-induced inhibition of CXCR4-CXCL12 melanoma chemotaxis was due to the specific inhibition of VEGFR2. To address this, a siRNA pool was used to specifically knock down VEGFR2 in CHL-1 and WM-164 metastatic melanoma cell lines and the effect on melanoma cell migration determined in the presence or absence of treatment with recombinant VEGF and pazopanib.

Knockdown of VEGFR2 in both CHL-1 and WM-164 metastatic melanoma cell lines was confirmed by the significant reduction in VEGFR2 mRNA by, on average 40% in CHL-1 and 32% in WM-164 cells compared to expression following transfection of cells with control non-targeting siRNA (Students T test, **P=0.090, and ***P=0.008, Figure 5.17 A).

Preliminary experiments in WM-164 B-RAF mutated metastatic melanoma cells additionally confirmed treatment with VEGF resulted in the significant increase in migration of untransfected cells in the absence of chemotactic stimulant (One-way ANOVA with Bonferroni post hoc correction, ***P<0.001, Figure 5.17 B). Interestingly, results also confirmed that the effects of transfection alone appeared to reduced WM-164 cell migration, with a significant reduction in migration of non-targeting control and VEGFR2 siRNA transfected cells compared to untransfected cells (One-way ANOVA with Bonferroni post hoc correction, ***P<0.001, Figure 5.17B). Pazopanib also significantly inhibited cell migration of WM-164 cells transfected with non-targeting siRNA in the presence or absence of treatment with VEGF (One-way ANOVA with Bonferroni post hoc correction, *P<0.05, Figure 5.17B), conversely, there was no reduction in WM-164 cell migration following transfection with VEGFR2 siRNA suggesting that pazopanib-induced inhibition of cell migration is mediated via VEGFR2 inhibition (One-way ANOVA, with Bonferroni post hoc correction, ns P>0.05, Figure 5.17B). Furthermore, a significant reduction in WM-164 cell migration was also observed following VEGFR2 mediated knockdown compared to that observed following transfection of cells with non-targeting siRNA, collectively suggesting VEGFR2 signalling is required for cell migration (One-way ANOVA with Bonferroni post hoc correction, *P<0.05, Figure 5.17B).

Migration studies were then repeated in CHL-1 and WM-164 cells cutaneous metastatic melanoma cells transfected with non-targeting or VEGFR2 siRNA in the presence or absence of VEGF and pazopanib. In both cell lines treatment of un-transfected cells with recombinant VEGF significantly increased melanoma cell chemotaxis (Kruskal-Wallis test with Dunn's multiple comparison post hoc correction, **P<0.01 and ***P<0.001 for CHL-1 and WM-164 respectively, Figure 5.17 C), thereby confirming the involvement of VEGF cell signalling in melanoma cell migration. Transfection of both cell lines with either non-targeting or VEGFR2 siRNA also significantly reduced VEGF-induced cell migratory ability, confirming again that transfection alone is able to inhibit melanoma cell migration (Kruskal-Wallis test with Dunn's multiple comparison post hoc correction, ***P<0.001, Figure 5.17 C). While in CHL-1 cells there was a non-significant trend for a reduction in cell migration following VEGFR2 siRNA transfection compared to non-targeting siRNA transfection (Kruskal-Wallis test with Dunn's multiple comparison post hoc correction, ns P>0.05), in WM-164 cells, knockdown of VEGFR2 resulted in a significant reduction in cell migration compared to cells transfected with non-targeting siRNA, (Kruskal-Wallis test with Dunn's multiple comparison post hoc correction, ***P<0.001, Figure 5.17B), suggestive of a role for VEGFR2 cell signalling in WM-164 cell migration, at least. Nevertheless, treatment of both cell lines with pazopanib following non-targeting siRNA transfection resulted in significant reduction of cell migration (Kruskal-Wallis test with Dunn's multiple comparison post hoc correction, ***P<0.0001, Figure 5.17 C), while there was no significant reduction in cell migration of either cell lines following knockdown of VEGFR2 (Kruskal-Wallis test with Dunn's multiple comparison post hoc correction, ns P>0.05, Figure 5.17 C), collectively indicating VEGFR2 is required for pazopanib induced-inhibition of melanoma migration.

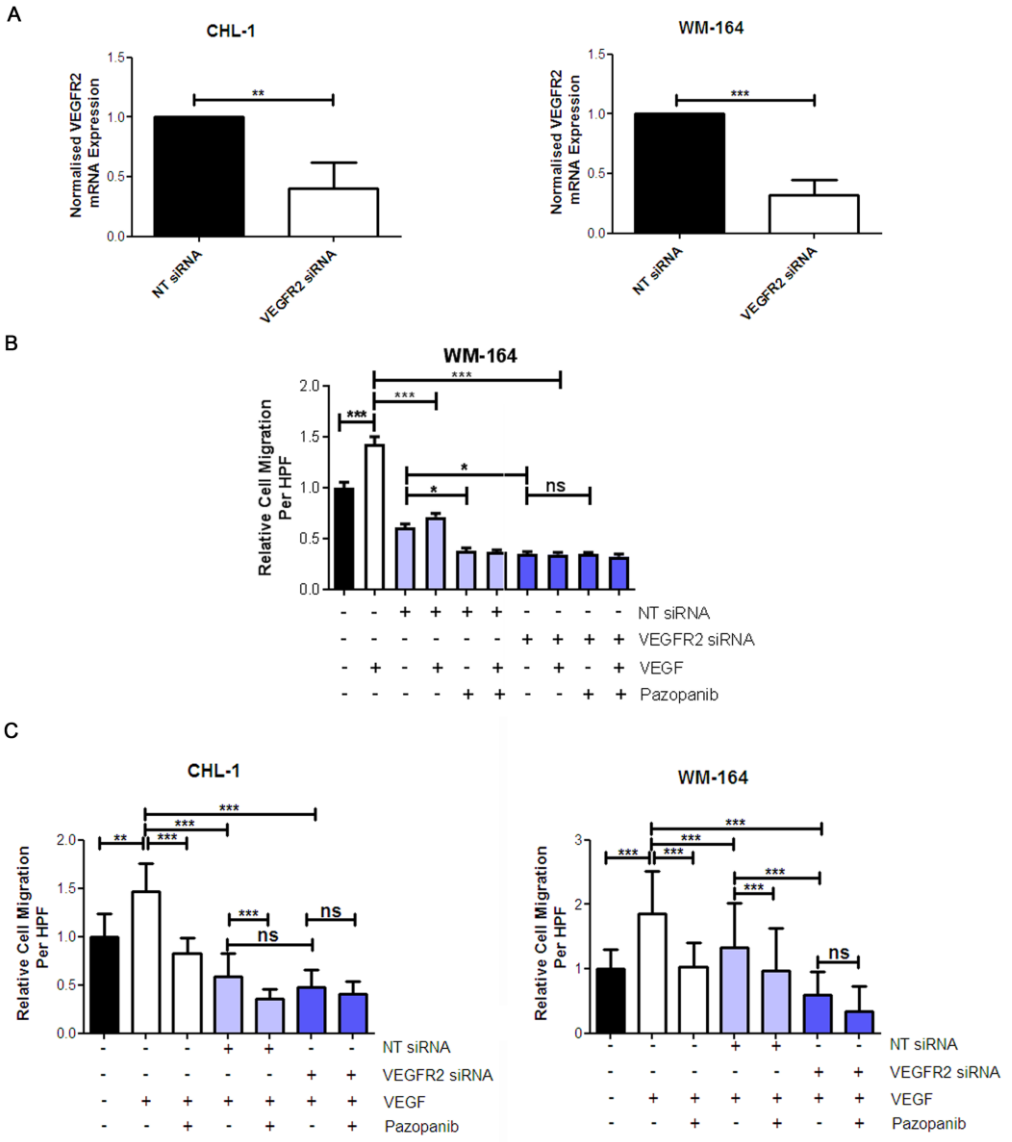

Figure 5.17 Knockdown of VEGFR2 Impairs Pazopanib-Induced Inhibition of Melanoma Cell Migration

*A.) Mean VEGFR2 mRNA expression in CHL-1 or WM164 metastatic melanoma cutaneous cell lines, 72 hours post transfection with VEGFR2 siRNA or non-target (NT) control siRNA. Each bar is mean VEGFR2 Cycle threshold normalised to 18s cycle threshold from 3 independent experiments (mean +SD N=3). Statistics acquired by Students T test (**P<0.01, **P<0.001). B.) Relative migration (Per high powered field, HPF) of WM164 cutaneous metastatic melanoma cells in the presence or absence of transfection with VEGFR2 siRNA or non-targeting (NT) siRNA, in the presence or absence of treatment with recombinant VEGF (10ng/ml) and pazopanib (1µM), for 16 hours. Each bar represents the mean HPF of 9 replicate filters +SD. Statistics acquired by One-way analysis of variance with Bonferroni post hoc correction (*P<0.05, ***P<0.001) C.) Relative migration per HPF of CHL-1 or WM164 cutaneous metastatic melanoma cells, in the presence or absence of transfection with VEGFR2 siRNA or non-targeting (NT) siRNA, in the presence or absence of treatment with recombinant VEGF (10ng/ml) and pazopanib (1µM), for 16 hours. Each bar represents the mean HPF derived from 9 replicate filters from 3 independent experiments + SD. Statistics acquired by Kruskal-Wallis test with Dunn's multiple comparison post hoc correction (ns P>0.05, **P<0.01, ***P<0.001).*

5.2.6 Combined MEK and VEGFR2 Inhibition Potentiates Inhibition of Cutaneous and Uveal Melanoma Cell Viability and CXCR4-CXCL12 Chemotaxis

Results revealed both MEK and VEGFR2 inhibition impairs melanoma cell viability and chemotaxis when trametinib and pazopanib are used as single agents (Section 4.2.3 and 5.2.5), however monotherapy targeting the MAPK pathway or angiogenesis has historically lead to a plethora of resistant mechanisms and reactivation of pro-survival downstream signalling pathways (Ellis and Hicklin, 2008a; Welsh *et al.*, 2016). Consequently attempts have been made to target the same pathways simultaneously or by combining drugs that target alternative pathways, resulting in increased efficacy and the reduced emergence of drug resistance (Lang *et al.*, 2008; Dankort *et al.*, 2009; Meng *et al.*, 2010; Flaherty *et al.*, 2012a; Carlino *et al.*, 2014). Experiments were therefore initiated, to investigate the potential for the increased efficacy of combined MEK and VEGFR2 inhibition on uveal or cutaneous melanoma CXCR4-CXCL12 chemotaxis.

Initially, the effect of combined trametinib and pazopanib on CHL-1 or WM-164 cutaneous or OM413 or UPMD2 uveal melanoma cell viability was assessed over 16 hours, in response to treatment with a clinically achievable dose range of both drugs and at a fixed dose ratio of 1: 1712.5 trametinib:pazopanib (nM), calculated from the cMax of both drugs. Results demonstrated there was no significant effect of single agent trametinib at any concentration on the inhibition of CHL-1 or OM413 cell viability (One-way ANOVA, P=0.2762 and P=0.6850 respectively, Figure 5.18 A and 5.20 A). Conversely, concentrations of trametinib at 2.5-10nM in WM-164 and 0.5-10nM in UPMD2 induced significant inhibition of cell viability (One-way analysis of variance with Dunnett's multiple comparison post hoc test, or Kruskal-Wallis test with Dunn's multiple comparison post hoc test, *p<0.05, **P<0.01, ***P<0.001, Figures 5.19 A and 5.21 A), highlighting the relative increased sensitivity of cutaneous and uveal melanoma cells bearing activating MAPK mutations to trametinib compared to B-RAF/N-Ras or GNAQ/11 wild-type cell lines.

Studies of single agent pazopanib-induced inhibition of melanoma cell viability, revealed dose dependant significant inhibition of CHL-1 and UPMD2 cells from 4.28 – 17.13µM (One-way ANOVA with Dunnett's post hoc correction, *P<0.05 and ***P<0.001 respectively, Figure 5.18B and 5.21B) and in WM-164 (Figure 5.19B) and OM413 (Figure 5. 20B) cells at doses 8.56-

17.12µM (One-way ANOVA with Dunnett's post hoc correction, **P<0.01 and ***P<0.001 respectively), collectively suggesting pazopanib-induced inhibition of melanoma cell viability is independent of B-RAF/N-Ras or GNAQ/GNA11 mutational status.

Next, the effects of combined trametinib and pazopanib were compared in which results demonstrated the significant inhibition of CHL-1, OM413 and UPMD2 cell viability in response to combined treatment over a fixed dose ratios of 2.5nM:4.28µM to 10nM:17.12µM (trametinib:pazopanib) (One-way ANOVA with Dunnett's post hoc correction, *P<0.05, **P<0.01, ***P<0.001, Figure 5.18 C, 5.20 C and 5.21 C), and in response to fixed dose ratio trametinib:pazopanib in WM-164 cells from 5nM:8.56µM to 10nM:17.12µM (One-way ANOVA with Dunnett's post hoc correction, ***P<0.001, Figure 5.10 C). Collectively these results demonstrate a dose-dependent inhibition of melanoma cell viability *in vitro* by dual MEK and VEGFR2 blockade with combined trametinib and pazopanib treatment.

To determine the significance of the effect of combined trametinib and pazopanib on the inhibition of cell viability with that derived by single agent treatment, one-way analysis of variance was performed with Bonferroni post hoc correction at each dose ratio comparing results derived using each drug alone (Figures 5.18-21 D and Appendix 3, Appendix Table 3.1-3.4). Results confirmed the dose dependant inhibition of cell viability in CHL-1, WM-164, OM413 and UPMD2 cell lines by single agent treatment (Figures 5.18-21D). Inhibition of CHL-1, WM-164 and OM413 cell viability was significantly potentiated by combined trametinib and pazopanib at the fixed dose ratios of 5nM:8.56µM and 10nM:17.12µM (trametinib:pazopanib) compared to single agents trametinib and pazopanib at the same concentrations (One-way ANOVA with Bonferroni post hoc correction, *P<0.05, **P<0.01,***P<0.001, Figures 5.18-20 D, and Appendix Table 3.1-3.3). However, in UPMD2 cells although combined trametinib:pazopanib potentiated trametinib-induced inhibition of cell viability at fixed doses of 5nM:8.56µM to 10nM:17.12µM (One-way ANOVA with Bonferroni post hoc correction, *P<0.05, **P<0.01,***P<0.001, Figure 5.21 D and Appendix Table 3.4), combined trametinib:pazopanib only potentiated pazopanib-induced inhibition of cell viability at a fixed dose ratio of 5nM:8.56µM (One-way ANOVA with Bonferroni post hoc correction, *P<0.001 Figure 5.21 D and Appendix Table 3.4) Collectively these data highlight the potentiation of the inhibition of melanoma cell viability by combined trametinib and pazopanib.

Since, there was no significant inhibition of cell viability by either single agent or combined at fixed dose ratios of 0.5nM:0.85μM, and 1nM:1.71μM trametinib:pazopanib, these drug combination ratios were therefore selected to evaluate the effects of combined MEK and VEGFR2 blockade on CHL-1, WM-164 and OM413 CXCR4-CXCL12 mediated chemotaxis.

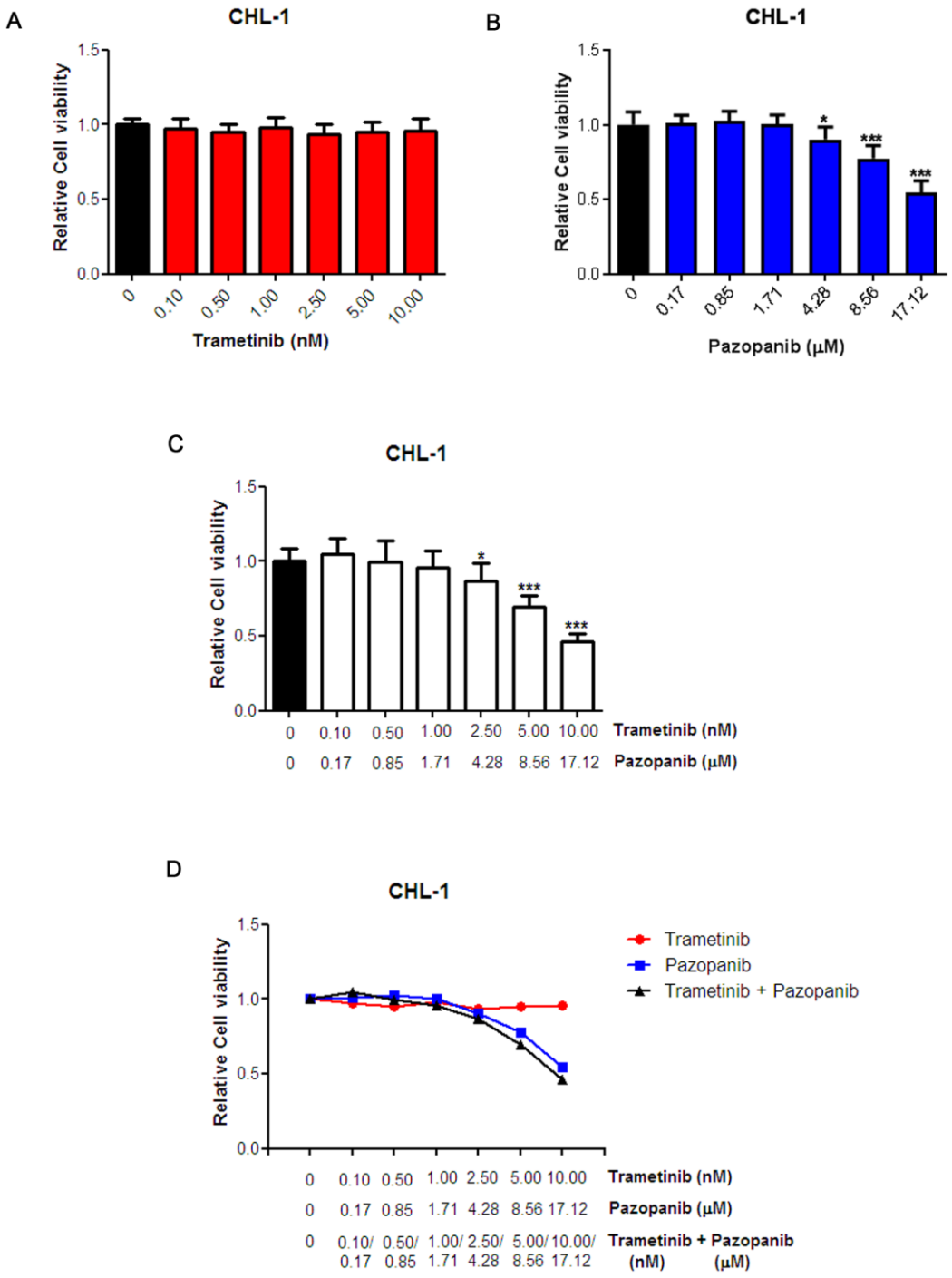

Figure 5.18 Combined Trametinib and Pazopanib Potentiates the Inhibition of CHL-1 Cutaneous Melanoma Cell Viability

*A.) Relative cell viability of CHL-1 cutaneous metastatic melanoma cells following treatment of 0.10-10nM trametinib or B.) following treatment of 0.17-17.12 μM pazopanib or C.) following combined treatment of trametinib and pazopanib at a fixed dose ratio of 1:1712 (relative to control untreated cells) for 16 hours. Each bar represents mean cell viability of 12 replicates from 3 independent experiments + SD. Statistics acquired by one-way ANOVA with Dunnett's post hoc correction *P<0.05 and ***P<0.001. D.) Relative cell viability of CHL-1 cutaneous metastatic melanoma cells following treatment for 16 hours with single agent trametinib (0.1-10nM), or pazopanib (0.17-17.12μM) or with both agents at a fixed dose ratio of 1:1712 (relative to untreated control) for 16 hours. Each point represents mean cell viability of 12 replicates from 3 independent experiments.*

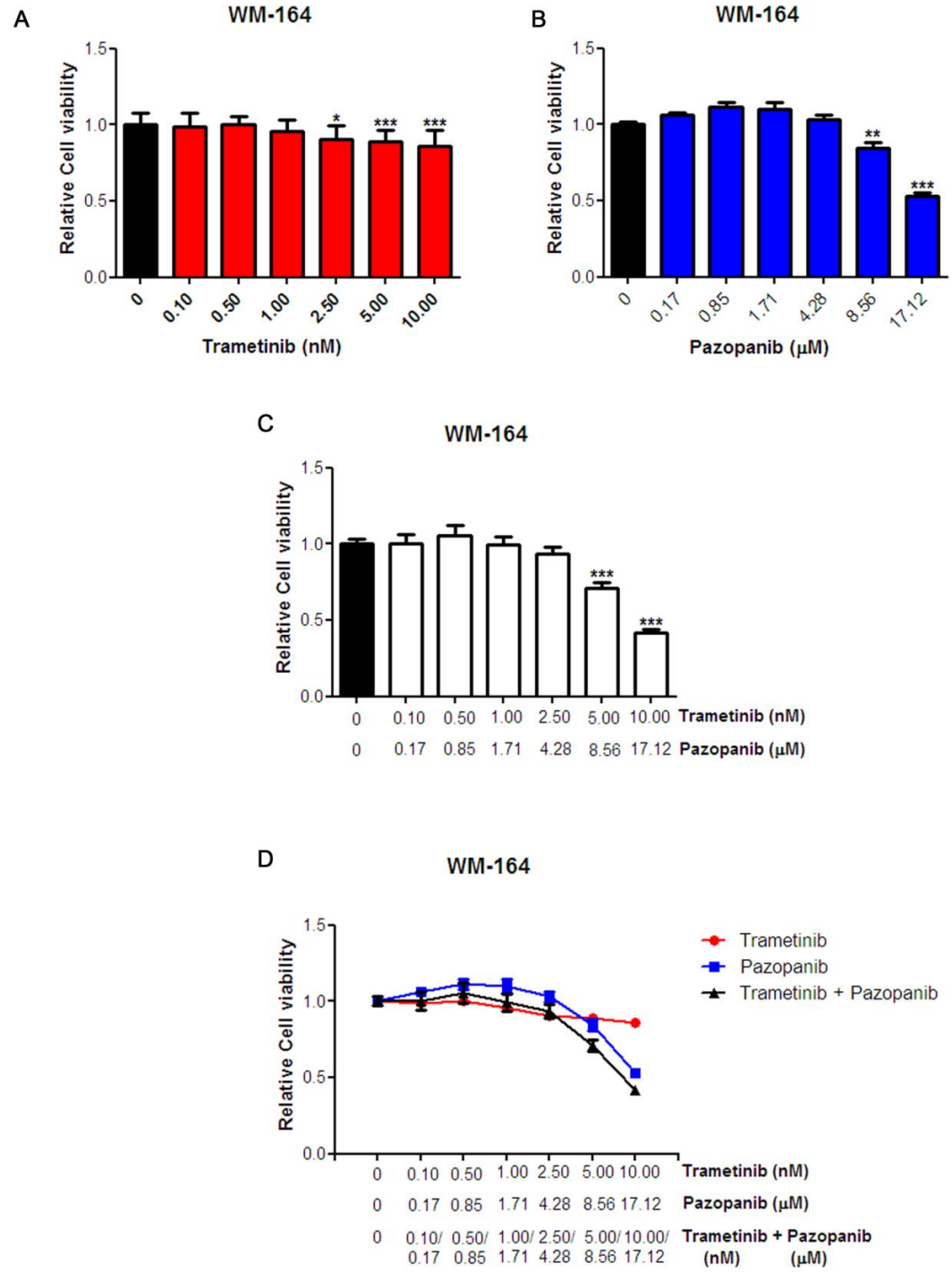

Figure 5.19 Combined Trametinib and Pazopanib Potentiates the Inhibition of WM-164 Cutaneous Melanoma Cell Viability

*A.) Relative cell viability of WM-164 cutaneous metastatic melanoma cells following treatment of 0.10-10nM trametinib or B.) following treatment of 0.17-17.12 µM pazopanib or C.) following combined treatment of trametinib and pazopanib at a fixed dose ratio of 1:1712 (relative to control untreated cells) for 16 hours. Each bar represents mean cell viability of 12 replicates from 3 independent experiments + SD. Statistics acquired by one-way ANOVA with Dunnett's post hoc correction **P<0.05, **P<0.01 and ***P<0.001. D.) Relative cell viability of WM-164 cutaneous metastatic melanoma cells following treatment for 16 hours with single agent trametinib (0.1-10nM), or pazopanib (0.17-17.12µM) or with both agents at a fixed dose ratio of 1:1712 (relative to untreated control) for 16 hours. Each point represents mean cell viability of 12 replicates from 3 independent experiments.*

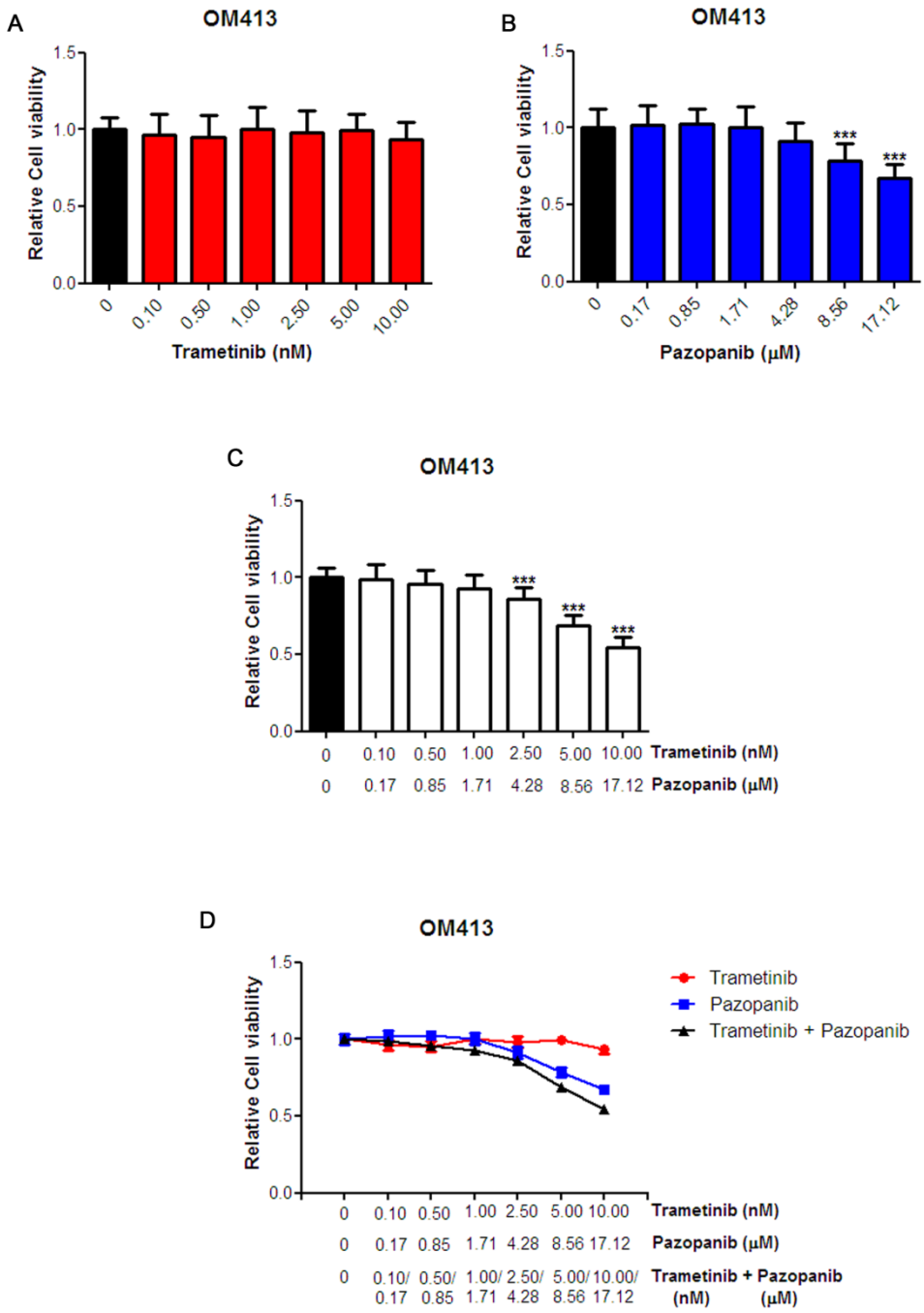

Figure 5.20 Combined Trametinib and Pazopanib Potentiates the Inhibition of OM413 Uveal Melanoma Cell Viability

*A.) Relative cell viability of OM413 uveal metastatic melanoma cells following treatment of 0.10-10nM trametinib or B.) following treatment of 0.17-17.12 μM pazopanib or C.) following combined treatment of trametinib and pazopanib at a fixed dose ratio of 1:1712 (relative to control untreated cells) for 16 hours. Each bar represents mean cell viability of 12 replicates from 3 independent experiments + SD. Statistics acquired by one-way ANOVA with Dunnett's post hoc correction ***P<0.001. D.) Relative cell viability of OM413 uveal metastatic melanoma cells following treatment for 16 hours with single agent trametinib (0.1-10nM), or pazopanib (0.17-17.12μM) or with both agents at a fixed dose ratio of 1:1712 (relative to untreated control) for 16 hours. Each point represents mean cell viability of 12 replicates from 3 independent experiments.*

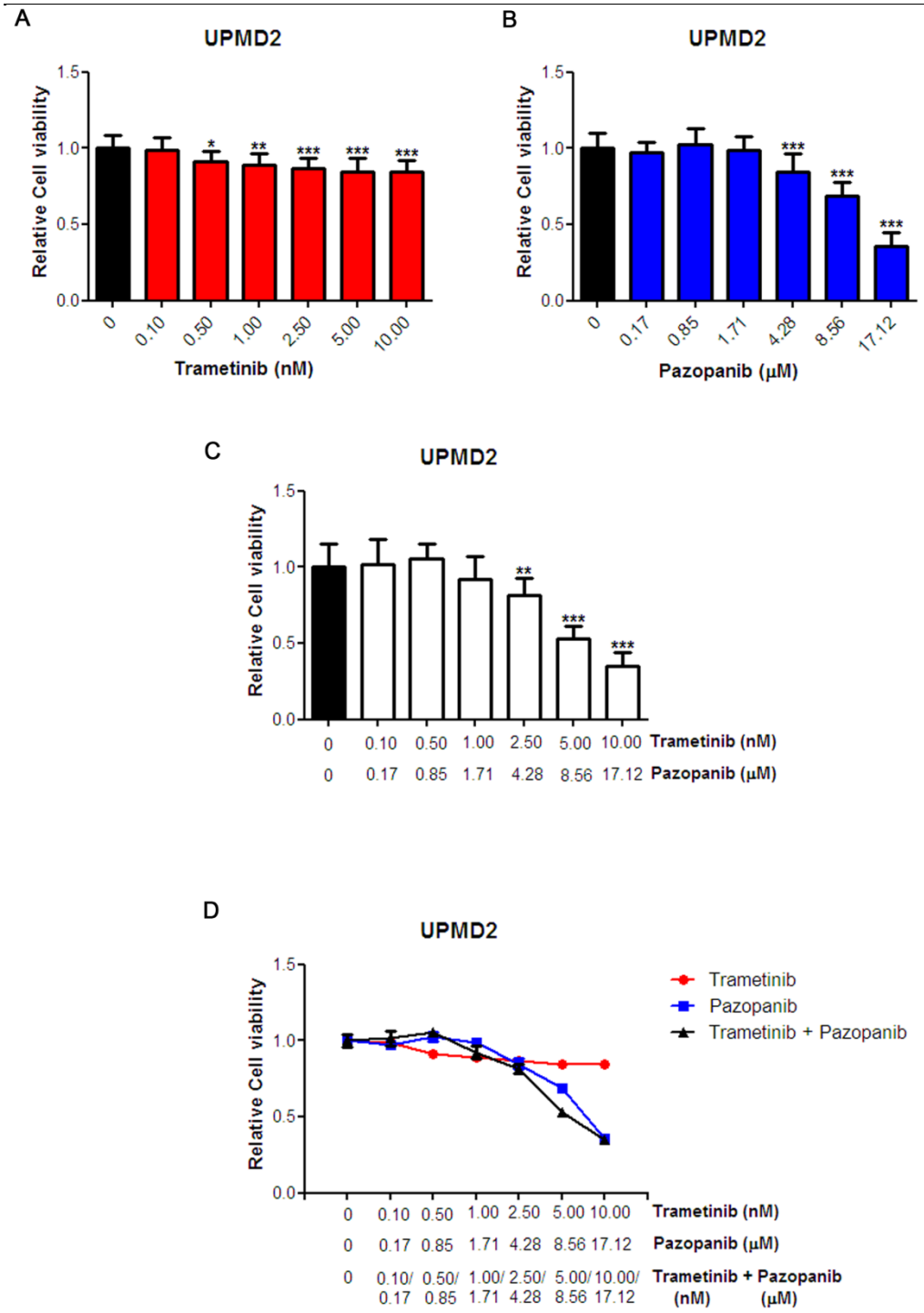

Figure 5.21 Combined Trametinib and Pazopanib Potentiates the Inhibition of UPMD2 Uveal Melanoma Cell Viability

*A.) Relative cell viability of UPMD2 uveal metastatic melanoma cells following treatment of 0.10-10nM trametinib or B.) following treatment of 0.17-17.12 µM pazopanib or C.) following combined treatment of trametinib and pazopanib at a fixed dose ratio of 1:1712 (relative to control untreated cells) for 16 hours. Each bar represents mean cell viability of 12 replicates from 3 independent experiments + SD. Statistics acquired by one-way ANOVA with Dunnett's post hoc correction *P<0.05, **P<0.01, and ***P<0.001. D.) Relative cell viability of UPMD2 uveal metastatic melanoma cells following treatment for 16 hours with single agent trametinib (0.1-10nM), or pazopanib (0.17-17.12µM) or with both agents at a fixed dose ratio of 1:1712 (relative to untreated control) for 16 hours. Each point represents mean cell viability of 12 replicates from 3 independent experiments.*

Studies of the effect of combined trametinib and pazopanib (at fixed dose ratios of 0.5nM:0.85µM, or 1nM:1.71µM, and 5nM:8.56µM trametinib:pazopanib) on CHL-1, and OM413 CXCR4-CXCL12 mediated chemotaxis revealed firstly, the significant inhibition of migration towards 10nM recombinant CXCL12 by 5nM trametinib and 8.56µM pazopanib as single agents (Kruskal-Wallis test with Dunn's multiple comparison post hoc test, *P<0.05 or ***P<0.001, Figure 5.22 A-B and 5.24 A-B), and also by single agent trametinib at 1nM and 5nM or single agent pazopanib at 1.71µM and 8.56µM in WM-164 cells (Kruskal-Wallis test with Dunn's multiple comparison post hoc test, *P<0.05, Figure 5.23 A-B). Further, in response to combined trametinib and pazopanib at all fixed dose ratios in all cells lines there was a significant inhibition of CXCR4-CXCL12 chemotaxis, with the exception of 0.5nM:0.85µM trametinib:pazopanib in OM413 cells (Kruskal-Wallis test with Dunn's multiple comparison post hoc test, **P<0.01, ***P<0.001, Figure 5.22C-5.24C).

To determine the significance of combined trametinib and pazopanib on the inhibition of CXCR4-CXCL12 mediated chemotaxis with that derived by single agent treatment, one-way analysis of variation was performed with Bonferroni post hoc correction at each drug ratio, comparing results derived from either drug alone (Figures 5.22 D - 5.24 D, and Appendix Table 3.5-3.7). Results demonstrated combined trametinib and pazopanib significantly potentiated the inhibition of CXCR4-CXCL12 mediated chemotaxis of CHL-1 and WM-164 cells over either drug alone at all fixed dose ratios (One-way ANOVA with Bonferroni post hoc correction, ***P<0.001, Figure 5.22 D-5.23 D and Appendix Table 3.5 and 3.6). While, in OM413 cells significant potentiation of the inhibition of CXCR4-CXCL12 mediated chemotaxis, was only observed with combined trametinib:pazopanib at fixed dose ratios of 1nM:1.71µM (One-way ANOVA with Bonferroni post hoc correction, *P<0.05 and **P<0.01, Figure 5.24 D, Appendix Table 3.7).

Collectively, these data demonstrate that in cutaneous and uveal metastatic melanoma cell lines the combination of trametinib and pazopanib significantly increases the inhibition of cell chemotaxis than either drug does alone, at concentrations that do not affect cell viability. Furthermore, these data additionally propose that dual inhibition of MEK and VEGFR2 maybe a more effective way of preventing cutaneous and uveal melanoma migration.

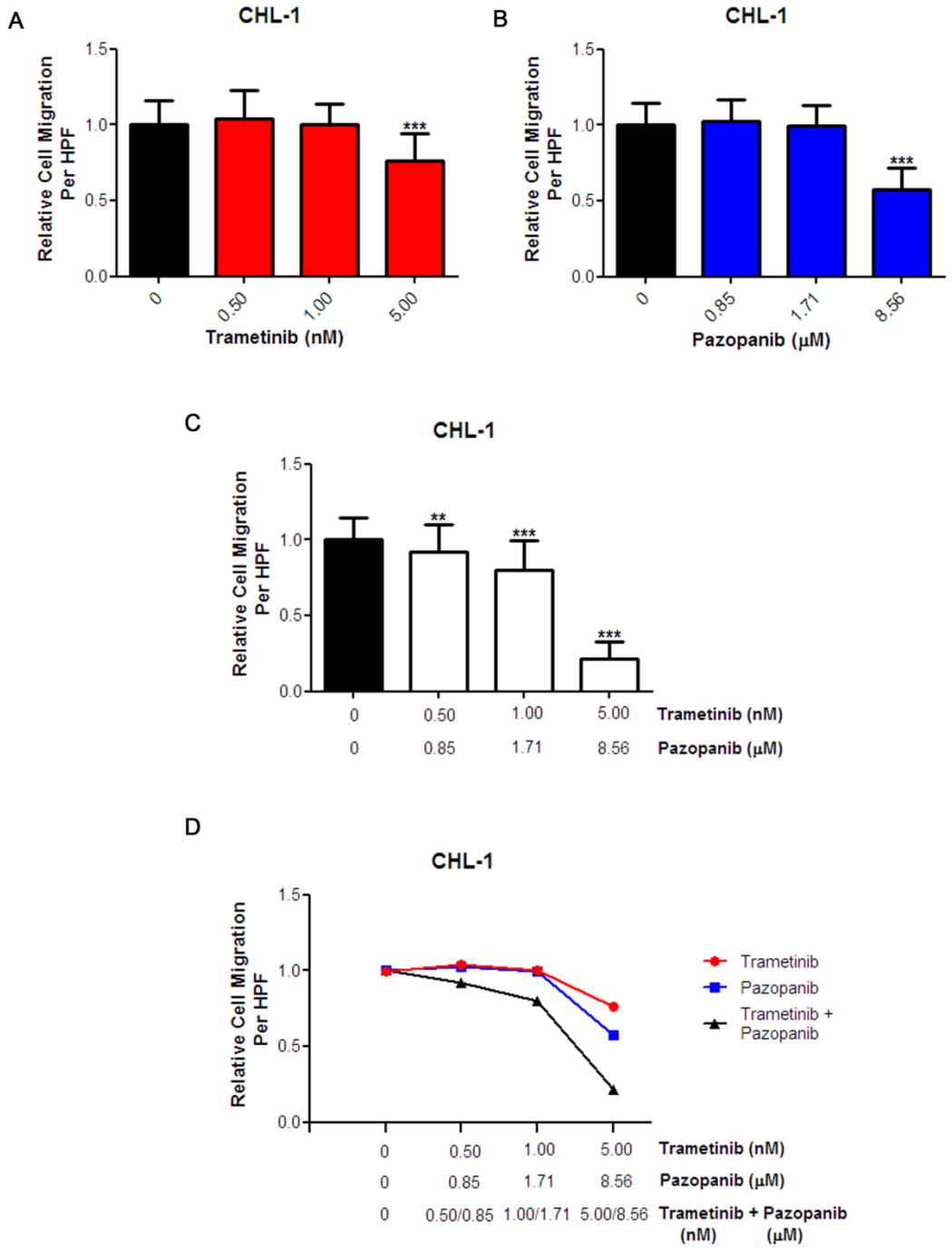

Figure 5.22 Combined Trametinib and Pazopanib Potentiates the Inhibition of CHL-1 Cutaneous Melanoma CXCR4-CXCL12 Chemotaxis

*Relative CHL-1 metastatic cutaneous melanoma cells migration (per high powered field, HPF) towards 10nM recombinant CXCL12 in the presence of A.) 0.50-5.00nM trametinib, or B.) 0.85-8.56μM pazopanib, or C.) in the presence of trametinib and pazopanib at a fixed dose ratio of 1:1712 for 16 hours, expressed relative to cell migration towards recombinant CXCL12 of untreated cells. Each bar represents the mean HPF of 6 replicate filters from 2 independent experiments + SD. Statistic acquired by Kruskal-Wallis test with Dunn's multiple comparison post hoc correction, where **P<0.01, ***P<0.001. D.) Relative CHL-1 metastatic cutaneous melanoma cells migration (per HPF) towards 10nM recombinant CXCL12 for 16 hours with single agent trametinib (0.5-5nM) or pazopanib (0.85-8.56μM), or with both agents at a fixed dose ratio of 1:1712.*

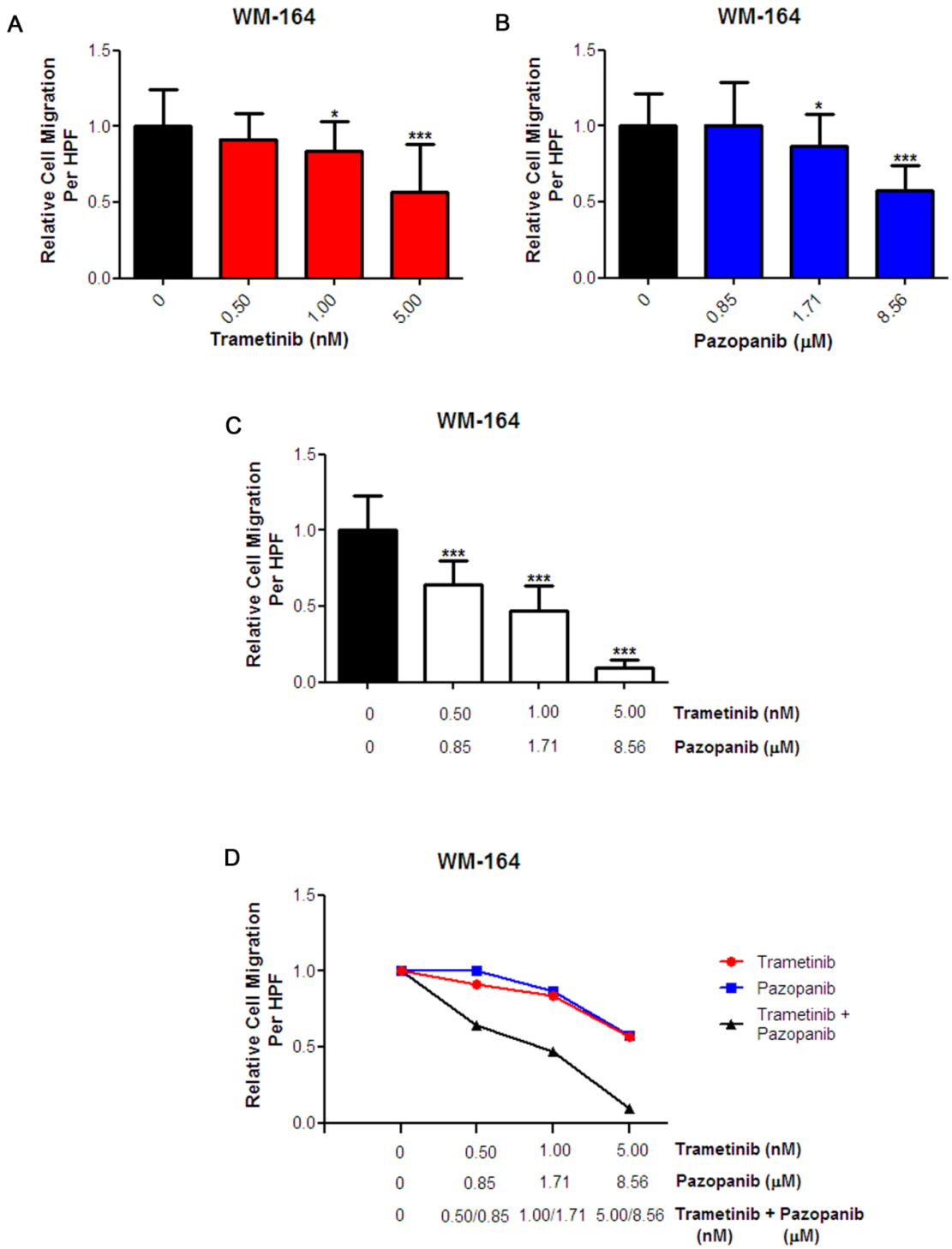

Figure 5.23 Combined Trametinib and Pazopanib Potentiates the Inhibition of WM-164 Cutaneous Melanoma CXCR4-CXCL12 Chemotaxis

*Relative WM-164 metastatic cutaneous melanoma cells migration (per high powered field, HPF) towards 10nM recombinant CXCL12 in the presence of A.) 0.50-5.00nM trametinib, or B.) 0.85-8.56μM pazopanib, or C.) in the presence of trametinib and pazopanib at a fixed dose ratio of 1:1712 for 16 hours, expressed relative to cell migration towards recombinant CXCL12 of untreated cells. Each bar represents the mean HPF of 6 replicate filters from 2 independent experiments + SD. Statistic acquired by Kruskal-Wallis test with Dunn's multiple comparison post hoc correction, where *P<0.05, ***P<0.001. D.) Relative WM-164 metastatic cutaneous melanoma cells migration (per HPF) towards 10nM recombinant CXCL12 for 16 hours with single agent trametinib (0.5-5nM) or pazopanib (0.85-8.56μM), or with both agents at a fixed dose ratio of 1:1712.*

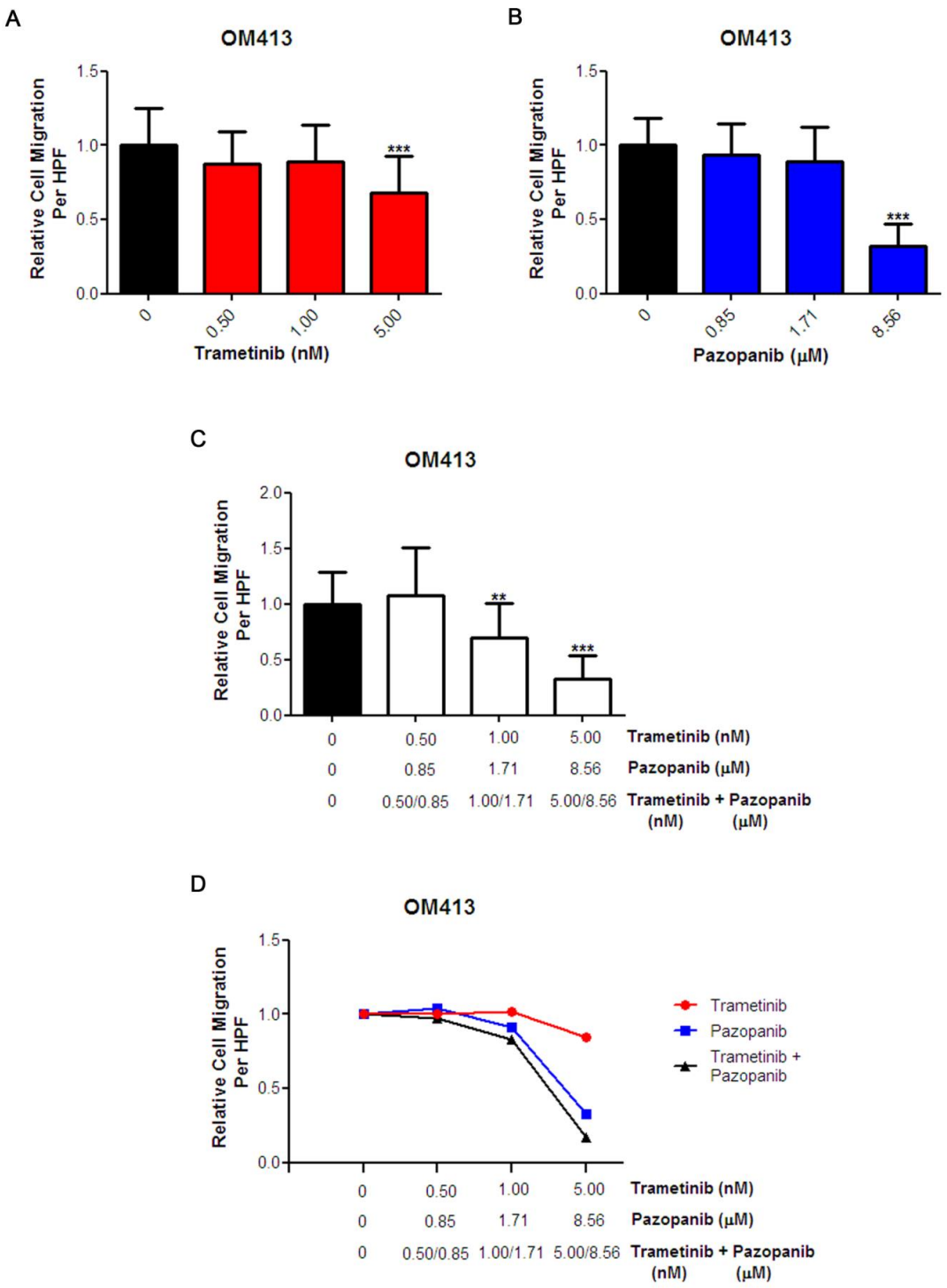

Figure 5.24 Combined Trametinib and Pazopanib Potentiates the Inhibition of OM413 Uveal Melanoma CXCR4-CXCL12 Chemotaxis

*Relative OM413 metastatic uveal melanoma cells migration (per high powered field, HPF) towards 10nM recombinant CXCL12 in the presence of A.) 0.50-5.00nM trametinib, or B.) 0.85-8.56μM pazopanib, or C.) in the presence of trametinib and pazopanib at a fixed dose ratio of 1:1712 for 16 hours, expressed relative to cell migration towards recombinant CXCL12 of untreated cells. Each bar represents the mean HPF of 6 replicate filters from 2 independent experiments + SD. Statistic acquired by Kruskal-Wallis test with Dunn's multiple comparison post hoc correction, where **P<0.01, ***P<0.001. D.) Relative OM413 metastatic uveal melanoma cells migration (per HPF) towards 10nM recombinant CXCL12 for 16 hours with single agent trametinib (0.5-5nM) or pazopanib (0.85-8.56μM), or with both agents at a fixed dose ratio of 1:1712.*

5.3 Discussion

Angiogenesis is an important step for melanoma progression, hence therapeutic attempts have been made to target VEGF ligand with bevacizumab the monoclonal antibody, however no improvement in overall survival of patients with cutaneous metastatic melanoma has been made (Corrie *et al.*, 2014). Such finding has led to current clinical interest in specific VEGFR anti-angiogenic therapy with agents such as pazopanib a new generation small molecule inhibitor, with particular efficacy to VEGFR2. In this context the analysis of VEGFR2 expression in primary melanoma tissues, may allow for biomarker identification and stratification of patients most likely to benefit from treatment with pazopanib. In cutaneous melanoma, the validity of targeting VEGFR2 expression by tumour cells is debatable since there is contradictory evidence for its expression. Although previous data report increased expression of VEGFR2 in cutaneous melanomas compared to benign naevi, supporting the emergence of an angiogenic phenotype facilitating tumour progression (Mehnert *et al.*, 2010), data from the present study did not corroborate these findings. In fact, the present data demonstrated little, if any difference between VEGFR2 expression in any AJCC stage primary melanoma and cutaneous naevi, or between primary melanomas that remained localised and those which subsequently metastasised (Figure 5.5 A and B). Such lack of differential VEGFR2 expression may perhaps be explained by extremely low levels of expression observed overall. Three quarters of all analysed primary cutaneous melanomas expressed VEGFR2 in <1% tumour cells with weak immunoreactivity, and it could be argued that this may reflect experimental noise or irrelevant expression since previous studies report only VEGFR2 expression of >10% are considered as positive expression (Liu *et al.*, 2008). Nevertheless the overall observed low levels of VEGFR2 expression aligns with results derived from a further large study cohort reporting detectable VEGFR2 expression in <10% of primary melanomas and thereby suggesting only a few patients would benefit from the anti-proliferative effects of anti-angiogenic therapies that target VEGFR2 (Molhoek *et al.*, 2011).

A common paradigm held for over 40 years is that angiogenesis is required for tumours to exceed a volume of 1-2mm^3 (Gimbrone *et al.*, 1972) however, there is evidence that angiogenesis is actually required at a much earlier pre-malignant stage in most cancers including melanoma. In this context, studies of pre-malignant benign melanocytic naevi and dysplastic melanocytic naevi demonstrate an increase in microvascular density occurs,

suggesting angiogenesis is active at early stages of tumour development (Barnhill RL et al., 1992). It may therefore be the case that VEGFR2 upregulation occurs at a pre-malignant stage and hence account for minimal changes in expression levels at later stages of melanoma progression.

Data from the present study additionally demonstrated a lack of any association between VEGFR2 expression in primary cutaneous melanomas and B-RAF/N-Ras mutational status (Figure 5.5 C). Although there are no reports of any such association, the presence of a B-RAF mutation has been correlated with micro and lymphovascular density, suggesting some association of the presence of an activating B-RAF mutation with markers of angiogenesis in cutaneous melanoma (Aung *et al.*, 2015). Furthermore, secretion of VEGF by melanoma cells is reported to be regulated, at least in part, by oncogene-mediated activation or tumour suppressor- mediated inactivation (Arbiser *et al.*, 1997; Arbiser, 2004), with supporting studies demonstrating MAPK-induced activation in cutaneous melanocytes, leads to tumourigenesis, VEGF and MMP secretion and an angiogenic switch (Govindarajan *et al.*, 2003). Collectively these reports thus further support a putative association between hyper-activation of MAPK signalling and angiogenesis, not corroborated by this study.

Data from the present study were again unable to find any correlation between CXCR4/CXCL12 and VEGFR2 expression. It may be that the level of VEGF secretion by melanomas is more correlative than VEGF receptor expression, since most studies indicating crosstalk between CXCR4-CXCL12 and VEGF-VEGFR2 cell signalling support a role for VEGF ligand (Bachelder *et al.*, 2002; Zagzag *et al.*, 2005; Hong *et al.*, 2006; Oda *et al.*, 2006; Ottaiano *et al.*, 2006; Franco *et al.*, 2010). Observations of strong VEGFR2 expression in capillary endothelial cells within melanomas or within the dermis, nevertheless suggest anti-angiogenic therapies may display some degree of targeting ability at the primary site (Figure 5.3). Interestingly, keratinocytes within the surrounding epidermis of primary melanomas expressed low levels of VEGFR2, a result also confirmed by the immunofluorescent expression of VEGFR2 observed in primary keratinocytes *in vitro*. Keratinocytes are known to express both VEGFR2 and VEGFR1 and use VEGF cell signalling in an autocrine manner to maintain their proliferative and migratory capacity in normal skin homeostasis (Man *et al.*, 2006). A significant positive correlation between VEGFR2 expression in the epidermis overlying melanomas and that expressed by tumour cells themselves was also observed (Figure 5.6 C),

suggesting that VEGFR2 expression maybe regulated by a global source within the tumour microenvironment. This could be the result of paracrine agents released by the tumour, including feasibly VEGF.

In Cutaneous melanoma like many cancers, the lymphatic vessels represent the major route for metastatic dissemination, with sentinel lymph nodes, serving as an important prognostic indicator. Little is known about the mechanism by which melanoma cells enter the lymphatic system; however, several important factors including VEGF-C and VEGF-D have been identified as important facilitators of lymphangiogenesis, where tumoural expression correlates with increased lymphatic density and the likelihood of lymph node metastasis (Schietroma *et al.*, 2003; Dadras *et al.*, 2005). The major receptor that binds VEGF-C and D is VEGFR3, and blocking this interaction negates the ability of VEGF-C and VEGF-D to promote lymphangiogenesis and metastasis to lymph nodes (Mandriota *et al.*, 2001b; Veikkola *et al.*, 2001). However, the N–terminal and C-terminal pro-peptides of VEGF-C and VEGF-D can undergo proteolytic cleavage to a mature form that bind VEGFR2 that may induce circumferential lymphatic vessel growth but not sprouting (Nagy *et al.*, 2002; Wirzenius *et al.*, 2007). Although the role of VEGFR3 on lymphatic endothelial cells is well established in the promotion of lymphangiogenesis, the role for VEGF receptors on tumour cells in this process is nevertheless ambiguous (Mehnert *et al.*, 2010). To elute a possible role for VEGFR2 expression by melanoma cells and lymph node metastasis, immunohistochemical expression was quantified in primary cutaneous melanomas and patient matched metastatic lymph nodes. Results revealed no significant difference between VEGFR2 expression in primary tumours and metastatic lymph nodes, suggesting that VEGFR2 expression in metastasised tumours within the lymph nodes is preserved at a similar level to that in primary tumours and thereby proposing a potential role for VEGFR2 in the maintenance of a metastatic phenotype (Figure 5.8). Interestingly levels of VEGFR2 expression in primary tumours varied between tumour cohorts, being greater in the Durham compared to the Newcastle cohort. However, it should be noted that the Durham cohort of primary cutaneous melanomas contained only eventual AJCC stage III tumours that subsequently metastasized to a regional lymph nodes and hence was not representative of a complete tumour cohort containing a range of differing AJCC stage tumours and tumours which remained localised. While the inclusion of eventual AJCC stage I/II tumours within the Durham melanoma cohort would be required to validate these findings, results from the analysis of this cohort nevertheless suggest VEGFR2 maybe

upregulated in the primary cutaneous tumours of patients who subsequently develop metastatic disease (Figure 5.9), and supports the notion that VEGFR2 promotes tumour progression and metastatic potential (Streit and Detmar, 2003).

Unlike skin, the choroid of the eye is a highly oxygenated microenvironment making the role of angiogenesis in uveal melanoma likely distinct from cutaneous melanoma (Stitt and Gardiner, 2002). Although the presence of VEGF in uveal melanoma tumours has been reported to correlated with poor prognostic indicators such as necrosis, scleral invasion, larger tumours, mixed and epithelioid cell type, suggestive of some clinicopathological value, there are however no reports of any association of VEGF expression by tumours with metastasis or patient survival (Sheidow *et al.*, 2000; Boyd *et al.*, 2002b; Yang *et al.*, 2014). Most uveal melanoma cell lines secrete abundant amounts of VEGF-A, that acts on VEGFR1 or VEGFR2 receptors to sustain autocrine proliferation and migration (Notting *et al.*, 2006; Logan *et al.*, 2013; Koch *et al.*, 2014b). However, the expression of VEGF receptors by primary tumours has only been identified in one study in which Stitt *et al* reported the expression of VEGFR1 and 2 as well as VEGF-A in enucleated eyes derived from both retinoblastoma and uveal melanomas, and with elevated expression also noted in the choroid, retina and iris compared to expression in normal eyes (Stitt *et al.*, 1998). Although Stitt *et al* did not quantify the level of VEGFR2 expression or relate expression to any clinicopathological features; this study nevertheless identified the potential for VEGFR2 upregulation in uveal melanomas and autocrine VEGF cell signalling. In the present study, although VEGFR2 expression was clearly and significantly increased in primary uveal melanomas (in albeit a small cohort) compared to expression by cutaneous melanomas, there was however, no apparent association with chromosome 3 status. Collectively these data thus suggest rational for anti-angiogenic therapy in uveal melanoma to prevent both neovascularisation as well as autocrine cell signalling.

In the present study, immunohistochemical expression of VEGFR2 in melanomas was detected in the cytoplasmic compartment, suggesting the receptor has been activated and is internalised upon ligand binding. However, the subcellular localisation of immunofluorescent VEGFR2 expression differed between melanoma cell lines, with most having equivocal cytoplasmic and nuclear expression, but in which WM-164 cutaneous and OMM2.3 uveal metastatic melanoma cell lines showed predominant nuclear expression. In contrast, VEGFR2 expression in the metastatic uveal melanoma cell line UPMD2 was predominantly

cytoplasmic. While not a primary aim, the diverse staining patterns for VEGFR2 expression in cutaneous or uveal melanoma cell lines warranted more investigation (Figure 5.12). The stark contrast in subcellular localisation of VEGFR2 between melanoma cell lines could represent receptor activation. Previous studies in both endothelial and cancer cells have demonstrated the translocation of VEGFR2 upon stimulation, to the cell nucleus where it may activate its own promoter *in vivo* (Blazquez *et al.*, 2006; Constantino Rosa Santos *et al.*, 2007; Domingues *et al.*, 2011), which would account for nuclear localisation of VEGFR2. Future studies to confirm this hypothesis could include ELISA assays to determine VEGF secretion by melanoma cell lines, or the treatment of cells with recombinant VEGF to determine any correlation with receptor activation and subcellular localisation.

Increasing the seeding density of melanoma cell lines incorporated in the present study also resulted in the increased immunofluorescent expression of VEGFR2 (Appendix 2). This may be mediated by increased VEGF availability secreted from a greater number of cells that may promote the nuclear localisation of VEGFR2 and constitutional transcriptional activation of the VEGFR2 promoter by the receptor itself (Domingues *et al.*, 2011). Remarkably, over time, however, even though the overall expression of VEGFR2 increased, nuclear subcellular localisation was lost and receptor expression became enhanced within the cytoplasm or most obviously at areas of cell-cell contact as culture confluence was reached. Studies have shown that VEGFR2 in endothelial cells accumulates at cell–cell contacts forming complexes with VE-cadherin/β-catenin, blocking the proliferative effect of VEGF, as a mechanism of cell contact inhibition (Hendrix *et al.*, 2001; Lampugnani *et al.*, 2006). This may therefore explain the accumulation of VEGFR2 expression at cell-cell contacts in cultures of confluent melanoma cells, although further studies are required to confirm this hypothesis (Hendrix *et al.*, 2001; Lampugnani *et al.*, 2006).

Collectively the immunohistochemical and immunofluorescent analysis of VEGFR2 expression in cutaneous and uveal melanoma in the present study, supported the rational for targeting VEGFR2 to prevent paracrine and possible autocrine VEGF signalling and led to subsequent studies with pazopanib. Treatment of both cutaneous and uveal melanoma cells with pazopanib resulted in dose dependent inhibition of cell viability, supporting studies of other VEGF receptor tyrosine kinase inhibitors (Eustace *et al.*, 2008; Li *et al.*, 2016). In addition, pazopanib potently inhibited cutaneous and uveal melanoma cell CXCR4-CXCL12 mediated

cell chemotaxis, at concentrations that did not affect cell viability, emphasising the capability for VEGFR2 inhibition to prevent melanoma migration, but also highlighting the crosstalk between these two signalling pathways. Interestingly the chemotaxis of the metastatic uveal melanoma cell line OM413 was most sensitive to pazopanib-induced inhibition, yet this cell line demonstrated the lowest levels of VEGFR2 expression, at both protein and mRNA level. This may indicate that the mechanism of action of pazopanib in this cell line, maybe through the targeting of a different tyrosine kinase receptor to VEGFR2. To explore further the mechanism of action by which pazopanib inhibits CXCR4-CXCL12 chemotaxis, siRNA was used to prevent translation of VEGFR2 in CHL-1 and WM-164 cutaneous melanoma cell lines. Results from these studies indicated VEGF treatment of these melanoma cell lines increased basal cell migration, confirming the ability of VEGF signalling to promote cell migration not only of endothelial cells but also of melanoma cells themselves (Erhard H *et al.*, 1997; Bougatef *et al.*, 2010). On the other hand, knockdown of VEGFR2 in WM-164 cells resulted in significantly reduced cell migration, suggesting that VEGFR2 cell signalling is also involved in WM-164 cell migration. However, this effect was not significant in B-RAF/N-Ras wild-type CHL-1 cells suggesting that VEGFR2 activation may not be the dominant migratory pathway for this cell line. Nevertheless, pazopanib only reduced cell migration of cells transfected with non-targeting control siRNA, confirming that pazopanib-mediated inhibition of cell migration is due to its effective VEGFR2 inhibition.

Both MEK inhibition and VEGFR2 inhibition reduced melanoma cell viability and chemotaxis as single agents (Section 5.2.6), however monotherapy targeting the MAPK signalling or angiogenesis has historically lead to a plethora of resistant mechanisms and reactivation of downstream signalling (Ellis and Hicklin, 2008a; Welsh *et al.*, 2016). It is frequently evidenced that a combination of drugs targeting the same pathways simultaneously or combining drugs that target alternative pathways is more efficacious with the reduced emergence of drug-induced resistance (Lang *et al.*, 2008; Dankort *et al.*, 2009; Meng *et al.*, 2010; Flaherty *et al.*, 2012a; Carlino *et al.*, 2014). Interestingly a recent paper by Friedman *et al* demonstrated that cediranib (a pan VEGF tyrosine kinase receptor inhibitor) was able to re-sensitise B-RAF inhibitor resistant melanoma cell lines, to the cytotoxic effect of PLX4720 (Friedman *et al.*, 2015). Furthermore Friedman *et al* demonstrated the synergistic effects of combined VEGFR antagonists and B-RAF inhibitors in a large panel of melanoma cell lines (Friedman *et al.*, 2015), supporting a potential rationale for combined MEK and VEGFR2 inhibition as a more

efficacious therapeutic strategy for metastatic melanoma compared to single agent therapy. Results from the present study additionally demonstrated the potentiation of combined trametinib and pazopanib on the inhibition of cutaneous and uveal melanoma cell viability, as well as the inhibition of CXCR4-CXCL12-mediated chemotaxis (Section 5.2.6). Whether or not these effects were synergistic or additive requires further analysis. In addition, it remains to be determined whether pazopanib has the capacity like trametinib to induce pro-survival autophagy to counteract its apoptotic capacity. Nevertheless, results derived from the present study of combined trametinib and pazopanib highlight the potential added benefits of dual MEK and VEGFR2 inhibition as an effective strategy to prevent melanoma migration and metastasis.

In summary, this chapter highlights VEGFR2 as not only an anti-angiogenic therapeutic target for endothelial cells but also as a target for cutaneous and uveal melanoma cells that promotes cell migration and which may be a feature of the metastatic melanoma phenotype. Furthermore, data highlight the capacity of pazopanib to inhibit CXCR4-CXCL12-mediated chemotaxis of melanoma cells through the inhibition of VEGFR2, an effect that can be enhanced by dual inhibition of MEK by trametinib.

5.4 Summary

- Primary cutaneous melanomas and cutaneous metastatic melanoma cell lines express VEGFR2.
- VEGFR2 expression is increased in primary cutaneous melanomas that subsequently metastasise to regional lymph nodes compared to expression in primary melanomas that remain localised.
- There is no correlation between VEGFR2 expression by cutaneous melanomas/melanoma cell lines and B-RAF/N-Ras mutational status.
- Metastatic uveal melanomas and uveal melanoma cell lines express VEGFR2.
- There is no correlation between VEGFR2 expression by uveal melanomas/melanoma cell lines and monosomy/disomy of chromosome 3
- Pazopanib inhibits cutaneous and uveal melanoma cell viability.
- Pazopanib inhibits CXCR4-CXCL12-mediated chemotaxis of cutaneous and uveal melanoma cells.
- Pazopanib targets VEGFR2 to prevent melanoma cell migration.
- Combined trametinib and pazopanib potentiate trametinib-induced inhibition of cutaneous and uveal melanoma cell viability.
- Combined trametinib and pazopanib significantly potentiates the inhibition of cutaneous melanoma CXCR4-CXCL12-mediated chemotaxis over and above either drug alone.
- Combined trametinib and pazopanib significantly potentiates the inhibition of uveal melanoma CXCR4-CXCL12-mediated chemotaxis compared to tametinib alone.
- Dual inhibition of MEK and VEGFR2 may be an effective strategy to limit melanoma migration and metastasis.

Chapter 6

Final Discussion and Concluding Remarks

80-90% of cutaneous and uveal melanomas bear genetic mutations in B-RAF/N-Ras or GNAQ/11 respectively, that occur early in melanoma pathogenesis and lead to hyper-activation of MAPK signalling pathway, driving cell cycle progression and autonomous cell proliferation of melanocytes (Davies *et al.*, 2002a; Van Raamsdonk *et al.*, 2010). The MAPK pathway is activated by signalling from several receptor tyrosine kinases including epidermal/platelet derived and fibroblast growth factor receptors, as well as the CXCR4-CXCL12 chemokine axis and VEGF-VEGFR receptor axis that provide melanomas with increased metastatic capabilities (Xia *et al.*; Robledo *et al.*, 2001; Wang *et al.*, 2008c). As a result, the MAPK pathway has become an attractive therapeutic target for both metastatic uveal and cutaneous melanoma, with MEK inhibition a corner stone to treatment of these tumours bearing activating mutations. However, although initially successful, the development of acquired resistance to MAPK inhibition in inevitable and with no clinically efficacious targeted treatments for B-RAF/N-Ras/GNAQ/GNA11 wild-type melanomas, metastatic disease is unvaryingly fatal and currently untreatable in both disease settings (Altekruse S.F *et al.*, 2010). Prognosis for patients with melanomas that progress from the primary site falls sharply, with tumours rapidly acquiring the ability to migrate, a key factor in driving tumour escape (Balch *et al.*, 2009a; S.B. Edge *et al.*, 2010). The ability to inhibit melanoma migration would not only provide a means to prevent tumour invasion within the primary tumour microenvironment, thereby reducing the likelihood of metastasis, but would also prevent metastatic spread itself. Defining novel strategies to impair melanoma migration is hence, an attractive therapeutic prospect.

The biggest player governing melanoma migration is the CXCR4-CXCL12 chemotaxis axis, with CXCR4 upregulated by melanomas and CXCL12 secreted from metastatic sites promoting organ specific metastasis (Muller *et al.*, 2001b; Murakami *et al.*, 2002; Toyozawa *et al.*, 2012). Yet, melanomas acquire other vital capabilities to facilitate tumour migration and the emergence of metastatic disease. Such capabilities include the process of angiogenesis, whereby the angiogenic mitogen, VEGF is secreted by tumour cells, and on binding to VEGF receptors on tumour associated endothelial cells promotes their migration, vital for new vessel development. However, it has been documented that melanomas themselves are able to upregulate the expression of VEGF receptors including VEGFR2, therefore enabling VEGF to drive tumour cell migration via an autocrine signalling mechanism (Mehnert *et al.*, 2010). Such

observations thus suggest both CXCR4-CXCL12 and VEGF-VEGFR2 signalling pathways are viable targets to inhibit melanoma migration. Given that components of both CXCR4-CXCL12 and VEGF-VEGFR2 pathways are upregulated in response to hypoxia, and correlations between CXCR4 and VEGF expression have been noted in melanoma, crosstalk between both pathways is anticipated, and perhaps unsurprising since the MAPK pathway is a common downstream signalling path to both axes (Bachelder *et al.*, 2002; Ceradini *et al.*, 2004; Zagzag *et al.*, 2005; Hong *et al.*, 2006; Oda *et al.*, 2006; Ottaiano *et al.*, 2006; Franco R *et al.*, 2010; Adamcic *et al.*, 2012). Understanding the role of the CXCR4-CXCL12 chemokine axis and VEGFR2 within primary melanomas, and the effect that these pathways and MAPK cell signalling have on melanoma migration, is the key aim of the present thesis, and will therefore inform on more efficacious combinational therapeutic approaches to limit primary tumour dissemination and the prevention of melanoma metastasis.

To endeavour to understand the currently undefined influence of CXCR4-CXCR7-CXCL12 and VEGFR2 signalling within primary melanomas and the tumour microenvironment, the expression of components of these axes and prognostic significance was first determined. CXCR4 was strongly expressed by the majority of primary uveal melanomas with differential expression seen by cutaneous melanomas as well as cutaneous and uveal melanoma cell lines, seemingly independent of mutational status, thereby confirming CXCR4 as a viable therapeutic target in all melanomas. Consistent with the association of high CXCR4 expression and the increased risk of metastasis in primary cutaneous melanomas, high CXCR4 expression (>50%) was found to be a putative prognostic biomarker for AJCC stage II cutaneous melanoma. This not only demonstrates the pivotal role that the CXCR4-CXCL12 axis plays in enabling the metastatic ability of locally invasive melanomas to exit from the primary site but also defines a 'high risk' melanoma AJCC stage II sub group, identifying those tumours that will eventually progress and allowing more predictive accuracy of metastatic progression at the time of excision. Contrasting to the role of CXCR4 in melanoma progression, CXCL12 expression by primary cutaneous and uveal melanomas was particularly low and did not influence disease progression from the primary site. However, this low expression of CXCL12 within the primary tumour seems logical given that abundant endogenous CXCL12 within the tumour would logically act to retain CXCR4 positive tumour cells, rather than facilitate their migration towards CXCL12 at distant sites. Studies do however suggest an important role for this low basal level of CXCL12 within primary tumours in driving pro-survival MAPK cell

signalling, allowing sustained tumour maintenance and survival, evidenced by the presence of autocrine CXCR4-CXCL12 cell signalling in cutaneous melanoma cell line WM-164. Together data from the present study suggest that targeting the CXCR4-CXCL12 chemokine axis within the primary tumour would not only prevent tumour cell migration but inhibit the activation of autocrine pro-survival MAPK cell signalling. Coupled with the role of the CXCR4-CXCL12 axis in primary tumours, the bidirectional interplay of this chemokine axis within the primary tumour and its microenvironment, likely influences tumour progression, but remains largely unexplored. CXCL12 was found to be abundantly expressed within both cutaneous and uveal melanoma tumour microenvironments, present within the epidermis and dermis of skin as well as within the retina and choroid of the eye. Interestingly the presence of increased levels of CXCL12 in the adjacent epidermis of primary cutaneous melanomas correlated with a increased time to tumour metastasis and cytoplasmic CXCR4 expression by tumour cells. Such findings suggest that high adjacent levels of CXCL12 are protective of metastasis with chronic activation of CXCR4 receptors within the primary tumour. It is hypothesised that the high CXCL12 gradient within the adjacent epidermis may act to retain CXCR4 positive tumour cells at the epidermal/dermal junction thereby promoting radial growth and delaying melanoma vertical invasion into the dermis and subsequent metastasis. For effective chemotaxis a gradient of CXCL12 stronger than that produced by the tumour or epidermis is required for the directional vertical migration of melanomas into the dermis. Given the potent secretion of CXCL12 from primary dermal fibroblasts and the presence of CXCR7 decoy receptor on dermal microvascular, suggests that these maybe key in modulating local CXCL12 concentrations within the tumour microenvironment and the promotion of directional melanoma migration. Transwell chemotaxis assays illustrated the ability of cutaneous metastatic melanoma cells to migrate towards supernatants derived from primary dermal fibroblasts. The use of an anti-CXCL12 neutralising antibody revealed that chemotaxis is specific to CXCL12 secreted within the primary dermal fibroblast supernatants. The scavenging of CXCL12 by CXCR7 decoy receptors on vascular endothelium has the ability to sharpen the extracellular chemokine gradient generated by dermal fibroblasts, possibly facilitating the chemotaxis of tumour cells in and out the blood vessels via modulation of local chemokine levels (Berahovich *et al.*, 2014). The detection of CXCR7 present on vessel endothelium within primary uveal melanoma tumours, hints that local chemokine levels may also be modulated in a similar fashion by CXCR7 expression within uveal melanomas. Taken together the role of the CXCR4-CXCR7-CXCL12 chemokine axis in primary melanomas highlights an intricate

relationship; intercalating variable expression of CXCR4 receptors by tumour cells with the secretion and modulation of CXCL12 within the tumour microenvironment, the summation of which may promote or hinder tumour progression.

The role of VEGFR2 in cutaneous and uveal melanoma primary tumours is far from understood. Although there is abundant evidence illustrating the role of VEGFR2 expressed by tumour associated endothelial cells promoting angiogenesis and lymphangiogenesis (Nagy *et al.*, 2002; Hong *et al.*, 2004b; Shibuya and Claesson-Welsh, 2006), as well as markers of angiogenesis or lymphangiogenesis linked with worse prognosis in both cutaneous and uveal melanoma (Foss *et al.*, 1996; Ilmonen S *et al.*, 1999; Mäkitie *et al.*, 1999; Kashani-Sabet *et al.*, 2002; Dadras *et al.*, 2005; Ly *et al.*, 2010), there is however little evidence specifically linking VEGFR2 expression by melanomas with disease progression. VEGFR2 expression by tumour cells is thought to provide autocrine VEGF-VEGFR2 cell signalling with subsequent enhancement of proliferation, survival and epithelial to mesenchymal transition (Adamcic et al., 2012; Ji et al., 2015; Scherbakov et al., 2016; Szabo et al., 2016). VEGFR2 was expressed by cutaneous and uveal melanoma cell lines at both the protein and mRNA level, with significantly higher expression detected in primary uveal melanomas compared to cutaneous primary melanomas of all AJCC stages. Although the level of expression of VEGFR2 in all primary cutaneous melanomas was extremely low, this was however, significantly increased in tumours that subsequently metastasised, particularly to lymph nodes, compared to those that remained localised (combined cohorts). This proposes that VEGFR2 expression may aid the emergence of a metastatic phenotype likely due to autocrine/endogenous VEGF-VEGFR2 cell signalling promoting cell survival and cell migration to lymph nodes, suggesting a role of VEGFR2 in lymphatic metastasis. In support of this hypothesis, chemotaxis assays using B-RAF/N-Ras mutated or wild-type cutaneous metastatic melanoma cell lines, demonstrated that stimulation with recombinant VEGF, increased cell migration, providing evidence for VEGF-VEGFR2-mediated promotion of melanoma migration. In addition to the expression of VEGFR2 on tumour cells, VEGFR2 was also strongly and consistently expressed by endothelial vasculature within skin and uveal melanomas. Taken together these data suggests that targeting VEGFR2 will not only inhibit angiogenesis of tumour associated vasculature but also may inhibit tumour autocrine VEGF-VEGFR2 cell signalling if present, and importantly prevent melanoma migration and subsequent metastasis.

While data from the present study demonstrated the contribution of CXCR4-CXCL12 and VEGFR2 signalling to tumour progression and the associations with a worse prognosis, there is a clear rationale to target both the CXCR4-CXCL12 chemokine axis and VEGFR2 to inhibit melanoma cell survival and migration, however the optimal means by which to approach this strategy must be carefully considered. While targeting the ligands of these pathways has provided proof of concept, positive clinical results haven't transpired (Liang *et al.*, 2004a; Corrie *et al.*, 2014). Worries over the effect of reduced CXCL12 and VEGF availability and immune regulation, as well as normal homeostatic processes and possible serious side effects with CXCR4 inhibition, suggests caution should be taken. Further, given that results derived from the present studies suggest higher epidermal expression of CXCL12 is actually preventative of cutaneous melanoma metastasis, directly targeting the CXCR4-CXCL12 axis may have undesired consequences. Results from the present studies also revealed increased nuclear CXCR4 expression and the down regulation of tumoural CXCL12 was associated with the presence of activating B-RAF/N-Ras mutations in cutaneous melanoma. Likewise, in uveal melanoma monosomy of chromosome 3, the strongest prognostic indicator of metastasis, was also associated with downregulation of CXCL12. Both increased tumoural CXCR4 and the downregulation of tumoural CXCL12 would aid tumour migration, indicating that hyper-activation of MAPK cell signalling in both cutaneous and uveal melanoma is associated with a more aggressive melanoma phenotype. These observations coupled with the presence of CXCR4-CXCL12 autocrine signalling also activating the MAPK pathway in cutaneous melanoma, suggests that inhibition of MAPK cell signalling maybe a novel way to inhibit the effects of CXCR4-CXCL12 cell signalling. The MEK inhibitor trametinib is currently used in clinical trials for advanced melanomas and unlike B-RAF inhibitors that are mutation specific inhibitors, trametinib is applicable for treatment of both B-RAF/N-Ras mutant cutaneous and GNAQ/GNA11 mutant uveal melanomas. In the present study trametinib at clinically achievable concentrations was able to significantly inhibit both B-RAF/N-Ras mutated and wild-type cutaneous melanoma chemotaxis towards recombinant CXCL12 and primary dermal fibroblast supernatants at concentrations in which there was no effect on the inhibition of cell viability. Likewise, OM413 (the GNAQ/11 wild-type uveal melanoma cell line) chemotaxis towards recombinant CXCL12 was also significantly inhibited by trametinib at concentrations that did not affect cell viability. Results thus indicate that MEK inhibition is a novel and efficacious means to prevent melanoma chemotaxis further highlighting the potential benefit of this strategy for both B-RAF/N-Ras mutant and wild-type cutaneous melanoma as well as

both GNAQ/GNA11 wild-type and mutant uveal melanoma, with MEK inhibition additionally preventing local invasion towards the dermis in the case of cutaneous melanoma, and further metastatic spread.

Results demonstrating VEGFR2 expression in primary cutaneous and uveal melanomas, and association of elevated VEGFR2 expression with metastasis of primary cutaneous melanomas, as well as the ability of VEGF to stimulate melanoma cell migration, advocates the use of VEGFR2 inhibition as a strategy to also prevent tumour migration. Data from the present study demonstrated the potent ability of pazopanib, a pan VEGFR2 tyrosine kinase inhibitor to inhibit the cell viability of both B-RAF/N-Ras or GNAQ/11 mutated or wild-type metastatic cutaneous and uveal melanoma cell lines, as well as inhibiting CXCR4-CXCL12 chemotaxis, at concentrations which had no effect on the inhibition of viability. Knockdown of VEGFR2 also confirmed VEGFR2 specific inhibition of melanoma migration by pazopanib, however the mechanistic effects of pazopanib on the inhibition of cell viability are unclear and maybe mediated by the inhibition of other tyrosine kinase receptors. Nevertheless, these investigations highlight the crosstalk between CXCR4-CXCL12 and VEGFR2 cell signalling, demonstrating both pathways promote melanoma cell migration, and that inhibition of just one of these pathways alone reduces migration. However, inhibiting only one pathway leaves an open opportunity for cell migration and MAPK cell signalling to be stimulated by the untargeted pathway. Given that there is potential to improve the efficacy of inhibition of cell viability and CXCR4-CXCL12 chemotaxis by use of both trametinib and pazopanib, studies were therefore undertaken to assess the potential efficacy of a combinational approach. In general, the combination of trametinib and pazopanib, potentiated the inhibition of cell viability induced by either drug alone, but notably resulted in significantly enhanced inhibition of CXCR4-CXCL12 chemotaxis, even at drug concentrations that had no effect on cell viability. Collectively, these data thus suggest dual MEK and VEGFR2 inhibition may be a more efficacious approach to inhibiting cutaneous and uveal melanoma cell viability and CXCR4-CXCL12 chemotaxis regardless of B-RAF/N-Ras or GNAQ/GNA11 mutational status.

Most targeted therapeutic approaches for melanoma, however powerful, ultimately given the heterogeneous nature of melanoma and ability to resist pro-apoptotic cell signalling lead to intrinsic or acquired drug resistance. In addition to defining more effective drug targets and more efficacious drug combinations, overcoming drug resistance is of equal importance in improving long term survival for patients with metastatic melanoma. Given the association of

activated autophagy in advanced cutaneous melanomas with a poorer prognosis (Ellis *et al.*, 2014b), its paradoxical role in tumour progression as well as in counteracting the pro apoptotic effects of chemotherapy (Mariño *et al.*, 2007; Hammerová *et al.*, 2012; Xie *et al.*, 2013) it was perhaps not surprising that autophagy was induced in cutaneous and uveal melanoma cell lines in response to treatment with trametinib. How to harness autophagy for the greatest therapeutic benefit is an area of much debate with evidence for both autophagy inhibition and exacerbation revealing beneficial effects in melanoma treatment (Mariño *et al.*, 2007; Hammerová *et al.*, 2012; Ambrosini *et al.*, 2013b; Xie *et al.*, 2013). Results from the present study however, revealed a clear sensitization of both uveal and cutaneous melanoma cells to the cytotoxic effects of trametinib by dual treatment with the lysosomal inhibitor chloroquine, suggesting dual autophagy and MEK inhibition maybe a viable strategy to increase the efficacy of MEK inhibitors such as trametinib. Nevertheless, such strategies require confirmation *in vivo*, including exploring the potential efficacy of more specific inhibitors of autophagy, such as new generation inhibitors of Vsp34 (Marsh T and Debnath J, 2015) or the potential for agents such as the cannabinoid Tetrahydrocannabinol (THC) that require autophagy induction /exacerbation for their cytotoxic effect (Armstrong et al., 2015).

In conclusion, while many aspects of cutaneous and uveal melanoma in terms of genetic background, pathogenesis, microenvironment and immune regulation contrast, the present study highlights great similarities between these two malignancies. Expression and dependence on key cell signalling pathways including CXCR4-CXCL12, VEGFR2 and MAPK signalling for melanoma cell survival, migration and disease progression, as well as similar responses to targeted pathway inhibition, suggests that many therapeutic interventions developed for cutaneous melanoma may be applicable to uveal melanoma. Uveal melanoma is generally thought to be the more aggressive form of melanoma with present data supporting this notion and specifically evidenced by the detection of higher CXCR4 and VEGFR2 expression levels in primary uveal tumours compared to cutaneous melanomas. With improved understanding of the melanoma microenvironment and how this interacts with tumour biology and disease progression, comes the opportunity to highlight critically required biomarkers to identify high risk patients and those most likely to benefit from novel stratified/precision based therapeutic interventions. Results from the present studies highlight CXCR4 expression as a biomarker to identify high risk AJCC stage II cutaneous melanomas, enabling earlier patient follow up. Inhibition of MAPK signalling is a likely

continued cornerstone for therapeutic intervention given its regulation in many aspects of melanoma tumorigenesis. However, single pathway targeted approaches are in the long term ineffective, emphasising the need for more tailored therapeutic approaches which take into account both the genetic and receptor profiles of individual tumours. In this context the present study highlights the potential benefits of novel combinational therapies for both B-RAF/N-Ras or GNAQ/GNA11 mutant or wild-type cutaneous/uveal melanomas directed at targeting the cross talk and interplay between multiple signalling mechanisms key to tumour metastasis and specifically, CXCR4-CXCL12 chemotaxis, autophagy, MEK and VEGFR2 signalling (Figure 6.1).

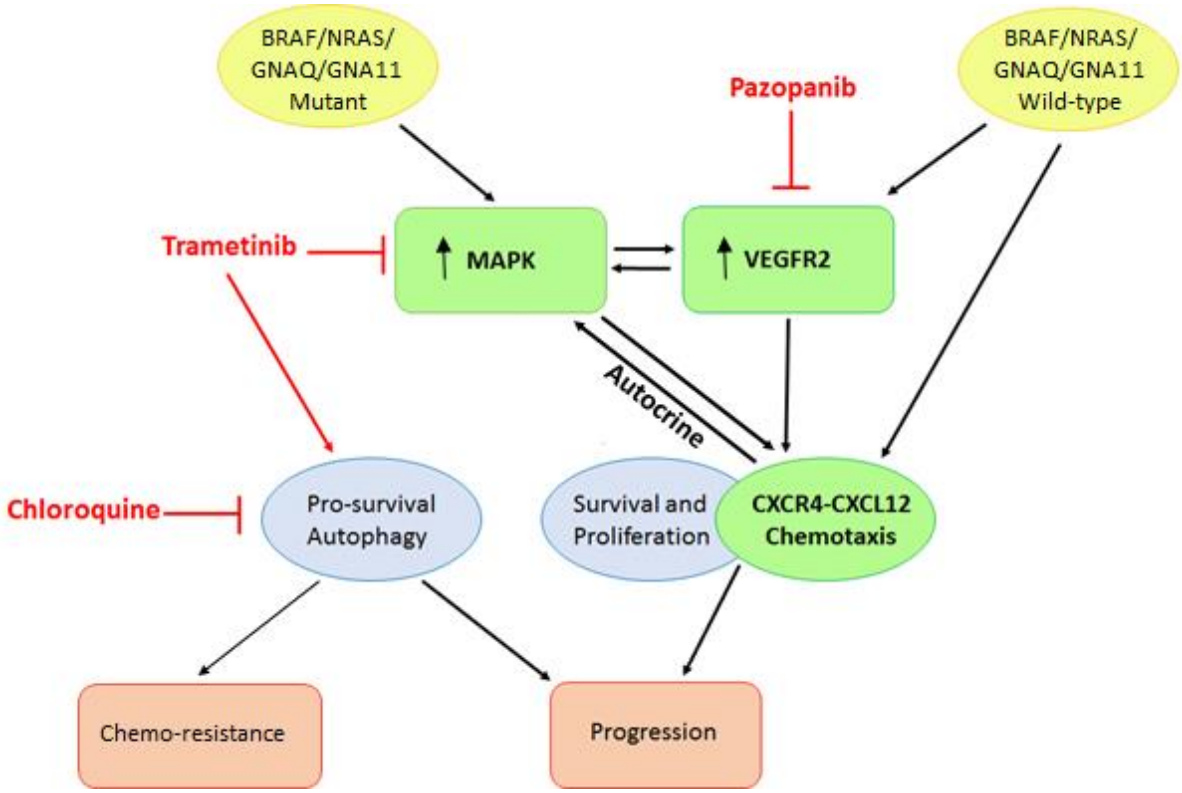

Figure 6.1 Novel Therapeutic Strategies to Target the Cross-talk of CXCR4-CXCL12 Chemotaxis, Autophagy, MAPK and VEGFR2 Cell Signalling in Cutaneous and Uveal Melanoma Metastasis

Schematic illustration of key signalling pathways: CXCR4-CXCL12 chemotaxis, autophagy, MAPK and VEGFR2 signalling that contribute to cutaneous and uveal melanoma survival, proliferation and migration and potential targeted approaches through which to prevent the cross talk and interplay of these key signalling mechanisms with trametinib, pazopanib and chloroquine to limit tumour chemo-resistance, progression and metastasis.

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Appendices

Appendices

Appendix 1

Characterisation of Uveal Melanoma Cell Lines

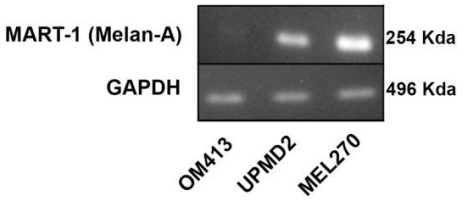
Although cutaneous melanoma cell lines are reportedly well characterised (Lovat *et al.*, 2008; Armstrong *et al.*, 2011), conflicting reports of misidentification highlighted the need to authenticate the selected uveal melanoma cell lines used in the present study (Folberg *et al.*, 2008). Metastatic uveal melanoma cell lines OM413, MEL270 and UPMD2 obtained from Professor R Marais (University of Manchester) were therefore characterised as being of melanocytic linage with the proposed mutations in GNAQ/GNA11.

Immunofluorescence studies confirmed the expression of the melanocytic proteins Melan-A and Mel-5 in all GNAQ/GNA11 mutant cell lines (illustrated for MEL270 and UPMD2 in Appendix Figure 1.1 Aii). However, no expression of either Melan-A or Mel-5 was detected in the reportedly GNAQ/GNA11 wild-type cell line, OM413. The expression of Melan-A mRNA was therefore determined by PCR analysis, revealing again, Melan-A mRNA expression in all GNAQ/GNA11 mutated cells, as well as, albeit weak, expression in OM413 cells (GNAQ/GNA11 wild-type) (in Appendix Figure 1.1 Ai and summarised in Appendix Figure 1.1 Bi).

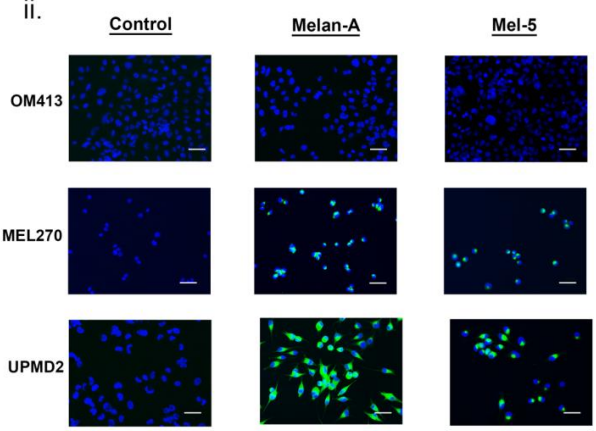
The GNAQ/GNA11 mutational status of all selected uveal melanoma cell lines was confirmed by PCR analysis and subsequent sequencing for single nucleotide polymorphisms in GNAQ or GNA11: GNAQ Q209, GNAQ R183, GNA11 Q209 and GNA11 R183, illustrated for GNAQ/GNA11 wild-type OM413, GNAQ mutant MEL270 harbouring a GNAQ Q209P mutation or UPMD2 with a GNA11 Q209L mutation.

A

i.



ii.

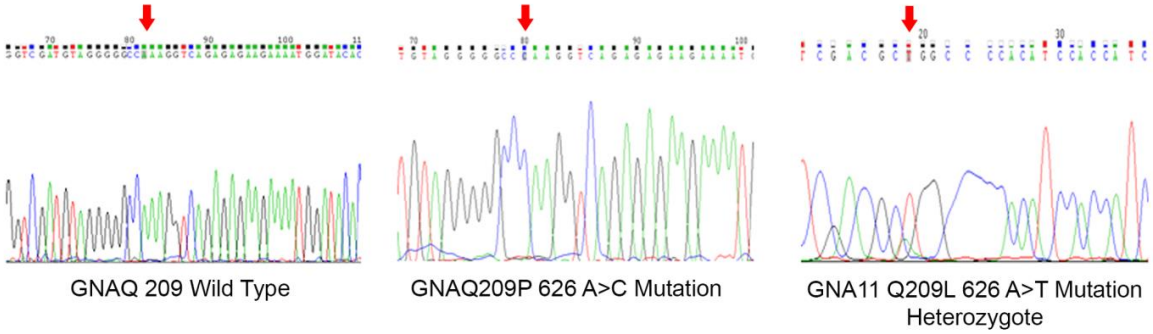


B

i.

Cell Line	Reported GNAQ/GNA11 Status	Melanocyte Marker Expression			Sequencing Analysis			
		Melan-A mRNA	Melan-A IF	Mel-5 IF	GNAQ Q209	GNAQ R183	GNA11 Q209	GNA11 R183
OM413	GNAQ/GNA11 WT	+ (weakly)	-	-	WT	WT	WT	WT
MEL270	GNAQ Q209P 626 A>C	+	+	+	Q209P 626 A>C	WT	WT	WT
UPMD2	GNA11 Q209L 626 A>T	+	+	+	WT	WT	Q209L 626 A>T (heterozygote)	WT

ii.



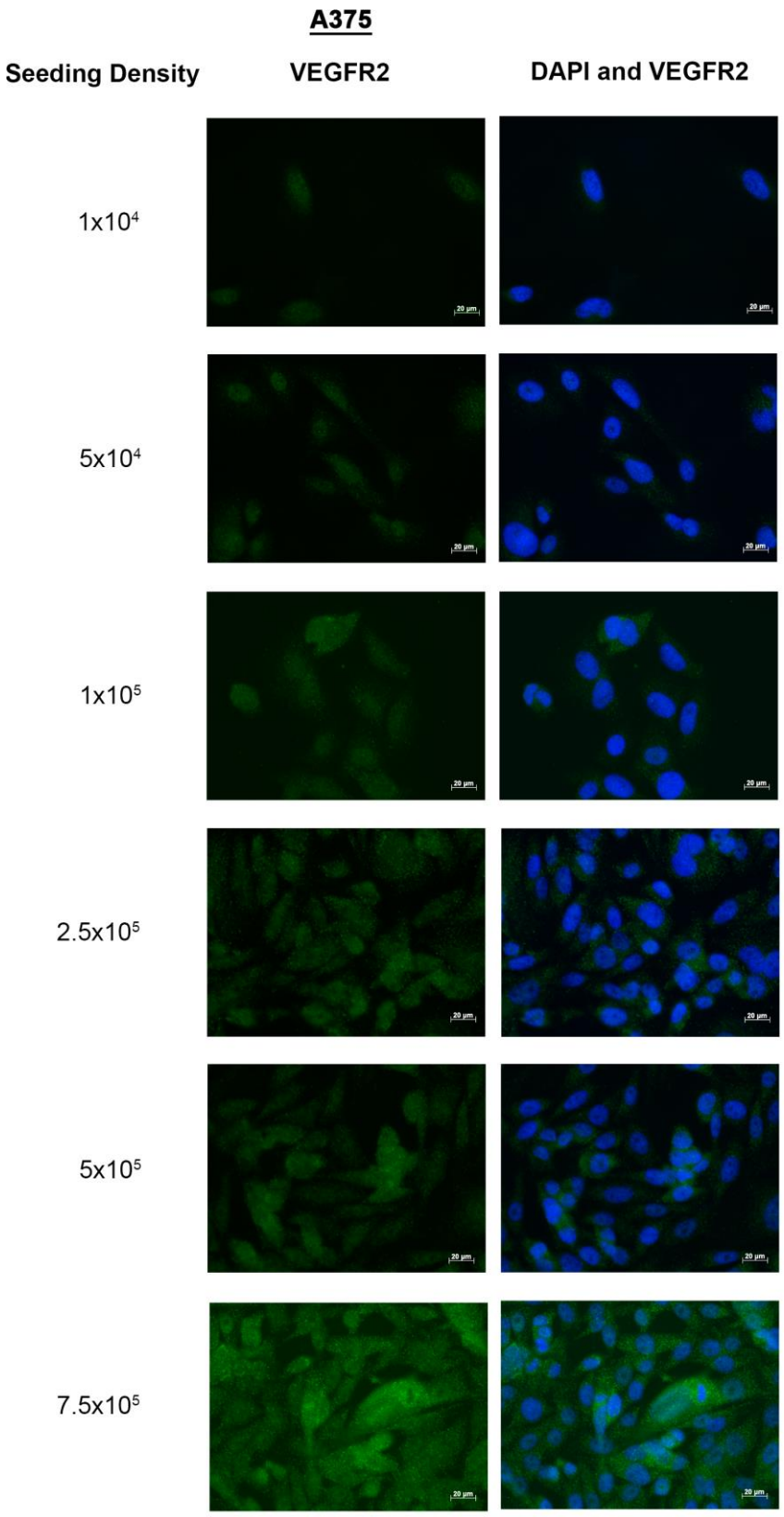
Appendix Figure 0.1 Characterisation of Metastatic Uveal Melanoma Cell Lines

A)i Representative image of gel electrophoresis of PCR products for MART-1 (Melan-A) mRNA or GAPDH (loading control) in OM413 (GNAQ/GNA 11 wild-type), MEL270 (GNAQ mutated) and UPMD2 (GNA11 mutated) human uveal melanoma cell lines. A.) ii Representative immunofluorescent images for the expression of melanocyte markers Melan-A, Mel-5 or null primary control in OM413, MEL270, and UPMD2 human uveal melanoma cell lines. Green depicts positive Melan-A or Mel-5 staining, blue is DAPI nuclear staining. Images taken by confocal microscopy with a magnification 20x scale bar = 50µm. B.)i Table of reported and determined uveal melanoma cell line characteristics and GNAQ/GNA11 mutational status of OM413, MEL270 and UPMD2 cells. B.) ii Representative sequencing plots of wild-type (OM413) or single nucleotide polymorphisms (SNP) at position 626 of GNAQ209 gene (MEL270) or position 626 of GNA11 Q209 gene (UPMD2) in human uveal melanoma cell lines.

Appendix 2

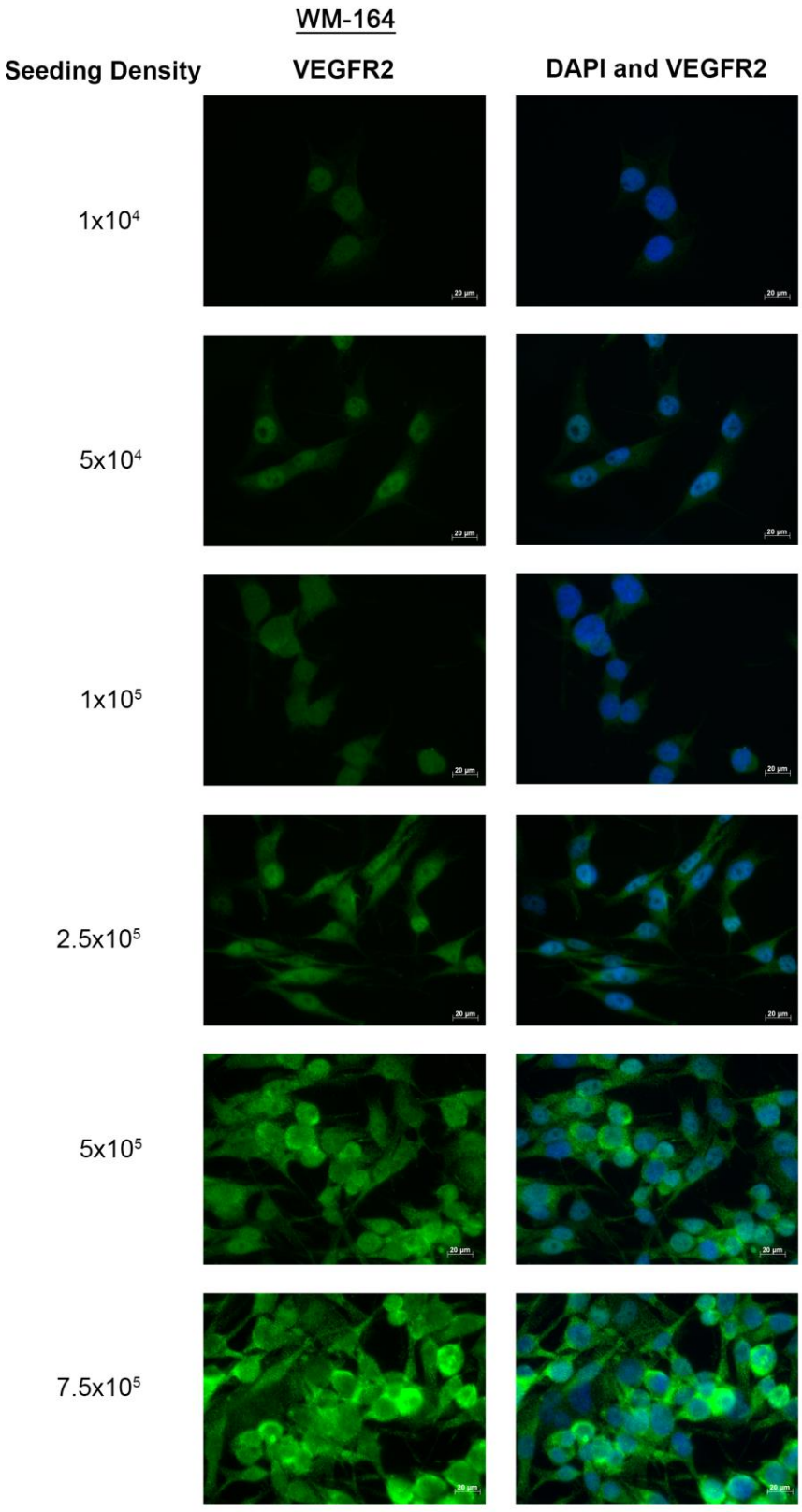
Increasing the Seeding Density of Cutaneous Melanoma Cells Increases the Expression of VEGFR2

Immunofluorescent analysis for the expression of VEGFR2 in cutaneous and uveal metastatic melanoma cell lines, revealed an apparent increase in expression in cells growing in large clusters compared to in cells growing as single cell colonies, implying VEGFR2 expression is modified by cell seeding density. To test this hypothesis A375, WM-164 and WM-1361 cells were seeded at increasing densities ranging from 1 X 10^4 to 7.5 X 10^5 /well of a 6 well plate, in a volume of 3mls normal cell culture media for 24 hours prior to the analysis and quantification of VEGFR2 fluorescence (Appendix Figure 2.1-2-2.4). Results revealed a significant increase in VEGFR2 expression in A375 and WM-1361 cells seeded at an original density of 7.5x10^5, and in WM-164 cells seeded at an original density of 2.5x10^5 compared to expression in cells seeded at the lowest seeding density of 1x10^4cells/well (One-way ANOVA with Dunnett's post hoc correction, **P<0.01 and ***P<0.001, Appendix Figure 2.4), suggesting VEGFR2 expression is enhanced with increasing cellular seeding density. To test whether increasing cellular seeding density impacted on VEGFR2 mRNA expression levels, qPCR analysis of VEGFR2 expression in WM-164 cells was also analysed following the seeding of cells for 24 hours over the same differing density range. Although qPCR analysis did not reveal any statistically significant changes in VEGFR2 mRNA expression with increasing cell seeding density (One-way ANOVA, with Dunnett's post hoc correction, P=0.1505, Appendix Figure 2.5), a similar trend for increased VEGFR2 expression with higher seeding densities was notable. Furthermore, the subcellular localisation of VEGFR2 in WM-164 and WM-1361 cells seemed to alter at higher seeding densities of 5x10^5 and 7.5x10^5cells/well where nuclear VEGFR2 expression appeared to be reduced and cytoplasmic expression increased, particularly at cell-cell junctions.



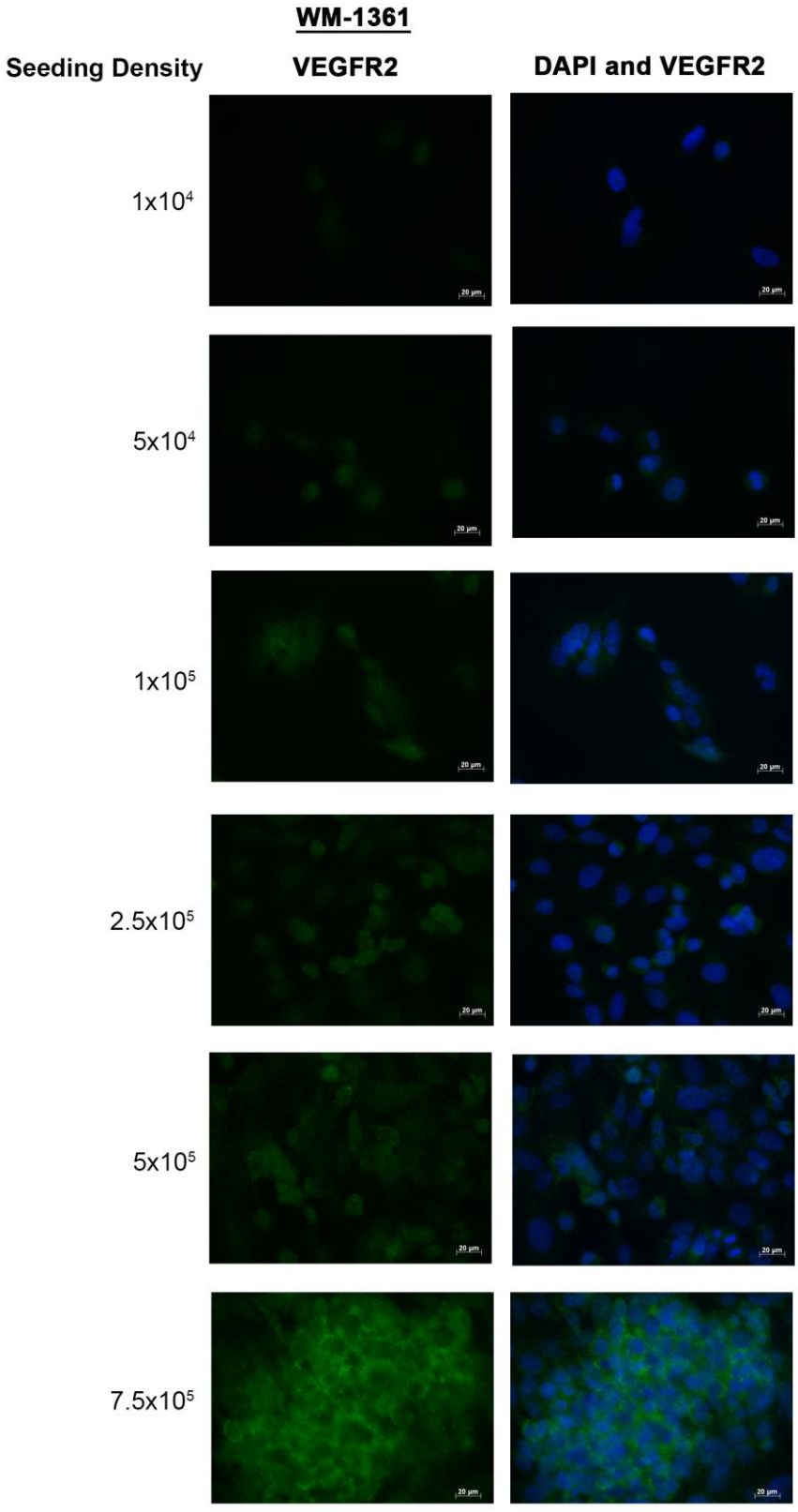
Appendix Figure 0.1 The Effect of Seeding Density on the Immunofluorescent Expression of VEGFR2 in A375 Cutaneous Metastatic Melanoma Cell Line

Representative images from 3 replicate experiments for the immunofluorescent expression of VEGFR2 or null primary control in cutaneous metastatic A375 melanoma cells seeded at 1 or 5x10^4, or 1-7.5x10^5 cells/well in a 6 well plate for 24 hours. Green depicts VEGFR2 positivity and blue DAPI nuclear staining. Images were acquired by confocal microscopy with a magnification 20x scale bar = 20µM.



Appendix Figure 0.2 The Effect of Seeding Density on the Immunofluorescent Expression of VEGFR2 in WM-164 Cutaneous Metastatic Melanoma Cell Line

Representative images from 3 replicate experiments for the immunofluorescent expression of VEGFR2 or null primary control in cutaneous metastatic WM-164 melanoma cells seeded at 1 or 5x10^4, or 1-7.5x10^5 cells/well in a 6 well plate for 24 hours. Green depicts VEGFR2 positivity and blue DAPI nuclear staining. Images were acquired by confocal microscopy with a magnification 20x scale bar = 20µM.



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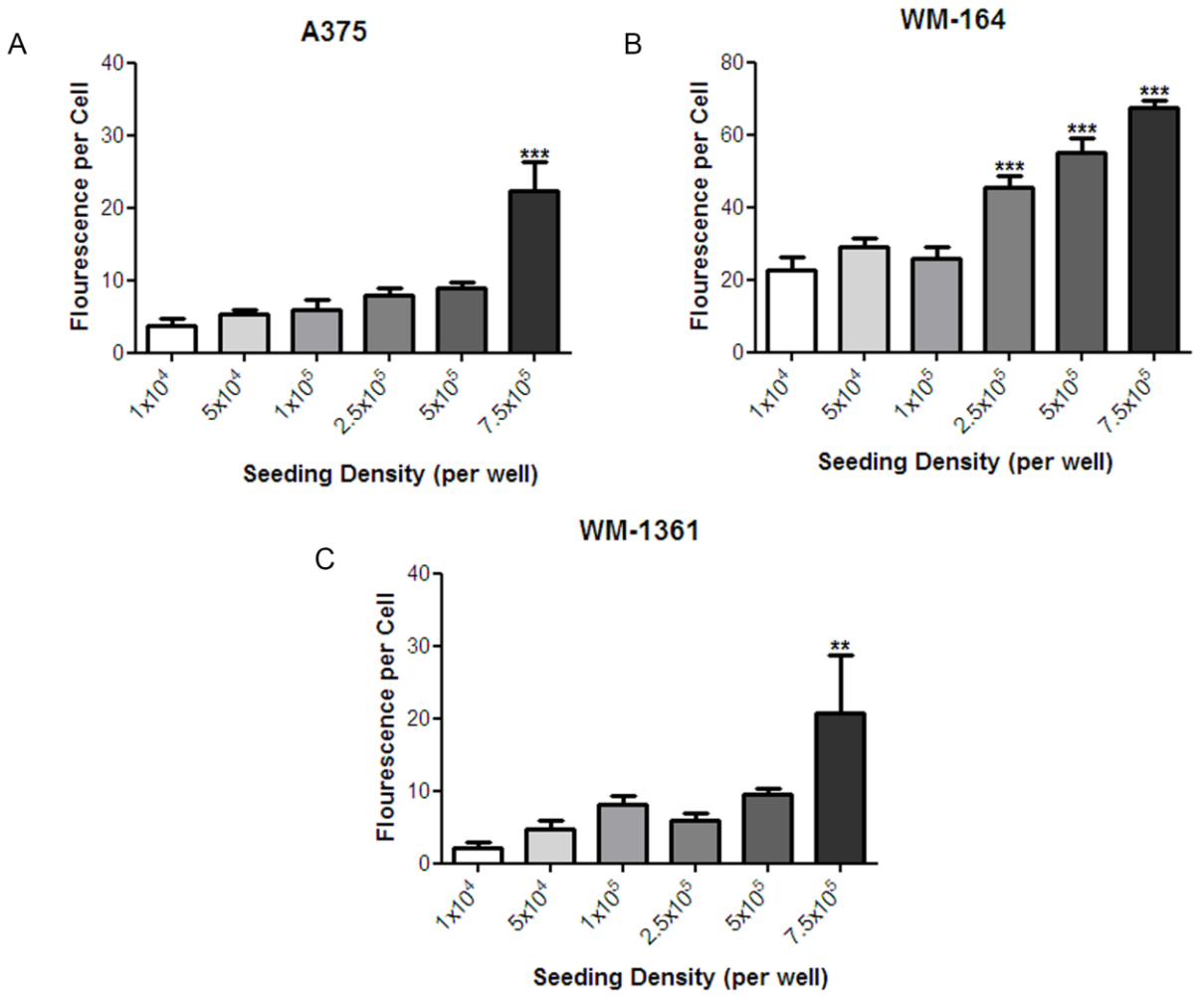
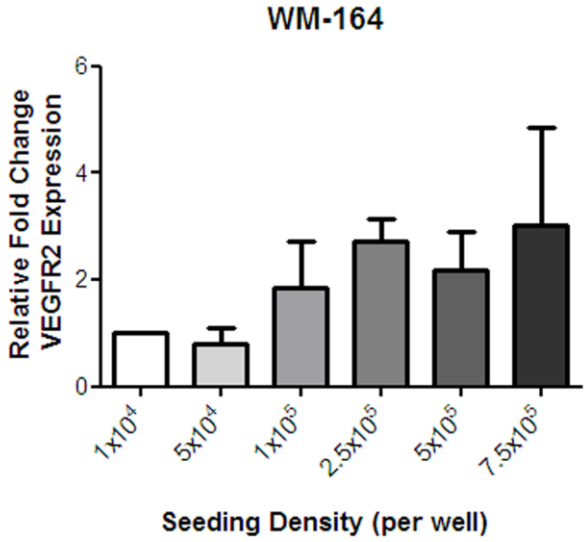
Appendix Figure 0.3 The Effect of Seeding Density on the Immunofluorescent Expression of VEGFR2 in WM-1361 Cutaneous Metastatic Melanoma Cell Line

Representative images from 3 replicate experiments for the immunofluorescent expression of VEGFR2 or null primary control in cutaneous metastatic WM-1361 melanoma cells seeded at 1 or 5x10^4, or 1-7.5x10^5 cells/well in a 6 well plate for 24 hours. Green depicts VEGFR2 positivity and blue DAPI nuclear staining. Images were acquired by confocal microscopy with a magnification 20x scale bar = 20µM.



Appendix Figure 0.4 Quantification of VEGFR2 Immunofluorescent Expression by Cutaneous Metastatic Melanoma Cell Lines Seeded at Different Density

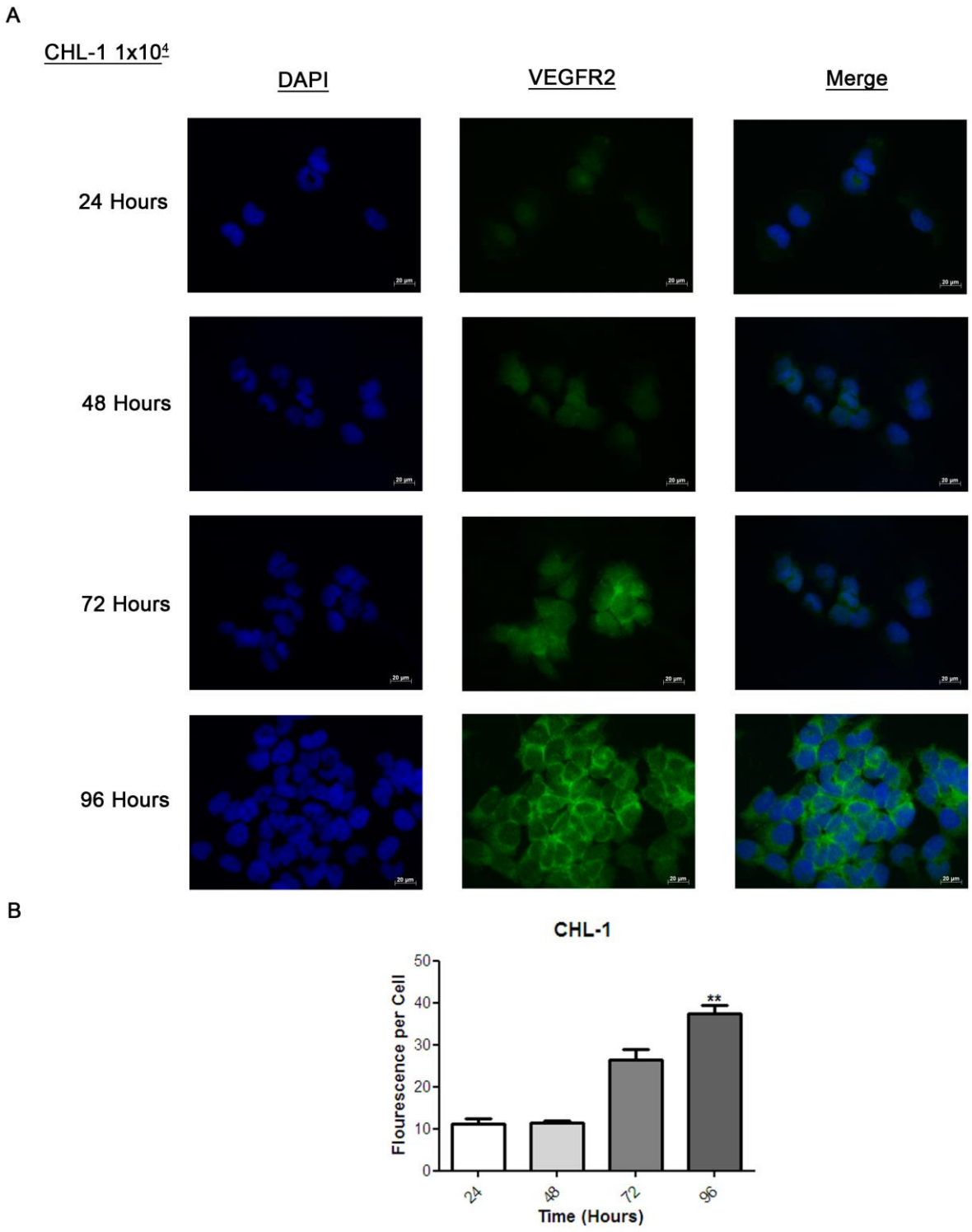
*Mean VEGFR2 fluorescence per cell within A.) A375, B.) WM-164, or C.) WM-1361 cutaneous metastatic melanoma cell lines seeded at $1x10^4$, $5x10^4$, $1x10^5$, $2.5x10^5$, $5x10^5$ and $7.5x10^5$ cells/well of a 6 well plate for 24 hours. Each bar is the mean VEGFR2 fluorescence per cell relative to DAPI, of 4 replicates from 2 independent experiments (mean +SD). Statistics acquired by One-way analysis of variance with Dunnett's post hoc correction, **P<0.01 and ***P<0.001.*



Appendix Figure 0.5 VEGFR2 mRNA Expression in WM-164 Metastatic Melanoma Cells According to Seeding Density

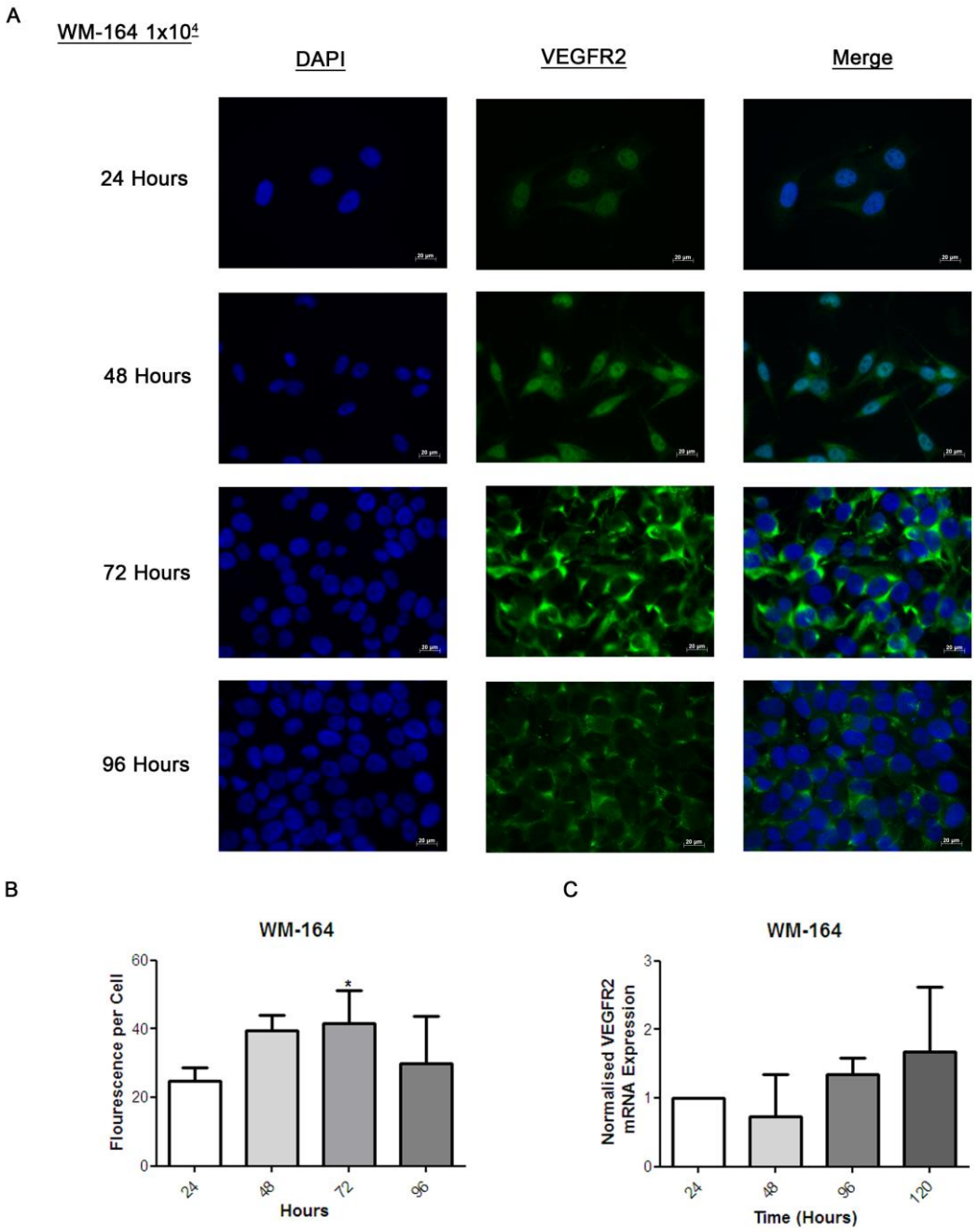
VEGFR2 mRNA expression in WM-164 cutaneous melanoma cell line seeded at $1x10^4$, $5x10^4$, $1x10^5$, $2.5x10^5$, $5x10^5$ and $7.5x10^5$ cells/well of a 6 well plate for 24 hours. Each bar is mean of VEGFR2 Cycle threshold for each sample normalised to 18s cycle threshold from 2 independent experiments (mean +SD N=2). Statistics acquired one-way analysis of variance with Dunnett's post hoc correction, all non-significant.

To determine if VEGFR2 expression also increased over time CHL-1, WM-164 and WM-1361 cutaneous metastatic melanoma cell lines were seeded at a low seeding density of $1x10^4$cells/well for 24, 48, 72 or 96 hours prior to the analysis and quantification of VEGFR2 immunofluorescent expression (Appendix Figure 2.6-2.8 A). Results revealed a significant increase in VEGFR2 expression in CHL-1, WM-164 and WM-1361 cells following culture for 96, 72 or 96 hours respectively compared to 24 hours (Kruskal-Wallis test with Dunn's multiple comparison post hoc correction, **P<0.01, *P<0.05, Appendix Figure 2.6-2.8 B). A similar although non-significant trend was also observed in WM-164 VEGFR2 mRNA expression levels (One-way ANOVA with Dunnett's post hoc correction, P=0.4820, Appendix Figure 2.7 C). Although immunofluorescent VEGFR2 expression increases over time to 96 hours in CHL-1 and WM-1361 cell lines, there was an apparent but non-significant reduction in VEGFR2 expression in WM-164 cells at 96 hours compared to 72 hours (Appendix Figure 2.7 B). Interestingly in all cell lines there was a very obvious change in VEGFR2 subcellular localisation from 72 hours to 96 hours, where the predominantly nuclear subcellular location of VEGFR2 at 72 hours appeared to be lost with strong accumulation of cytoplasmic VEGFR2, particularly seen at cell to cell contacts at 96 hours, a pattern mirrored with higher seeding densities of cells at 24 hours as previously described (Appendix Figure 2.1-2.5).



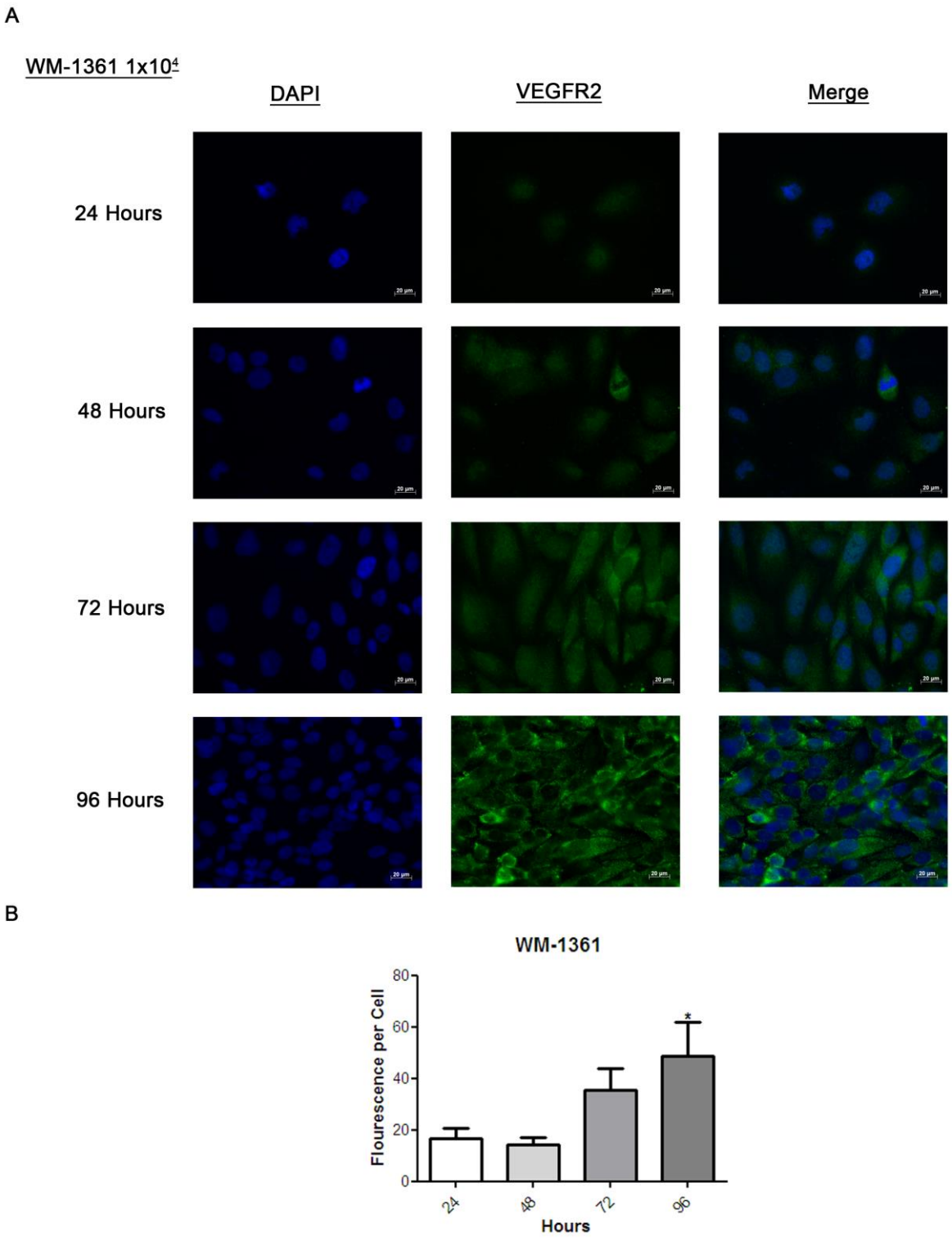
Appendix Figure 0.6 The Effect of Culture Time on the Immunofluorescent Expression of VEGFR2 in the Cutaneous Metastatic Melanoma Cell Line CHL-1

*A.) Representative images from 2 replicate experiments for the immunofluorescent expression of VEGFR2 or null primary control in CHL-1 cutaneous melanoma cells seeded at a density of 1x10^4 cells/well of a 6 well plate and incubated for 24, 48, 72, 96 hours. Green depicts VEGFR2 positivity and blue DAPI nuclear staining. Images were acquired by confocal microscopy with a magnification 20x scale bar = 20µm B.) Mean VEGFR2 fluorescence per cell at 24, 48, 72 or 96 hours' incubation. Each bar is the mean VEGFR2 fluorescence per cell relative to DAPI, of 8 replicates from 2 independent experiments (mean +SD). Statistics acquired by Kruskal-Wallis test with Dunn's multiple comparison post hoc correction, **P<0.01.*



Appendix Figure 0.7 The Effect of Culture Time on the Immunofluorescent Expression of VEGFR2 in the Cutaneous Metastatic Melanoma Cell Line, WM-164

*A.) Representative images from 2 replicate experiments for the immunofluorescent expression of VEGFR2 or null primary antibody control in cutaneous WM-164 cells seeded at a density of 1x10^4 cells/well in a 6 well plate for 24, 48, 72, 96 hours. Green depicts VEGFR2 positivity and blue DAPI nuclear staining. Images were acquired by confocal microscopy with a magnification 20x scale bar = 20µm B.) Mean VEGFR2 fluorescence per cell at 24, 48, 72 or 96 hours' incubation. Each bar is the mean VEGFR2 fluorescence per cell relative to DAPI, of 8 replicates from 2 independent experiments (mean +SD). Statistics acquired by Kruskal-Wallis test with Dunn's multiple comparison post hoc correction, *P<0.05. C.) VEGFR2 mRNA expression WM-164 cells seeded at a density of 1x10^4 cells/well in a 6 well plate for 24, 48, 72 or 96 hours. Each bar is mean of VEGFR2 Cycle threshold for each sample normalised to 18s cycle threshold from 2 independent experiments (mean +SD N=2). Statistics acquired one-way analysis of variance with Dunnett's post hoc correction, all non-significant.*



Appendix Figure 0.8 The Effect of Culture Time on the Immunofluorescent Expression of VEGFR2 in the Cutaneous Metastatic Melanoma Cell Line, WM-1361

*A.) Representative images from 2 replicate experiments for the immunofluorescent expression of VEGFR2 or null primary antibody control in cutaneous WM-1361 cells seeded at a density of 1x10^4 cells/well in a 6 well plate for 24, 48, 72, 96 hours. Green depicts VEGFR2 positivity and blue DAPI nuclear staining. Images were acquired by confocal microscopy with a magnification 20x scale bar = 20µm B.) Mean VEGFR2 fluorescence per cell at 24, 48, 72 or 96 hours' incubation. Each bar is the mean VEGFR2 fluorescence per cell relative to DAPI, of 8 replicates from 2 independent experiments (mean +SD). Statistics acquired by Kruskal-Wallis test with Dunn's multiple comparison post hoc correction, *P<0.05.*

Appendix 3

One-way Analysis of Variance of Combined MEK and VEGFR2 Inhibition on Cutaneous and Uveal Melanoma Cell Viability and CXCR4-CXCL12 Chemotaxis

To assess if combined efficacy of trametinib and pazopanib on the inhibition of melanoma cell viability or CXCR4-CXCL12 chemotaxis compared to the efficacy of either drug alone, a one-way analysis of variance was performed at each drug concentration, comparing trametinib, pazopanib or trametinib and pazopanib. The results of each individual ANOVA with significance values are tabulated below, all significant differences in cell viability (Appendix Table 3.1-3.4) or chemotaxis are highlighted in blue (Appendix Table 3.5-3.7).

CHL-1 Cell Viability			
Drug Concentrations	Drug Combinations	ANOVA (P value)	Dunnett's Post Hoc Correction (P value)
0.10nM Trametinib (T) 0.17µM Pazopanib (P)	T vs P	0.1063 (ns)	0.4326
	T vs T+P		0.089
	P vs T+P		0.6237
0.50nM Trametinib (T) 0.85µM Pazopanib(P)	T vs P	0.1869 (ns)	0.1622
	T vs T+P		0.5473
	P vs T+P		0.6950
1.00nM Trametinib (T) 1.71µM Pazopanib (P)	T vs P	0.4503 (ns)	0.7268
	T vs T+P		0.8695
	P vs T+P		0.4219
2.50nM Trametinib (T) 4.28µM Pazopanib (P)	T vs P	0.2420 (ns)	0.7052
	T vs T+P		0.2129
	P vs T+P		0.6320
5.00nM Trametinib (T) 8.56µM Pazopanib (P)	T vs P	***P<0.001	***P<0.001
	T vs T+P		***P<0.001
	P vs T+P		0.0482 *P<0.05
10.00nM Trametinib (T) 17.12µM Pazopanib (P)	T vs P	***P<0.001	***P<0.001
	T vs T+P		***P<0.001
	P vs T+P		0.0183 *P<0.05

Appendix Table 0.1 One-way Analysis of variance of CHL-1 Cell Viability in Response to Trametinib and Pazopanib Alone or in Combination

WM-164 Cell Viability			
Drug Concentrations	Drug Combinations	ANOVA (P value)	Dunnett's Post Hoc Correction (P value)
0.10nM Trametinib (T) 0.17µM Pazopanib (P)	T vs P	0.2606 (ns)	0.2393
	T vs T+P		0.9392
	P vs T+P		0.4987
0.50nM Trametinib (T) 0.85µM Pazopanib(P)	T vs P	0.993 (ns)	0.0836
	T vs T+P		0.5489
	P vs T+P		0.5659
1.00nM Trametinib (T) 1.71µM Pazopanib (P)	T vs P	0.0201 (*P<0.05)	0.0162 *P<0.05
	T vs T+P		0.7823
	P vs T+P		0.1265
2.50nM Trametinib (T) 4.28µM Pazopanib (P)	T vs P	0.0185 (*P<0.05)	0.0145 *P<0.05
	T vs T+P		0.7488
	P vs T+P		0.1300
5.00nM Trametinib (T) 8.56µM Pazopanib (P)	T vs P	0.0001 (***P<0.0001)	0.5022
	T vs T+P		***P<0.0001
	P vs T+P		0.0075 **P<0.01
10.00nM Trametinib (T) 17.12µM Pazopanib (P)	T vs P	<0.0001 (***P<0.0001)	***P<0.0001
	T vs T+P		***P<0.0001
	P vs T+P		0.0093 **P<0.01

Appendix Table 0.2 One-way Analysis of variance of WM-164 Cell Viability in Response to Trametinib and Pazopanib Alone or in Combination

OM413 Cell Viability			
Drug Concentrations	Drug Combinations	ANOVA (P value)	Dunnett's Post Hoc Correction (P value)
0.10nM Trametinib (T) 0.17µM Pazopanib (P)	T vs P	0.4764 (ns)	0.4436
	T vs T+P		0.8338
	P vs T+P		0.8098
0.50nM Trametinib (T) 0.85µM Pazopanib(P)	T vs P	0.2298 (ns)	0.2502
	T vs T+P		0.8098
	P vs T+P		0.9939
1.00nM Trametinib (T) 1.71µM Pazopanib (P)	T vs P	0.2146 (ns)	0.3499
	T vs T+P		0.9984
	P vs T+P		0.2466
2.50nM Trametinib (T) 4.28µM Pazopanib (P)	T vs P	0.0441 (ns)	0.3058
	T vs T+P		0.3520
	P vs T+P		0.4987
5.00nM Trametinib (T) 8.56µM Pazopanib (P)	T vs P	***P<0.001	***P<0.001
	T vs T+P		***P<0.001
	P vs T+P		0.0426 *P<0.05
10.00nM Trametinib (T) 17.12µM Pazopanib (P)	T vs P	***P<0.001	***P<0.001
	T vs T+P		***P<0.001
	P vs T+P		0.0069 **P<0.01

Appendix Table 0.3 One-way Analysis of Variance of OM413 Cell Viability in Response to Trametinib and Pazopanib Alone or in Combination

UPMD2 Cell Viability			
Drug Concentrations	Drug Combinations	ANOVA (P value)	Dunnett's Post Hoc Correction (P value)
0.10nM Trametinib (T) 0.17µM Pazopanib (P)	T vs P	0.6416 (ns)	0.9649
	T vs T+P		0.7859
	P vs T+P		0.6325
0.50nM Trametinib (T) 0.85µM Pazopanib(P)	T vs P	0.0013 (**P<0.01)	0.0123 *P<0.05
	T vs T+P		0.0016 **P<0.01
	P vs T+P		0.7311
1.00nM Trametinib (T) 1.71µM Pazopanib (P)	T vs P	0.1031 (ns)	0.0913
	T vs T+P		0.7406
	P vs T+P		0.3379
2.50nM Trametinib (T) 4.28µM Pazopanib (P)	T vs P	0.4277 (ns)	0.8076
	T vs T+P		0.3943
	P vs T+P		0.7597
5.00nM Trametinib (T) 8.56µM Pazopanib (P)	T vs P	***P<0.0001	***P<0.001
	T vs T+P		***P<0.001
	P vs T+P		***P<0.001
10.00nM Trametinib (T) 17.12µM Pazopanib (P)	T vs P	***P<0.0001	***P<0.001
	T vs T+P		***P<0.001
	P vs T+P		0.9857

Appendix Table 0.4 One-way Analysis of Variance of UPMD2 Cell Viability in Response to Trametinib and Pazopanib Alone or in Combination

CHL-1 Cell Chemotaxis			
Drug Concentrations	Drug Combinations	ANOVA (P value)	Dunnett's Post Hoc Correction (P value)
0.50nM Trametinib (T) 0.85µM Pazopanib(P)	T vs P	***P<0.001	0.8457
	T vs T+P		0.0004 ***P<0.001
	P vs T+P		0.0038 **P<0.01
1.00nM Trametinib (T) 1.71µM Pazopanib (P)	T vs P	***P<0.001	0.9742
	T vs T+P		***P<0.0001
	P vs T+P		***P<0.0001
5.00nM Trametinib (T) 8.56µM Pazopanib (P)	T vs P	***P<0.001	***P<0.0001
	T vs T+P		***P<0.0001
	P vs T+P		***P<0.0001

Appendix Table 0.5 One-way Analysis of Variance of CHL-1 Cell Chemotaxis in Response to Trametinib and Pazopanib Alone or in Combination

WM-164 Cell Chemotaxis			
Drug Concentrations	Drug Combinations	ANOVA (P value)	Dunnett's Post Hoc Correction (P value)
0.50nM Trametinib (T) 0.85µM Pazopanib(P)	T vs P	***P<0.001	0.8457
	T vs T+P		0.0004 ***P<0.001
	P vs T+P		0.0038 **P<0.01
1.00nM Trametinib (T) 1.71µM Pazopanib (P)	T vs P	***P<0.001	0.9742
	T vs T+P		***P<0.0001
	P vs T+P		***P<0.0001
5.00nM Trametinib (T) 8.56µM Pazopanib (P)	T vs P	***P<0.001	0.9792
	T vs T+P		***P<0.0001
	P vs T+P		***P<0.0001

Appendix Table 0.6 One-way Analysis of Variance of WM-164 Cell Chemotaxis in Response to Trametinib and Pazopanib Alone or in Combination

OM413 Cell Chemotaxis			
Drug Concentrations	Drug Combinations	ANOVA (P value)	Dunnett's Post Hoc Correction (P value)
0.50nM Trametinib (T) 0.85µM Pazopanib(P)	T vs P	0.008 **P<0.01	0.7131
	T vs T+P		0.0990
	P vs T+P		0.0631
1.00nM Trametinib (T) 1.71µM Pazopanib (P)	T vs P	0.0003 ***P<0.001	0.9992
	T vs T+P		0.0011 **P<0.01
	P vs T+P		0.0014 **P<0.01
5.00nM Trametinib (T) 8.56µM Pazopanib (P)	T vs P	P<0.0001 ***P<0.001	***P<0.001
	T vs T+P		***P<0.001
	P vs T+P		0.9829

Appendix Table 0.7 One-way Analysis of Variance of OM413 Cell Chemotaxis in Response to Trametinib and Pazopanib Alone or in Combination

List of Published Manuscripts and Abstracts Arising From This Thesis

List of Published Manuscripts Arising from This Thesis

McConnell A.T, Ellis R, Pathy B, Plummer R, Lovat PE, O'Boyle G.

The prognostic significance and impact of the CXCR4/CXCR7/CXCL12 axis in primary cutaneous melanoma.

British Journal of Dermatology, 2016 Dec;175(6):1210-1220.

List of Published Abstracts Arising from This Thesis

A.T. McConnell, R. Ellis, G. O'Boyle, R. Plummer, M. Wright, and P.E. Lovat
Nuclear CXCR4 expression in advanced B-RAF/N-Ras mutant melanomas is associated with a more aggressive phenotype
Pigment Cell and Melanoma Research 2013: 26(6)
Poster presentation at Society for Melanoma Research, Philadephia, USA (November 2013)

A.T. McConnell, R.Ellis, S. Verykiou, G. O'Boyle, M. Wright, R. Plummer and P.E. Lovat
Prognostic Significance of CXCR4 Expression in Cutaneous Melanoma
Oral presentation at The British Society for Investigational Dermatology Annual Meeting, Newcastle (April 2014)

A.T. McConnell, G. O'Boyle, M. Wright, R. Plummer and P.E. Lovat
Targeting MEK to Prevent CXCR4-CXCL12 Chemotaxis in Cutaneous Melanoma
Oral presentation at The British Society for Investigational Dermatology Annual Meeting (Southampton May 2015)

A.T McConnell, F.Botelho, S. Verykiou, E. Woodward, R. Plummer, T. Sandinha, and P.E. Lovat
Concomitant blockade of MEK and autophagy potentiates uveal melanoma cell death.
Pigment Cell and Melanoma Research 2015: 28(6)
Poster presentation at Society for Melanoma Research, San Francisco, USA (November 2015)

A. T. McConnell, R. Ellis, B. Pathy, T. Sandinha, R. Plummer, and P.E. Lovat
CXCR4-CXCR7-CXCL12 signalling promotes cutaneous and uveal melanoma metastasis.
Pigment Cell and Melanoma Research 2015: 28(6)
Poster presentation at Society for Melanoma Research, San Francisco, USA (November 2015)