A CXCR1/CXCR2 AND HETEROLOGOUS GPCRS ANTAGONIST IN MELANOMA DEVELOPMENT

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By

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ABSTRACT

Being the most aggressive human skin cancers, melanoma has always occurred with a poor prognosis. It is responsible for 80% of skin cancer. Treatments for melanoma include surgical removal, and radio- and chemotherapy, which are not effective toward the advanced stages of the disease. Only three chemotherapy drugs, hydroxyurea, dacarbazine and interleukin-2, are currently approved by the Food and Drug Administration for metastatic melanoma, and the therapeutic response rate is only 5%-20%. Thus, there is a need for novel therapies that can target tumours, especially when the tumour cells become refractory to chemotherapy.

ELR–CXC chemokines with a Glutamine – leucine – arginine (ELR) motif (for example, interleukin-8/CXCL8) are able to chemoattract neutrophils during inflammation responses via their receptors, CXCR1 and CXCR2, which can be expressed by human malignant tumour cells, keratinocytes, endothelial cells, and fibroblasts. CXCR1 and CXCR2 play very important roles in melanoma by promoting tumour cell proliferation, angiogenesis, and metastasis. They are also involved in the tumour's becoming refractory to chemotherapy.

An ELR–CXC chemokine antagonist developed by our lab, $CXCL8_{(3-72)}$K11R/G31P (G31P), effectively blocks CXCR1- and CXCR2- induced inflammatory responses, and further antagonizes the functions of heterologous G protein–coupled receptor's (GPCR). The tumour–associated GPCRs, along with ELR–CXC chemokines and their receptors, have been shown to simultaneously increase in several tumour models, including melanoma. Thus, given the knowledge regarding the importance of the ELR-CXC chemokines and heterologous GPCRs' in melanoma and G31P's ability to block ELR-CXC chemokines and at least some heterologous GPCRs, we hypothesize that G31P is a viable therapeutic option for melanoma cancers by virtue of its success in blocking tumour progression in mouse models.

Our data indicated that ELR-CXC chemokine antagonism with G31P had no significant impact on tumour growth or tumour-induced angiogenesis, which suggested that blockade of CXCR1 and CXCR2 alone was insufficient to block tumour development in this melanoma mouse model. Evaluation of other tumour-related parameters (e.g., angiogenic patterns and stress protein level) are recommended as a means of determining what parameters beyond CXCR1 and CXCR2 signaling are important in tumour progression in our matrigel model.

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5-FU	5-Fluorouracil
ACT	adoptive T cell transfer
C5a	complement component 5a
DARC	the murine duffy antigen receptor
DC	dendritic cell
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptors
ELR	glutamic acid-leucine-arginine
EMT	epithelial-to-mesenchymal transition
ER	estrogen receptors
ERK	extracellular signal-regulated kinase
ETAR	endothelin A receptor
FBS	fetal bovine serum
FGF2	basic fibroblasts growth factor
FGFR1	fibroblast growth factor receptor 1
FITC	fluorescein isothiocyanate
fMLP	formyl-methionyl-leucyl-phenylalanine
GPCRs	G protein–couple receptors
GRK	G protein-coupled receptor kinase

GRP	gastrin-releasing peptide
Her 2	human epidermal factor receptor
IL-6	interleukin-6
ILGF	insulin-like growth factor I
IP3	inositol triphosphate
JAK/STAT	Janus kinase/Signal transducer and activator of transcription
JNK	c-Jun N-terminal kinase
KSHV	Kaposi's sarcoma-associated herpesvirus
LIX	LPS-inducible CXC chemokine
LPA	lysophosphatidic acid
LTB4	leukotriene B4
M-CSF	macrophage colony-stimulating factor
MAPK	mitogen-activated protein kinase
MIP 2	macrophage inflammation protein 2
MMP	matrix metalloproteinase
MVD	micro-vessel density
NAP-2	neutrophil-activating peptide-2
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cell
PaCa	pancreatic cancer
PAF	platelet-activating factor
PAR-1	protease-activated receptor-1

PCa	prostate carcinoma
PDGF	placenta-derived growth factor
PECAM	platelet endothelial cell adhesion molecule
PI3K	phosphatidylinositol 3-kinase
PIN	prostate intraepithelial neoplasia
PTEN	phosphatase and tensin homolog
qRT-PCR	quantitative real-time polymerase chain reaction
SCC	squamous cell carcinoma
SEM	standard error of the mean
SMS	senescence-messaging secretome
TAM	tumour-associated macrophages
TGF	tumour growth factor
TNF	tumour necrosis factor
TYPR-2/Dct	tyrosinase-related protein-2/dopachrome tautomerase
VEGF	vascular endothelial cells growth factor

CHAPTER1: INTRODUCTION AND LITERATURE REVIEW

1.1.INTRODUCTION

Melanoma is the most aggressive human skin cancer, and it carries a poor prognosis (Garbe, Terheyden et al. 2008). According to data, it is one of the most common cancers in Canada: the incidence of melanoma has increased dramatically from approximately 1/1500 in the 1930s to 1/85 for women and 1/67 for men now. It is responsible for 80% of skin cancer deaths (Joshua 2012). The main therapeutic options for melanoma are surgical removal, and radio- and chemo-therapy, which are not highly effective during the advanced stages of this disease (Garbe, Terheyden et al. 2008). Currently, only three chemotherapeutic drugs, hydroxyurea, dacarbazine and interleukin-2, have been approved for the treatment of metastatic melanoma (Mansfield and Markovic 2009), and sadly none of these are highly effective. Thus, there is a need for new therapies that can target tumours, especially when they become refractory to chemotherapy.

The ELR–CXC chemokines (e.g., interleukin-8/CXCL8) are CXC-family chemokines that express an "ELR (Glutamic acid-leucine-arginine)" motif, each of which can chemoattract and activate neutrophils during the inflammatory response. The "ELR" motif is essential for the ligands' function (Moser, Dewald et al. 1993). Their receptors, CXCR1 and CXCR2, are expressed by several types of human cells, such as keratinocytes, endothelial cells, fibroblasts, neutrophils, and, of course, at least some types of malignant tumour cells (Varney, Singh et al. 2011). ELR-CXC chemokines and their receptors play very important roles in melanoma development, where they promote tumour cell proliferation, angiogenesis, metastasis, and the cells becoming refractory to chemotherapy (Vandercappellen, Van Damme et al. 2008). For instance, the binding of CXCL8 to either the CXCR1 or 2 induces tumour cell proliferation and survival. This binding also promotes tumour angiogenesis by activating endothelial cell proliferation, survival, migration (Li, Varney et al. 2005), and the up-regulation of other angiogenic factors such as matrix metalloproteinase (MMP)-2, -9, and vascular endothelial cell growth factor (VEGF) (Vandercappellen, Van Damme et al. 2008). It has been pointed out that fully-humanized neutralizing antibodies toward CXCL8 inhibit human melanoma cell growth, invasion and angiogenesis in a mouse model (Huang, Mills et al. 2002).

Our lab developed an ELR–CXC chemokine antagonist, $CXCL8_{(3-72)}$K11R/G31P (G31P), which not only blocks CXCR1- and CXCR2-induced inflammatory responses with high efficiency, but also antagonizes at least some heterologous G protein–coupled receptors (GPCRs) at the same time (Zhao, Town et al. 2009). Importantly, numerous GPCRs, such as CXCR4, the melanocortin-type 1 receptor (MC1R), endothelin receptors, the protease-activated receptor-1 (PAR-1), platelet-activating factor (PAF) receptor and the metabotropic glutamate receptor 1 (GRM1) are involved in tumour development via the facilitation of tumour cell transformation, proliferation, migration, invasion, and angiogenesis (Lee, Wall et al. 2008). Tumour-associated GPCRs display increased expression in several primary as well as metastatic tumours, including melanoma (Li, Huang et al. 2005).

G31P has been shown to block tumour progression, including angiogenesis, in mouse models of human prostate cancer, murine hepatoma and murine hepatocellular carcinoma (Liu, Peng et al. 2012, Wei, Chen et al. 2014, Li, Khan et al. 2015). Thus, given the important roles of the ELR-CXC chemokines and heterologous GPCRs in tumour development, it might be predicted that G31P should be able to block melanoma tumour progression, at least in part through its effects on angiogenesis.

1.2. LITERATURE REVIEW

1.2.1. Cancer introduction

Cancer refers to malignant tumours that are characterized by abnormal growth, and the tendency to spread or invade through blood vessels or the lymph system. Although extensive research has been conducted, cancer is still one of the most notorious killers in the world. According to Siegel and his colleagues, the three most deadly cancers in men are lung and bronchial cancer, prostate cancer, and colorectal cancer, while in women they are lung and bronchial cancer, breast cancer, and colorectal cancer (Siegel, Ma et al. 2014). Siegel *et al.* reported that in 2014, the number of new cancer cases in the United States will reach 1,665,540, and there will be 585,720 cancer-attributable fatalities, corresponding to almost 1,600 deaths per day in the United States alone (Siegel, Ma et al. 2014).

An array of factors are responsible for cancer: 5-10% of cancers are induced directly by genetic factors. The remaining 90-95% of cancers can be triggered by unhealthy lifestyles and environmental factors, such as cigarette smoking, unhealthy diet (over-consumption of fat and red meat), stress, obesity, physical inactivity, environmental pollutants, sun exposure, aging, as well as chronic infections (Heidenreich, Bellmunt et al. 2011). Around 15 years ago, Hanahan and Weinberg summarized that although more than 100 various types of human cancers had been found, the majority of them shared six common acquired hallmarks: (1) sustained proliferative signalling, (2) evasion of growth suppressors, (3) resistance to cell death, (4) enabling of replicative immortality, (5) induction of angiogenesis, and (6) activation of invasion and metastasis (Hanahan and Weinberg 2000). With our ongoing efforts in cancer research, some other traits have been added to this list, including reprogramming of energy metabolism, evasion of immune destruction that is involved in immune cell recruitment, formation of tumour-related microenvironments (Menendez, Joven et al. 2013), an unstable genome (Negrini, Gorgoulis et al. 2010), tumour-related inflammation (Colotta, Allavena et al. 2009), and blood vessel abnormalities (De Bock, Cauwenberghs et al. 2011).

Generally, therapeutic options might be different when they are designed to target different types and stages of cancer. These options include surgical removal, chemotherapy, radiation therapy, and immunotherapy. In addition, hyperthermia (Baronzio, Parmar et al. 2014), stem cell (Sharkis, Jones et al. 2012), and blood product transfusion therapy have also been effective in treating cancer. Although multiple therapeutic choices exist, the side-effects

from some of these, especially chemotherapy, are severe and common, while the treatment outcomes remain unsatisfactory. In breast cancer schemes, for example, surgery is usually the primary course of action, to be followed by radiotherapy, which might also be combined with other therapies such as chemotherapy, hormonal/endocrine therapy (Gho, Steele et al. 2013). Although benefits from these treatments are indeed well-documented, side-effects such as cardiovascular damage, moist desquamation, pneumonitis and pulmonary fibrosis are grievous and extensive (Killander, Anderson et al. 2014). In prostate cancer, the first stage of the disease is less aggressive, so active surveillance is more recommended than aggressive therapies (Heidenreich, Bellmunt et al. 2011). However, androgen-deprivation therapy is almost always the cornerstone of treatment for patients who have already entered advanced stages of prostate cancer, are at intermediate or high risk of recurrence, are still in early stages but receiving the radiation therapy, or are experiencing recurrent disease after surgery (Planas Morin, Celma Domenech et al. 2014). Androgen deprivation is able to curb tumour development to some extent, but side-effects include sexual dysfunction, depression, issues with thermoregulation and other changes affecting quality of life (Cary, Singla et al. 2014). Not surprisingly, these side-effects persist for prolonged periods. Davis and Kelly illustrated that the side-effects occurring as a result of prostate cancer therapy last as long as 10 years, but even after that quality of life issues were still important (Davis, Kelly et al. 2014). Moreover, researchers have realized that these aggressive therapies might even induce increased risk of other, secondary cancers (Kleinerman, Smith et al. 2013).

The medical options for melanoma include surgical removal, radio- and chemo- therapy, and/or combinations of those, although none of these is highly effective in advanced melanoma (Garbe, Terheyden et al. 2008). Common side-effects, such as thrombotic microangiopathy and capillary leak syndrome (Tseng, Citrin et al. 2014) are often present. Another issue in melanoma management is that the chemotherapeutic options are limited - for about two decades, only dacarbazine and interleukin-2 (IL-2) had been approved by the U.S. Food and Drug Administration (FDA) for advanced/metastatic melanoma chemotherapy (Mansfield and Markovic 2009). Acting as an alkylating reagent, dacarbazine still serves today as the first-line chemotherapy drug in advanced melanoma treatment, even though only 15–20% of patients respond well to it. However, even those patients who do respond to dacarbazine therapy soon develop resistance to this agent. Hydroxyurea chemotherapy was also approved by the FDA,

but it was not widely used (Gogas, Kirkwood et al. 2007). In 2011 two first-in-class agents, Ipilimumab, an immune-stimulating agent, and Vemurafenib, a v-raf murine sarcoma viral oncogene homolog B1 (BRAF) inhibitor, were approved by the FDA for melanoma therapy, although the side-effects of these drugs impose significant clinical limitations (Trinh, Davis et al. 2014). Hence, there is an urgent need to develop new therapeutic options. Chemokines and their receptors antagonists have gained increasing attention since they play a vital role in tumour development.

1.2.2. Chemokines and their receptors

Chemokines (chemotactic cytokines) are a group of small-molecule proteins (8-10 kDa) that were first recognized in the context of their abilities to regulate leukocytes' trafficking and localisation in both normal and inflamed tissues. Four families of chemokines have been classified based on the relative position of two conserved cysteine residues (CXC, CC, CX_3C and C) in the amino sub-terminus of the molecule. Chemokine receptors all belong to the GPCR family of cell surface molecules, each of which shares a common structure whereby they span the membrane seven times (i.e., a seven-transmembrane structure). Chemokines play important roles in normal as well as inflammation-associated physiology and pathology through their abilities to induce the recruitment or maturation of neutrophils, macrophages, dendritic cells, B cells, and T cells (Rossi and Zlotnik 2000).

In humans, the ELR–CXC chemokines include CXCL1-3 and 5-8 (growth-related oncogene [GRO]-α, -β, and -γ; epithelial cell neutrophil-activating peptide-78 [ENA-78]; granulocyte chemotactic protein-2 [GCP-2]; neutrophil-activating peptide-2 [NAP-2]; and IL-8, respectively) (Chemokine 2002). CXCL8, a ligand for both the CXCR1 and CXCR2 for example, is an 8.5 kDa protein that is cleaved into 72- (in monocytes and microphages) or 77- (in non-immune cells) amino acid peptides. The 72-amino acid form of CXCL8 has a higher affinity for its receptors (Rodriguez, Miller et al. 1992). Many types of cells secrete CXCL8, and among them, macrophages and monocytes represent the main sources. CXCL8 secretion can be triggered by diverse factors, e.g., lipopolysaccharide (LPS), bacterial or early inflammatory factors such as TNF α, IL-1 (Standiford, Kunkel et al. 1990), and it is sensitive to oxidants (DeForge, Preston et al. 1993). CXCL8's signaling through CXCR1 and CXCR2 receptors provokes neutrophil activation and chemotaxis. Shape changes, respiratory burst, up-

regulation of CD11b, and the exocytosis of granule contents, as well as calcium flux are detected in CXCL8-stimulated neutrophils (Baggiolini, Walz et al. 1989). Generally, CXCL8 can be detected in the early stage of inflammatory responses, but its expression can also persist for days or even weeks (DeForge, Fantone et al. 1992). Thus, CXCL8 performs a substantial role in a number of neutrophilic acute or chronic inflammatory diseases. In rodents, ELR-CXC chemokines include CXCL1 (keratinocyte-derived chemokine or KC), CXCL2 (macrophage inflammation protein (MIP)-2), CXCL5 (LPS-inducible CXC chemokine or LIX), and CXCL7 (neutrophil-activating peptide-2 or NAP-2).

Both ELR-CXC chemokine receptors, CXCR1 and CXCR2, can be activated by CXCL8 with high affinity, although CXCL6 only does so with lower affinity (Zhao, Town et al. 2009). CXCR1 and CXCR2 are expressed widely on neutrophils, monocytes, macrophages, basophils, eosinophils, T cells, and endothelial cells. They are also detectable in other types of cells, such as pancreatic islets cells, thyroid C cells, scattered Kulchitsky cells of the bronchi, and neuroendocrine cells in the stomach (Tecimer, Dlott et al. 2000). Of course, numerous tumour cells also express CXCR1 and CXCR2 as well. In rodents, all of the ELR-CXC chemokines bind to the murine CXCR2 (Sanz and Kubes 2012). Although a CXCR1 homolog in rodents has been identified and cloned, its function still needs to be verified (Fu, Zhang et al. 2005).

Chemokines activate their receptors through their extracellular N-terminus and extracellular loops (Fernandez and Lolis 2002). After being activated, their intracellular domains of these receptors relinquish association with their G-protein α, β and γ monomers, allowing formation of the second messengers inositol triphosphate (IP3) and diacylglycerol (DAG), which leads to cytoplasmic calcium mobilization and multiple downstream signalling cascades, including phosphatidylinositol 3-kinase (PI3K)/Akt, Ras/mitogen-activated protein kinase (MAPK), and janus kinase/signal transducer and activator of transcription (JAK/STAT) pathways activation (Balkwill 2012). Generally, signalling through CXCR1 and CXCR2 has a prominent role in the recruitment of immune cells, especially neutrophils, thus it is an indispensable component in neutrophilic inflammatory diseases such as psoriatic epidermis, sepsis, chronic obstructive pulmonary disease (COPD), and diabetes (Erdem, Pay et al. 2005). CXCL8 is also involved in human immunodeficiency virus (HIV)-1 infection wherein it facilitates passage of HIV-infected monocytes-derived macrophages' (MDM) across the blood-

brain barrier through CXCR1 and CXCR2, which leads to the HIV-1-related central nervous system disease (Mamik and Ghorpade 2014).

Although CXCR1 and CXCR2 share the same basic seven-transmembrane structure and 77% sequence homology at the amino acid level (Wuyts, Proost et al. 1998), structural differences between CXCR1 and CXCR2 still exist, especially in their extracellular and intracellular loops and NH2-terminal domain. It is known that the NH2-terminal domain is critical to ligand- and receptor-binding (Clark-Lewis, Kim et al. 1995). Moreover, differences in the COOH-terminal domains of CXCR1 and CXCR2 are found as well (Jones, Dewald et al. 1997), and these structural differences explain the discrepancies between CXCR1 and CXCR2 signalling and biological functions (Richardson, Pridgen et al. 1998). For example, CXCR1 mainly interacts with G protein-coupled receptor kinase (GRK) 2, whereas CXCR2 mainly conducts its function through GRK6 (Singh, Raghuwanshi et al. 2014). CXCR1, but not CXCR2, is the receptor that fosters the neutrophil respiratory burst and the activation of phospholipase D, whereas the CXCR2 is key for MMP-9 release (Chakrabarti and Patel 2005). In tumour development, CXCR2, not CXCR1, is primarily involved in endothelial cell chemotaxis, and therefore angiogenesis (Schraufstatter, Trieu et al. 2003), but CXCL8 signalling through CXCR1 still has responsibilities in tumour cell trans-endothelial migration (Ramjeesingh, Leung et al. 2003). As such, targeting CXCR1 and CXCR2 is regarded as a promising strategy for the treatment of numerous diseases.

1.2.3. Abnormal chemokines and their receptors in tumour development

Being found on various tumour cells and tumour-associated stromal cells, chemokines and their receptors have important roles in tumour development including melanoma. It has been shown that the mutation of chemokines or/and their receptors impacts directly on tumour development.

1.2.3.1. Mutant chemokines and their receptors

It is well known that numerous cancers are induced by mutations. P53, for example, which is known as "the guardian of the genome" (Efeyan and Serrano 2007) is encoded by the human *pt53* gene. Functioning as a critical tumour repressor gene, it regulates apoptotic processes and genomic stability in normal cells. In cancer, P53 is linked to tumour growth,

angiogenesis, and metastasis as well as to chemo-resistance (Shi, Nikulenkov et al. 2014). As a dominant-negative allele, either inactivation or mutation in P53 breaks its tumour suppressor functions. Actually, these changes in P53 impact chemokines and their receptors, and thus tumour development. Cytokines and chemokines are two of the targets of tumour-induced P53 mutations. Yeudall *et al.* illustrated that the gain-of-function P53 mutation strengthened expression of several CXC chemokines (such as CXCL5, -8 and -12), and thus increased the tendency toward tumour cell migration and metastasis (Yeudall, Vaughan et al. 2012), whereas wild-type P53 suppresses the expression of CXCR4 (the receptor for the lymph nodes homing chemokine SDF-1/CXCL12) in breast cancer (Mukherjee and Zhao 2013). As stated by Mehta et al, (Mehta, Christopherson et al. 2007), higher CXCR4 expression is usually correlated with enhanced tumour invasion, metastasis, and poor prognosis. Another example is the somatic mutation, V600E in B-Raf kinase, which is common in melanoma (Sun, Wang et al. 2014), and generally enhances CXCL8 expression and thus tumour progression (Bourcier, Griseri et al. 2011). Remarkably, inhibition of this B-Raf mutation impairs CXCL8 secretion from melanoma cells, and spurs anti-tumour immune responses via the INF-γ, CCL4 and TNFα pathways in melanoma patients (Wilmott, Haydu et al. 2014). Additionally, for prostate cancer, phosphatase and tensin homolog (PTEN) gene inactivation prevails even in the early stage, wherein it up-regulates CXCL8, promoting disease development by facilitating tumour cell proliferation and survival (Maxwell, Coulter et al. 2013). Mutations in chemokines and their receptors can also prompt normal cell alterations. For example, the mutated CXCL8 receptor CXCR2 (Asp138 by Val in the second intracellular loop) enhances cellular proliferation as well as transformation in the mouse NIH 3T3 embryonic fibroblast (Burger, Burger et al. 1999).

Interestingly, mutated chemokines and their receptors take part in cell senescence, a state wherein cells are still biologically functional but have lost their ability to divide. It is known that several states, such as oxidative stress, telomere dysfunction, DNA damage, and oncogene activation foster senescence. Although oncogene-induced senescence is an important defense against oncogenesis, the acquisition of the senescence-associated secretory or senescence-messaging secretome (SMS) phenotype (Ohtani and Hara 2013) is regarded as detrimental since it promotes tumour development. The SMS phenotype supports the production of numerous factors that boost tumour development, such as proteases, cytokines, and growth factors, including IL-6 and CXCL8 (Coppe, Patil et al. 2008). Being critical DNA

damage-related cellular senescence mediators, CXCR2 and its CXCL1 and CXCL2 ligands are up-regulated in a number of cell types under genotoxic or oxidative stress conditions, which enhance cellular senescence (Guo, Liu et al. 2013). In addition, in NCI-H1395 lung adenocarcinoma cells a point mutation of CXCR2 reinforces tumour progression by disrupting the senescence-associated tumour-suppressive actions (Acosta and Gil 2009).

Some single nucleotide polymorphisms in chemokine genes can enhance cancer risk by inducing the development of cancer. For instance, a CXCL8 (-251T>A) single nucleotide polymorphism accounts for inflammation-related risk in sporadic colorectal cancer (Landi, Moreno et al. 2003), while in breast cancer decreases in overall- and disease-free survival are significantly associated with CXCL8 (-251) A and/or the CXCR2 (+1208) T alleles. This indicates that polymorphisms in CXCL8 and CXCR2 genes are strongly linked to breast cancer risk and disease progression (Snoussi, Mahfoudh et al. 2010). Ethnicity-associated single nucleotide polymorphisms in chemokines might also explain distinct cancer risks in different populations. Singh, Jaiswal and their collaborators demonstrated that CXCR2 and the variant genotype (C1280T) contributed to the higher prevalence of bladder cancer in north Indian populations (Singh, Jaiswal et al. 2014).

1.2.1.1. Enhanced expression of ELR-CXC chemokines and receptors

In general, compared to their normal counterparts, malignant cells are able to express abnormally enhanced levels of some chemokine receptors, and that is correlated with tumour progression. In fact, many of these, including intensified expression of ELR-CXC chemokines and their receptors, are predictors of diseases, especially cancer development. In the early stage of alveolar neoplastic lesions, the strengthened expression of CXCR2 was discovered in a mouse model and correlated with a poor prognosis (Wislez, Fujimoto et al. 2006). In human prostate cancer, elevated levels of CXCR1 and/or CXCR2, as well as CXCL8, are associated with tumour progression, especially in advanced stage Gleason pattern grade 3 and/or 4 tumours. Among prostate cancer patients, those who were diagnosed with higher grade tumours also show higher level expression of CXCL1 (Miyake, Lawton et al. 2014), while CXCL5 levels are regarded as an independent prognostic factor in nasopharyngeal carcinoma (Zhang, Xia et al. 2013). Overexpression of CXCL5 is also associated with the late stage of gastric cancer (Park, Park et al. 2007), while in metastatic lung adenocarcinoma (Saintigny,

Massarelli et al. 2013) and breast cancer (Singh, Simoes et al. 2013), overexpression of CXCR2 predicts a poor prognosis. Similar up-regulation of CXCR1 and/or CXCR2 as well as their ligands is observed in numerous other human cancers, such as melanoma, colon and colorectal cancer, nasopharyngeal cancer, laryngeal squamous cells cancer, esophageal cancer, oral squamous cell carcinoma, ovarian cancer, and intrahepatic cholangiocellular carcinoma (Zarogoulidis, Katsikogianni et al. 2014).

1.2.2. Functions of ELR-CXC chemokines and their receptors

Altered chemokine signalling can aggravate tumour development by impinging on several cancer-related pathways such as c-myc, NF-κB, FAS/FAS ligand, HER2, EGR1, FOXO3A, and KRAS (Balkwill 2012). These pathways can control tumour cell proliferation, inhibition of apoptosis, angiogenesis, invasion, metastasis, and the likelihood of the cells becoming hormone- and/or chemotherapy-resistant. These pathways themselves can modify expression of chemokines and their receptors. Oncogenic mutation of KRAS or EGFR intensifies CXCL8 expression in non-small cell lung cancer, and that promotes tumour cell proliferation and migration (Sunaga, Imai et al. 2012). More specifically, in breast cancer, the combination of TNFα and Ras signalling leads to the expression of CXCL8, and thus prompts tumour development via the Mek, NF-κB and AP-1 pathways (Leibovich-Rivkin, Liubomirski et al. 2014).

The CXCR1, CXCR2 and NF-κB signalling pathways have gained increasing attention because they participate in tumour development. Playing important roles in both innate and acquired inflammatory responses, NF-κB participates in the activation of several tumour-related genes. In prostate cancer, the activation of NF-κB elevates the expression of the anti-apoptotic protein Bcl-2, so it induces anti-apoptotic effects in tumour cells (Catz and Johnson 2001). In addition to its anti-apoptotic effects, overexpression of Bcl-2 assists angiogenesis by activating the VEGF promoter, leading to production of a more stable version of VEGF mRNA (Iervolino, Trisciuoglio et al. 2002). NF-κB is also involved in the formation and maintenance of tumour-associated microenvironments. Zhu and Woll demonstrated that NF-κB activation resulted in polarization of macrophages to a M2 phenotype, which exacerbates tumour development through secretion of various tumour-promoting cytokines (Zhu and Woll 2005).

Not surprisingly, as an important biomarker in cancer, CXCL8 signalling through CXCR1 and CXCR2 reinforces NF-κB signalling (Wilson, Purcell et al. 2008). More interestingly, NF-κB binds to the CXCL8 promoter, upregulating expression of CXCL8 (Zhu and Woll 2005) as a positive feedback loop.

In addition to inducing the signalling through its own pathways, ELR-CXC chemokine signalling through CXCR1 and CXCR2 also support tumour development by interacting with other receptors. Research showed that in non-small cell lung cancer, CXCL8 causes tumour cell proliferation via the transactivation of epidermal growth factor receptors (EGFR), and this proliferation is inhibited by either CXCL8 antibodies or EGFR tyrosine kinase inhibitors (Luppi, Longo et al. 2007). The details of ELR-CXC chemokine facilitation of cancer development will be discussed below.

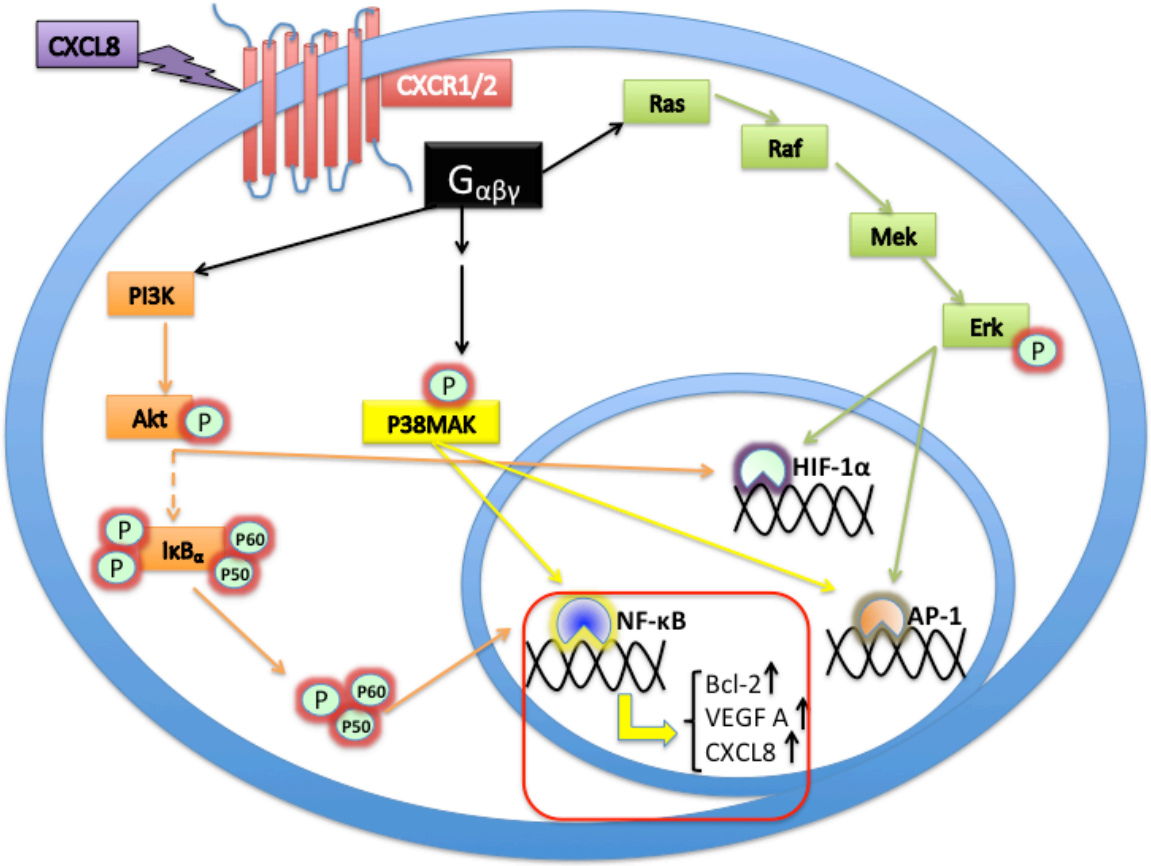


Figure 1.1 Main CXCL8 signal pathways through CXCR1 and CXCR2 in inducing cancer cell survival.

NF-κB, HIF-1 α and AP-1 are the key transcription factors involved in CXCL8-induced cancer cell survival. NF-κB can be activated through Akt and P38MAK pathway whereas HIF-1 α can be activated through Akt and Erk. Erk as well as P38MAK activates AP-1. Several genes will be regulated through these activated transcription factors. For example, the activation of NF-κB induces the up-regulations of Bcl-2, VEGF A, and CXCL8, which not only promote caner cell survival, but also proliferation, angiogenesis and metastasis.

1.2.2.1. Proliferation

ELR-CXC chemokine signalling through the CXCR1 and CXCR2 stimulates tumour cell proliferation chiefly via paracrine and autocrine pathways (Zhu and Woll 2005). By and large, the autocrine pathway is uncommon in normal cells. According to Sparmann, Bar-Sagi and co-researchers, ELR-CXC chemokines fuel tumour cell proliferation principally via phosphatidylinositide 3-kinases PI3K/Akt, the extracellular signal-regulated kinases Raf/MEK/ERK and Ras pathways (Sparmann and Bar-Sagi 2004). In liver tumours, the expression of CXCL8 from $CD133^+$ stem-like tumour cells promotes tumour growth and angiogenesis via the MAPK pathway (Tang, Ma et al. 2012). Cell cycling proteins, such as Cyclin D1 and B1, and thus cell proliferation, are regulated through these pathways (Zarogoulidis, Katsikogianni et al. 2014). Moreover, the infiltration of immune cells such as neutrophils induced by CXCL8 production also contributes to tumour growth via their secretion of several growth factors (Tazzyman, Barry et al. 2011). As noted above, other receptors that are involved in tumour cell proliferation might also be transactivated via ELR-CXC chemokines (Luppi, Longo et al. 2007).

Intratumoral hypoxia is fairly common in solid tumours, and that can push tumours to more detrimental phenotypes. Hypoxic stress encourages epithelial-to-mesenchymal transition (EMT) processes, maintaining cancer stem cell populations and the formation of cancer-related inflammatory microenvironments (Bao, Ahmad et al. 2012). According to Rapisard and Melillo's research, hypoxia is also associated with tumour cells becoming radio- and chemo-resistant through up-regulation of several factors, such as HIF-1, NF-κB and their downstream molecules (Maxwell, Gallagher et al. 2007), which spur tumour cell proliferation, angiogenesis, invasion, and metastasis. These molecules are also involved in tumour-specific transformed glycolysis and tumour-related de-differentiation processes (Semenza 2011). Not surprisingly, CXCR1 and CXCR2, as well as their ligands, are important factors in both the HIF-1 and NF-κB pathways. Consequently, CXCR1 and CXCR2 are engaged in tumour cell proliferation, angiogenesis, metastasis, and survival in hypoxic conditions (Maxwell, Gallagher et al. 2007). Liu and his colleagues reported that in cervical cancer, hypoxia induces enhanced expression of CXCL8, CXCR1, and HIF-1α, which facilitates cancer cell proliferation and anti-apoptotic activities (Liu, Xie et al. 2014). Interestingly, tumour growth-induced hypoxia itself is also

strongly associated with angiogenesis - tumours alleviate hypoxic stress through the formation of new blood vessels, which brings nutrients and oxygen to the primary tumour site.

Several genes are involved in hypoxia-induced angiogenesis. For example, in non-small cell lung cancer, the hypoxic environment prompts the up-regulation of VEGF A, which has an important role in angiogenesis through its abilities to activate the MEK/ERK and PI3K pathways (Jackson, Zhou et al. 2010). In breast cancer, hypoxia strongly fosters angiogenesis via increased expression of osteopontin (OPN), a hypoxia-responsive gene that contributes to the activation of the integrin-linked kinase (ILK)/Akt-mediated nuclear factor, NF-κB and HIF1α-dependent VEGF A expression (Raja, Kale et al. 2014). Transfection of tumour cells for increased CXCL8 expression leads to tumour cell proliferation, migration, and significant chemo-resistance in several colon cancer cell lines (Ning, Manegold et al. 2011).

1.2.2.2. Metastasis

Undoubtedly, ELR-CXC chemokines are also involved in tumour metastasis. It has been demonstrated that in a breast cancer bone metastasis model, CXCL8 secretion directly induces osteoclastogenesis and bone resorption (Bendre, Margulies et al. 2005). The use of CXCL8 antibodies increases the survival rate in tumour-challenged mice, which suggests that anti-CXCL8 treatment might have beneficial effects toward bone-related events associated with breast cancer (Kamalakar, Bendre et al. 2014). ELR-CXC chemokines also invigorate tumour metastasis by stimulating tumour cell migration and adhesion. For example, it was reported that the expression of CXCL1 and CXCR2 reinforces tumour cell migration and invasion in gastric cancer (Cheng, Wang et al. 2011). Furthermore, CXCL8 assists in tumour cell adherence to endothelial cells and the extracellular matrix (Ju, Sun et al. 2012).

Lymphangiogenesis, another crucial factor that causes metastasis in cancer, also involves the ELR-CXC chemokines. The activation of the NF-κB pathway induces CXCL1 expression by lymphatic endothelial cell (LEC), which supports LEC migration and tube formation. The CXCL1 receptor, CXCR2, is correlated with a metastatic tendency in gastric cancer (Xu, Zhang et al. 2012). Likewise, CXCL1 and CXCL2 signalling through CXCR1 and CXCR2 facilitate tumour metastasis and chemo-resistance via a three-component paracrine network: endothelial cells, tumour cells, and myeloid cells (Acharyya, Oskarsson et al. 2012). In lung cancer, knockdown of CXCR2 impedes tumour cell invasion *in vitro* and the metastatic

potential *in vivo* (Saintigny, Massarelli et al. 2013). In non-small cell lung cancer, removal of CXCR2 signalling increases tumour necrosis and diminishes lung metastases (Keane, Belperio et al. 2004). Additionally, Varney and his colleagues (Varney, Singh et al. 2011) pointed out that the blocking CXCR1 and CXCR2 constrains human colon cancer liver metastasis. In addition to affecting tumour cells directly, chemokines also stimulate tumour metastasis through regulation of tumour-associated immune cells. For example, ELR-CXC chemokine expression by macrophages provokes tumour cell invasion as well as migration via the fibroblast growth factor receptor 1 (FGFR1) pathway (Bohrer and Schwertfeger 2012).

1.2.2.3. Angiogenesis

In 1971, the theory that tumour development depended on the new blood vessel formation was first put forward by Folkman, a pioneer in tumour angiogenesis research (Folkman 1971). Further research demonstrated that tumours were still able to receive oxygen and nutrients simply via the diffusion pathway when their diameter is less than 2 mm. However, larger tumours require new blood vessel formation in order to release metabolic wastes and to supply oxygen and nutrients to support tumour development (Flier, Underhill et al. 1995).

The first stage of blood vessel formation is characterized by the recruitment of endothelial cells or progenitors. The process was named after vasculogenesis, and the concept of angiogenesis is slightly different from that of vasculogenesis. Angiogenesis mainly refers to the formation of vasculature via the sprouting of endothelial cells, meaning that the new blood vessels sprout from existing vessels rather than forming brand-new ones (Hu and Cheng 2009). However, in addition to endothelial sprouting, other types of angiogenic patterns such as the co-option (Vajkoczy, Farhadi et al. 2002), wherein tumours acquire their vasculatures by recruiting existed capillaries, intussusceptive microvascular growth (Ribatti and Djonov 2012), which refers to the insertion of connective columns to make two vessels from one existing vessels, postnatal vasculogenesis, which is characterized by the involvement of endothelial progenitor cells in tumour angiogenesis (Dome, Hendrix et al. 2007), and glomeruloid angiogenesis, the aggregation of microvessels surrounded by basement membrane (Brat and Van Meir 2001). In addition, vascular mimicry, has also been specified as leading to cancer progression. Vascular mimicry was originally observed in melanoma. It was identified with

the malignant tumour cells' presenting endothelial cells markers. These endothelial-like tumour cells were able to form new blood vessels or/and connect with the endothelial cell-linked vessels to bring blood perfusion to the tumours even without the involvement of endothelial vessels (Seftor, Hess et al. 2012).

In normal conditions, angiogenesis is a highly hierarchical and controlled process. It is regulated by various pro- and anti-angiogenic factors such as those associated with the VEGF family, the angiopoietin family, VE-Cadherin, TGFβ, CXCL8, etc. However, once the balance of pro- and anti- angiogenic factors shifts towards angiogenesis, the "angiogenic switch" is turned on and angiogenic activities thrive (Bach, Uddin et al. 2007). Interestingly, although angiogenesis occurs in normal physiological processes, such as wound healing and female reproductive cycles, there were some differences in physiological and pathological angiogenesis. For example, leukocyte adhesion is prominent in pathological angiogenesis (Ishida, Usui et al. 2003). In addition, tumour-driven endothelial cells exhibit unique tumour-related phenotypes that are different from normal endothelial cells. More specifically, tumour vessels are usually characterized by uncontrolled permeability, abnormal sprouting patterns, a defective endothelial monolayer, and intercellular gaps (Dudley 2012). All of these lead to reinforced tumour metastasis, failures in delivery of chemotherapy drugs, and resistance to anti-vascular therapies. The abnormal and immature vessels induced by tumours are one of the other hallmarks of solid tumours (Metheny-Barlow and Li 2003).

1.2.2.3.1. VEGF A

Many ligands and receptors, such as the VEGF/VEGFR pathway, the fibroblast growth factor 2 (FGF2) and its receptors-FGFR1 as well as FGFR2, the epidermal growth factor (EGF) and its receptors, delta-like ligand 4 (DLL4)/Notch, CXCL8 and CXCR1 as well as CXCR2, angiopoietins and their receptors, are involved in angiogenesis (Dimova, Popivanov et al. 2014). Among them, VEGF family members, especially VEGF A, are the most widely known. VEGF family members are also important in embryogenesis, skeletal growth, and the maintenance of reproductive functions. So far, six members are included in this family: VEGF A, B, C, D, E, and the placenta-derived growth factor (PlGF) (Ferrara, Gerber et al. 2003). Three tyrosine kinase receptors serve as their receptors: VEGFR1 (flt-1), VEGFR2 (kinase insert domain receptor: KDR/flk-1) and VEGFR3 (flt-4) (Bahram and Claesson-Welsh 2010). VEGF A

(commonly simply referred to as VEGF), VEGF B, and PlGF mainly interact with VEGFR1 and 2, whereas VEGF C and D interact with VEGFR3 (Wang, Taylor et al. 2014). VEGF A is primarily associated with angiogenesis, whereas VEGF C and D are regarded as lymphatic endothelial factors that regulate lymphatic angiogenesis (Bahram and Claesson-Welsh 2010). Lymphangiogenesis contributes to tumour lymph node metastasis, and thus tumour development as well (Karpanen and Alitalo 2008). Because VEGF C and D are critical lymphangiogenic factors, they also promote tumour metastasis and modify the tumour-associated microenvironments (Alitalo, Proulx et al. 2013). Although its function is not clear, VEGF B, has been reported to aid the development of endometrial cancer via the maintenance of host-tumour immuno-responses through VEGFR1 (Holland, Day et al. 2003).

VEGF A is the molecule that initiates angiogenesis and tunes the permeability of blood vessels. It also makes the selection of the tip cell that is characterized by the extended filopodia in response to VEGF A gradients, and stalk cells, the cells that are adherent to pericytes (Matsumoto and Ema 2014) to facilitate the migration of newly-formed blood vessels. Generally, VEGF A is secreted by endothelial cells and serves as a mitogen and autocrine survival factor for endothelial cells, but VEGF A from pericytes is also able to send survival signals to endothelial cells to maintain angiogenesis in a paracrine way. Owing to its functions, the VEGF A gene is widely expressed in many adult tissues and organs, such as the lungs, kidneys, adrenal glands, heart, livers, and the stomach mucosa (Lynn, Roland et al. 2010).

Importantly, tumour cells express both VEGF A and its receptors in ovarian, breast, colon, and prostate cancers, and melanoma (Matsumoto and Ema 2014). Like endothelial cells and pericytes, tumour-associated immune cells such as neutrophils, monocytes and macrophages, which can be chemoattracted through VEGF A and its receptors, can generate VEGF A as well (Machado, Andrade et al. 2014). VEGF A expression leads to angiogenesis directly in tumours. Importantly, it also leads to the expression of other important tumour-related angiogenic factors such as angiopoietin 1 and 2 from endothelial cells (Oh, Takagi et al. 1999). Moreover, it also supports the epithelial-mesenchymal transition (EMT) process. It was illustrated by Gonzalez-Moreno and colleagues that in the prostate intraepithelial neoplasia (PIN)-like C3 (1)/Tag-derived Pr-111 cell line, the overexpression of VEGF A is correlated with the transition from PIN to the more invasive carcinoma. The added VEGF A generates the acquisition of EMT features with the up-regulation of mesenchymal markers such as N-

cadherin, Snail1, Snail2 (Slug), and vimentin, and with the down-regulation of E-cadherin (Gonzalez-Moreno, Lecanda et al. 2010).

The EMT process in tumour development is assigned to malignant epithelial cells that lose their polarity, express epithelial markers and convert into dedifferentiated mesenchymal cells. These epithelial cells express mesenchymal markers such as N-cadherin, vimentin or fibronectin (Fan, Zheng et al. 2013). Regulated mainly through the TGFβ pathway (Moustakas and Heldin 2014), the EMT process breaks cell-cell adhesion and allows tumour cells to depart from their primary sites, thus it contributes to the maintenance of tumour stem cells, tumour invasion, metastasis, chemo-resistance and vascular mimicry (Fan, Zheng et al. 2013).

ELR-CXC chemokines and VEGF A function synergistically to maintain angiogenesis and facilitate the EMT process. For example, in hepatocellular carcinoma (HCC), it was reported that the activation of the neurotensin (NTS)/CXCL8 pathway was involved in the EMT process, with the expression of CXCL8 predicting a poor prognosis (Yu, Ren et al. 2013). Sobolik, *et al.* demonstrated that the tumours with EMT features that co-express CXCR2, CXCR4, CXCR7, CXCL1, CXCL8, CCL2, IL-6 and GM-CSF display more aggressive phenotypes, and blockade of either CXCR4 or CXCR2 is helpful in limiting the tumour's invasion and metastasis (Sobolik, Su et al. 2014). In colorectal cancer, EMT phenotypes and the cooperation of CCL20 and CXCL8 increase liver metastasis, such that expression either of CCL20 or CXCL8 indicates a poor prognosis, but blockade of either one alone is not sufficient to block tumour development (Cheng, Li et al. 2014). Researchers also demonstrated that in tumour angiogenesis, CXCL8-induced VEGF A secretion augments expression of the anti-apoptotic protein Bcl-2, which stimulates endothelial cells to produce more CXCL8 in a positive-feedback loop (Nor, Christensen et al. 2001). In uveal melanoma, CXCL8 and VEGF A act synergistically to maintain angiogenesis (Lattanzio, Tonissi et al. 2013). Furthermore, in a 'transgenic adenocarninoma in the mouse prostate' (TRAMP) model, VEGF A expression is correlated with other tumour-promoting angiogenic factors, e.g., angiopoietin 2, platelet endothelial cell adhesion molecule (PECAM)-1, VE-cadherin, Tie 1, the KDR as well as Flt-1 (Shih, Robinson et al. 2002).

Mutations and polymorphisms of VEGF A genes are directly associated with cancer risk. For example, VEGF A and FLT1 (VEGFR1) genes are associated with susceptibility to breast cancer (Beeghly-Fadiel, Shu et al. 2011), and VEGF A polymorphisms are also linked to

lung cancer risk (Lee, Lee et al. 2005). In addition, a mutation in the VEGF A regulatory region has a close relationship with risk of bladder cancer (García-Closas, Malats et al. 2007). Over-expression of VEGF A is common in cancer patients and it usually implies more aggressive phenotypes and a poor prognosis. In endometrial carcinoma, VEGF A expression is correlated with the density of micro-blood vessels (Wang, Taylor et al. 2014), while serum levels of VEGF A and Bcl-2 are correlated with disease development in melanoma patients (Tas, Duranyildiz et al. 2008). The VEGF A pathway plays crucial roles not only in solid tumours (Park do, Thomas et al. 2015), but also in hematologic malignancies (Paesler, Gehrke et al. 2012) such as acute myeloid leukemia (AML), since it extends the micro-vessels' density (MVD).

Blocking the VEGF A curbs angiogenesis, and thus tumour development. According to Sun and Blaskovich *et al.*, the blockade of the VEGF A via GFA-116, a small synthetic molecule that prevents the binding of the VEGF A to its receptor, inhibited tumourigenesis, angiogenesis and lung metastasis (Sun, Blaskovich et al. 2004). When VEGF A antibodies were combined with chemotherapy drugs (Adamcic, Skowronski et al. 2012) or ionizing radiation therapy (Gorski, Beckett et al. 1999), the increased anti-tumour effects that occurred both *in vitro* and *in vivo* were realized through effects on both tumour and endothelial cells. However, anti-VEGF A therapy still has its limitations (Sitohy, Nagy et al. 2012) in that not all tumour endothelial cells or tumour cells themselves are VEGF A-dependent (Vasudev and Reynolds 2014). Thus, patients may develop resistance to anti-VEGF A therapy soon after the tumour is first exposed to the treatment (Lu and Bergers 2013). At the same time, several side-effects, such as hypertension, proteinuria, and impaired wound healing have been reported during anti-VEGF A therapies (Vasudev and Reynolds 2014). Furthermore, while anti-VEGF A therapy did lower blood flow, blood supply or the density of vessels in cancer, it can bring on a severe hypoxia that can induce more aggressive cancer phenotypes (Keunen, Johansson et al. 2011). Consequently, anti-VEGF A treatment strategies must be considered carefully and thoroughly.

1.2.2.3.2. Angiopoietin 1

In addition to the VEGF family, the angiopoietin (Ang) family and their receptors — Tie receptors on endothelial cells - play critical roles in tumour angiogenesis. The angiopoietin

family includes four members, angiopoietins 1-4 (Valenzuela, Griffiths et al. 1999). They are glycoproteins that mainly control the development and the stability of blood vessels (Fagiani and Christofori 2013). The angiopoietins and their receptors regulate blood vessel formation, changes in vascular permeability and maturation, as well as lymphatic vessel development under normal physiological conditions. The angiopoietins are also involved in tumour-associated inflammation, angiogenic remodelling and vascularization (Eklund and Saharinen 2013).

Two tyrosine kinase receptors, Tie 1 (TIE) and 2 (TEK), which were originally defined as orphan receptors, interact with angiopoietins. Tie 1 continues to be an orphan receptor, but interestingly, it was illustrated that the removal of Tie 1 curbs tumour angiogenesis (D'Amico, Korhonen et al. 2014). According to several reports, Tie 1 is able to regulate the activities of Tie 2 via heterodimerization (Yun, Lee et al. 2013). Thus, Tie 1 is engaged in tumour angiogenesis via interactions with VEGF A, the angiopoietins and the Notch pathway and Tie 2. Tie 2, which is chiefly expressed by endothelial cells, is the receptor for angiopoietins 1-4. Although mainly expressed by endothelial cells, Tie 2 expressed by monocytes also participates in tumour development. Forget and fellow researchers reported that angiogenesis was enhanced through up-regulation of $CD14^+$ Tie 2-positive monocytes following macrophage colony-stimulating factor (M-CSF) stimulation (Forget, Voorhees et al. 2014).

Angiopoietin 1 is one of the ligands of Tie 2 receptor. Pericytes, smooth muscle cells, and fibroblasts are the main sources of this Tie 2 ligand, which affects endothelial cells, mainly via the paracrine pathway (Nasarre, Thomas et al. 2009). Angiopoietin 1 induces Tie 2 phosphorylation and the activation of PI3K-AKT, Raf-MEK-extracellular signal-regulated kinase (ERK) as well as the STAT pathway (Tsai and Lee 2009), each of which is involved in the survival of endothelial cells, as well as vessel stabilization, maturation, and the maintenance of the structural integrity (Tsai and Lee 2009). Angiopoietin 1 is indispensable for the remodelling and stabilization of newly-formed vessels (Holash, Maisonpierre et al. 1999), where it supports adhesion between mural cells and endothelial cells (von Tell, Armulik et al. 2006). It also regulates mesenchymal cell migration (Metheny-Barlow, Tian et al. 2004). Moreover, angiopoietin 1 is able to adjust blood vessel leakage by acting on the cytoskeleton, loosening endothelial cell junctions in inflamed vascular beds (Baffert, Le et al. 2006). Indeed, both VEGF A and angiopoietin 1 promote endothelial cell survival, although they exert

different activities in the control of cell junctions. Angiopoietin 1 maintains vascular junction stability by sequestering the RhoA-specific guanine nucleotide exchange factor Syx, whereas VEGF A leads to the translocation of Syx to foster junction disassembly (Ngok, Geyer et al. 2012). Consequently, angiopoietin 1 has anti-permeability as well as anti-inflammatory effects. It is constitutively expressed at low levels as a stabilisation signal in normal tissues (Holash, Maisonpierre et al. 1999) and it is necessary for embryo development. Embryonic over-expression of angiopoietin 1 increases the diameter of normal vessels, although neither ephemeral over-expression in normal adult tissues nor prolonged over-expression in tumours has an impact on the diameters of blood vessels (Reiss 2010).

In addition to its impact on endothelial cells, angiopoietin 1 signalling through Tie 2 also has affects other cells. For example, neutrophils express the Tie 2 receptor as well, such that exposure of neutrophils to angiopoietin 1 increases CXCL8 expression (Neagoe, Dumas et al. 2012). Angiopoietin 1 also influences mouse primary skeletal myoblasts, promoting cell proliferation, migration, and differentiation, which suggests that it might participate in myogenesis of striated muscles (Lee, Woo et al. 2013). In Alzheimer's disease, significant up-regulation of hypoxia-induced angiopoietin 1 is observed, with the levels of angiopoietin 1 found to be negatively correlated with cognitive function (Schreitmuller, Leyhe et al. 2012). Moreover, it was demonstrated that angiopoietin 1 takes part in neurogenesis, resulting in the activation of the stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) MAPK pathway in axonogenesis. (Rosa, Goncalves et al. 2010).

Although much research has been conducted, the function of angiopoietin 1 in tumour development is still not fully understood, and remains controversial. According to the literature, the expression of angiopoietin 1 is closely related to tumour development. For example, in the melanoma mouse model, the expression of angiopoietin 1 in tumour tissue was much higher than in normal tissues such as the liver and the spleen (Pomyje, Zivny et al. 2001), while a significant association was observed between angiopoietin 1 genetic variants and the overall survival rates in surgically-treated colorectal cancer patients (Dai, Wan et al. 2012). In the case of non-small cell lung carcinoma, increased mRNA levels of Tie 2, angiopoietin 1, the VEGF A and CD31 were verified in cancer patients as opposed to the healthy population (Takahama, Tsutsumi et al. 1999). It appears that expression of angiopoietin 1 supports tumour development by enhancing the growth of mature blood vessels that are covered by pericytes

(Machein, Knedla et al. 2004). In addition to its impact on endothelial cell-related angiogenesis in tumours, angiopoietin 1 acts directly upon on tumour cells too. In HeLa cells, angiopoietin 1 anti-sense modified tumour cells express less angiopoietin 1 and exhibit slower tumour growth, reduced angiogenesis, and enhanced apoptosis (Shim, Teh et al. 2001). Tanja and Holopainen pointed out that angiopoietin 1 is able to facilitate tumour cell entry into the circulation and thus increases metastasis (Holopainen, Huang et al. 2009). However, some researchers have shown that angiopoietin 1 inhibits tumour development. In the mouse hepatic colon tumour model, over-expression of angiopoietin 1 leads to retarded angiogenesis by decreasing micro-vessel density, thereby inhibiting tumour development (Stoeltzing, Ahmad et al. 2003). Hawighotst, Skobe and their colleagues showed that in human squamous cell carcinoma (SCC) xenografts, the expression of angiopoietin 1 facilitates blood vessel maturation. Moreover, the stable over-expression of angiopoietin 1 more than 70% inhibited tumour growth when compared to the lower angiopoietin 1 expression control in human A431 SCCs cell lines (Hawighorst, Skobe et al. 2002).

1.2.2.3.3. Angiopoietin 2

Apart from angiopoietin 1, angiopoietin 2 is another important molecule that manipulates angiogenesis. Angiopoietin 2 has 60% of homology at the amino acid level with angiopoietin 1. Acting as the natural antagonist to angiopoietin 1, angiopoietin 2 mainly regulates blood vessel regression through promoting the detachment of endothelial cells from vessel walls (Maisonpierre, Suri et al. 1997). Like angiopoietin 1, angiopoietin 2 is essential for lymphatic vessel formation - removal of angiopoietin 2 results in abnormal lymphatic vessels and the failure of lymphangiogenesis (Zheng, Nurmi et al. 2014). Above and beyond its impact on pericyte detachment and lymphangiogenesis, angiopoietin 2 is also important in the production of cytokines induced by vascular leakage. Angiopoietin 2-deficient mice display altered vessel structures (i.e., with a wider basement membrane) and dampened vascular responses toward vascular leakage-associated agents, such as histamine, bradykinin and the VEGF A (Benest, Kruse et al. 2013). Owing to its function in normal adult tissues, angiopoietin 2 is generally expressed at a very low level, and restricted to vascular remodelling sites. But angiopoietin 2 is released from endothelial cell Weibel-Palade bodies within minutes of cellular stimulation (Fiedler, Scharpfenecker et al. 2004). Interestingly, it was reported that

angiopoietin 2 is expressed constitutively in human and murine skeletal myoblasts after H_2O_2 stimulation, where it induces skeletal myoblast survival, migration and differentiation (Mofarrahi and Hussain 2011).

In tumour vessels, expression of angiopoietin 2 leads to immature blood vessels that are characterized by lower pericyte coverage, and thus to the suppression of tumour angiogenesis (Fagiani, Lorentz et al. 2011). However, in some cases, angiopoietin 2 could be a Tie 2 activator and promote angiogenesis. It has been shown that blockade of angiopoietin 2 with antibodies suppresses tumour growth and vascularity (Daly, Eichten et al. 2013). As a matter of fact, angiopoietin 2 promotes angiogenesis in the presence of high levels of VEGF A (Holash, Maisonpierre et al. 1999, Hu and Cheng 2009). The combination of angiopoietin 2 antibodies and the VEGF A blocker Aflibercept decreases tumour vascularity significantly more than the use of a single reagent (Daly, Eichten et al. 2013), which implies that the VEGF A and angiopoietin 2 might work synergistically. In advanced castration-resistant prostate cancer, higher expression level of angiopoietin 2 and VEGF was found when compared to hormone-naïve (HN) prostate cancer, with the higher expression leading to blood vessel stabilization (Tomic, Gustavsson et al. 2012). Angiopoietin 2 is involved in tumour metastasis as well. It raises the numbers of lymph node and lung metastases through its effects on endothelial disaggregation and thereby tumour cell translocation and migration to target organs (Holopainen, Saharinen et al. 2012). It also facilitates tumour metastasis through the activation of MMP-2 and ECM degradation (Zhang, Zhou et al. 2013). Additionally, angiopoietin 2 attracts Tie 2-expressing macrophages and monocytes to tumour sites, and it induces the differentiation of regulatory T cells. Consequently, angiopoietin 2 also maintains tumour-associated microenvironments and immunosuppression (Riabov, Gudima et al. 2014).

In many malignancies, expression of angiopoietin 2 is correlated with disease development. In melanoma-bearing mice, angiopoietin 2 is expressed at abnormally higher levels in the spleen, livers and bone marrow than in normal mice. (Pomyje, Zivny et al. 2001). This suggests that tumour development might alter the pattern of angiopoietin 2 expressions even in remote tissues. In patients with metastatic melanoma, serum levels of angiopoietin 2 are correlated with tumour progression (Helfrich, Edler et al. 2009). Moreover, in patients with multiple myeloma, higher levels of angiopoietin 2 are strongly correlated with other angiogenic factors, such as interleukin-6 (IL-6), platelet-derived growth factor-AB (PDGF-AB), resulting

in higher bone marrow micro-vascular density (MVD) (Pappa, Alexandrakis et al. 2014). The level of angiopoietin 2 also correlates with disease development in non-small cell lung cancer such that it has been used as a useful prognostic marker. Similar correlations have been observed in acute myeloid leukemia, colorectal cancer, and thyroid cancer (Fagiani and Christofori 2013).

Blocking angiopoietin 2 has been a promising strategy in treating cancer. In a hepatocellular carcinomas model, the administration of antibodies to angiopoietin 2 restrains the VEGF A- and angiopoietin 2-induced tumour cell proliferation in human umbilical vein endothelial cells *in vitro*. *In vivo*, this antibodies treatment decreased tumour weight, the expression of the endothelial marker CD31, micro-blood vessel densities, and lung metastases (Zhang, Zhang et al. 2014). Although angiopoietin 2 is increasingly gaining attention as a potential inhibitor of tumour angiogenesis, its mechanisms of action still need to be explored (Gerald, Chintharlapalli et al. 2013).

1.2.2.3.4. Angiopoietin 1, angiopoietin 2, VEGF A and angiogenesis

VEGF A, angiopoietin 1, and angiopoietin 2 work in concert to promote angiogenesis. In general, mature blood vessels are made up of endothelial cells and perivascular cells, with the endothelial cells comprising the inner lining of the vessels, and perivascular cells enveloping the vascular tube surfaces, where they function as a supporter of the vessel walls. Perivascular cells also called pericytes, vascular smooth muscle cells or mural cells (Bergers and Song 2005), are able to send survival signals to endothelial cells (Franco, Roswall et al. 2011). Other types of blood vessels, such as capillaries, arterioles, and venules can have different components (Jain 2003).

When angiogenic sprouting begins, VEGF A is released, which regulates cell-cell junctions and vascular leakage. The latter is important since it provides endothelial cells with increased contact with angiogenic stimulants and facilitates tumour cell migration out of their primary site (Le Guelte, Dwyer et al. 2011). After such stimulation, endothelial cells release angiopoietin 2, thus destabilizing the vessel's integrity. Angiopoietin 2 also promotes the detachment of pericytes from the endothelium, creating gaps that allow for the growth of vessels toward pro-angiogenic factors' gradients such as VEGF A, PDGF, TGFβ-1 and TNF α, and thereby provisional tube formation. This regression and proliferation process is

characterized by the low levels of angiopoietin 1, but high angiopoietin 2 and VEGF A expression (Bach, Uddin et al. 2007). In addition, VEGF A and FGF stimulate the migration of endothelial cells to form stronger branches (Chung, Lee et al. 2010). FGF, EGF, PDGF and other growth factors are also act in the recruitment of the progenitor cells and pericytes that produce mature blood vessels. In the last stage, angiopoietin 1 is released from the pericytes to elicit further stabilization as well as maturation of blood vessels. (Brudno, Ennett-Shepard et al. 2013). In the end, as mentioned above, angiopoietin 1 will be expressed continuously by vessel endothelial cells at a low level as a stabilisation signal (Holash, Maisonpierre et al. 1999).

Importantly, tumour-associated angiogenesis differs from the normal angiogenic sprouting. In tumour-induced angiogenesis, multiple types of cells are recruited to support the angiogenesis, owing to tumour-induced inflammatory responses. According to Ishida *et al.*, high-level leukocyte adhesion occurs in pathological, but not in physiological angiogenesis (Ishida, Usui et al. 2003). Additionally, tumour vessels are usually built in a chaotic way rather than being highly organized as they are in normal situations, thus the structure and morphology of the blood vessels are usually abnormal in tumour-induced angiogenesis (e.g., tumour vessels display a mosaic-like morphology and have an imperfect endothelial cell lining, with wider intercellular junctions (Jain 2003). Research has also shown that endothelial cells in mosaic-like tumour vessels did not express such common endothelial cell markers as CD31 and CD105 (Chang, di Tomaso et al. 2000).

The functions of VEGF A, angiopoietin 1 and 2, especially angiopoietin 1, are controversial and complicated. Their expression patterns are also different in different cancers. Some investigator have begun to look into the ratio of angiopoietin 1 to angiopoietin 2 when evaluating tumour development (Welti, Loges et al. 2013). In some cancers, this ratio is related to disease development. For instance, in the late stages of melanoma, a decreased serum angiopoietin 1 to angiopoietin 2 ratio occurs, and that predicts tumour progression (Gardizi, Kurschat et al. 2012). In brain tumours, blockade of VEGFR induces an increased ratio of angiopoietin 1 and 2, and that is correlated with reduced tumour vessel diameters, but also to an enhanced radiosensitivity of tumour cells (Winkler, Kozin et al. 2004). The ratio of angiopoietin 1 and 2 is also used as a prognostic marker in some other diseases, such as febrile neutropenia, lung injury, and Waldenstrom macroglobulinaemia (Thomas and Augustin 2009).

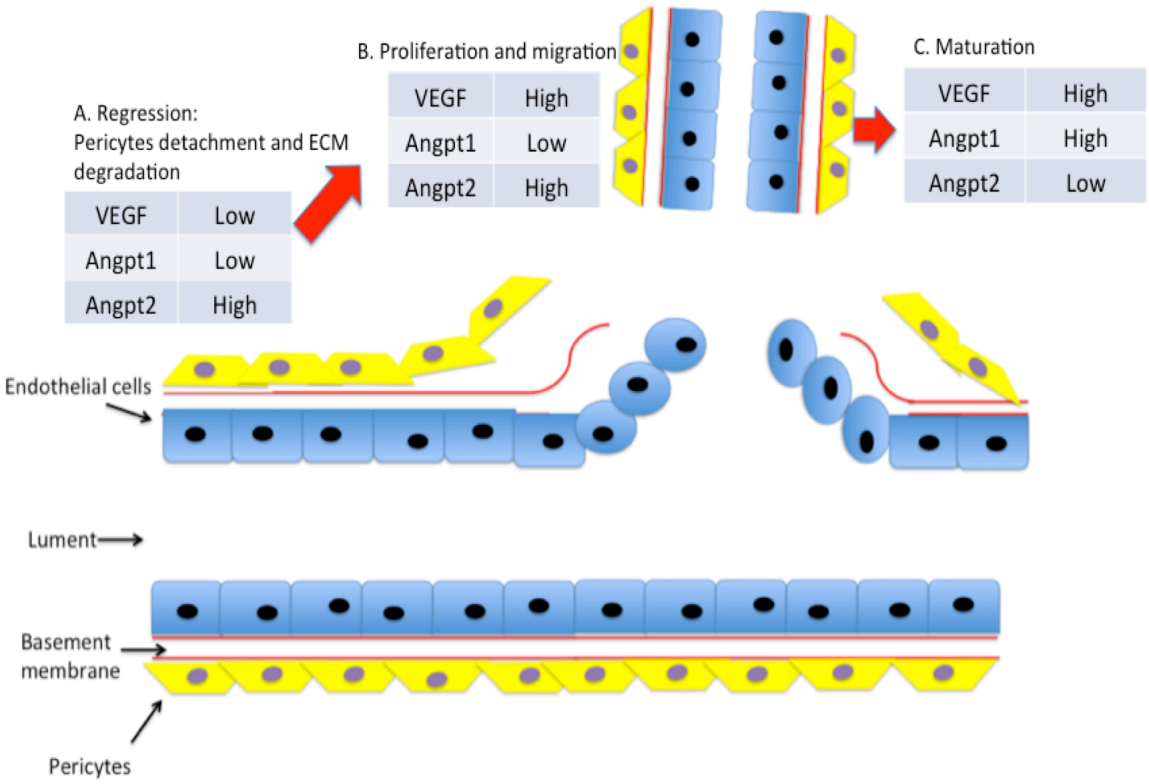


Figure 1.2 Expression of angiopoietins and VEGF A in the development of tumour vessels.

Three main stages of tumour vessel formation exist, including regression, proliferation & migration, and maturation. The two angiopoietins and VEGF A play different roles in these stages, with the levels of angiopoietin 2 and VEGF A changing through these different stages of vessel development.

1.2.2.3.5. ELR-CXC chemokines in angiogenesis

The ELR-CXC chemokines also play important roles in angiogenesis. Indeed, they are all angiogenic factors either by themselves or upon interaction with the other molecules that promote angiogenic processes. Chemokine signalling directly impacts the vascular endothelium (Kiefer and Siekmann 2011), activation of which is characterized by the secretion of angiopoietin 2, the detachment of smooth muscle cells, and vascular leakage (Saharinen, Eklund et al. 2011), each key features in cancer development. For example, in non-small cell lung cancer, removal of CXCR2 signalling increases tumour necrosis and diminishes lung metastases (Keane, Belperio et al. 2004). ELR-CXC chemokines such as CXCL8 are able to foster tumour angiogenesis directly through their influence on the proliferation, survival, and migration of endothelial cells (Li, Varney et al. 2005). ELR-CXC chemokines also facilitate tumour angiogenesis by adjusting the permeability of endothelial cells. For example, CXCL8 down-regulates tight junction molecules such as zonnual occludens (ZO)-1, claudin-5 and occludin in the endothelium, so permeability increases, and thus supports tumour angiogenesis as well as metastasis (Yu, Huang et al. 2013). At the same time, ELR-CXC chemokines also cooperate with other angiogenic factors. Li and Varney *et al.* reported that CXCL8 induces the expression of other angiogenic factors such as MMP-2 and -9, and the VEGF A from endothelial cells in the autocrine pathway (Li, Varney et al. 2005) as well as in the paracrine pathway (Schruefer, Lutze et al. 2005). The MMP family is also one of the most important groups involved in regulating tumour invasion and metastasis through their abilities to break down the extracellular matrix. In melanoma, the combination of CXCL8, VE-cadherin, endothelial differentiation gene 1 (EDG-1), MMP-2, and fibronectin-1, along with galectin-3, promote tumour angiogenesis as well as vascular mimicry (Mourad-Zeidan, Melnikova et al. 2008). Cyclooxygenase (COX)-2 converts arachidonic acid to prostaglandins and their related eicosanoids. Performing important roles in inhibiting apoptosis and intensifying angiogenesis, expression of COX-2 is elevated in several types of cancers. Interestingly, Chan, Ogino and colleagues suggested that regular use of anti-inflammatory drugs such as aspirin decreases colorectal cancer risk by interfering with the over-expression of COX2 (Chan, Ogino et al. 2007). Importantly, neutralizing antibodies against CXCL5 and CXCL8 can also inhibit enhanced human non-small cell lung tumour growth under COX2-over-expressing conditions (Pold, Zhu et al. 2004).

The murine duffy antigen receptor (DARC) is a decoy receptor for CXC or CC chemokines. Expressed on leukocytes and endothelial cells, the enhanced DARC expression curbs tumour-associated angiogenesis in the DARC-transgenic melanoma mouse model (Horton, Yu et al. 2007). Over-expression of DARC is associated with better prognosis, decreased metastatic potential and neovascularization in breast cancer, thyroid cancer , non-small-cell long carcinoma and melanoma (Horton, Yu et al. 2007), whereas the decreased expression of DARC seen in African-Americans might be associated with their enhanced mortality to prostate cancer (Shen, Schuster et al. 2006).

1.2.2.3.6. Anti-angiogenic therapy as a therapeutic option

The advantages of using anti-angiogenic drugs for treating cancer are obvious. Reducing angiogenesis decreases the chemotherapeutic drug-resistance of tumour cells, since the treatment is more targeted to genetically stable endothelial cells than to unstable tumour cells. Tumour cells have plastic genomes. Moreover, abnormal changes in chromosomes (Rodero, Combadière et al. 2015), such as deletions, rearrangements, missegregations, amplified genes, and mutations in tumour cells induce cells that are drug-resistant during and after chemotherapy (Kerbel 1997). Furthermore, anti-angiogenic therapy is believed to decrease severe side-effects, since it is more specific to tumour-associated endothelial cells than to normal cells (Shen, Zhou et al. 2013). For example, chemotherapy might damage "bystander" tissues since it does not specifically target tumour cells, and such damage may facilitate tumour metastasis (Ebos and Kerbel 2011).

Several anti-angiogenic strategies have been developed (Dome, Hendrix et al. 2007), although we have yet to satisfactorily establish the treatment effects of any anti-angiogenic drugs. Moreover, while not all the tumours are sensitive to anti-angiogenic treatments (Vasudev and Reynolds 2014), no single anti-angiogenic medication has proven adequate to block tumour development in cancer patients (Eklund, Bry et al. 2013), suggesting that combined therapies may be necessary. In fact, the combination of anti-angiogenic drugs with other anti-tumour therapies leads to better outcomes (Ribatti, Nico et al. 2010). For example, in the mouse melanoma model, combining anti-VEGF A plus adoptive T cell transfer (ACT)-based immunotherapy increases the infiltration of transferred killer T cells into the tumours.

The blockade of the VEGF A increased the ACT treatment's cytotoxic effect toward tumour cells significantly (Shrimali, Yu et al. 2010).

1.2.3.2. Resistance to chemo- or hormone-therapy

Chemo-resistance contributes greatly to tumour-related mortality. Chemokines and their receptors, e.g., CXCL8, CXCR1 as well as CXCR2, contribute substantially to the development of chemotherapeutic drug resistance. In the PC-3 human prostate cancer cell, the mRNA levels of CXCL8, CXCR1 and CXCR2 are up-regulated in a time-dependent way under hypoxia (Maxwell, Gallagher et al. 2007) and chemotherapy (Wilson, Purcell et al. 2008). The increasing expression of CXCL8 not only facilitates tumour cell chemoresistance, but also augments radio-resistance in several prostate cancer cell lines (Xu, Fang et al. 2012). In triple-negative breast cancer patients, CXCL8 and CXCR1 expression stimulate the PI3K isoform through the PI3K-γ and JAK2-STAT5 pathways, thus leading to resistance to PI3K-mTOR inhibitors (Abraham 2012). Moreover, repeated exposure to chemotherapy drugs results in enhanced expression of chemokines and their receptors, while blockade of signalling through CXCR1 and 2 improves the effects of chemotherapy. The anti-metabolite drug 5-fluorouracil (5-FU), for example, enhances the expression of CXCL8 as well as CXCR1 and 2 in metastatic human prostate cancer, while blocking CXCL8 signalling intensifies 5-FU's efficiency via inhibition of Bcl-2 (Wilson, Maxwell et al. 2012). In human melanoma cell lines, repeated administration of the first-line chemotherapy drug dacarbazine spurs expression of CXCL8 and the VEGF A and induces a reinforced tumourigenicity and higher metastatic potentials *in vivo* (Varney, Li et al. 2003). Similarly, in breast cancer, the chemotherapy drug docetaxel promotes up-regulation of CXCL8 and expands the cancer stem cell population, survival and maintenance of which contributes significantly to tumour cells' becoming resistant to chemotherapy (Ginestier, Liu et al. 2010).

Interestingly, signalling through CXCR1 and CXCR2 can transactivate other signalling pathways, such as the human epidermal growth factor (EGF) receptor, AKT, and ERK1/2 in several different cancers (Singh, Farnie et al. 2013). In breast cancer, up-regulation of CXCL8 is reportedly associated with low expression of estrogen receptors (ER), another predictor of poor prognosis among patients (Singh, Simoes et al. 2013). In androgen-responsive prostate cancer cell lines, interactions between CXCL8 and CXCR1, as well as CXCR2, lowers the

effects of bicalutamid, an anti-androgen reagent (Araki, Omori et al. 2007). It has also been observed that CXCL1 signalling through CXCR2 results in the cleavage of heparin-binding EGF-like growth factor (HB-EGF), causing transactivation of the EGF receptor and promoting tumour cell proliferation (Bolitho, Hahn et al. 2010). Moreover, in prostate cancer, up-regulation of CXCL8 intensifies the expression of CXCR7 and is linked to the activation of the EGFR and cancer development (Singh and Lokeshwar 2011).

1.2.2.4. CXCR1 and CXCR2 in tumour microenvironments

It is known that undiagnosed infections leading to chronic inflammation can induce malignancies directly. Oncogene activation, on one hand, can trigger inflammatory cascades while, on the other hand, the inflammatory cascades contribute to the increased cancer risk and the formation of tumour-associated microenvironments. Several types of cells, such as tumour-associated neutrophils, macrophages and fibroblasts, mesenchymal stem cells, lymphocytes, and endothelial cells are recruited into tumour sites by chemokines, and these cells become secondary sources of additional chemokines (Lazennec and Richmond 2010).

1.2.2.4.1. Neutrophils

Even though tumour-associated neutrophils contribute both pro- and anti-tumoural activities in tumour microenvironments, the presence of neutrophils always indicates a poor prognosis in patients (Gregory and Houghton 2011). Neutrophils acquire different pro- or anti-tumoural phenotypes in tumour microenvironments driven either by tumour cells or T helper cells (Galdiero, Garlanda et al. 2013). The neutrophils' pro-tumoural 'N2' phenotype, which presents as cells that secrete collagenase, MMPs, chemokines, and arginase, reduces cytotoxic effects by restraining the function of T cells (Fridlender and Albelda 2012). ELR-CXC chemokines and their receptors, CXCR1 and CXCR2, are able to recruit immune cells, mainly neutrophils, into inflammatory sites. In melanoma tumour-bearing mice, CXCL1, CXCL2 and CXCL5 induce neutrophil recruitment into tumour sites (Jablonska, Wu et al. 2014). After being recruited, neutrophils themselves become a second resource of cytokines and growth factors, including MMPs and CXCL8. In murine malignant mesothelioma AB12 tumour-bearing mice, intensified expression of CXCL1, CXCL2 and CXCL5 mRNA is associated with neutrophil recruitment, and reducing this neutrophilic input increases the activity of $CD8^+$ T

cells, curbing tumour growth (Fridlender, Sun et al. 2009). In the A549 lung tumour model, tumour growth is inhibited by preventing neutrophil infiltration with the CXCR2-specific small molecule inhibitor AZ10397767 (Tazzyman, Barry et al. 2011).

1.2.2.4.2. Macrophages

Tumour-associated macrophages (TAMs) also contribute significantly to tumour development. Macrophages can acquire a polarized 'M2' phenotype that supports tumour progression, wherein they decrease their antigen-presenting capabilities, and hence have lower cytotoxic functions, but at the same time they strengthen their secretion of suppressive cytokines, such as IL-10 and TGFβ, but also MMPs, thymidine phosphorylase (TP), urokinase-type plasminogen activator (uPA), adrenomedullin (ADM), and angiogenic factors such as VEGF A and FGF2 (Riabov, Gudima et al. 2014). Thus, M2 macrophages promote tumour growth and angiogenesis, and foster tumour-associated immunosuppression. Interestingly, tumour-associated macrophages also support lymphangiogenesis and the trans-differentiation of lymphatic endothelial cells through their expression of the lymphatic endothelium marker lymphatic vessel endothelial hyaluronan receptor (LYVE-1) (Riabov, Gudima et al. 2014). The M2 macrophage phenotype can also induce Th_2 differentiation in T cells, and that can promote tumour development in various types of cancers (Gabitass, Annels et al. 2011). ELR-CXC chemokines induce the recruitment of tumour-associated immune cells and regulate the function and positioning of M2 tumour-associated macrophages (Mantovani, Sozzani et al. 2002). Promisingly, inhibition of NF-κB, a key component in ELR-CXC chemokine signalling, pushes tumour-associated macrophages to convert back into a classical cytotoxic phenotype (Sica, Larghi et al. 2008).

1.2.2.4.3. Fibroblasts

Tumour-associated fibroblasts and their related chemokine networks also play critical roles in tumour progression, and the ELR-CXC chemokines have a close relationship with tumour-associated fibroblasts. Matsuo and his colleagues (Matsuo, Ochi et al. 2009) demonstrated that CXCL12 from fibroblasts induces enhanced CXCL8 expression by pancreatic cancer cells, thus promoting tumour development. Interestingly, expression of CXCL12 by fibroblasts is elevated when the fibroblasts were co-cultured with pancreatic

tumour cells. In addition, tumour cell-derived CXCL8 and fibroblast-derived CXCL12 stimulate human umbilical vein endothelial cell proliferation and invasion, thus facilitating tumour angiogenesis (Matsuo, Ochi et al. 2009).

1.2.2.4.4. Dendritic cells

Both myeloid and plasmacytoid dendritic cells are found in tumour sites. Immature dendritic cells express several chemokine receptors, such as CCR1, CCR2, CCR5, CCR6, as well as CXCR1, and maturation causes rapid expression of CXCL8 (Sallusto, Palermo et al. 1999). Both immature DCs and mature DCs produce and are responsive to CXCL8. It has been shown that the infiltration of neutrophils and CD123-positive dendritic cells, which results in the production of T regulatory cells in the tumour microenvironment, forecasts a poor prognosis in primary melanoma (Jensen, Schmidt et al. 2012). Interestingly, CXCL8 produced by tumour cells alters the migration of dendritic cells without impacting DC-mediated T cell stimulation (e.g., it induces retention of intra-tumoural dendritic cells) (Alfaro, Suarez et al. 2011).

1.2.2.4.5. Mast cells

Mast cells express CXCR1 as well as CXCR2, such that they can be chemoattracted by ELR-CXC chemokines, including CXCL8 (Nilsson, Mikovits et al. 1999). Mast cells are involved in maintaining and remodelling tumour-associated microenvironments. Mast cell infiltration predicts a poor prognosis in several cancers (Pappa, Tsirakis et al. 2014). Like tumour-associated macrophages and neutrophils, tumour-associated mast cells also polarize to a M2 phenotype, with enhanced expression of pro-inflammatory factors such as heparin, histamine, CXCL8, VEGF A and IL-17 (Huang, Lei et al. 2008). They thereby support tumour cell proliferation, angiogenesis, tumour-induced-immunosuppression and tissue damage through their degranulation and release of these cytokines and chemokines. In pancreatic cancer, mast cells are recruited into tumour sites via the β-islet cells *myc* pathway, where they are an abundant source of angiogenic factors such as CXCL8, VEGF A, TGFβ, TNFα and MMP-9 (Soucek, Lawlor et al. 2007). It has been reported that mast cell density is correlated with the expression of the VEGF A, GRO-α, and ENA-78 in multiple myeloma (MM), and that their presence is indicative of activated angiogenic processes and disease progression (Pappa,

Tsirakis et al. 2014). Mast cells are also able to promote tumour development by interacting with other cells, such as effector T cells, dendritic cells, B cells and regulatory T cells (Yang, Zhang et al. 2010). Mast cell infiltration is associated with increases in mRNA levels of Foxp3 and TGFβ in tumours, and consequently of $CD4^{+}CD25^{+}$ $Foxp3^{+}$ T regulatory cells, and thus fosters tumour-induced immunosuppression (Huang, Lei et al. 2008).

1.2.2.4.6. Cancer stem cells

Abnormal expression of chemokines is also fundamental to maintaining populations and the functions of cancer stem cells. As a group of stem-like cells, cancer stem cells are the specific sub-population of tumour cells that are equipped with a self-renewal function. They are involved in the induction of functional heterogeneities in solid tumours, and play a significant role in tumour development. They are responsible for the chemo-resistant characteristics of breast, prostatic, lung, brain, liver, colonic, pancreatic, and mesenchymal cancers and melanoma (Visvader and Lindeman 2008). The ELR-CXC chemokine receptors, CXCR1 and CXCR2, are involved in the development of cancer stem cells. In breast cancer, over-expression of CXCR1 occurs in ALDEFLUOR-positive cancer stem cells when compared to the other tumour cells in the same tumour masses, but CXCL8, CXCR1 and CXCR2 expression are each important to both the type of cancer stem cell present, and the maintenance of their relative proportions within tumours (Singh, Farnie et al. 2013).

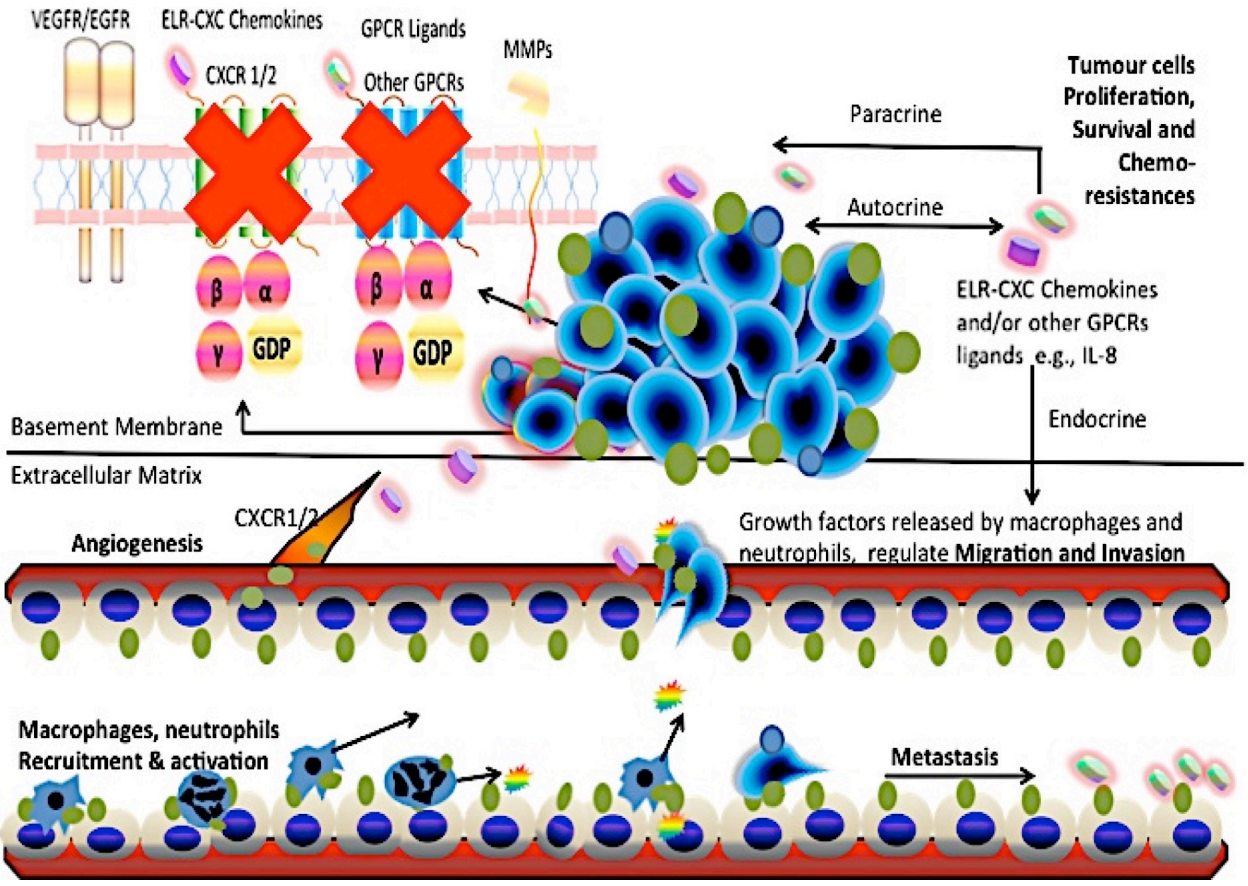


Figure 1.3 Roles of the ELR-CXC chemokines, CXCR1 and CXCR2 in tumour development.

ELR-CXC chemokines signal through the CXCR1 and CXCR2 to promote tumour cell proliferation, survival, angiogenesis, metastasis and chemo-resistance. Tumour cells, immune cells such as neutrophils and macrophages, and vessel endothelial cells express CXCR1 and CXCR2. ELR-CXC chemokines are able to impact on these different cells via autocrine, paracrine and endocrine pathways to promote tumour development.

1.2.3. The inhibition of CXCR1 and CXCR2 as a tumour therapy

Because the ELR-CXC chemokines have such great effects on tumour development, a number of molecules that target the ELR-CXC chemokines or their receptors have been developed and, to varying extents, they are able to restrain tumour development. For example, two small molecule antagonists (SCH-527123, SCH-479833) targeting CXCR1 and CXCR2 have been developed. Oral delivery has prevented human colon cancer from metastasizing to the liver (SCH-527123, 27-42% inhibition; SCH-479833, 19-49% inhibition), and up to 60% reduced the proliferation and migration of tumour cells (Varney, Singh et al. 2011). Small interfering RNA (siRNA) toward CXCR1 and CXCR2 curbs cell proliferation, survival, invasion and migration in human melanoma cell lines (Singh, Sadanandam et al. 2010). In human intrahepatic cholangiocelluar carcinoma, blockage of CXCR2 through siRNA prevents tumour development *in vitro* and *vivo* (Sueoka, Hirano et al. 2014). Repertaxin, a small molecule allosteric CXCR1 and CXCR2 antagonist, similarly prevents ELR-CXC chemokine signalling. The usage of Repertaxin, singly or in combination with CXCR1 antibodies, is able to deplete cancer stem cells *in vitro* and *in vivo* in a humanized SCID mouse model of breast cancer (Ginestier, Liu et al. 2010). However, although Repertaxin prevents CXCL8-induced calcium flux in neutrophils, it does not inhibit the binding of CXCL8 to its receptors on purified rat neutrophils or the activation of neutrophils induced by ligands for heterologous GPCR, such as fMLP, PAF or LTB_4 (Souza, Bertini et al. 2004). Additionally, even though several CXCR1 and 2 antagonists have been created, overall they lack sufficient receptor affinity to be clinically useful (Jones, Dewald et al. 1997). Antibodies toward CXCL8 have also been developed. In human melanoma xenograft models, fully humanized neutralizing antibodies toward CXCL8 inhibit tumour growth and angiogenesis (Varney, Li et al. 2003). CXCL8 antibodies have had some effects on the inhibition of tumour development, but Waugh and Wilson illustrated that the single usage of antibodies was insufficient in clinical application, probably owing to over-expression and redundancy among tandemly-expressed ELR-CXC chemokines (Waugh and Wilson 2008).

More promisingly, G31P, developed by Fang Li and Gordon, (Li and Gordon 2001) not only effectively blocks CXCR1- and CXCR2-dependent inflammatory responses (Fox, Gordon et al. 2011), but also antagonizes heterologous GPCRs such as those for C5a, fMLP, and LTB_4 in human neutrophils (Zhao, Town et al. 2009). G31P also exhibits potent anti-tumour effects

in some tumour models. In a humanized SCID mouse prostate cancer model, G31P is able to regulate cancer cell viability, adhesion and migration, as well as tumour progression, angiogenesis and metastasis (Liu, Peng et al. 2012). In a mouse hepatoma model, either the combination of the common chemotherapy drug cisplatin or the use of G31P by itself reduces tumour growth (Wei, Chen et al. 2014). Furthermore, still in the mouse H22 hepatoma model, G31P treatments not only inhibit tumour cell proliferation both *in vitro* or *vivo*, but also reduced side-effects such as acute renal failure (Li, Khan et al. 2015). Radiotherapy is a commonly used therapeutic option in many types of cancers and in the mouse model, G31P usage can postpone the emergence of respiratory distress and decrease radiation-induced alveolitis (Fox, Gordon et al. 2011), and that implies that the combination of G31P with radiotherapy might achieve better results in cancer treatments.

1.2.4. Altered GPCR signalling in tumour development

Not surprisingly, altered non-CXCR1 or CXCR2 GPCR signalling also impact tumour growth, angiogenesis, metastasis and immuno-tolerance in different cancers. Mutated GPCR are associated with variations in cancer risk (Lappano and Maggiolini 2012). For example, according to Beaumont, Newton et al., mutated human melanocortin-1 receptor gene (MC1R) induces the skin-cancer-related phenotype, characterized by red hair and fair skin (Beaumont, Newton et al. 2005). The abnormal expression of other GPCRs is also correlated with chronic tumour-associated inflammation. Other tumour-associated GPCRs, such as CXCR4, the follicle-stimulating hormone receptor (FSH-R), and thrombin, lysophosphatidic acid (LPA), gastrin-releasing peptide (GRP), endothelin and prostaglandin receptors also increased in several types of primary as well as metastatic tumours (Li, Huang et al. 2005). For instance, enhanced expression of CXCR4 is a predictor of poor prognosis in several human cancers including melanoma (Gil, Seshadri et al. 2013). CXCL12, the ligand for CXCR4, intensifies the proliferation and migration of melanoma cells. Inhibition of stromal CXCR4 also prevents lung metastasis in melanoma (D'Alterio, Barbieri et al. 2012). The interaction of CXCL12 and ELR-CXC chemokines and their receptors induces invasion and angiogenesis in pancreatic cancer (Varney, Li et al. 2003). In androgen-dependent prostate cancer, tumour cells are able to proliferate with the help of ELR-CXC chemokines, even in the absence of the steroid-like hormone. This indicates that crosstalk between ELR-CXC chemokines and the androgen

receptor is involved in the resistance to hormone ablation therapy (Kasina and Macoska 2012). Interestingly, ELR-CXC chemokines such as CXCL8 are able to activate the Kaposi's sarcoma-associated herpesvirus (KSHV)/human herpesvirus 8-encoded GPCRs - the KSHV virus is important to the pathogenesis of Kaposi's sarcomas and lymphomas (Gershengorn, Geras-Raaka et al. 1998). Another example is the LPA receptors, which are expressed in human colorectal cancer cells, and an elevated LPA2/LPA1 ratio is significantly associated with malignant transformation (Shida, Watanabe et al. 2004). In human pancreatic cancer, higher-mobility malignant cells have higher levels of the LPA receptor 1 compared to those with the lower migratory abilities (Yamada, Sato et al. 2004). Gastrin-releasing peptide (GRP) acts as an autocrine factor in tumour development, inasmuch as it is linked to tissue lesions and neoplasms in the human prostate (Markwalder and Reubi 1999). Furthermore, the expression of endothelin-1 (ET-1)/endothelin A receptor (ETAR) prompts proliferation, angiogenesis, metastasis and epithelial-mesenchymal transition (EMT) processes in human ovarian cancer via an autocrine pathway, but they are also engaged in the chemo-resistance via increasing MAKP and Akt signalling (Bagnato and Rosano 2012).

GPCRs are also able to collaborate with non-GPCR to support tumour development. For instance, crosstalk between GPCRs and growth factor receptors is important to the progression of colon, lung, breast, ovarian, prostate, and head and neck tumours (Lappano and Maggiolini 2011). In addition, crosstalk between the GPCRs and insulin or the insulin-like growth factor I (IGFI) receptor (IGFIR) is involved in the development of diverse malignancies. In pancreatic cancer, crosstalk between the IGFI and GPCRs induces enhanced DNA synthesis and enhanced cell proliferation. The anti-diabetic drug metformin prevents tumour cell proliferation by interfering with this crosstalk pathway in a xenograft model (Rozengurt, Sinnett-Smith et al. 2010).

1.2.5. The CXCR1/2 Antagonist CXCL8 $_{(3-72)}$ K11R/G31P

CXCL8 $_{(3-72)}$ K11R/G31P (G31P) is an antagonist of the CXCR1 and CXCR2, but also heterologous GPCRs on CXCR1 and/or CXCR2-positive cells. It was first developed as an analogue of bovine CXCL8. CXCL8 is homodimer in solution (Clore, Appella et al. 1990). Its monomeric unit displays a flexible NH_2 terminal region followed by three anti-parallel β-strands and a COOH-terminal α-helix. Numerous studies had illustrated that modest amino-

terminal CXCL8 truncation can increase its receptor affinity (Clark-Lewis, Dewald et al. 1994). Clark-Lewis and colleagues reported that both 3-72 and 4-72 amino-truncated forms of CXCL8 was more active than full length (1-72) CXCL8 in neutrophil elastase release assays, whereas further truncated (i.e., a.a. 7-72) CXCL8 displayed no detectable activities in neutrophil chemotaxis assays, which suggested that NH_2 terminal residues Glu^4-Leu^5-Arg^6 (ELR motif) is essential for receptor binding and neutrophils-stimulated activities (Clark-Lewis, Schumacher et al. 1991). Bovine $CXCL8_{(3-73)}$K11R (incorporating a lysine 11 to arginine substitution, to increase receptor affinity) was developed as a super-agonist, and shown to be highly effective in inducing neutrophil β-glucuronidase release and chemotaxis (Li and Gordon 2001). A G31P substitution was then introduced into $CXCL8_{(3-73)}$K11R in order to delete the molecule's receptor-stimulatory properties, as determined in neutrophil elastase release assays . Bovine $CXCL8_{(3-73)}$K11R/G31P proved to dramatically antagonize bovine CXCR1- and CXCR2-related activities (Li, Zhang et al. 2002). One low dose of bovine G31P is able to antagonize neutrophil responses to endotoxin stimulation in cattle for more than 2 days (Li, Zhang et al. 2002).

G31P is able to block severe inflammatory responses in different models and species, including *Klebsiella* pneumonia in guinea pigs (G31P dose, 500 μg/kg) (Wei, Peng et al. 2013), radiation-induced lung responses and fibrosis in mice (G31P dose, 500 μg/kg every second day), (Fox, Gordon et al. 2011), ischemia reperfusion in the intestine in rats (G31P dose, 500 μg/kg) (Zhao, Town et al. 2010), aspiration pneumonia in guinea pigs (G31P dose, 250 μg/kg) (Zhao, Town et al. 2010), airway inflammation induced by the endotoxin exposure (G31P dose, 100 μg/kg) in swine (Gordon, Zhang et al. 2009). Human CXCL8 shares 78% of sequence homology with bovine CXCL8 (Podechard, Lecureur et al. 2008), such that a human form of G31P: CXCL8 (3-72) K11R/G31P was also developed, and shown to conduct its antagonizing function at multiple levels, including epithelial cells, neutrophils, and alternate G protein-coupled receptors (Zhao, Town et al. 2009). As discussed above, more promisingly, G31P has also been shown to block tumour progression, including angiogenesis, in mouse models of human prostate cancer, murine hepatoma and murine hepatocellular carcinoma (Liu, Peng et al. 2012, Wei, Chen et al. 2014, Li, Khan et al. 2015). Taken together, it is clear that the ELR-CXC chemokines and tumour-associated GPCRs play critical roles in tumour development, and especially in angiogenesis.

CHAPTER 2: HYPOTHESIS AND OBJECTIVES

2.1. Hypothesis

The purpose of this thesis is to verify whether or not signalling through CXCR1 and/or CXCR2, as well as through heterologous GPCRs, plays an important role in tumour development, at least in part through their impact on angiogenesis. We hypothesize that blockade of CXCR1 and 2 will curb tumour development and angiogenesis in a murine subcutaneous Matrigel B16-10 melanoma tumour cell model.

2.2. Objective

1. Evaluate tumour progression through assessments of mouse health, tumour volumes, and tumour weights.

2. Assess tumour-bearing Matrigel plug vascularization, as determined using intravascular FITC-dextran to visualize blood vessels and contents.

3. Assess the expression of angiogenic mediators and ELR-CXC chemokine in tumour-bearing Matrigel plugs as a measure of tumour angiogenesis.

CHAPTER 3: BLOCKADE OF CXCR1 AND CXCR2 IN TUMOUR ANGIOGENESIS

3.1 INTRODUCTION

Angiogenesis is an essential aspect of normal physiology, inasmuch as it is important in processes such as embryo development, reproduction and wound-healing, among many others. It is also involved in the pathogenesis of many diseases, as well as tumour development (Flier, Underhill et al. 1995).

Much research has been conducted, and numerous models have been set up to assess angiogenesis both *in vitro* and *in vivo*. This includes assays inspecting endothelial cell proliferation, migration, tube formation and wound-healing, organotypic organ culture (e.g., aortic ring) assays, the mouse cornea model, the rat mesentery window assay, zebra fish models, chick chorioallantoic membrane (CAM) assays and the Matrigel plug assay (Auerbach, Lewis et al. 2003). Each of these assays has their own advantages and disadvantages. *In vitro* assays are sometimes more easily controlled, repeatable, and labour- and time-saving, but might not reflect the integrated blood vessel formation processes correctly, whereas *in vivo* assays represent the dynamic more vividly and more accurately reflect the full-spectrum of biological processes. However because individual differences exist, *in-vivo* assays are less easily reproduced since they will be impacted by all the variables that resident in the intact animal. Generally, it is believed that *in vivo* angiogenesis assays are more physiologically-relevant than *in vitro* assays. Although there are no definite standard rules for angiogenesis observations, the selection of appropriate assays is very important and a combination of multiple assays is usually recommended (Irvin, Zijlstra et al. 2014).

The Matrigel plug assay provides an elegant way to look into tumour angiogenesis *in vivo* at the gross or molecular levels and it is relatively easily quantified. Matrigel is a commercially-available mixture of solubilized extracellular matrix (ECM) proteins, derived from Engelbreth-Holm-Swarm (EHS) mouse sarcoma, which remains in a liquid state when the temperature is below 4°C, but solidifies rapidly at body temperatures (Benton, Kleinman et al. 2011). Basement membrane extracellular matrix (ECM) components are produced by epithelial, endothelial, fat and smooth muscle cells, and consist of laminin, type IV collagen, perlecan (a heparan sulfate glycoprotein), nidogen (a glycoprotein), proteases, growth factors, and other proteins. The main function of the ECM is to support and maintain cell growth. In tumours, the cancer stem cell population resides within an ECM milieu, from which it

establishes the tumour-associated microenvironment and facilitates angiogenesis as well as lymphangiogenesis. The components of the ECM can vary by tissue type and tumour developmental stages - the components of the tumour ECM are usually deregulated and disorganized (Lu, Weaver et al. 2012). Since 1972, a number of tumour models have been set up to investigate the roles of the ECM in tumour development and diseases. In 1977, Orkin and his colleagues found that tumours produced different types of matrices compared to normal cells. In 1986, Matrigel was shown to be biologically active, facilitating tube formation by endothelial cells. Also, melanoma cells that were co-cultured with Matrigel display more intense, earlier pigment formation than melanoma cells cultured without Matrigel (Kleinman, McGarvey et al. 1986). Later, the Matrigel assay was developed to test tumour cell invasion, with the discovery that HT1080 tumour cells were able to invade Matrigel whereas normal cells did not (Albini, Iwamoto et al. 1987).

Over the years the Matrigel assay has been used widely and successfully in investigations of tumour-induced angiogenesis. In general, for the Matrigel assay tumour cells are mixed with liquid Matrigel on ice and then injected subcutaneously into mice. The cellular plugs solidify rapidly and subsequently vascularize as the oxygen and nutrients demands of the growing tumours increases over time. After the plug is taken out, the angiogenic process can be measured by an array of assays, including determination of the extent of vascularity (the FITC-dextran assay), quantification of hemoglobin (Johns, Freay et al. 1996), the measurement of blood flow and blood pressure in tumours. In addition, the cells or cellular components (e.g., proteins, mRNA) can be extracted from the plugs for further characterization.

3.2. METERIALS AND METHODS

3.2.1. Animals

Female C57BL/6 mice (7-8 week-old) were purchased from Charles River Labs (Quebec) and maintained in the Lab Animal Services Unit, College of Medicine, University of Saskatchewan. The animals were held on a 12 h light: 12 h dark cycle and provided with food (LabDiet®, St Louise, MO) and water *ad libitum*. All the animal handling procedures employed in these experiments were approved by the University of Saskatchewan's Animal Research Ethics Board in accord with the Canadian Council on Animal Care Guidelines for humane animal use.

3.2.2. Generation of the Matrigel model

The highly metastatic murine melanoma cell line, B16-10, which was a kind gift from Dr. Jim Xiang's lab at the University of Saskatchewan, was used for our experiments. Tumour cells were cultured in RPMI 1640 medium (Thermo Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS; Mediatech, Manassas, VA), 0.1 mg/mL streptomycin (Invitrogen, Burlington, ON), 1% 2-mercaptoethanol, 1% L-glutamine, 1% sodium pyruvate, 100 μg/mL kanamycin (each from Sigma Chemical Co, St. Louis, MO) in a humidified incubator (Thermo Scientific, Waltham, MA) at 37°C in a 5% of CO_2 atmosphere. Cells were recovered from flasks using 0.5% Trypsin/2nM ethylenediamine tetraacetic acid (EDTA) (Life technology, Carlsbad, CA) when they had achieved ≈ 80% confluence. The trypsin was diluted with RPMI 1640 medium/10% FBS, and then the cells were diluted to the proper concentration (8×10^6 /mL) after being centrifuged. All cells were kept for the same number of passages. The extra cells would be collected and frozen at -80 °C freezer (Isotemp® Fisher Scientific, Waltham, MA) for the repeated experiment. Finally, the cells were washed with PBS and kept on ice until the Matrigel injections were prepared.

Tumour cells in PBS (final concentration: 4×10^6 /mL) were mixed 1:1 with the Matrixgel™ Basement Membrane Matrix Phenol-Red Free (9 mg/mL; BD Biosciences, San Diego, CA) (Salcedo, Ponce et al. 2000). The tumour cell/Matrigel mixture (200 μL) was injected subcutaneously on the shaved backs of the mice on two sites. At the same time, the 200 μL of the negative control (9 mg/mL the Matrigel only with PBS) was also injected on a lower back site of the same mice.

3.2.3. G31P injections

G31P was generated using a standard protocol, as noted (Li, Zhang et al. 2002), and then was stored at -80°C to prevent any degradation. The G31P used throughout this work was diluted in saline from the same batch of 1 mg/mL low-LPS G31P (EU/mL=0.1347184). We had noted previously that G31P is able to block severe inflammatory responses in different models and species, including *Klebsiella* pneumonia (Wei, Peng et al. 2013) and radiation-

induced lung responses and fibrosis, both in mice (G31P dose, 500 µg/kg every second day), (Fox, Gordon et al. 2011), mesenteric artery ischemia reperfusion injury in rats (G31P dose, 500 µg/kg) (Zhao, Town et al. 2010), aspiration pneumonia in guinea pigs (G31P dose, 250 µg/kg) (Zhao, Town et al. 2010), and airway inflammation induced by the endotoxin exposure (G31P dose, 100 µg/kg) in swine (Gordon, Zhang et al. 2009).

In our matrigel model, mice were administered G31P i.p. at the standard rodent dose of 500 µg/kg body weight three times each week (as determined previously in the lab; Dr. J Gordon, personal communication), beginning on the day of Matrigel injection (day 0). This route of G31P administration has been shown to effectively block inflammatory responses (Fox, Gordon et al. 2011, Wei, Peng et al. 2013) and tumour progression, including angiogenesis, in mouse models of human prostate cancer, murine hepatoma and murine hepatocellular carcinoma (Liu, Peng et al. 2012, Wei, Chen et al. 2014, Li, Khan et al. 2015).

3.2.4. Mouse health and the Matrigel measurement

The body weight (g) and temperature (°C) of each mouse was obtained using a lab scale (Sartorius BL1500, UK) and mouse thermometer (ThermoWorks MicroTherma 2, ThermoWorks, Lindon, UT). The mice were sacrificed using CO_2 and the plugs were taken out according to the Matrigel suppliers recommendation (Ohashi, Yokoyama et al. 2006). Weights (g) and volumes (mm^3) of the embedded tumour plugs were acquired after the Matrigel plugs were taken out on day 6 or 14. Calipers (Black Rock Tools™ Digital Caliper, Canada) were used to measure the sizes of plugs across three dimensions, and the equation: tumour volume = $0.5 \times L \times W \times H$, where L is the greatest length, W is the greatest width, and H is the greatest depth or height of the tumour, was employed in calculating the final tumour volumes (Monga, Wadleigh et al. 2000).

3.2.5. Fluorescein isothiocyanate (FITC)-dextran labeling

High molecule weight FITC-dextran (molecular weight: 20 kDa, Sigma-Aldrich, St. Louis, MO) was injected into the mice tail veins of mice (100 µl of 25 mg/ml) 20 minutes prior

to euthanization (Chander, Foster et al. 2007). After sacrifice, the Matrigel plugs were taken out for weighing, photography and fluorescence quantification.

3.2.6. Photography of tumour vasculatures

Photographs of the tumour vasculature were obtained using an Olympus SZX16 dissecting stereoscope equipped with visible and fluorescence (FITC) filters (Olympus, Melville, NY). The photographs of the Matrigel plugs were taken immediately after the plugs were removed from the animals. We used exposure times of 200 ms under multiple magnifications (0.5 ×, 1.0 ×, 1.6 ×, 2.0 ×) in order to obtain clear photographs of the plugs.

3.2.7. Quantification of FITC-dextran

After the Matrigel plugs were photographed, an average portion of each plug (Day 6: plugs with cells: 79.1 % of the total weight of the plugs, negative control: 56.90 % of the total weight of the plugs; Day 14: 97.56 % of the total weight of the plugs, negative control: 61.11 % of the total weight of the plugs) was transferred into a 1.5 mL tube with 1 (day 6) or 2 mL (day 14) of dispase solution (BD Biosciences, San Diego, CA), while another portion of each plug was processed for mRNA extraction using RNeasy kits (QIAGEN, Chatsworth, CA). For quantification of FITC-dextran, the dispase /Matrigel plugs were incubated for 16 hr at 37° C in the dark, after which the contents were centrifuged (Allegra 6R® Centrifuge, BECKMAN, Mississauga, ON) at 1924 × g (Chander, Foster et al. 2007), and the levels of FITC in the supernatants were quantified using a microplate reader (NOVOstar® BMG Lab Technologies, Midland, ON) equipped with a 485 nm excitation filter and a 520 nm emission filter. This data was compared with a FITC-dextran standard curve (0.06 – 250 μg/mL). The FITC-dextran concentrations were normalized with the serum FITC-dextran, and then were divided by the weight of each Matrigel plug. Thus, vasculature content is presented as the volume of blood per gram of Matrigel plug (mm^3/g).

3.2.8. Quantitative Real-time PCR (qRT-PCR)

3.2.8.1. RNA Isolation

Immediately after being collected, Matrigel samples for RNA extraction were stored in RNAlater™ RNA Stabilization Reagent (Qiagen, Chatsworth, CA), with no more than 30 mg Matrigel/mL of RNALater solution, at room temperature for ≤1 week before use. The total RNA was extracted using a spin column-RNeasy® RNA Mini Kit (Qiagen, Chatsworth, CA) according to the suppliers protocol; the average RNA yield for each Matrigel sample was 810 ng/ μ L mRNA, with A260/280 ratios of ≈ 2.1 (Maseko, Howell et al. 2014). All the samples were stored at -80˚C until processed for analysis.

3.2.8.2. cDNA synthesis

The RNA was diluted to 15 ng/μL in RNase-free water for cDNA synthesis when it was ready. Each 20 μL cDNA reaction contained: 4 μL qScript™ cDNA SuperMix (Quanta BioSciences, Gaithersburg, MD), 6.67 μL RNA template (15 ng/μL), and 9.33 μL RNase-free H_2O. The cDNA reaction was run using a CFX96 Touch™ Real-time-PCR Detection System (Bio-Rad, Hercules, CA), with run cycles of: 4 min at 25°C, 30 min at 42°C, and 5 min at 85°C, and then the reactions were allowed to sit at 4°C. RNase-free water was added into each tube (180 μL) to bring the total volumes to 200 μL once the incubation was completed.

3.2.8.3. Primer design and Real-time-PCR

Primer sequences of mouse β-Actin, dopachrome tautomerase (Dct), MIP2, VEGF A (sequences were confirmed through the NCBI online tool Primer-BLAST) as well as angiopoietin 1 (Rosa, Goncalves et al. 2010) and angiopoietin 2 (Marteau, Valable et al. 2012) were listed in Table 3.1. Positive controls used in the assay included mouse heart (angiopoietin 1 and VEGF A; (Bi, Drake et al. 1999), uterine horns (angiopoietin 2)(Maisonpierre, Suri et al. 1997), LPS-induced inflammatory lung tissues (36 hr responses; MIP2) (Abraham, Carmody et al. 2000, Matzer, Baumann et al. 2001) and B16-10 tumour cells (Dct).

The reagents used for the Real-time-PCR reactions (20 μL total volumes) included: 10 μL PerfeCTaTM SYBR® Green FastMixTM, Low ROXTM (Quanta BioSciences,

Gaithersburg, MD), 1 µL Primer 1, 1 µL Primer 2, and 3 µL RNase-free H2O. To 15 µL of this solution we added 5 µL of cDNA template, with no cDNA template added to the non-template control (NTC). The real-time-PCR reactions were run in a CFX9 Touch™ Real-time-PCR Detection System (Bio-Rad, Hercules, CA), with the cycles set as: 1 cycle of 30 sec at 95°C; 45 cycles of 1 sec at 95°C, and 30 sec at 60°C; and one cycle of 1 min at 95°C, 30 sec at 65°C, and 30 sec at 95°C. After data were collected and were normalized to mouse β-actin, the relative levels of mRNA were calculated using the ΔΔCt equation (Ct GOI - Ct nom = ΔCt; ΔCt sample - ΔCt Calibrator = ΔΔCt; Relative quantity = $2^{-\Delta\Delta Ct}$) (Adhikary and Eilers 2005). The Ct value is the numbers of qRT-PCR cycles at which the fluorescence signal (i.e., DNA amplification within the PCR reaction) crosses a threshold of detection; that value correlates with the initial amount of primer template within the reactions mixtures. Thus, expression levels of genes are presented as $2^{-\Delta\Delta Ct}$.

Table 3.1 Primer sequence for Real-time-PCR

Gene name	Forward sequences	Reverse sequences
β-Actin	AGAGGGAAATCGTGCGTC	CAATAGTGATGACCTGGCGGT
Dct	GAGAAACAACCCTTCCACAGATG	ACCAACTGGAGCTTCTTTCCTCT
CXCR 2	CATCCGTTTGAGGGTCGTA	GCCAGCAGAGCAGGAAGACA
MIP 2	AAACATCCAGAGCTTGAGTGTGAC	GCCTTGCCTTTGTTCAGTATCTT
VEGF A	GAGTACCCCGACGAGATAGAGTACA	TCTCCTATGTGCTGGCTTTGGT
Angiopoietin 1	TGCATTCTTCGCTGCCATTC	ATTGCCCATGTTGAATCCGGT
Angiopoietin 2	TTAGCACAAAGGATTCGGACAAT	GGACCACATGCGTCAAACC

3.3.Statistical analysis

All experiments were run twice (ie, one repeat). All results presented are the pooled data from the two independent experiments and are expressed as mean ± Standard Error of the Mean (SEM) (n=10 mice/group). Graphpad Prism 6® was used for statistic analysis. Non-parametric unpaired Mann-Whitney T-tests were conducted when comparing two groups. Differences were considered significant when $p < 0.05$.

CHAPTER 4: EXPERIMENT RESULTS

4.1 Mouse health and tumour growth in the Matrigel model

4.1.1. Mouse body weights

To confirm how the tumour development would impact mouse's health, we observed mouse body weights and body temperatures. These are two critical parameters for clinical scores evaluations and humane endpoint decisions (Orellana-Muriana 2013). Hence, we observed mouse body weights (Fig. 4.1) and temperatures (Fig. 4.2) on days 5, 7, 12, and 14 after the mice were injected with B16-10 melanoma cells in Matrigel. Each paired Matrigel plug with tumour cells and the negative control plugs (the Matrigel with PBS only) were from the same mice. According to Fig. 4.1, mouse body weights increased gradually in both the G31P-treated (Day 0: 19.960 ± 0.304 g; Day 14: 22.800 ± 0.396 g, $p < 0.0001$) and untreated groups (Day 0: 19.869 ± 0.202 g; Day 14: 22.489 ± 0.338 g, $p < 0.0001$) from day 0 to day 14. Normal mice (age-matched, unmanipulated healthy mice) exhibited increased body weights from day 0 to day 14 as well (Day 0: 19.180 ± 0.563; Day 14: 21.250 ± 0.476 g, $p = 0.0196$). So both the G31P-treated and untreated mice showed increased body weights from day 0 to day 14 owing to the growth of tumour and normal weight gain. Moreover, tumour-challenged mice also showed significantly higher means in the measurement of body weights when compared to normal mice on day 14 in both the treated (G31P treated: 22.489 ± 0.338 g; Normal mice: 21.250 ± 0.476 g, $p = 0.0218$) and untreated groups (untreated: 22.800 ± 0.396 g; Normal mice: 21.250 ± 0.476 g, $p = 0.0501$). But there was no significant difference in body weights of the G31P-treated and untreated groups on day 14 ($p = 0.5613$). The G31P treatment did not impact mouse body weights, but the body weights of all three groups were in a normal range.

4.1.2. Mouse body temperatures

Along with body weights, mouse body temperatures were also obtained on days 0, 5, 7, 12, and 14. It can be seen from in Fig. 4.2 that there was no difference in body temperatures in the G31P-treated and untreated groups (G31P-treated: 37.820 ± 0.114 °C; untreated: 38.002 ± 0.180 °C, $p = 0.3645$). However, interestingly, it seems that the B16-10 tumour cell-challenged mice displayed significantly higher body temperatures than mice with no tumour cell challenge in both the G31P-treated (G31P-treated: 37.820 ± 0.114 °C; control: 37.340 ± 0.156 °C, $p = 0.0445$) and untreated groups (untreated: 38.002 ± 0.180 °C, $p = 0.0173$) on day

14. According to Sanchez-Alavez and colleagues (Sanchez-Alavez, Alboni et al. 2011), although there were fluctuations in mouse body temperatures, they were still in a normal range. G31P did not impact mouse body temperatures even on day 14 ($p \geq 0.05$).

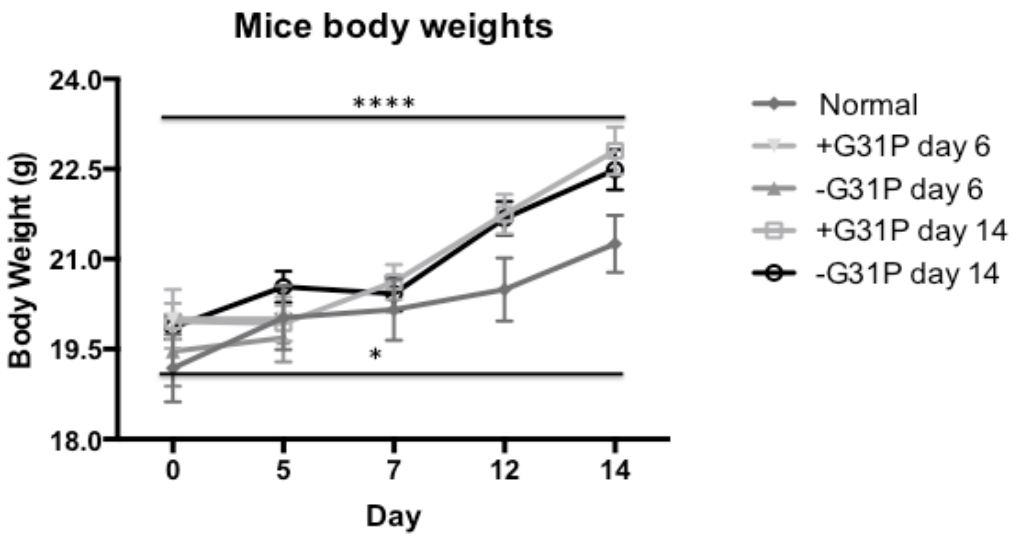


Figure 4.1 The G31P treatment did not alter mouse body weights.

Mouse body weights increased with time in the G31P-treated, untreated and normal groups (unmanipulated, healthy mice). Mouse body weights increased gradually in both the G31P-treated and untreated groups from day 0 to day 14 (***, $p < 0.001$). The G31P treatment did not impact body weights, even on day 14 ($p \geq 0.05$). Mann-Whitney T-test was conducted, n =10 mice/group.

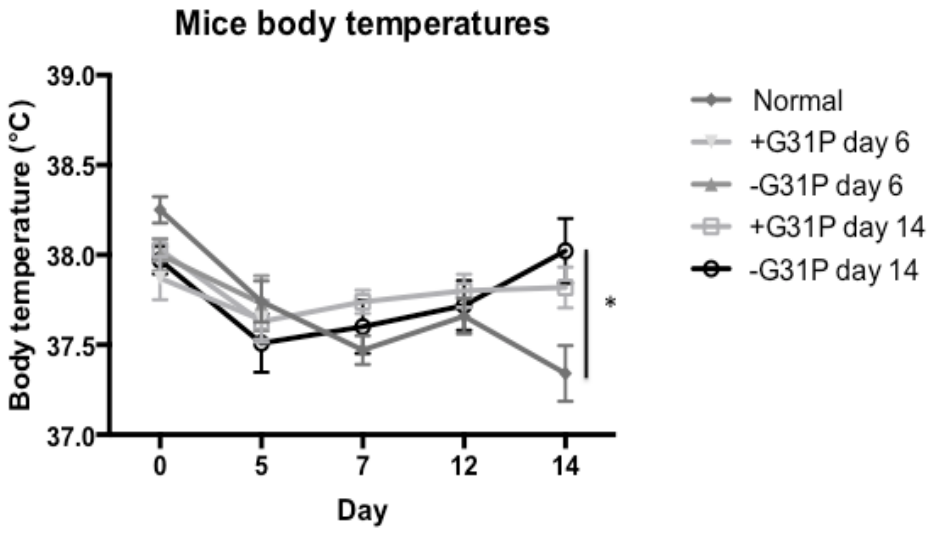


Figure 4.2 The impact of the G31P treatment on mouse body temperatures.

Mouse body temperatures were acquired on days 0, 5, 7, 12, and 14. There was no difference in body temperatures in the G31P-treated and untreated groups ($p \geq 0.05$). B16-10 tumour cell-challenged mice displayed significantly higher body temperatures than mice that were not challenged with tumour cells (unmanipulated, healthy mice). (*, $p < 0.05$). Mann-Whitney T-test was conducted. n = 10 mice/group.

4.1.3. Volumes of the Matrigel plugs

Volumes of the Matrigel plugs were also determined to evaluate tumour growth (Fig. 4.3). The Matrigel plugs were taken out of the mice after euthanization on both day 6 and 14. The volumes of the Matrigel plugs increased gradually as the tumours contained within grew. Statistically significant differences were found when comparing day 6 plugs to those from day 14, since the tumours were growing in both the G31P-treated (50.511 ± 6.737 mm^3 on day 6 to 620.043 ± 141.333 mm^3 on day 14, $p < 0.0001$) and untreated groups (49.100 ± 5.457 mm^3on day 6 to 720.477 ± 130.318 mm^3 on day 14, $p < 0.0001$). The Matrigel plugs without tumour cells were set as the negative control (Day 6: G31P treated: 25.644 ± 3.462 mm^3, Untreated: 29.053 ± 3.979 mm^3; Day 14: G31P treated: 22.280 ± 3.185 mm^3; Untreated: 27.017 ± 3.448 mm^3). However, there were no significant differences between the G31P-treated or untreated groups in terms of tumour volumes either on day 6 or on day 14 ($p \geq 0.05$). The G31P treatments did not have a discernible impact on tumour volumes.

4.1.4. Weights of the Matrigel plugs

The weights of the Matrigel plugs with B16-10 melanoma cells were also investigated as another indicator of tumour growth. The data (Fig. 4.4) indicates, that similar to the tumour volumes, the weights of the Matrigel plugs rose with time in both the G31P-treated (0.091 ± 0.011 g on day 6 to 1.123 ± 0.171 g on day 14, $p < 0.0001$) and untreated groups (0.101 ± 0.105 g on day 6 to 1.090 ± 0.168 g on day 14, $p < 0.0001$) likely due to the tumour growth in the plugs. The Matrigel plugs without tumour cells were set as the negative control (Day 6: G31P-treated: 0.061 ± 0.007 g, Untreated: 0.054 ± 0.009 g; Day 14: G31P-treated: 0.050 ± 0.005 g; Untreated: 0.059 ± 0.003 g). Still, there were no differences in the G31P-treated and untreated groups on day 6 ($p = 0.6453$) and day 14 ($p = 0.9851$), so it can be concluded that G31P did not impact tumour growth because the weights and volumes of tumour plugs did not change significantly with the G31P treatment versus without G31P.

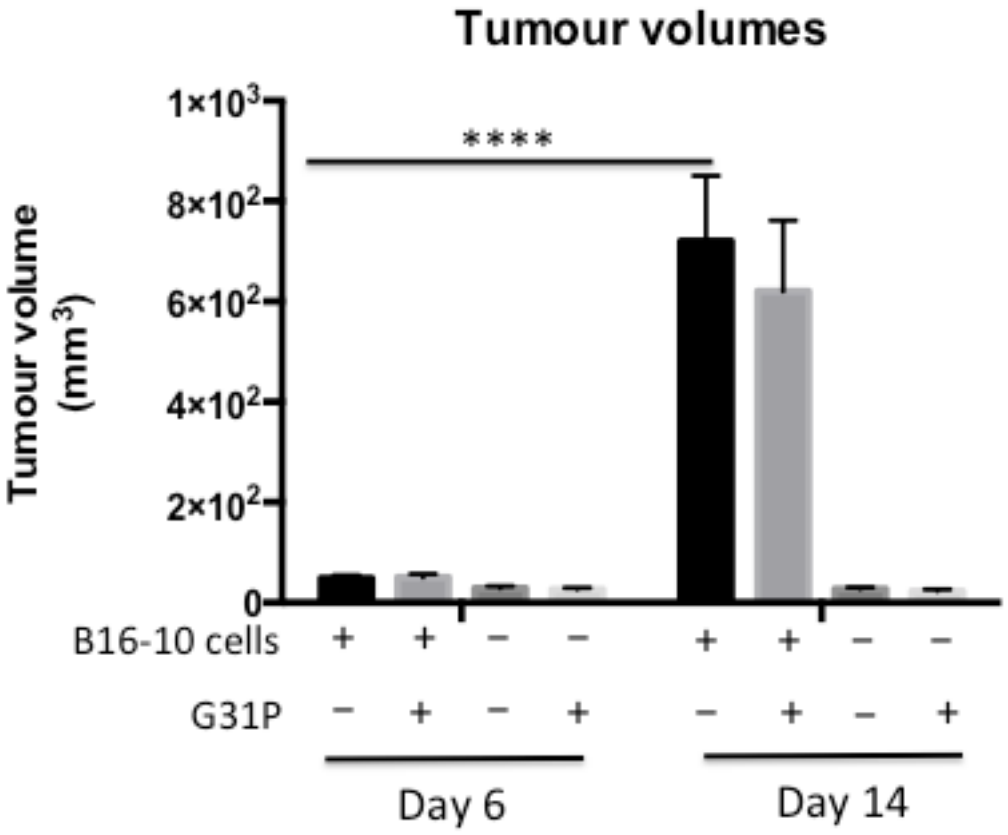


Figure 4.3 Volumes of melanoma cell-containing Matrigel plugs from untreated and G31P-treated mice.

Matrigel plugs were measured using caliper after euthanizasion on day 6 and 14 and the volume was calculated using the equation described in the materials and methods section: tumour volume = 0.5 × L × W × H, where L is the greatest length, W is the greatest width, and H is the greatest height of the tumour. Matrigel plugs without tumour cells were set as the negative control. The volumes of the Matrigel plugs increased significantly from day 6 to day 14. ****, $p < 0.0001$, relative to tumour cell-bearing untreated mice. The G31P treatment did not have a discernible impact on tumour volumes ($p \geq 0.05$). Mann-Whitney T-test was conducted. n = 10 mice/group.

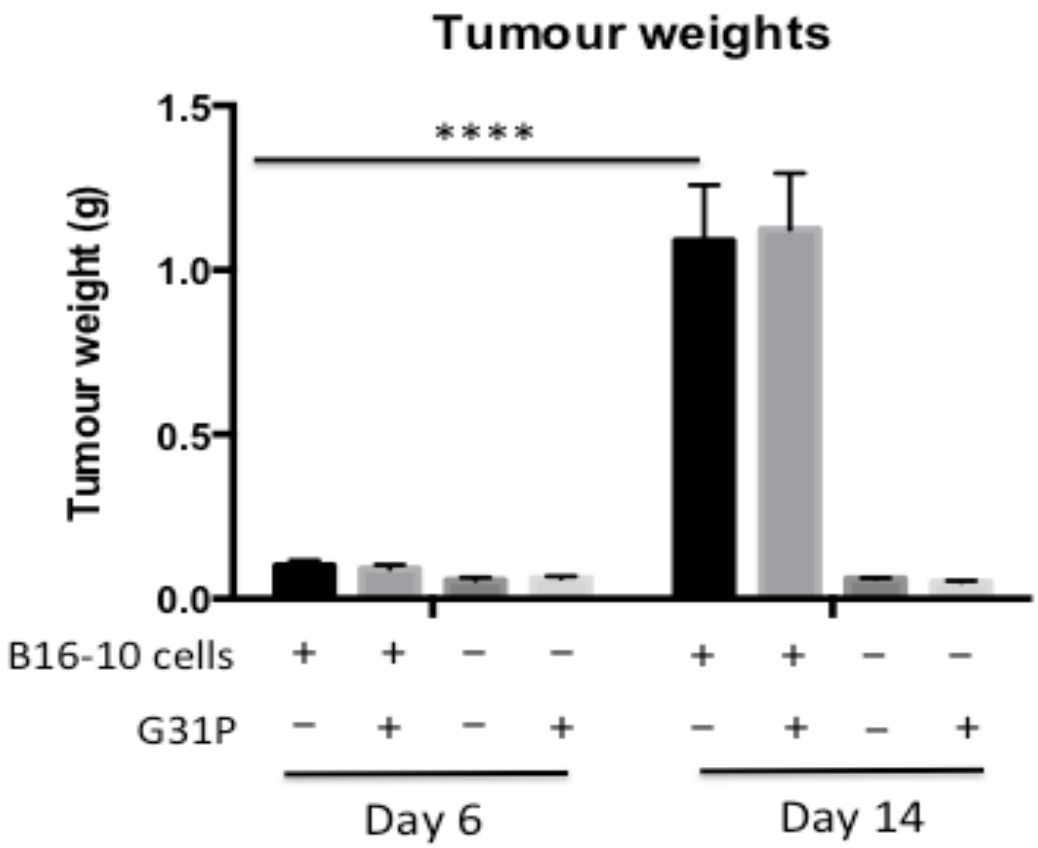


Figure 4.4 Weights of melanoma cell-containing Matrigel plugs from untreated and G31P-treated mice.

Weights of the Matrigel plugs were obtained after removal from mice. Matrigel plugs without tumour cells were set as the negative control. The weights of the Matrigel plugs increased significantly from day 6 to day 14. ****, $p < 0.0001$, relative to tumour cell-bearing untreated mice. Similarly, the G31P treatments did not impact tumour weights ($p \geq 0.05$). Mann-Whitney T-test was conducted. n = 10 mice/group.

4.1.5. The expression of tumour marker Dct

We next employed real-time PCR to verify the effects of G31P on tumour growth in the Matrigel plugs, first assessing expression of the mouse melanoma tumour marker Dct. We found that the tumour cell-loaded Matrigel plugs contained Dct mRNA, confirming that the tumour cells had grown successfully within the plugs (Fig. 4.5). There was an up-regulation of Dct from day 6 (1.002 ± 0.228) to day 14 (9.823 ± 2.821) in untreated group plugs *($p = 0.0002$)*. Similarly, there were no differences in tumour cell expression of Dct between the G31P-treated and untreated group animals on either day 6 or 14 (each, $p \geq 0.05$), which indicated that melanoma cells grew successfully in this model and that the G31P treatment did not affect tumour cell expression of Dct.

4.1.6. The expression of ELR-CXC chemokines receptors CXCR2

After looking into the tumour marker Dct, expression of the mouse ELR-CXC chemokines receptor, CXCR2, was also assessed (Fig. 4.6). The CXCR2 plays a critical role in murine melanoma development. It is the target of the G31P treatment, therefore it was important to confirm that the melanoma cells in this model express CXCR2 We found that the B16-10 embedded tumour cells did express CXCR2 (Fig. 4.6), that this expression did not change from day 6 to day 14 ($p \geq 0.05$), and that the G31P treatment did not make any difference in the expression of CXCR2 on either day 6 or day 14 ($p \geq 0.05$). Thus, the G31P treatment did not significantly impact CXCR2 expression.

4.1.7. The expression of mouse ELR-CXC chemokines MIP2

The expression of one of mouse ELR-CXC chemokines, the well-known tumour growth and angiogenic factor MIP2, was assessed (Fig. 4.7). In this study, on day 6, we confirmed that the B16-10 tumour cell-containing Matrigel plugs (1.000 ± 0.253) had significantly higher levels of MIP2 mRNA than the LPS-induced inflammatory lung tissues (0.029 ± 0.002). In the untreated group, there was a decrease in MIP2 expression from day 6 to 14 (Day 6: 1.000 ± 0.253; Day 14: 0.083 ± 0.064, $p = 0.0005$). As for G31P treatment effects, it was assumed that the use of G31P might block the MIP2 autocrine pathway in tumour cells

and perhaps also impact the recruitment of tumour-associate immune cells (e.g., macrophages and neutrophils), thus effecting paracrine MIP2 secretion. However mice receiving G31P treatments did not display a significant change in MIP2 expression on either day 6 (G31P-treated: 0.743 ± 0.252; Untreated: 1.000 ± 0.253, $p = 0.4225$) or day 14 (G31P-treated: 0.038 ± 0.012; Untreated: 0.083 ± 0.064, $p = 0.5106$). Hence, expression of the mouse ELR-CXC chemokine MIP2 did not change significantly under G31P treatment in this model.

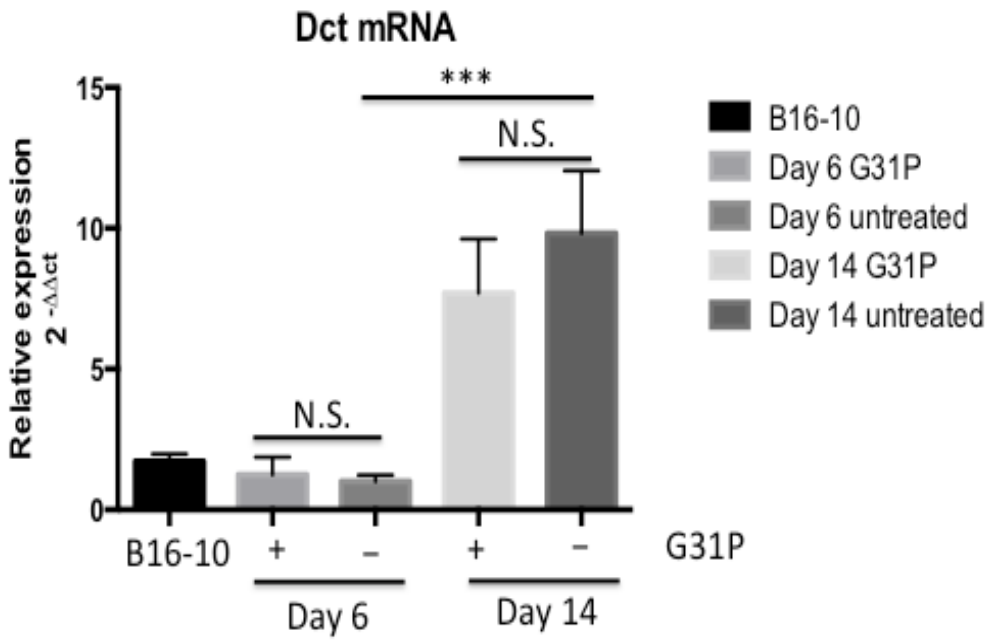


Figure 4.5 The impact of G31P treatment on expression of the melanoma tumour marker Dct in melanoma cell-containing Matrigel plugs.

Total RNA was taken from the Matrigel plugs on day 6 and 14 and the expression of Dct mRNA was determined by qRT-PCR, as noted in the materials and methods section. There was an up-regulation of Dct from day 6 to day 14 in both the G31P-treated and untreated group Matrigel plugs ($p = 0.0002$), but there were no differences between the G31P-treated and untreated group on either day 6 or 14 ($p \geq 0.05$). The negative control Matrigel plugs without tumour cells, was not presented in the graph since, by definition, these samples would not contain meaningful levels of mRNA. B16-10 murine melanoma tumour cells were used as the positive control. ***, $p < 0.001$ versus the indicated comparator. (n = 10 mice/group). The day 6 no G31P treatment samples were arbitrarily assigned the qRT-PCR reference value of 1..

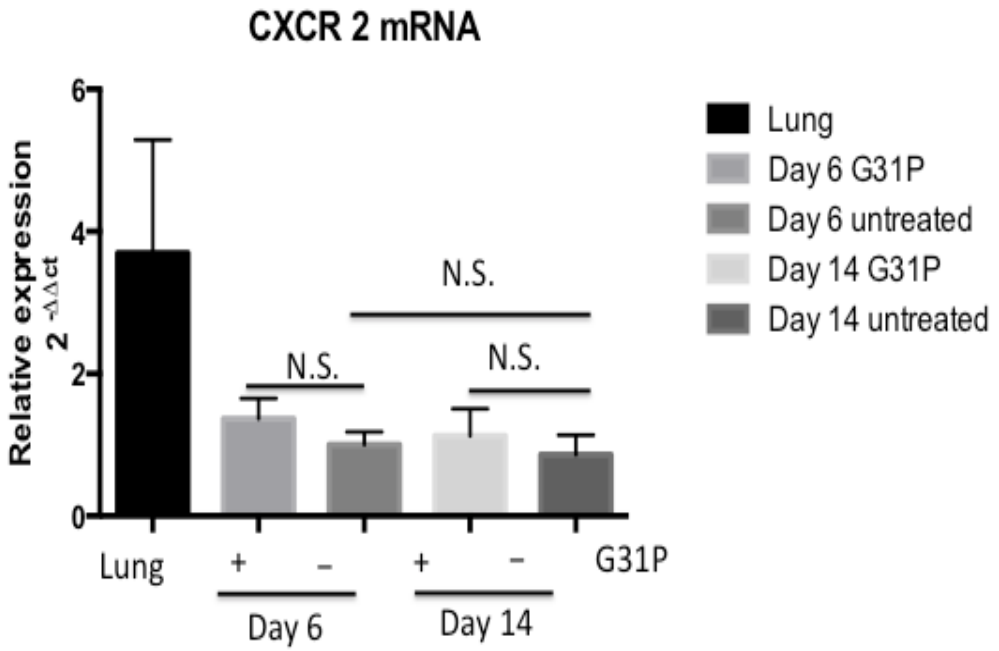


Figure 4.6 The impact of G31P treatment on expression of mouse the ELR-CXC chemokine receptor CXCR2 in melanoma tumour cell-bearing Matrigel plugs.

The expression of CXCR2 was assessed by RT-PCR. Expression of CXCR2 in the Matrigel plug did not change from day 6 to day 14 (control group, $p \geq 0.05$), and G31P did not make any difference in the expression of CXCR2 on either day 6 or day 14 ($p \geq 0.05$). LPS-induced lung tissue was used as the positive control for CXCR2. NS, $p \geqslant 0.05$ versus the indicated comparator. (n = 10 mice/group). The day 6 no G31P treatment samples were arbitrarily assigned the qRT-PCR reference value of 1. The negative control, The negative control Matrigel plugs without tumour cells, was not presented in the graph since, by definition, these samples would not contain meaningful levels of mRNA.

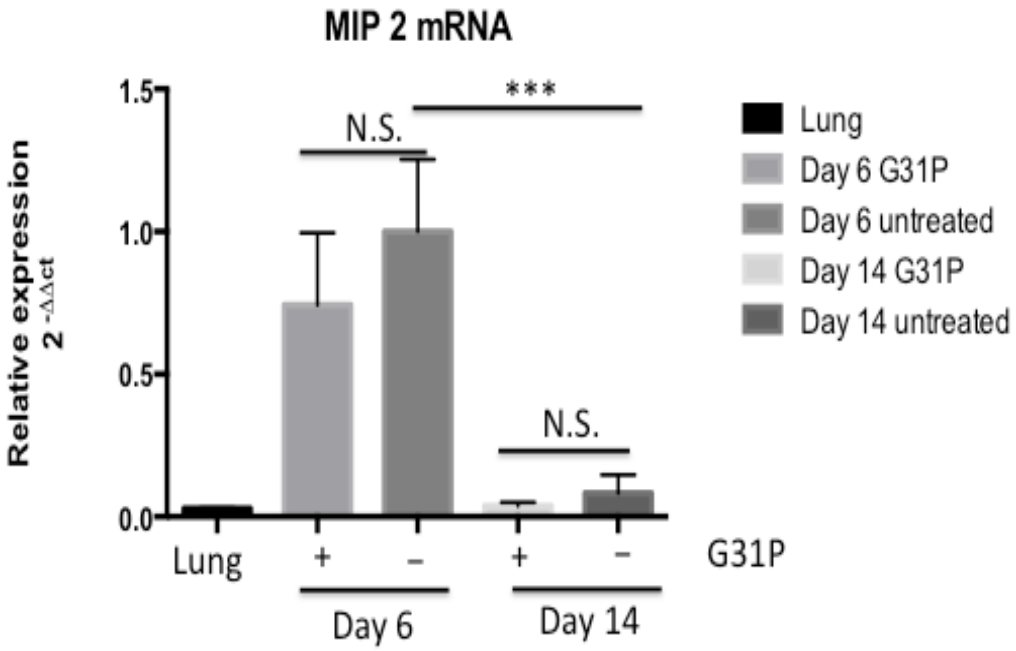


Figure 4.7 Expression of mouse ELR-CXC chemokines MIP2 did not change under the G31P treatment.

The expression of MIP2 was tracked on day 6 and 14. Expression of MIP2 in the Matrigel plug decreased significantly from day 6 to 14 (control group, $p < 0.001$), and G31P did not make any difference in the expression of MIP2 on either day 6 or 14 ($p \geq 0.05$). LPS-induced lung tissue was used as the positive control for MIP2. NS, $p \geq 0.05$ versus the indicated comparator. (n = 10 mice/group). The negative control Matrigel plugs without tumour cells, was not presented in the graph since, by definition, these samples would not contain meaningful levels of mRNA. Mann-Whitney T test was conducted. The day 6 no G31P treatment samples were arbitrarily assigned the qRT-PCR reference value of 1..

4.2 Assessing the vascular bed in B16 melanoma-bearing Matrigel plugs

4.2.1. Vasculature contents in the Matrigel plugs

After observing tumour growth, the angiogenic processes in the Matrigel plugs were then looked into, as we were questioning if G31P treatments would prevent the angiogenic processes in the Matrigel plugs. Angiogenesis was evaluated by detecting the blood vessel contents and the expression of several angiogenic factors. To directly evaluate the effects of G31P in tumour angiogenic processes, we used both gross anatomic and fluorescence quantification approaches. In general, others have reported that blood vessels in Matrigel plugs can be visualized (Akhtar, Dickerson et al. 2002). The photomicrographs in Fig. 4.8 depict representative vascular structures in the Matrigel plugs in both G31P-treated and untreated mice in this experiment.

High molecular weight FITC-labelled dextran would be evenly dispersed within the blood vessels after it's injection. Importantly, this molecule could not pass through the vessel walls owing to its high molecular weight, such that blood vessels can be visualized by tracking the FITC fluorescence. Fig. 4.8 shows representative Matrigel plugs in both the G31P-treated (left half of the panel) and untreated groups (right half of the panel), with plugs containing no tumour cells (negative controls) depicted in the half of the panels. It can be seen from Fig. 4.8 that on day 6 no obvious vascular structures were observed in both the G31P-treated and untreated tumour cell-containing Matrigel plugs, although some Matrigel-associated autofluorescence can be seen. Similarly, under white light conditions, there was no discernible sign of vascularization. With tumour development, obvious blood vessel branches with green fluorescence was detected on Dy 14, when it was clear from the white light photographs that the black melanoma pigment filled the Matrigel plugs. Although vasculatures were visible, G31P's effects were hard quantified using this approach, so the following quantitative FITC-dextran quantification assay was conducted.

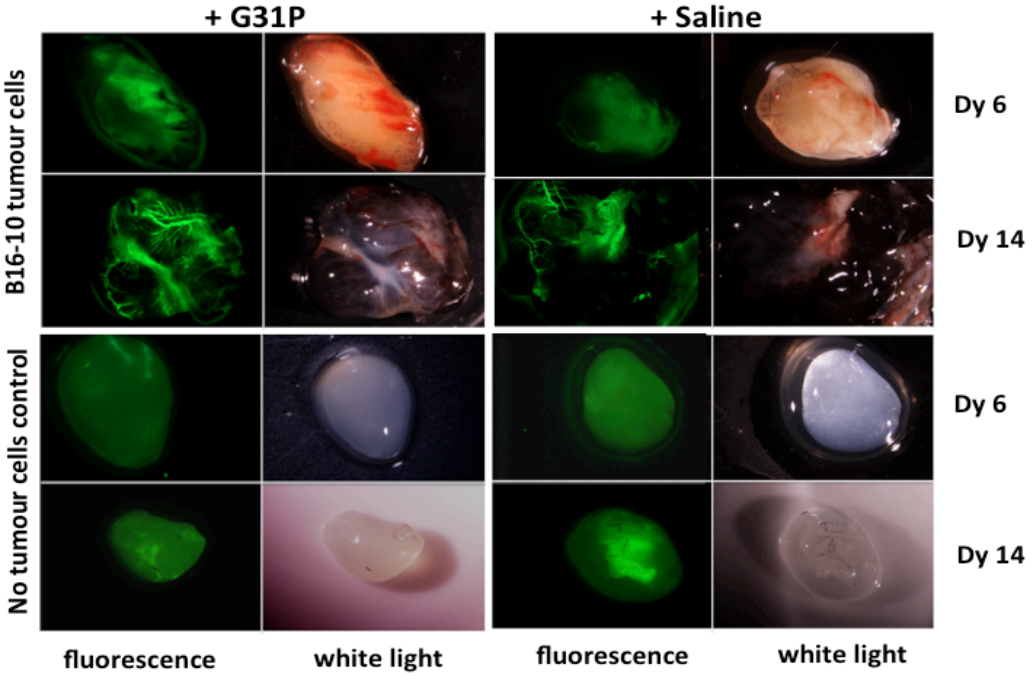
4.2.2. Quantification of FITC-dextran within the Matrigel plugs

After photographing the plug, the more objective FITC-dextran quantification assay was used to quantify the vascular contents of the plugs. As mentioned above, high molecule FITC-labelled dextran was injected i.v. into the mice, such the amount of FITC-dextran in each

Matrigel plug could be obtained by measuring the florescence in the digested plugs. Those florescence values were then converted to concentrations using a FITC-dextran standard curve. For more accurate evaluation, the FITC-dextran concentration in each plug was normalized with the FITC-dextran concentration in the serum of each mouse, and then divided by the weight of each Matrigel plug, such that the final vasculature content would be shown as the serum volume in per gram of the Matrigel plug.

In the experiment, FITC-dextran was not detected in the plugs of either the G31P-treated and untreated groups on day 6, and that outcome correlated well with the photographic data depicted in Fig. 4.8. As the tumours grew, there was a significant increase in vascular content from day 6 (none detected) to 14 in the untreated groups (1.307 ± 0.330 mm^3/g, $p < 0.0001$). This enhanced angiogenic process also corresponds with the increased weights (Fig. 4.4) and volumes (Fig. 4.3) of the Matrigel plugs. This confirms that, in this model, tumour growth is associated with the formation of the new blood vessels. The Matrigel plugs without tumour cells were set as the negative control (Day 14: G31P-treated: 0.029 ± 0.001 mm^3/g, Untreated: 0.069 ± 0.016 mm^3/g). There were no statistical differences in vessel volumes between the G31P-treated and untreated groups on day 14 (G31P-treated group: 1.215 ± 0.224 mm^3/g, Untreated: 1.307 ± 0.330 mm^3/g, $p = 0.5346$) (Fig. 4.8), indicating that G31P did not influence tumour-mediated blood vessel formation.

A.



B.

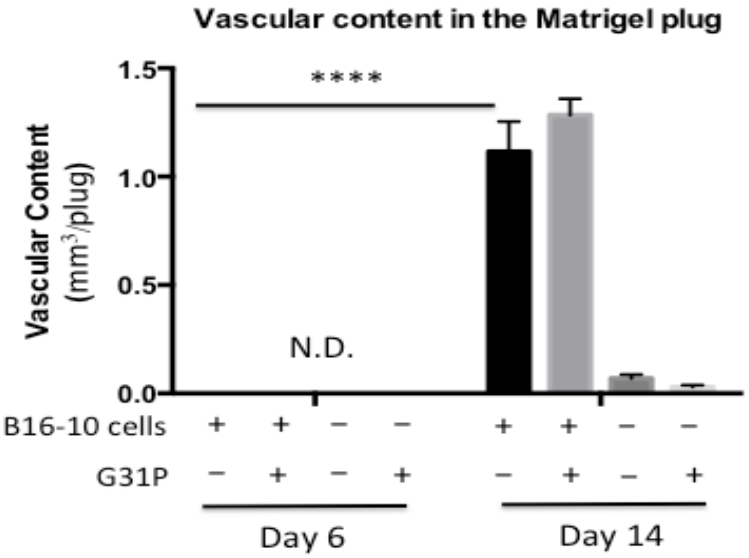


Figure 4.8 Assessments of the vascularity of tumour cell-containing versus control Matrigel plugs.

(A) Photomicrographs were captured either with a GFP filter (fluorescence; excitation filter 460 - 490 nm; emission filter, 510 nm) and without (white light) to visualize FITC-dextran-containing blood vessels in the Matrigel plugs taken from G31P-treated or saline-treated animals on day 6 or 14. Matrigel plugs with PBS only were set up as negative control. High molecule weight FITC-dextran was injected into mice i.v. 20 minutes before euthanasia. The clear vasculatures branches were displayed with lighter green lines while the background of tumour plugs was black. On day 6, there was no obvious angiogenic activities, but on day 14, branching blood vessels were clearly visible. These photographs were chosen from the represented Matrigel plugs. (B) Objective measures of vascular volume were obtained by quantifying the amounts of FITC-dextran in the Matrigel plugs in mice and comparing those data to FITC-dextran standard curves and the circulating levels of FITC-dextran in each experimental mouse, as described in the methods section. Matrigel plugs without tumour cells were set as the negative control. Expression of vascular content in the Matrigel plug increased significantly from day 6 to day 14 (control group, $p < 0.0001$), and G31P did not have a significant impact on this parameter on either day 6 and 14 ($p \geq 0.05$). NS, $p \geq 0.05$ versus the indicated comparator (untreated). (n = 10 mice/group). Mann-Whitney T test was conducted.

4.2.3. The expression of angiogenic factor VEGF A

Three other angiogenic factors, VEGF A, angiopoietin 1 and angiopoietin 2 were detected by real-time PCR. In this study, the expression of VEGF A (Fig. 4.9) in the Matrigel plug was lower than in the positive control heart tissue (Heart tissue: 2.520 ± 0.152; Day 6: 1.000 ± 0.241, $p = 0.0080$). Additionally, VEGF A levels did not change from day 6 to day 14 (Day 6: 1.000 ± 0.241; Day 14: 0.803 ± 0.073, $p = 0.7655$), which did not correlate with the expression tendency of MIP2 and the angiogenic process captured by both photography and FITC-dextran quantification. G31P treatments did not significantly change the expression of VEGF A in the plugs on either day 6 (G31P treated: 0.843 ± 0.143; Untreated: 1.000 ± 0.241, $p = 0.8144$) or day 14 (G31P treated: 0.775 ± 0.0073; Untreated: 0.803 ± 0.0073, $p = 0.7065$). It can be concluded that G31P did not impact expression of VEGF A in the Matrigel plugs.

4.2.4. The expression of angiogenic factor angiopoietin 1

In addition to VEGF A, angiopoietin 1 is also a crucial factor in vessel development and maturation in melanoma (Helfrich and Schadendorf 2011). We evaluated the expression of angiopoietin 1 in our Matrigel plugs by qRT-PCR (Fig. 4.10), as we had done with VEGF A. It can be seen from the graph that the expression of angiopoietin 1 did not change significantly from day 6 to day 14 (Day 6: 1.000 ± 0.135; Day 14: 7.339 ± 2.727, $p = 0.8872$). Although mice showed higher mean angiopoietin 1 values on day 14 (7.339 ± 2.727), there was no statistical difference from day 6, as we had observed with VEGF A. In this model, both the expression of VEGF A and angiopoietin 1 did not change from day 6 to day 14. Similar to MIP2 and VEGF A, the G31P treatment did not affect expression of angiopoietin 1 on either day 6 (G31P treated: 2.054 ± 0.410; Untreated: 1.000 ± 0.135, $p = 0.0545$) and 14 (G31P treated: 8.121 ± 0.480; Untreated: 7.399 ± 2.727, $p = 0.8208$).

4.2.5. The expression of angiogenic factor angiopoietin 2

Along with angiopoietin 1, the expression of angiopoietin 2 was also tracked in this experiment (Fig. 4.11). We found that the expression of angiopoietin 2 increased significantly from day 6 to day 14 (Day 6: 1.000 ± 0.393; Day 14: 3.463 ± 0.318, $p = 0.0006$), which was in

accord with the observed increase in tumour vascularization, the enhanced expression of tumour marker Dct, and tumour progression, but G13P treatments did not change the expression of angiopoietin 2 on either day 6 (G31P treated: 0.703 ± 0.385; Untreated: 1.000 ± 0.393, $p = 0.9355$) or 14 (G31P treated: 4.182 ± 0.465; Untreated: 3.463 ± 0.318, $p = 0.3493$).

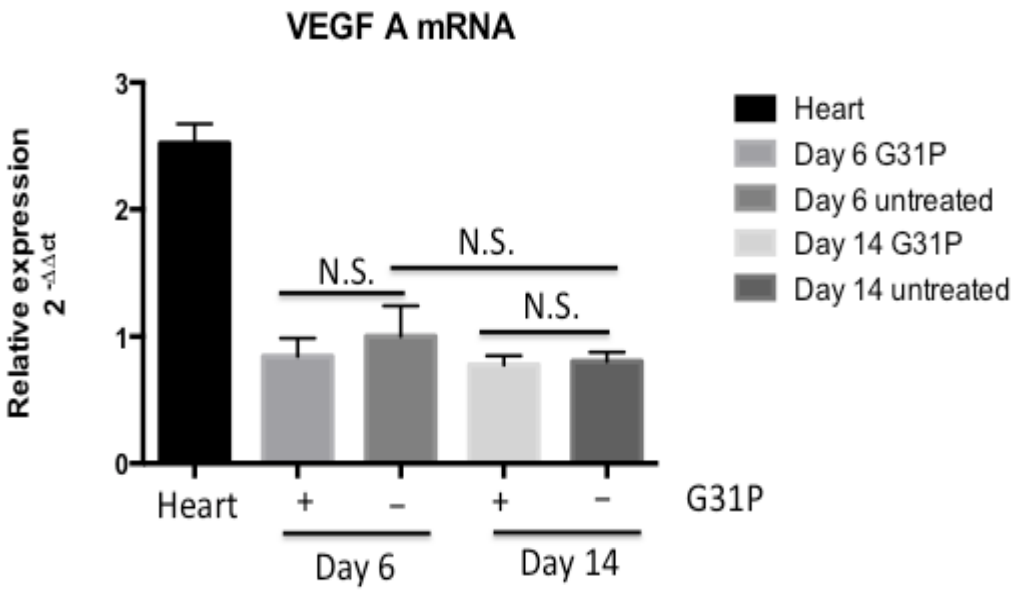


Figure 4.9 The G31P treatment did not change the expression of the angiogenic factor VEGF A.

Expression of the angiogenic factor VEGF A in B16-10 melanoma cell-bearing Matrigel plugs was compared in the G31P-treated and untreated mice on day 6 and 14. Heart tissue was used as the positive control. Expression of VEGF A did not change from day 6 to day 14 ($p \geq 0.05$), and G31P had no significant impact on VEGF A expression on either day 6 or 14 ($p \geq 0.05$). n = 10 mice/group. The negative control Matrigel plugs without tumour cells, was not presented in the graph since, by definition, these samples would not contain meaningful levels of mRNA. Mann-Whitney T test was conducted. The day 6 no G31P treatment samples were arbitrarily assigned the qRT-PCR reference value of 1..

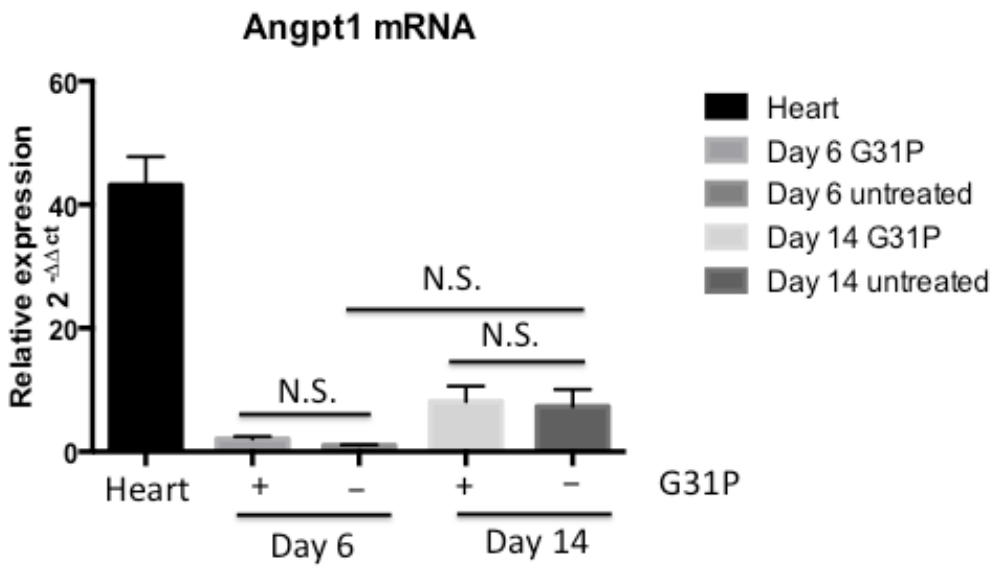


Figure 4.10 The expressions of mouse angiogenic factor angiopoietin 1 did not change under the G31P treatment.

The expression of angiopoietin 1 in melanoma tumour cell-bearing Matrigel plugs was compared to heart tissue on day 6 and 14, using mice that had been treated with or without the G31P. Heart tissue was used as the positive control. The expression of angiopoietin 1 did not change from day 6 to day 14 ($p \geq 0.05$), and G31P had no significant impact on this expression on either day 6 or 14 ($p \geq 0.05$). n = 10 mice/group. Mann-Whitney T test was conducted. The negative control Matrigel plugs without tumour cells, was not presented in the graph since, by definition, these samples would not contain meaningful levels of mRNA. The day 6 no G31P treatment samples were arbitrarily assigned the qRT-PCR reference value of 1..

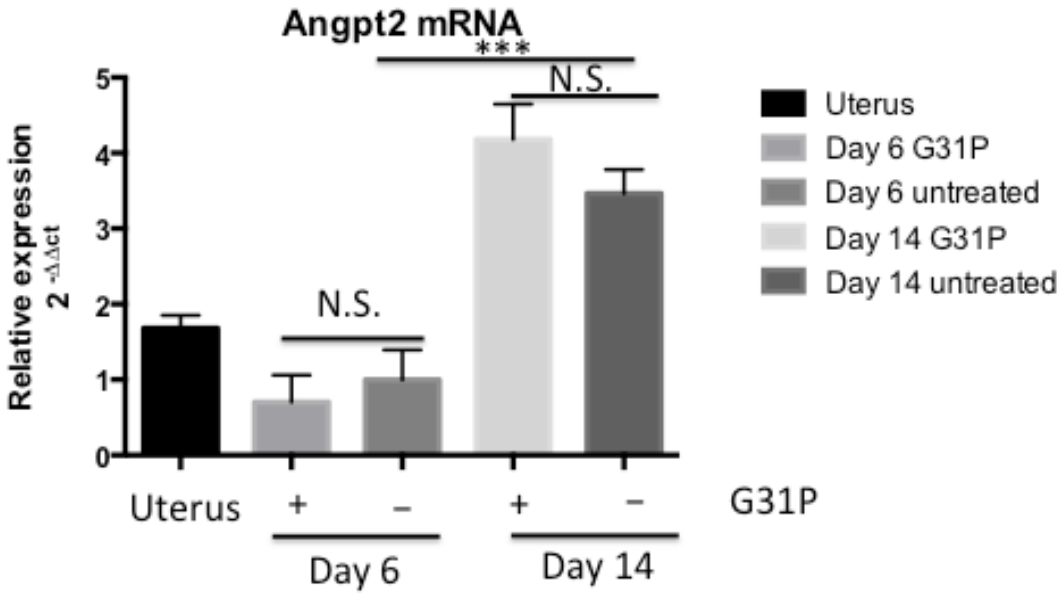


Figure 4.11 The expression of mouse angiogenic factor angiopoietin 2 with/without the G31P treatment.

The expression of angiopoietin 2 in the Matrigel plugs was tracked on day 6 and 14 in mice that were, or were not treated with G31P. Uterine tissue from a normal female mouse was used as the positive control. The expression of angiopoietin 2 increased significantly from day 6 to day 14 ($p < 0.001$), although G31P did affect this expression on either day 6 or 14 ($p \geq 0.05$). n = 10 mice/group. Mann-Whitney T test was conducted. The negative control Matrigel plugs without tumour cells, was not presented in the graph since, by definition, these samples would not contain meaningful levels of mRNA. The day 6 no G31P treatment samples were arbitrarily assigned the qRT-PCR reference value of 1..

4.3. Final comments

The purpose of this thesis was to investigate the effects of ELR-CXC chemokine blockade on melanoma tumour growth and angiogenesis in a subcutaneous Matrigel model. According to our experiment results, ELR-CXC chemokine blockade did no affect melanoma tumour growth and tumour angiogenesis in this model.

CHAPTER 5: DISCUSSION

We looked at the impact on treating melanoma tumour cell-bearing mice with a potent ELR-CXC chemokine antagonist, G31P, as determined by assessing the animals' body weights and temperatures, tumour volumes and weights, the development of patent tumour vasculature, and the expression of an array of tumour and angiogenesis-related markers.

Our data showed that mouse body temperatures remained the same from day 0 to 14 but, as expected, the animals' body weights climbed gradually from day 0 to day 14 in B16-10 tumour cell-challenged mice. In pilot experiments the pigment of B16-10 melanoma cells first became discernible in the Matrigel plugs around day 6, but by day 14 the Matrigel plugs were opaque with black pigment. *In vitro*, melanin pigment becomes discernible in melanoma cells when the cells became aggregated, as if in a tumour mass (S. Jiang, unpublished observation), while in our tumour model the mRNA levels of the pigment-related enzyme and tumour marker Dct also increased significantly from day 6 to day 14. Taken together, our data indicated that the tumours grew successfully within our mice, with obvious tumour progression coming after day 6, such that both tumour growth and vascularization processes had markedly developed by day 14. Under the fluorescence microscope, plugs collected on day 6 did not display discernible vascularity, but by day 14 they displayed clear vascular branching structures containing substantial FITC-dextran. Similarly, the quantification of the FITC-dextran contents revealed significant increases from day 6 (non-detected) to day 14. In a pilot experiment in which tumours were allowed to progress to day 16, some of the mice exhibited marked drops in body temperature, low responsiveness to physical provocation and increased lethargy (i.e., the humane termination endpoint) (Paster, Villines et al. 2009), such that that pilot experiment was terminated. Hence, two time points, day 6 and day 14 were chosen for subsequent experiments in this model. In conclusion, all of this physical data clearly indicated that the tumour cells inside the Matrigel were thriving, and that that was accompanied angiogenic activities that were readily discernible by day 14.

Having established the Matrigel model, we next explored whether signalling through the ELR-CXC chemokines affected tumour growth in this model, examining the impact of G31P treatments on a number of parameters related to tumour growth (i.e., body weights and temperatures, tumour volumes and weights, Dct mRNA levels and vascularization). In short,

we saw that none of these parameters were affected by the G31P treatments, at least as determined on days 6 and 14. Nevertheless, we did confirm that in the cells within the Matrigel plugs expressed CXCR2, G31P's direct target, which is known to be associated with tumour angiogenesis (Addison, Daniel et al. 2000). A number of cell types, including B16-10 tumour cells themselves, endothelial cells, and tumour-associated immune cells such as neutrophils, dendritic cells, and macrophages, express the CXCR2 (Jablonska, Wu et al. 2014). In this model, the cells within the Matrigel plugs expressed CXCR2, and that did not change from day 6 to day 14.

We next turned our attention to the angiogenic processes in our Matrigel model, initially using fluorescent microscopy to assess blood vessel formation (i.e., presence of FITC-dextran-containing vascular structures). There had been no obvious vessel formation on day 6 but vessels were readily discernible on day 14, and our FITC-dextran quantification data confirmed this as well. On day 6, the levels of FITC-dextran in the Matrigel plugs were too low to detect, such that FITC-dextran data was only obtained on day 14. Thus, vascularization appeared to progress hand-in-hand with tumour growth, as might be predicted.

We also investigated the expression of several angiogenic factors using qRT-PCR approaches. Our data indicated that one of mouse ELR-CXC chemokines, the well-known angiogenic factor MIP2, was highly expressed within the day 6, but not day 14, B16-10 cell-bearing Matrigel plugs. In humans, ELR-CXC chemokine (e.g., CXCL8) expression is correlated with melanoma progression, metastatic potential and angiogenesis (Ribatti, Nico et al. 2010). In mouse models of melanoma, MIP2 plays a key role in tumour development, inducing tumour cell proliferation, angiogenesis, and metastasis. MIP2 actives the NF-κB pathway, thereby inducing the activation of several other tumour-related regulators, and fostering melanoma progression (Dhawan and Richmond 2002). In this model, interestingly, there was a significant decrease in MIP2 expression from day 6 to day 14, whereas we observed angiogenic processes to increase from day 6 to day 14. Moreover, while the majority of cells within this Matrigel plug would have been B16-10 tumour cells, other types of cells (e.g., endothelial cells) would have been involved in tumour progression within the Matrigel plug as well. Unfortunately, using the approaches we employed, it was impossible to distinguish the contribution of each cell type to the mRNA levels in each factor. Moreover, we

did not assess the expression of any other ELR-CXC chemokines (e.g., KC, LIX) in the Matrigel plugs, although these chemokines are often but not always tandemly expressed, such that they could have contributed to tumour progression in our model.

We also detected the expression of another three important angiogenic factors, VEGF A, angiopoietin 1 and angiopoietin 2. Generally, healthy melanocytes do not express VEGF A receptors whereas malignant human melanoma cells do so (Gitaygoren, Halaban et al. 1993), which suggests that VEGF A might impact melanoma cells via an autocrine pathway. It is known that enhanced VEGF A expression is correlated with an increased potential for metastasis (Streit and Detmar 2003), although whether expression of VEGF A is a viable prognostic tool in melanoma is controversial (Helfrich and Schadendorf 2011). Not all melanoma cell lines secrete VEGF A, such that different lines may respond differently to anti-VEGF A treatments. In this model, tumour cells-embedded in Matrigel plugs did express VEGF A and angiopoietin 1, but the mRNA levels remained unchanged from day 6 to 14. The function of angiopoietin 1 in tumour development is controversial. In this study, similar to VEGF A, expression of angiopoietin 1 remained unchanged (untreated group) from day 6 to day 14, which is in agreement with other data regarding its correlations with VEGF A (Saharinen, Eklund et al. 2011). Unlike either VEGF A or angiopoietin 1, the expression of angiopoietin 2 increased significantly from day 6 to day 14. Others have reported that angiopoietin 2 acts as an autocrine factor in melanoma patients. Indeed, enhanced circulating levels of angiopoietin 2, which is strongly expressed in tumour tissues, are correlated with the disease progression and decreased patient survival (Helfrich, Edler et al. 2009). Thus, our data agree with the human data relating to melanoma progression and angiopoietin 2 expression. Similar to the tumour progression results we observed in G31P-treated mice, G31P did not affect the expression of the angiogenesis-related parameters we investigated. Vascularization and expression of the angiogenic factors MIP2, VEGF A, angiopoietin 1 and angiopoietin 2 in the Matrigel plugs were unchanged by the G31P treatments on both days 6 and 14. Thus, we concluded that blocking the ELR-CXC chemokines did not affect angiogenic processes in this Matrigel model. G31P is a highly effective anti-inflammatory agent in multiple contexts, as noted above, but in this model G31P failed to achieve significant outcomes in parameters reflecting both tumour growth and angiogenesis. This is interesting because we have alternate evidence showing that G31P has very substantial protective effects in a B16-10 melanoma

model wherein the cells are injected i.v., such that they seed the lungs with tumours (J. Town 2008).

Tumour blood vessels themselves are known to be highly unstable and heterogeneous, such that they incorporate at least 6 distinct types of vessels (Nagy, Chang et al. 2010). They undergo abnormal angiogenic process and are characterized by odd vessel morphology (De Bock, Cauwenberghs et al. 2011). Although G31P treatment did not impact any of the parameters we investigated, question remain regarding potential alternate impacts for the G31P treatments. For example, it is open to question whether blockade of CXCR1, CXCR2 and heterologous GPCRs would normalize the blood vessels generated in the tumours, which has been reported to improve hypoxic conditions, and make drug delivery via the vasculature more efficient. The normalization of tumour vessels has been a promising therapeutic strategy in treating cancer (Goel, Wong et al. 2012). Such normalization might not interfere tumour volumes, weights or blood contents, but might nevertheless be beneficial to drug delivery. Indeed, we have reported already that G31P treatments significantly improve the efficacy of cisplatin chemotherapy in a mouse model of hepatoma (Wei, Chen et al. 2014, Li, Khan et al. 2015).

Nevertheless, we must conclude that in our mouse Matrigel melanoma tumour model, blocking the ELR-CXC chemokines by itself is insufficient to alter angiogenesis or tumour growth. Clinically, it has also been discussed that single agent anti-angiogenic therapy is inadequate to prevent tumour development (Eklund, Bry et al. 2013), with combined therapy being highly recommended. Certainly, we would like to continue to work in this model, assessing additional parameters relating to the roles of the ELR-CXC chemokines in melanoma models. For example, we never did study the angiogenic patterns utilized in the vascularization of our tumours in the present study.

Angiogenesis routinely refers to the formation of blood vessels mainly via endothelial sprouting from an existing vessel toward the stimulatory microenvironment (Hu and Cheng 2009). However, other patterns, such as co-option of pre-existing vessels, intussusceptive microvascular growth, postnatal vasculogenesis, glomeruloid angiogenesis, and vasculogenic mimicry (Dome, Hendrix et al. 2007, Seftor, Hess et al. 2012) are also described in tumour development. These patterns utilize different biological processes, including expression of

distinct angiogenic factors (Young, Fernando et al. 2014). In this study, we did not assess which angiogenic patterns were used, or how abnormal the blood vessels were, which complicates the analysis of the relationships between VEGF A, angiopoietin 1 and angiopoietin 2. One possible solution would be to confirm tumour angiogenic patterns by use of two-photon (Kitahara, Morikawa et al. 2010) or transmission electron (Kerbel and Folkman 2002) microscopy. In addition to such structural observation of tumour vessels (Kitahara, Morikawa et al. 2010), measurements of blood flow and pressure and micro-blood vessels density (MVD) inside tumours, could also be used to classify angiogenic patterns (Tozer 2014).

Importantly, melanoma tumours have been described as having lower blood flow relative to some other tumours, probably owing to their association with the skin, a 'lower metabolism' organ. It has been speculated that this might explain why the late stages of melanoma were insensitive to oxygen stress and became more aggressive (Fried and Arbiser 2008). Multiple investigators have noted that vascularity was not an independent prognostic factor for melanoma (e.g., (Busam, Berwick et al. 1995)). Thus, it might be that, compared to other tumour models, B16-10 melanoma cells required less vascularization to survive, such that they might be less sensitive to G31P therapy. Consequently, it is still uncertain whether G31P treatments would achieve better outcomes in tumour models that are more dependent on neoangiogenesis, especially endothelial sprouting. For example, G31P did achieve better outcomes in hepatoma and prostate cancer models ((Liu, Peng et al. 2012, Wei, Chen et al. 2014, Li, Khan et al. 2015), as well as in intravenous (Town et al) and intradermal (J. Gordon, unpublished observation) B16-10 melanoma models.

We see potential for a number of factors to have confounded our analyses. For example, while the FITC-dextran quantification assay for vascularity provided information regarding the volume of the new vasculature within the Matrigel plugs, as noted we did not explore the details of the blood vessels (i.e., vessel components, diameters, and morphology), and that might have been important information to have obtained. Moreover, in tumour-associated microenvironments, cells are often stressed owing to hypoxia, although chemotherapy would also induce stress among the dying cancer cells (Sukkurwala, Martins et al. 2014). We do not know if the G31P treatment would have impacted this stress condition, such that future studies

should probably take expression of stress factors such as heat-shock proteins (Downes, Wolf et al. 2014) into account.

Statistics and sample sizes were also discussed in this thesis. In general, the parametric test is designed for the data that fit in the distribution patterns. According to statistic principles, the greater the sample sizes, the more normal the distribution pattern will be. But due to the difficulties in handling large amounts of animals, animal ethics, and financial considerations, usually only 5-10 animals (Kitahara, Morikawa et al. 2010) are used in cancer-related studies. But theoretically, the determination and classification of distribution patterns are based on large sample sizes. Consequently, in this study, the non-parametric T-test, the Mann-Whitney T-test was employed, which is used for the comparison of two groups (G31P-treated and untreated). This choice of method is believed to decrease the statistical bias as much as possible.

Regarding sample sizes, too large sample sizes lead to the waste of recourses, whereas insufficient sample sizes result in inaccurate outcomes. Generally, there are two ways for calculating animal sample sizes. One of these is power calculation, which is the most powerful and scientific method (Charan and Kantharia 2013). A free software called G power, which is based on Cohen's principle is recommended by Charan and Kantharia. For in-depth discussion regarding this method, please see (Cohen 2013). The other is called "resource equation" method, which is simpler but coarse. However, it is useful for when the effect size (differences between the means of each group) and standard deviation are unknown, or there are multiple experimental endpoints, or complex statistical procedures are used for analysis (Charan and Kantharia 2013). In this case, an E value will be calculated to determine the sample sizes: E = Total number of animals - Total number of groups. The sample sizes that keep E value from 10 to 20 are appropriate. If E value is lower than 10, then the sample sizes might be inadequate to achieve statistically meaningful results. However, if E is more than 20, the unnecessary waste might be induced (Charan and Kantharia 2013).

After reviewing the literatures, there will be scientific and statistical basis if the calculation was only based on "resource equation" method, which was 6 animals per group ($E = 2 \times 6 - 2 = 10$). Since the more accurate sample sizes calculations are recommended, according to Naduvilath, John and Dandona, simple sample sizes calculation can be done manually via two different equations based on Cohen's principle (Naduvilath, John et al. 2000).

In this experiment, we have five animals per group in with/without the G31P treatment group. All the data came from two independent experiments, so the total sample size is 10. According to the equation, we had a sufficient large sample size. But if the experiment was repeated independently again, more than 5 animals in each independent experiment is recommended. Besides, if more than two unpaired groups are taken into consideration in our future research, the more accurate calculation can be achieved through the usage of the online software (http://biomath.info/power/ttest.htm).

CHAPTER 6: ACKNOWLDEGEMENT

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CHAPTER 7: REFERENCES

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