

MECHANISMS OF COLORECTAL CANCER PATHOGENESIS: ROLE OF HYPOXIA

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by

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Abstract

Colorectal cancer (CRC) is the third most common cancer worldwide. Hypoxia plays a pivotal role in cancer, regulating cellular processes such as angiogenesis via the Hypoxia Inducible Factor (HIF) pathway. HIF-1 α and HIF-2 α , isoforms of the α -subunit, were previously thought to be functionally redundant, but mounting evidence supports their divergent roles in many cancers. In CRC their relative roles remain unclear. This study aimed to elucidate their relative contribution to hypoxic regulation of CRC using the Caco-2 cell-line. *Ex vivo* cultures of primary cells isolated from CRC tissue were used to validate the Caco-2 data.

Caco-2 cells were stimulated with hypoxia (1% O₂) or the hypoxia-mimetic dimethylxaloylglycine (DMOG), with normoxia (21% O₂) controls. Expression of known hypoxia-induced genes was quantified by polymerase chain reaction (PCR) and a PCR-based array was used to further characterise angiogenic genes. Protein expression was determined using Western Blotting and ELISA. The effect of selective HIF-isoform knockdown on gene expression was evaluated. Tumour-derived cultures (TDCs) were established using tissue obtained from surgically resected CRC specimens. mRNA expression of epithelial cell markers (Ep-CAM, VE-Cadherin) was quantified by Q-PCR, and protein expression of the CRC tumour marker carcinoembryonic antigen (CEA) measured by ELISA. TDCs were exposed to hypoxia, and gene expression relative to normoxia was quantified by Q-PCR and PCR-based array.

In Caco-2 cells, hypoxia upregulated both HIF isoform proteins, inducing genes involved in angiogenesis (VEGF, ANGPTL-4, EFNA-3, TGF- β 1), metabolism (CA-IX, GLUT-1) and apoptosis (BNIP-3), with mRNA changes reflected at protein level. Three novel hypoxia-induced angiogenesis genes (ANGPTL-4, EFNA-3, TGF- β 1) were identified. Hypoxia-induced ANGPTL-4, BNIP-3 and TGF- β 1 expression was reduced by siHIF-1 α only, while EFNA-3 and VEGF expression was reduced by both siHIF-1 α and siHIF-2 α . TDCs expressed epithelial CRC cell markers, and showed similar hypoxia-induced angiogenesis gene expression to Caco-2 cells, although there was significant inter-donor variability.

Statement Of Originality

All the experiments described in this report have been performed by me unless otherwise stated, and are, to the best of my knowledge, original research which has not been carried out elsewhere.

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List Of Abbreviations

Ab	antibody	DTT	dithiothreitol
Abs	absorbance	EGF	epidermal growth factor
ACTB	actin beta	EGF-R	epidermal growth factor receptor
ANGPT	angiopoietin	ECM	extracellular matrix
ANGPTL	angiopoietin-like protein	EDTA	ethylenediaminetetraacetic acid
ANOVA	one-way analysis of variance	ELISA	enzyme-linked immunoadsorbent assay
AP-1	activator protein-1	EMEM	Eagle's minimum essential medium
APC	adenomatous polyposis coli	EMT	epithelial-mesenchymal transition
APER	abdomino-perineal resection of rectum	Ep-CAM	epithelial cell adhesion molecule
ARD1	acetyltransferase-ADP-ribosylation factor domain	EPO	erythropoietin
ARNT	aryl hydrocarbon receptor nuclear translocator	FACS	fluorescence-activated cell sorting
ARP	acidic ribosomal protein	FAP	familial adenomatous polyposis
ASR	age-standardised rates	FBS	foetal bovine serum
ATE	arterial thromboembolic event	FDA	food and drug administration
ATP	adenosine triphosphate	Fe ²⁺	ferrous ion
Bax	Bcl-2-associated X protein	Fe ³⁺	ferric ion
BCA	bicinchoninic acid	FEEL-1	fasciclin, EGF-like, laminin-type EGF-like and link domain-containing scavenger receptor 1 fibroblast growth factor basic FGF receptor
Bcl-2	B-cell lymphoma 2	FGFb	factor inhibiting HIF
bHLH	basic helix-loop-helix	FGFR	FMS-like tyrosine kinase 1
BM	basement membrane	FIH	fluoro-uracil
bp	base pairs	FLT-1	glyceraldehydes-3-phosphate dehydrogenase
BNIP-3	Bcl2/adenovirus E1B 19d-interacting protein	FU	guanosine diphosphate
BSA	bovine serum albumin	GAPDH	gastro-intestinal glucose transporter
CA-IX	carbonic anhydrase 9	GDP	guanosine triphosphate
CAM	cell adhesion molecule	GI	hypoxia
CBP	CREB-binding protein (cAMP response element-binding protein)	GLUT	hypoxia associated factor
CCL	chemokine (C-C motif) ligand	GTP	heart and neural crest derivatives expressed 2
CEA	carcinoembryonic antigen	H	Hank's buffered salt solution
CD	cluster of differentiation	HAF	hypoxia-inducible factor
cDNA	complementary DNA	HAND-2	human microvascular endothelial cells
CIMP	CpG island methylated phenotype	HBSS	hereditary non-polyposis colorectal cancer
CIN	chromosomal instability	H ₂ SO ₄	heme oxygenase
CNS	central nervous system	HCl	hypoxanthine
CO	carbon monoxide	HIF	phosphoribosyltransferase 1
CoCl ₂	cobalt chloride	HMEC	heparanase
COX	cyclooxygenase	HNPCC	hypoxia responsive element
CRC	colorectal cancer	HO	horseradish peroxidase
CRT	chemo-radiotherapy	HPRT-1	human umbilical vein endothelial cells
CSG	Caco-2 signature gene	HPSE	inflammatory bowel disease
Ct	threshold cycle	HRE	intercellular adhesion molecule
dCt	delta Ct	HRP	interstitial fluid pressure
ddCt	delta delta Ct	HUVEC	immunoglobulin
C-TAD	COOH-terminal TAD	IBD	insulin-like growth factor
CXCL	chemokine (C-X-C motif) ligand	ICAM	immunohistochemistry
DCC	deleted in colorectal cancer	IFP	interleukin
DEPC	diethylpyrocarbonate	Ig	interferon
DFO	desferrioxamine	IGF	c-Jun N-terminal kinase
DMEM	Dulbecco's modified Eagle's medium	IHC	kilo Dalton
DMOG	dimethyloxaloylglycine	IL	kinase insert domain receptor
DMSO	dimethyl sulfoxide	IFN	
DNA	deoxyribonucleic acid	JNK	
dNTP	deoxyribonucleotide triphosphate	kDa	
DP	2,2'-dipyridyl	KDR	
DRE	digital rectal examination		
dsRNA	double-stranded RNA		

LV	leucovorin	RR	response rate
MAC	modified Astler-Coller	RT	reverse transcription
MAPK	mitogen activated protein kinase	RTKI	receptor tyrosine kinase
MCP	monocyte chemoattractant protein		inhibitor
		SAR	swine anti-rabbit antibody
MDSC	myeloid-derived suppressor cells	SCID	severe combined immunodeficiency
MLH-1	MutL-homolog 1	SD	standard deviation
M-MLV	moloney murine leukemia virus	SDS	sodium dodecyl sulphate
MMP	matrix metalloproteinase	SEM	standard error of mean
MoAb	monoclonal antibody	siRNA	short interfering RNA
MRI	magnetic resonance imaging	SMA	smooth muscle cell actin
mRNA	messenger RNA	STAB-1	stabilin 1
miRNA	microRNA	TAD	transactivation domain
MSI	microsatellite instability	TAE	tris-acetic acid-EDTA buffer
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide	TAM	tumour-associated macrophage
		TBP	TATA-binding protein
MVD	micro-vascular density	TBS	tris-buffered saline
ND	not detected	TDC	tumour-derived culture
N	normoxia	TEK	tyrosine kinase, endothelial
NFκB	nuclear factor κB	TGF	transforming growth factor
NICE	national institute for clinical excellence	TIMP	tissue inhibitors of metalloproteinases
		Tm	melting temperature
NO	nitric oxide	TMB	3,3',5,5'-tetramethylbenzidine
NOS	nitric oxide synthase	TME	total mesorectal excision
NSCLC	non-squamous non-small cell lung cancer	TNF	tumour necrosis factor
		TSG	TDC signature gene
N-TAD	NH ₂ -terminal TAD	U	untreated
OD	optical density	UC	ulcerative colitis
ODD	oxygen dependent degradation	uPAR	urokinase plasminogen activator receptor
OG	oxoglutarate		
OS	overall survival	V	volt
p-value	probability	VE-Cad	vascular endothelial cadherin
PAGE	polyacrylamide gel electrophoresis	VEGF	vascular endothelial growth Factor
PAS	Per-ARNT-Sim	VEGF-R	VEGF receptor
PBS	phosphate-buffered saline	vHL	von Hippel-Lindau tumour suppressor
PBST	phosphate-buffered saline plus Tween20		
(Q)-PCR	(quantitative) polymerase chain reaction	VM	vasculogenic mimicry
		VTE	venous thromboembolic event
PDGF	platelet-derived growth factor	XIAP	X-linked inhibitor of apoptosis protein
PDGF-R	platelet-derived growth factor receptor		
PECAM	platelet endothelial cell adhesion molecule		
PFS	progression-free survival		
PHD	prolyl hydroxylase domain		
PLAU	plasminogen activator, urokinase		
PLXDC-1	plexin domain containing 1		
PMSF	phenylmethylsulfonyl fluoride		
PoAb	polyclonal antibody		
PROK-2	prokineticin 2		
PTEN	phosphatase and tensin homologue deleted on chromosome ten		
PVDF	polyvinylidene difluoride membrane		
R	correlation coefficient		
R ²	correlation coefficient squared		
RA	rheumatoid arthritis		
RAM	rabbit anti-mouse antibody		
RCC	renal cell carcinoma		
RNA	ribonucleic acid		
ROS	reactive oxygen species		

Chapter 1

1 INTRODUCTION

The global importance of cancer has been apparent since the early part of the 20th century. In 1939 the renowned American pathologist Professor James Ewing said: “public interest in the cancer problem is now at the highest point in history” (Newman 1939). Over 30 years later, President Richard Nixon declared what came to be known in the US as the “war on cancer” with the signing of the National Cancer Act in 1971, and US spending on cancer research rose from \$15 billion in 1972 to \$75 billion in 2005 (Cutler 2008). By 2008, the annual global spend on cancer research had reached almost \$2 trillion (Eckhouse et al. 2008). In the same year, there were an estimated 12.7 million new cancer cases and 7.6 million cancer-related deaths worldwide, with the greatest disease burden falling on the developing world (56% of incidence and 63% of deaths) (American Cancer Society 2011). Cancer has the most devastating economic impact of any cause of death, with an estimated global cost of close to a trillion US dollars (approximately 1.5% of global GDP) (American Cancer Society 2010). Largely due to a growing and ageing global population, a continual rise in the global cancer burden is projected, and it has been estimated that annual figures will reach 30 million new cases and 13 million deaths by 2030 (Ferlay et al. 2010).

In the USA, a decline in both cancer incidence and mortality has been reported since the 1990s (Eheman et al. 2012). In the UK and Europe, although incidence continues to rise, mortality rates are declining (Coleman et al. 2008; Olsen et al. 2008; Mistry et al. 2011). These improving trends are the result of intense research activities that have led to significant advances in the prevention, detection and treatment of cancer. Our understanding of the molecular basis for the development and progression of cancer has increased profoundly over the past 50 years, but there remains significant scope for further progress.

An important advance in the understanding of cancer has been the appreciation that rather than being simply the consequence of the disorganised, uncontrolled growth of randomly mutated cells, the development of a tumour requires the organised and rather sophisticated adaptation of normal physiological processes by cancer cells. This allows them to resist programmed cell death (apoptosis), evade the immune system, and survive unfavourable nutritional and metabolic conditions resulting from increased energy demands. Furthermore, cancer cells alter the structure and function of surrounding host cells and extracellular matrix components, furthering their survival, for example by stimulating the development of new blood vessels (angiogenesis). This manipulation of the tumour microenvironment results in an increase in tumour mass, allows invasion outside the cellular compartment of origin, and culminates in the spread to distant locations that is invariably fatal.

By the time a clinically significant tumour has developed, therefore, a highly complex series of alterations in normal cellular processes has occurred. This results in the acquisition of what have been described as the hallmarks of cancer: unlimited replicative potential, evasion of apoptosis, self-sufficiency in growth signals, insensitivity to growth inhibitors, stimulation of angiogenesis, tissue invasion and metastasis, reprogramming of energy metabolism and evasion of immune-mediated destruction (Hanahan and Weinberg 2011). A better understanding of the underlying molecular mechanisms has revolutionised approaches to cancer treatment. Where total surgical excision of the tumour is not possible, treatment has historically relied on the high turnover of cancer cells, aiming to cause fatal DNA damage using ionised radiation or cytotoxic drugs. Rapidly dividing cancer cells are disproportionately affected by this approach, but the inevitable collateral damage to normal cells results in significant side-effects. Elucidation of both normal and cancer cell biology at the molecular level has allowed the development of targeted therapies that have significantly greater specificity to cancer cells, resulting in improved efficacy and reduced toxicity. A notable success story has been the development of antiangiogenesis drugs such as bevacizumab (Avastin TM), which in the last decade has transformed the treatment of metastatic colorectal cancer (CRC). This monoclonal antibody targets Vascular Endothelial Growth Factor (VEGF), a highly potent stimulator of angiogenesis.

All organisms that rely on aerobic metabolism have tightly regulated mechanisms by which cells respond to episodes of low oxygen tension (hypoxia) commonly encountered in the course of normal physiology. Angiogenesis is a critical mechanism by which organisms adapt to this, and hypoxia is one of the most potent stimuli for angiogenesis. Due to a high rate of relatively disorganised growth, most solid tumours are characterised by hypoxia, and it is now well established that this drives specific cellular pathways and mechanisms including angiogenesis (Harris 2002). Cancer cells that can adapt in this way not only survive but thrive in a hypoxic environment, leading to advanced disease and decreased survival.

This work focuses on hypoxia-driven angiogenesis in CRC. The role of key regulatory proteins of the hypoxia pathway in the angiogenic response of a CRC cell-line is investigated, and the angiogenic response to hypoxia of primary cell cultures derived from individual patients' tumours is examined.

1.1 Colorectal Cancer

1.1.1 Epidemiology

With an estimated 1.2 million new cases and 610,000 deaths globally in 2008, Colorectal Cancer (CRC) is the third most common cancer worldwide (American Cancer Society 2011). It is the second most common cause of cancer death in economically developed countries. In the UK, CRC accounts for 13% of all new cancer diagnoses (41,142 in 2009) and 10% of all cancer deaths (16,013 in 2010) (Cancer Research U. K. 2012). Geographical variation of both incidence and mortality is seen, age-standardised rates (ASR) for both being higher in more developed than in less developed countries (ASR per 100,000 in 2008 was 37.7 *versus* 12.1 for incidence, and 15.1 *versus* 6.8 for mortality) (Ferlay et al. 2010). Both incidence and mortality are also related to ethnicity. In the US, both are highest in African-Americans and lowest in Asian/Pacific Islanders, with intermediate rates for Hispanics and Caucasians (Eheman et al. 2012). In Israel, CRC is the leading cause of cancer death in the ethnic Jewish community, and incidence is five times higher than Palestinians in the same country (Rozen et al. 1987). Furthermore, incidence is higher in Ashkenazy Jews than in Sephardic Jews (Chang and Morris 2006). Factors contributing to these geographic and ethnic variations include genetics, economics (e.g. access to medical care), lifestyle and environmental factors.

CRC risk increases with age, with 72% of cases arising in people aged 65 or older in the UK. Incidence is similar for men and women up to the age of 50, after which it is more common in men, with the widest gap being between the ages of 65 and 74 (male:female ratio 17:10) (Garden 2006). 69% of cancers arise in the colon and 31% in the rectum and anus. A previous diagnosis of bowel cancer also increases the lifetime risk of developing a new metachronous tumour (Hayat 2007). First-degree relatives of patients with a history of colonic adenoma or carcinoma have a two- to threefold increased risk of CRC; the relative risk increases with the number of affected relatives, and with younger age at diagnosis in those relatives (Boland and Bresalier 2006).

1.1.2 Aetiology

Numerous observational studies have suggested an association between diet and incidence of CRC. That reduced fibre intake is associated with a higher CRC risk was first suggested in 1971 by Burkitt who noted reduced CRC incidence in West African populations who had high fibre intake (Burkitt 1971). Increased consumption of fruits and vegetables, and reduction in animal fat and red meat intake have subsequently been reported to reduce CRC risk (Willett 1989). In recent years, some of these findings have been called into question. A review by Willet reported that energy intake, or more precisely energy balance, had the most powerful and consistent dietary influence on CRC risk (Willett 2001). Six modifiable dietary/lifestyle risk

factors were identified: high body mass index (BMI), inadequate exercise, high alcohol consumption (combined with low folic acid intake), smoking and red meat consumption.

The association between cancer and chronic inflammation was first noted in 1863 by Virchow, and tumours have been described as “wounds that do not heal” (Lujan et al. 2002; Jacob and Salky 2005). The tumour-associated inflammatory infiltrate described by Virchow, composed of leucocytes, macrophages and lymphocytes, provides a rich source of cytokines, growth factors and angiogenic factors which enhance the malignant potential of tumours (Garden 2006). Chronic infections are implicated in the aetiology of approximately 15% of cancer deaths worldwide (American Cancer Society 2011). In addition, anti-inflammatory drug use reduces the risk of CRC and other tumours (Giacchetti et al. 2000; Kabbinavar et al. 2005b), and the therapeutic targeting of inflammatory cells and mediators in cancer is a promising area of research (Yan et al. 2006; Balkwill and Mantovani 2010). CRC is associated with the inflammatory bowel diseases (IBDs) Ulcerative Colitis (UC) and Crohn’s Disease, which are characterised by episodic and often progressive bowel inflammation. The risk is proportional to the severity, duration, and extent of colonic involvement. Overall prevalence of CRC is higher in UC than in Crohn’s (3.7% versus 1%) (Kabbinavar et al. 2005d; Goldberg 2006). The risk increases significantly with time, with the cumulative probability of developing CRC in UC being 2% by 10 years, 8% by 20 years and 18% by 30 years (Venook et al. 2005). While UC is confined to the large bowel, Crohn’s can affect any part of the alimentary tract, and is associated with an increased risk of small bowel cancer (Hurwitz 2004). Epithelial dysplasia is an important marker of risk of malignancy in IBD, and is found in 90% of IBD-related CRC (Fearon and Vogelstein 1990).

Colorectal polyps commonly occur sporadically in small numbers, but may also be part of hereditary syndromes associated with large numbers of polyps throughout the colon. Polyps may be neoplastic or non-neoplastic, with only the former (also known as adenomas) having malignant potential. Traditionally, three histological types of adenomas were described: tubular adenomas, the most common (75%) but with the lowest malignant potential (5%); villous, the least common (10%), but with the greatest malignant potential (40%); and intermediate tubulovillous adenomas (Umetani et al. 2000). Malignant potential is also directly related to polyp size (Church 2004; Conlin et al. 2005). Traditional adenomas develop via the canonical adenoma-carcinoma sequence (Figure 1.1 and section 1.1.3). A further group of neoplastic polyps, known as serrated adenomas, has been identified in recent years. Before their malignant potential was recognised they were classed as (non-neoplastic) hyperplastic polyps (Levine 1997; Russo et al. 2005). They were then thought of as a variant of “traditional” villous or tubulovillous adenomas, but are now considered a distinct histological entity. They arise via the serrated pathway, a molecular pathway distinct from the classical adenoma-carcinoma sequence. They are subdivided into sessile-serrated adenomas/polyps (SSA/Ps), which have a flat morphology and are more commonly found in the

proximal colon, and traditional serrated adenomas (TSAs), which are pedunculated and found predominantly in the distal colon.

Adenomas and carcinomas show similar anatomical distribution, with adenomas occurring earlier. Foci of CRC are often present in otherwise benign polyps, and the risk of CRC is proportional to degree of cellular atypia or dysplasia, and to the number of adenomas present. Perhaps the strongest evidence that carcinomas arise from adenomas is that systematically removing adenomatous polyps as part of an endoscopic screening programme decreases the incidence and mortality of CRC (Boland and Bresalier 2006).

Although over 70% of CRC occurs sporadically (Kaz and Brentnall 2006; Lakatos and Lakatos 2006) our understanding of the genetic basis of CRC is largely derived from the study of hereditary conditions which predispose individuals to CRC. The best-described are Familial Adenomatous Polyposis (FAP) and Hereditary Non-Polyposis Colorectal Cancer (HNPCC) which account for 3-5% of all CRC. FAP is an autosomal dominant condition characterised by the development of multiple (typically 500-2500) benign colonic and rectal adenomas. Colonic polyps usually first occur in adolescence, increasing in number and size with age. Virtually all individuals with FAP develop cancer by the age of 55, and the average age of diagnosis is 42 (20 years earlier than in sporadic cases). FAP is caused by mutations in the adenomatous polyposis coli (APC) gene. This is an initial step in the adenoma-carcinoma sequence, also known as the chromosomal instability (CIN) Pathway. The most severe mutations lead to the development of over 5000 polyps and a mean age at cancer diagnosis of 35 years; the least severe develop less than 100 polyps and are diagnosed at a mean age of 50 or 60 years. HNPCC (Lynch Syndrome), is also inherited in an autosomal dominant fashion. There are two types – type I is associated with an 80% lifetime risk of developing CRC; type II is associated with reproductive tract and extra-colonic tumours. In contrast to FAP, the development of HNPCC is characterised by defects in DNA repair which leads to Microsatellite Instability (MSI) (discussed in next section).

1.1.3 Molecular Genetics

The CIN pathway, first proposed by Fearon and Vogelstein (Fearon and Vogelstein 1990), describes the sequential transition from healthy colonic epithelium, to increasingly dysplastic adenoma and finally to CRC, by progressive accumulation of sporadic chromosomal mutations. The initial mutation is in the adenomatous polyposis coli (APC) gene, a tumour suppressor gene found on chromosome 5q. This is followed during the adenoma phase by mutations in the K-ras oncogene (chromosome 12p). p53 mutations (chromosome 17p), and deletions in DCC (“deleted in colorectal cancer”) and SMAD (chromosome 18q) occur latest, during the transition to carcinoma.

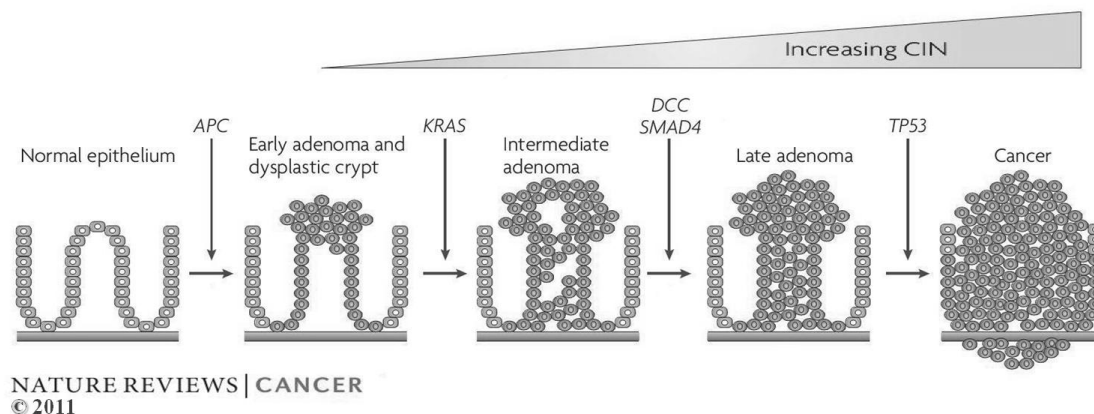


Figure 1.1 Adenoma-Carcinoma Sequence

The canonical adenoma-carcinoma (CIN) pathway describes a series of stepwise mutations in the development of CRC from normal epithelium (modified from Walther et al. 2009).

It has been suggested that functional loss of APC is a prerequisite for progression towards malignancy, and APC mutations are seen in up to 80% of sporadic carcinomas (Kinzler and Vogelstein 1996; Lüchtenborg et al. 2004). The APC protein inhibits the Wnt signalling pathway which is involved in embryogenesis and normal physiological processes as well as cancer. APC mutation leads to increased transcription of genes implicated in CRC carcinogenesis such as VEGF and matrix metalloproteinases (MMPs) (Zhang et al. 2001; Schneikert and Behrens 2007; Wu et al. 2007).

p53 is a transcription factor that has been described as the “cellular gatekeeper for growth and division” (Levine 1997). p53 mutations are associated with a variety of cancers and are seen in 40-60% of sporadic CRC (Webley et al. 2000; Conlin et al. 2005; Russo et al. 2005). The normal p53 protein mediates repair of damaged DNA, and causes apoptosis where such damage cannot be repaired. It has also been implicated in angiogenesis. In CRC tissue, mutant p53 expression is associated with increased markers of angiogenesis such as VEGF expression and microvascular density (MVD) (Kondo et al. 2000), and gene-transfer of wild-type p53 can suppress VEGF expression and angiogenesis in CRC cell-lines (Bouvet et al. 1998). There is some controversy in the literature as to whether p53 mutations in CRC are associated with a better or poorer prognosis (Remvikos et al. 1992; Adrover et al. 1999), and outcomes may be dependent on the cellular location of mutated p53 (Sun et al. 1992), and on the type of mutation (Webley et al. 2000; Russo et al. 2005).

Mutations in the K-ras oncogene are found in a number of cancers, primarily those of gastro-intestinal (GI) origin, and have been reported in up to 50% of CRC (Umetani et al. 2000; Kikuchi et al. 2009). The RAS proteins (K-ras, H-ras and N-Ras) are part of a large superfamily of low molecular weight GTP-binding proteins. They are self-inactivating signal transducers for several pathways controlling cell growth. In its inactive state RAS is bound to GDP. Transient

activation occurs when signalling from cell-surface receptors promotes substitution of GDP with GTP, which in turn leads to activation of downstream pathways (e.g. PI3K/Akt and Raf/MEK/ERK pathways) (Lowy and Willumsen 1993; Downward 2003). Self-inactivation can occur through the intrinsic ability of RAS to hydrolyse GTP back to GDP, a process accelerated by GTPase activating proteins (GAPs) (Bourne et al. 1990). RAS mutations inhibit this self-regulation both by reducing its intrinsic hydroxylase activity and by inhibiting GAPs (Lin et al. 2000; Brand and Wheeler 2012) so that mutant RAS proteins remain in the activated GTP-bound state. In CRC, K-ras mutations result in enhanced proliferation (van Houdt et al. 2010), reduced apoptosis (Ward et al. 1997), enhanced proteolysis and binding of tumour cells to extracellular matrix (Pollock et al. 2005), and promotion of tumour angiogenesis via VEGF upregulation (Zhang et al. 2001). K-ras mutations are associated with poor prognosis in stage II (Belly et al. 2001) and stage III disease (Ahnen et al. 1998), and are predictive of poor response to chemotherapy (Karapetis et al. 2008b; Van Cutsem et al. 2009). K-ras has a central role in the epidermal growth factor (EGF) receptor signalling pathway which is important in CRC pathogenesis (Lockhart and Berlin 2005). K-ras mutations activate EGF receptor (EGFR) signalling independently of ligand stimulation. Chemotherapeutic agents that inhibit EGFR, e.g. cetuximab and panitumumab, can improve outcomes in advanced CRC (Cunningham et al. 2004; Jonker et al. 2007), but response is poor in the presence of K-ras mutation (Amado et al. 2008; Karapetis et al. 2008a).

Chromosome 18q, which is mutated in 70% of primary CRC, is the locus of the tumour suppressor genes DCC and SMAD4 (Fearon and Vogelstein 1990). DCC is a cell surface receptor which is markedly reduced or absent in advanced CRC (Fearon et al. 1990), and induces apoptosis when not bound to its ligand netrin-1 (Mehlen and Fearon 2004). It was recently shown that DCC mutation leads to reduced apoptosis, and the development of an invasive and metastatic phenotype in CRC and breast cancer (Castets et al. 2012; Krimpenfort et al. 2012). SMADs act as signal transducers for the TGF- β superfamily (Miyazono 2000). SMAD4 deficiency in CRC cell-lines enhances VEGF expression, apoptosis resistance and cell migration (Papageorgis et al. 2011).

While the CIN pathway is a feature of FAP, the micro-satellite instability (MSI) pathway is associated with HNPCC. Microsatellites are normal simple sequence repeats (SSRs) of 1 to 4 DNA base pairs which are of a set length in every individual. Defective DNA-repair due to failure of mismatch repair enzymes causes shortening or lengthening of these repeats. MSI is a feature of 15% of CRC, with 3% being associated with HNPCC (Boland and Goel 2010). In sporadic cancers MSI results from hypermethylation of the MLH1 gene promoter (Kane et al. 1997), which leads to the rapid development of additional mutations. Colorectal tumours with MSI have distinctive features, including a tendency to arise in the proximal colon, lymphocytic infiltrate,

and poor differentiation. They have a slightly better prognosis than colorectal tumours without MSI and do not have the same response to chemotherapeutics.

While it was previously assumed that almost all colorectal carcinomas arose via the CIN pathway, it is now clear that this pathway accounts for only approximately 60% of colon carcinoma (Jass 2007). Most of the remaining 40% develop via the more recently described serrated pathway which leads to CpG island methylated phenotype (CIMP) carcinoma (approximately 35%), with the remaining 5% arising via the MSI pathway. The serrated pathway is initiated by mutation of BRAF which leads to inhibition of normal apoptosis and the development of serrated lesions (Snover 2011). Such lesions are prone to methylation of the CpG island promoter regions resulting in the epigenetic silencing of a number of genes. The best characterized of these is the MLH1 gene, which is the gene silenced in sporadic MSI carcinomas. Following silencing of MLH1, serrated lesions become MSI positive and are prone to the rapid development of additional mutations.

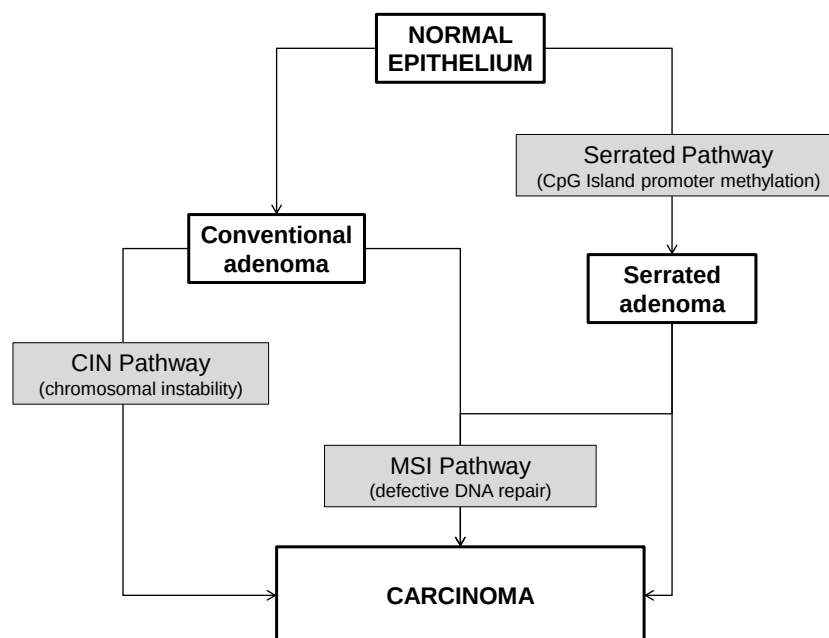
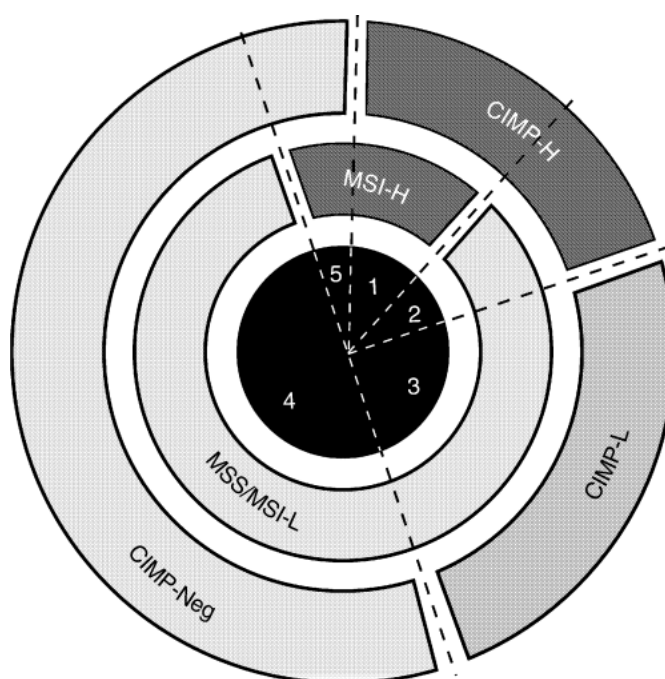


Figure 1.2 Genetic Pathways of CRC Tumorigenesis

Schematic representation of genetic pathways leading from normal epithelium to CRC.

Thus CRC can develop via multiple pathways (Figure 1.2). In a recent review by Jass, a classification of 5 molecular types of CRC as defined by MSI and CIMP status (high, low or negative) is described, which corresponds to clinical and morphological features (Jass 2007). This underlines the importance of recognising the heterogeneity of CRC, which has important implications for the management of this disease.



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Figure 1.3 CRC classification based on CIMP and MSI status.

Schematic representation of 5 molecular subtypes of CRC based on CpG island methylator phenotype (CIMP) status (H, high; L, low; Neg, negative) and DNA microsatellite instability (MSI) status (H, high; L, low; S, stable). The size of each segment of the black circle approximates to the frequency of each group. The groups (and their frequencies) are as follows: 1, CIMP-H, MSI-H (12%); group 2, CIMP-H, MSS/MSI-L (8%); 3, CIMP-L, MSS/MSI-L (20%); 4, CIMP-Neg, MSS/MSI-L (57%); 5, CIMP-Neg, MSI-H (3%). Groups 1-3 arise via the serrated pathway, group 4 via the CIN pathway, and group 5 corresponds to HNPCC. Figure from (Jass 2007).

1.1.4 Clinical Presentation and Diagnosis

Although diagnosis in asymptomatic patients is increasing since the National Bowel Cancer Screening Programme was introduced in 2006, the majority of CRC is still diagnosed in symptomatic patients. Symptoms include abdominal pain, a palpable mass, vomiting, change of bowel habit, rectal bleeding and tenesmus, and they vary according to the site of the tumour (Majumdar et al. 1999). Other non-CRC-specific symptoms include weight loss and iron-deficiency anaemia due to occult blood loss. Up to 20% of patients present as emergencies with perforation causing peritonitis or with obstruction (Koehne et al. 1999). Metastatic disease is present at first diagnosis in as few as 10% of patients (Cancer Research U. K. 2011), but ultimately develops in up to 60% of patients, with the liver (approximately 50%) and lung (10%) being the most common sites (Geoghegan and Scheele 1999).

Following an initial clinical examination, a broad range of diagnostic studies can be employed in the evaluation of suspected CRC. 40-80% of patients with rectal cancers present with masses palpable by digital rectal examination (DRE) (Shallow et al. 1955; McSherry et al. 1969),

Rectosigmoid cancers which account for 36% of all CRC can be detected by DRE and/or rigid sigmoidoscopy. Cancers sited within 60cm of the anal verge are amenable to examination by flexible sigmoidoscopy. These constitute the majority of cancers causing rectal bleeding and/or a change in bowel habit. Patients presenting with 'red flag' symptoms consistent with proximal cancers, such as otherwise unexplained iron-deficiency or change of bowel habit, should be assessed by colonoscopy. In patients diagnosed with potentially curable CRC, the incidence of synchronous lesions is in the order of 4-5%, and therefore completion colonoscopy is important to visualise the entire length of colon (Finan et al. 1987; Barillari et al. 1990).

Crucial to the management of CRC is accurate clinical, radiological and histopathological staging, since this is the most accurate predictor of outcome, and forms the basis of decisions about the most appropriate treatment for the patient. Pre-operative staging in the form of computed tomography (CT) of the thorax, abdomen and pelvis allows accurate determination of local and distant extent of disease. In rectal cancers, pelvic magnetic resonance imaging (MRI) and endoanal ultrasound allow assessment of local tumour infiltration, which helps to determine whether curative excision with tumour-free margins is feasible.

98% of cancers within the large bowel are adenocarcinomas, with rarer tumours comprising of carcinoid, primary and secondary lymphoma and leiomyosarcoma. Macroscopically, the tumours can appear as papilliferous, ulcerating, annular circumferential lesions, diffusely infiltrating and colloid tumours producing excess mucus. The Dukes' classification of tumours was introduced in 1932, and categorises CRC lesions by depth of invasion and local metastasis into stages A, B and C (Dukes 1932), with a fourth stage D subsequently introduced to describe distant metastasis (Whittaker and Goligher 1976). The modified Astler-Coller classification further subdivided stages B and C (Astler and Collier 1954). While the Dukes' stage remains in common use clinically, these two classification systems have been superseded by the more precise TNM (Tumour, Node, Metastasis) classification, which was first introduced in 1977 and is currently in its 7th Edition (Figure 1.4) (American Joint Committee on Cancer 2010). Table 1.1 illustrates the importance of staging as a predictor of survival. Patients presenting with Dukes' A CRC are almost twice as likely to survive for 5 years after diagnosis compared to those presenting with Dukes' C, and only 1 in 15 patients with distant metastasis (Dukes' D) survive this long.

Primary Tumor (T)

- TX** Primary tumor cannot be assessed
T0 No evidence of primary tumor
Tis Carcinoma in situ: intraepithelial or invasion of lamina propria
T1 Tumor invades submucosa
T2 Tumor invades muscularis propria
T3 Tumor invades through the muscularis propria into pericolorectal tissues
T4a Tumor penetrates to the surface of the visceral peritoneum
T4b Tumor directly invades or is adherent to other organs or structures

Regional Lymph Nodes (N)

- NX** Regional lymph nodes cannot be assessed
N0 No regional lymph node metastasis
N1 Metastasis in 1–3 regional lymph nodes
N1a Metastasis in one regional lymph node
N1b Metastasis in 2–3 regional lymph nodes
N1c Tumor deposit(s) in the subserosa, mesentery, or nonperitonealized pericolic or perirectal tissues without regional nodal metastasis
N2 Metastasis in 4 or more regional lymph nodes
N2a Metastasis in 4–6 regional lymph nodes
N2b Metastasis in 7 or more regional lymph nodes

Distant Metastasis (M)

- M0** No distant metastasis
M1 Distant metastasis
M1a Metastasis confined to one organ or site (for example, liver, lung, ovary, nonregional node)
M1b Metastases in more than one organ/site or the peritoneum

ANATOMIC STAGE/PROGNOSTIC GROUPS					
Stage	T	N	M	Dukes*	MAC
0	Tis	N0	M0	—	—
I	T1	N0	M0	A	A
	T2	N0	M0	A	B1
IIA	T3	N0	M0	B	B2
IIB	T4a	N0	M0	B	B2
IIC	T4b	N0	M0	B	B3
IIIA	T1–T2	N1/N1c	M0	C	C1
	T1	N2a	M0	C	C1
IIIB	T3–T4a	N1/N1c	M0	C	C2
	T2–T3	N2a	M0	C	C1/C2
	T1–T2	N2b	M0	C	C1
IIIC	T4a	N2a	M0	C	C2
	T3–T4a	N2b	M0	C	C2
	T4b	N1–N2	M0	C	C3
IVA	Any T	Any N	M1a	(D)**	—
IVB	Any T	Any N	M1b	(D)**	—

Figure 1.4 Staging of CRC

Figure shows definition of TNM Classification (TNM 7), and comparison of TNM, Dukes' and Modified Astler-Coller (MAC) Staging of CRC. * Dukes' B is a composite of better (T3 N0 M0) and worse (T4 N0 M0) prognostic groups, as is Dukes' C (any TN1 M0 and any T N2 M0). ** The original Dukes classification did not include Dukes' D. Modified from AJCC Cancer Staging manual 7th Edition (American Joint Committee on Cancer 2010).

A number of molecular biomarkers that are predictive of response to chemotherapy have been identified in recent years, and are the subject of continued research (Tejpar et al. 2010). The evaluation of these is increasingly a part of routine pathological evaluation of resected tumours (Engstrom et al. 2009b; Engstrom et al. 2009a). K-ras status is the best established, as mutations are associated with resistance to EGFR blockade (Amado et al. 2008; Karapetis et al. 2008a). An association with BRAF mutations and poor anti-EGFR response has also been reported (Tol et al. 2009). Reliable biomarkers that are predictive of response bevacizumab therapy are yet to be identified (Jubb and Harris 2010).

Table 1.1 Dukes' Staging and CRC Survival

Percentage of cases and 5-year relative survival rates by Dukes' stage at diagnosis for CRC patients diagnosed in England (1996-2002) (Cancer Research U. K. 2011). (* Dukes' D incidence of up to 25% has been reported (Fong et al. 1996; Rees et al. 2008)).

Dukes' Stage at diagnosis	Percentage of cases	5-year relative survival
A	8.7%	93.2%
B	24.2%	77.0%
C	23.6%	47.7%
D	9.2% *	6.6%
Unknown	34.3%	35.4%

1.1.5 Treatment

1.1.5.1 Surgery

Decisions regarding patient management should be discussed by a multidisciplinary team comprised of a panel of specialists with clinically relevant skills and expertise, in order for all patients to benefit from suitably informed oncological and surgical opinions. The mainstay of treatment is surgical resection. The goals of surgery are en-bloc resection with adequate margins of normal colon proximal and distal to the tumour, removal of regional lymph nodes, and restoration of bowel continuity. Accomplishment of these goals optimises the chance of preventing locoregional recurrence of the disease, and provides better quality of life for patients. For colonic lesions, segmental resection of the bowel with its regional lymph nodes is the accepted practice, but there has been a recent trend towards extended right hemicolectomy for tumours of the transverse colon and splenic flexure. In rectal cancers, careful removal of the tumour by total mesorectal excision (TME) ensures removal of all locoregional lymph nodes while preserving autonomic nerves and limiting blood loss. Although the technique can increase operating time and surgical complications, it significantly reduces local recurrence rates, and as part of an anterior resection or abdomino-perineal excision of rectum (APER), TME is considered the gold standard (MacFarlane et al. 1993; Quirke et al. 2009). Intersphincteric resection is an alternative to APER for low rectal tumours, and is significantly preferable in terms of patients' quality of life. It allows primary coloanal anastomosis avoiding a permanent colostomy, preserves sphincter function, and reduces the risk of urinary and sexual dysfunction (Schiessel et al. 1994; Martin et al. 2012b).

For small rectal cancers and polyps with small areas of invasive cancer, local excision is safe providing that clear resection margins are demonstrated at histological examination (Whiteway et al. 1985). Excision by transanal endoscopic microsurgery (TEM) has gained popularity, with data suggesting that this is at least as good as traditional transanal resection (Middleton et al. 2005; Zacharakis et al. 2007). Preoperative assessment of depth of invasion and nodal disease by MRI and endoanal ultrasound is paramount in these patients, and histopathological examination of some of these locally excised lesions may identify a proportion of patients who will require more radical surgery.

The presence of distant metastasis predicts significantly poorer prognosis. In recent years, resection of liver metastases has gained significant ground, and is offered to patients with up to four lesions providing they are confined to one lobe of an otherwise healthy liver, the volume to be resected is less than 70% of total liver volume, and there is no unresectable extra-hepatic disease (Zdenkowski et al. 2012). Up to 23% of patients with liver metastases have resectable lesions, and neoadjuvant chemotherapy (oxaliplatin, irinotecan) can increase this by a further 12% (Adam et al. 2004). Survival rates of 30% at 5 years and 25% at 10 years can be achieved with this strategy (Gallagher and Kemeny 2010). Where logistics allow, simultaneous resection of the primary and secondary lesions may be performed by colorectal and hepato-biliary surgeons. Resection of lung metastases can also be undertaken in patients with controlled primary disease who are fit for thoracotomy. 5-year survival rates of 50% have been reported in patients with solitary lesions, although outcomes are less favourable where there are multiple unilateral lesions (40%) and worse still with bilateral metastases (12.5%) (Inoue et al. 2004).

1.1.5.2 Chemo-radiotherapy

Significant improvements in survival rates for late-stage CRC (Dukes' C and D) have been achieved in recent years largely due to the use of (neo-)adjuvant chemo- and radiotherapy, which can be used in combination (chemoradiotherapy (CRT)). Traditional chemotherapeutic agents consist of cytotoxic drugs such as topoisomerase-inhibitors (e.g. irinotecan) and anti-metabolites (5-fluorouracil (5-FU), which is usually used in combination with leucovorin (LV)). More recently, platinum-based drugs (oxaliplatin) and the FU pro-drug capecitabine have shown promise (Arkenau et al. 2008). These drugs are generally used in combinations designed to minimise toxicity while maintaining efficacy.

While the use of pre-operative (neo-adjuvant) CRT might be thought to introduce the risk of progression by delaying surgery, it can in fact improve outcomes, as first demonstrated by the Swedish Rectal Cancer Trial (1997). Patients with resectable rectal cancers who received short-course radiotherapy (SCRT; 1 week of treatment) before "traditional" (non-TME) anterior resection or APER were found to have a significant reduction in local recurrence compared to

surgery alone (11% *versus* 27%). Improvement in survival rates was also seen (overall survival (OS) 58% *versus* 48% at 5 years; cancer-specific survival 74% *versus* 65% at 9 years). The benefits in both local recurrence and OS persisted at a median follow-up of 13 years (Folkesson et al. 2005). Results from the Dutch TME Trial showed that SCRT also improves local recurrence rates when this technique (which in itself reduces local recurrence compared to traditional surgery) is used, although significant survival benefits were not seen (Kapiteijn et al. 2001; Peeters et al. 2007).

For patients with advanced locoregional (T3 or T4) rectal disease, long course (4-5 weeks) pre-operative radiotherapy given in combination with 5-FU has been shown to be effective in down-staging tumours, improving resectability and allowing sphincter-preserving surgery (Sauer et al. 2004; Bosset et al. 2005). Indeed 15-20% of patients receiving long-course chemoradiotherapy (LCCR) show a pathological complete response (pCR), i.e histological examination of the resected specimen shows no viable tumour cells (Chan et al. 2005; Quah et al. 2008). There is some controversy as to whether pCR confers a survival advantage (Onaitis et al. 2001; Pucciarelli et al. 2004; Chan et al. 2005). However in a systematic review and meta-analysis which included 16 studies and 3363 patients, Martin et al. reported that compared to partial and non-responders, complete responders had lower local and distant recurrence rates (odds ratio (OR) 0.25 and 0.23 respectively), and better disease-free (OR 4.33) and overall survival (OR 3.28) (Martin et al. 2012a). The study reported a mean local recurrence rate of 0.7% (range 0 – 2.6%) in patients with pCR, suggesting that the risk of local recurrence is all but eradicated. This raises the possibility of such patients having less radical surgery such as local excision (Callender et al. 2010), or even being managed with an expectant (“watch and wait”) approach, by which surgery is offered once a recurrence has been detected (Habr-Gama et al. 2004; Habr-Gama et al. 2006). However, although complete responders have a four-fold reduction in distant metastases, a significant risk remains (8.7%) despite the apparent disappearance of the primary. It may be that some patients showing pCR would benefit from adjuvant chemotherapy to reduce this risk, but identifying which these would be remains a challenge.

CRT therefore offers hope to the large subgroup of patients (up to 55% of all CRC) who present late with advanced disease (Campbell et al. 2001), with potential curative resection following adequate downstaging of the tumour (Adam et al. 2008; Fleming et al. 2011). Furthermore, post-operative chemotherapy in the form of 5-FU as a single agent, or combined with oxaliplatin, improves disease-free survival and OS for patients with lymph node positive disease (Wolmark et al. 2000; Andre et al. 2004; NICE 2005).

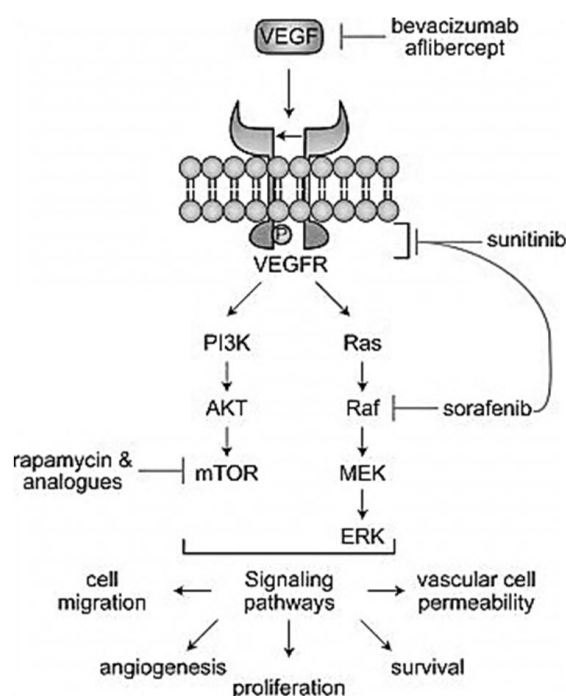
1.1.5.3 Novel Biological Agents

The management of patients with inoperable or recurrent disease, or who are resistant to CRT, remains challenging. Much work is focussed on the development of new treatments aimed at retarding tumour growth and/or causing tumour regression. The last decade has seen the development of novel therapeutic agents that target specific cellular pathways. This is exemplified by the monoclonal antibody bevacizumab (Avastin®), which inhibits VEGF. VEGF is the key effector of angiogenesis, a process that is critical in the pathogenesis of cancer, as discussed in detail below (section 1.2). Endothelial cells are central to angiogenesis, and are co-opted by tumours in order to develop the new blood vessels required to sustain rapid growth (Folkman 1971; Folkman and Shing 1992) (section 1.2.2). VEGF is a potent promoter of endothelial cell survival, mitogenesis, migration, differentiation, as well as regulating the function of other cell-types such as smooth muscle and haematopoietic cells. It plays a central role in both physiological and pathological angiogenesis. In CRC, expression of VEGF increases with progression along the adenoma-carcinoma pathway (Wong et al. 1999; Kondo et al. 2000). Increased expression of VEGF and its receptor KDR (Kinase Dependent Receptor) is associated with metastasis and poor prognosis (Ishigami et al. 1998; Lee et al. 2000; Harada et al. 2001). VEGF expression and MVD, a measure of angiogenesis, is strongly associated with depth of invasion, lymph node metastasis and liver metastasis (Nakasaki et al. 2002).

These observations, which are paralleled in several other cancers, were first made in the 1990s, and provided strong evidence that VEGF may be a therapeutic target. The first clinical confirmation of this came in clinical trials of bevacizumab, which was shown to improve overall survival in metastatic CRC (mCRC) when used in combination with traditional chemotherapy (Kabbinnavar et al. 2003; Hurwitz et al. 2004). Bevacizumab was subsequently licensed for use in non-squamous non-small cell lung cancer (NSCLC), glioblastoma, metastatic renal cell carcinoma (RCC) and breast cancer (Scott 2007; Planchard 2011; Melichar et al. 2012; Patel et al. 2012). While bevacizumab is specific to VEGF-A (the most dominant VEGF isomer in CRC), the newer aflibercept binds VEGF-A and VEGF-B, as well as placental growth factor (PlGF). Aflibercept improves survival in mCRC even in patients who have previously failed bevacizumab treatment (Van Cutsem et al. 2012). In the wake of promising results with bevacizumab therapy, the development of several VEGF-inhibiting drugs with different modes of action has followed (Figure 1.5). These include the receptor tyrosine kinase inhibitors (RTKIs), such as sunitinib, a VEGF-receptor RTKI (used in RCC and gastro-intestinal stromal tumours (GIST)) (Gan et al. 2009). Other drugs inhibit downstream VEGF-activated pathways: the Raf/MEK/ERK pathway is inhibited by sorafenib (which also inhibits VEGFR), used in RCC and hepatocellular carcinoma (Escudier et al. 2007; Llovet et al. 2008); and the PI3K/AKT/mTOR pathway is inhibited by temsirolimus (a rapamycin analog), used in RCC (Amato 2011; Ibrahim et al. 2012). Regorafenib,

a new inhibitor that targets several RTKs (VEGFR, PDGFR, FGFR) as well as downstream pathways (e.g. Raf), has shown efficacy when used as monotherapy in refractory mCRC (Grothey et al. 2012), and received FDA approval in September 2012. Bevacizumab is discussed in detail in Section 0.

Another newly developed class of agents inhibits the ErbB signalling pathway (Hynes and MacDonald 2009). EGFR is a member of the ErbB receptor tyrosine kinase family, and EGF binding promotes clonal expansion of transformed cells, and modulates cellular adhesion, differentiation and apoptosis, enhancing tumourigenicity and malignant progression (Yarom and Jonker 2011). Human cancers can overactivate the ErbB signalling pathway by overproduction of ligands and/or corresponding receptors, or expression of constitutively activated receptors. Overexpression of both EGF and EGFR is seen in CRC (Salomon et al. 1995; Messa et al. 1998), and is associated with poorer prognosis (Karameris et al. 1993; Spano et al. 2005; Zlobec et al. 2007). Cetuximab is a chimeric monoclonal anti-EGFR antibody, whereas the newer panitumumab is the first fully human anti-EGFR monoclonal antibody. Both have been shown to improve outcomes in mCRC in phase III trials (Cunningham et al. 2004) (Jonker et al. 2007; Peeters et al. 2010).



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Figure 1.5 Inhibitors of VEGF signalling

VEGF binding to VEGFR, a receptor tyrosine kinase, leads to receptor dimerization and subsequent auto-phosphorylation of the receptor complex. Interaction with cytoplasmic signalling molecules activates pathways leading to a variety of tumourigenic processes including angiogenesis. Examples of clinical inhibitory compounds are shown (see text). Aflibercept is used in age-related macular degeneration (PI3K indicates phosphoinositide 3-kinase; mTOR, mammalian target of rapamycin; MEK, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated kinases.) (modified from Cook and Figg 2010)

1.2 Angiogenesis

Angiogenesis is the formation of new blood vessels from existing vasculature (as distinct from vasculogenesis, the formation of new blood vessels *de novo*). Angiogenesis plays an important role in normal adult human physiology. It is normally quiescent, only coming into play in specific circumstances, such as in wound healing, and in the female reproductive cycle. It is tightly regulated by angiogenic and antiangiogenic (angiostatic) factors, the latter being dominant in the “normal” state. VEGF is a key regulator of angiogenesis, and its role in both physiological and pathological angiogenesis discussed in a detailed review by Chung and Ferrara (Chung and Ferrara 2011). Loss of intrinsic control of angiogenesis tips the balance in favour of angiogenic factors (the so-called “angiogenic switch”), leading to pathological chronic inflammatory conditions and fibroproliferative disorders (for example in ophthalmic and rheumatic diseases). In cancer cells this allows exploitation of the various control mechanisms which regulate angiogenesis resulting in improved survival, increased proliferation and growth, and enhanced tumour spread. In order to appreciate the mechanisms by which cancers transform these regulatory pathways to promote angiogenesis, an understanding of the regulatory systems underlying physiological angiogenesis is necessary.

1.2.1 Physiological Angiogenesis

Sprouting angiogenesis occurs in several well-characterised stages, with each stage requiring coordinated signals between various growth factors and receptors (summarised in Figure 1.6). The first step of angiogenic remodelling of mature vasculature requires disengagement of endothelial cells from surrounding supporting cells. This destabilisation process is driven by a progressive shift in angiopoietin (ANGPT) expression from ANGPT-1, which maintains vessels in a quiescent state, to ANGPT-2 which encourages pericyte detachment (Maisonpierre et al. 1997). This detachment renders endothelial cells accessible to angiogenic stimuli, such as VEGF and FGF-2, which activate endothelial cells, resulting in release of proteases such as MMP, plasminogen activators (uPA and tPA) (Millauer et al. 1993; Pepper 1997), and interstitial collagenase (Unemori et al. 1992). These proteases degrade and remodel the endothelial cell basement membrane and surrounding extracellular matrix (Moses 1997), and new matrix synthesized by stromal cells is then laid down. This, in combination with the proliferative (Gospodarowicz et al. 1987; Connolly et al. 1989; Gospodarowicz et al. 1989) and pro-migratory effects (Terranova et al. 1985; Dimmeler et al. 2000) of VEGF and FGF-2, allows migration of endothelial cells which create finger-like projections into the region to be vascularised. Invasion and migration of endothelial cells through extracellular matrix (ECM) comprising of Type I collagen, vitronectin, fibronectin, and fibrin requires cell-matrix interactions, which are mediated by integrins such as $\alpha_v\beta_3$ (Cheresh 1987). Integrin $\alpha_v\beta_3$ mediates endothelial cell attachment,

spreading, and migration (Leavesley et al. 1993), and its importance for endothelial cell function is reflected by its transient expression on tips of sprouting endothelial cells, and on newly formed blood vessels in granulation tissue (Clark et al. 1996; Li et al. 2003b). As the endothelial cells coalesce together to form tube-like structures, mesenchymal cells proliferate and migrate along the abluminal surface of the endothelium under the influence of platelet derived growth factor-B (PDGF-B), maturing to form pericytes and smooth muscle cells (Conway et al. 2001). Presence of an enveloping layer of pericytes inhibits further endothelial cell proliferation and migration, signalling the end of new blood vessel formation (Beck and D'Amore 1997). Finally, ECM is deposited around the new vessel under the control of TGF- β , secretion of which is regulated by interactions between endothelial cells and the vascular smooth muscle cell-pericyte complex. This signals the final stages of vessel stabilisation and maturation (Beck and D'Amore 1997).

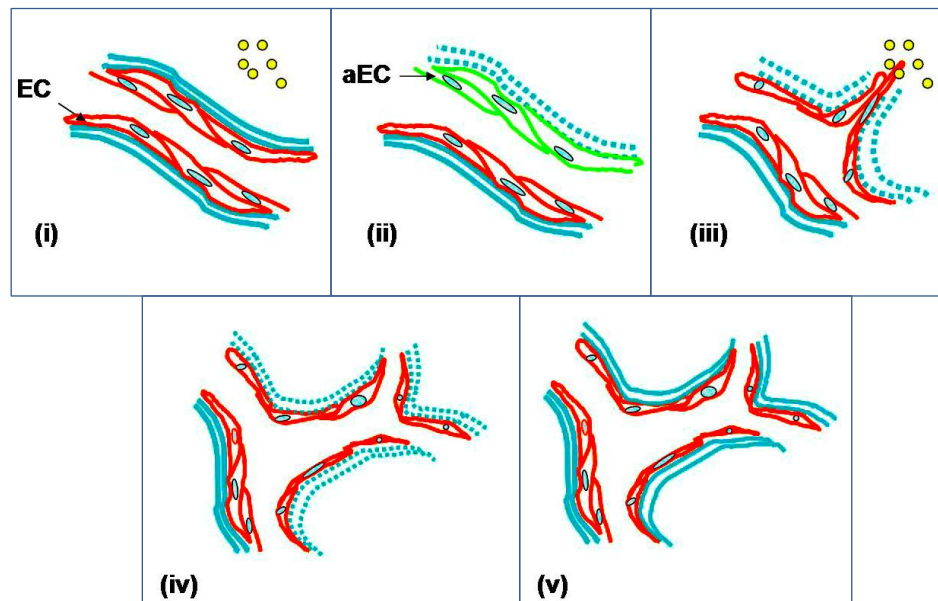


Figure 1.6 Sprouting Angiogenesis

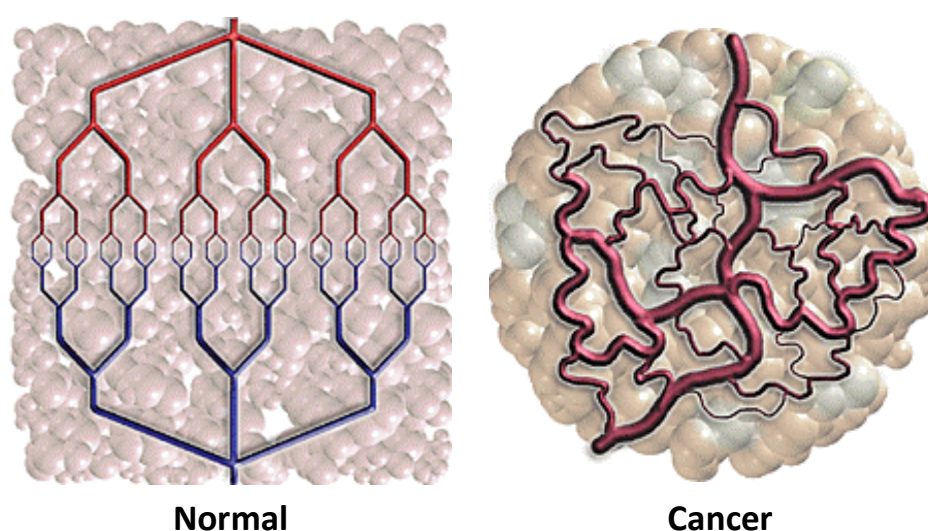
Sprouting angiogenesis: formation of blood vessels is a multi-step process, which includes (i) reception of angiogenic signals (yellow circles) by endothelial cells (EC); (ii) retraction of pericytes from the abluminal surface of capillary and secretion of protease from activated endothelial cells (aEC) and proteolytic degradation of basement membrane (blue dashed line) and extracellular matrix; (iii) chemotactic migration of EC under the induction of angiogenic stimulation; (iv) proliferation of EC and formation of lumen/canalisation by fusion of formed vessels with formation of tight junctions; (v) recruitment of pericytes and deposition of new basement membrane and initiation of blood flow (modified from Yue et al. 2007).

1.2.2 Angiogenesis in Cancer

The fine balance between pro- and anti-angiogenic factors is disrupted in pathological conditions such as psoriasis, ophthalmic and rheumatic diseases where inflammation recruits inflammatory cells causing release of pro-angiogenic factors and proteases. The pivotal role of angiogenesis in the development of tumours was demonstrated by Folkman in the early 1970s who showed that for continued growth, a tumour which exceeds 1-2mm³ in volume requires its

own blood supply (Folkman 1971). Signals that trigger the angiogenic switch include metabolic stress, genetic mutations that activate certain oncogenes or inactivate tumour suppressor genes, and the presence of an immune or inflammatory response (Carmeliet and Jain 2000). For most of these causative factors, VEGF is the common effector inducing angiogenesis, especially in the presence of other angiogenic allies such as nitric oxide synthase (NOS), PDGF-B and ANGPT-2 (Carmeliet and Jain 2000). Hypoxia, a feature of most solid tumours, also potently and consistently induces VEGF expression by recruiting hypoxia inducible factor (HIF)-dependent or HIF-independent mechanisms (see below).

Once the balance is tipped in favour of angiogenesis, the process of sprouting angiogenesis described previously ensues. Unlike normal blood vessels, tumour vessels are structurally and functionally abnormal Figure 1.7 (Jain 1994; Jain 2001).



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Figure 1.7 Normal versus abnormal (cancer) vessel architecture

Normal vasculature (left) is composed of mature vessels arranged in an ordered manner. Abnormal tumour vasculature (right), is composed largely of immature vessels with increased permeability, vessel diameter, vessel length, vessel density, tortuosity and interstitial fluid pressure. (Modified from Jain 2001)

Abnormal perivascular cells, and an excess of endothelial cells with weak inter-endothelial junctions, contribute to formation of tortuous irregular networks of dilated and hyperpermeable blood vessels (Gazit et al. 1997; Baish and Jain 2000; Hashizume et al. 2000; Dejana et al. 2009). Further, the mechanical stress from the solid mass of proliferating cancer cells leads to compression of the vessel lumen (Padera et al. 2004). The combination of raised IFP and areas of vascular collapse leads to regional hypoxia and acidosis within tumours (Goethals et al. 2006; Goel et al. 2011).

In the face of increased oxygen demand by the growing tumour therefore, oxygen

delivery is in fact reduced, creating a positive feedback loop in which the resultant hypoxia encourages development of more dysfunctional vessels. Other sequelae of tumour angiogenesis include increased risk of metastasis, and resistance to therapeutic agents. Impaired integrity of the vascular endothelium increases access of tumour cells in the primary site to the circulation, and hence transport to distant sites (Tien et al. 2001; Galaup et al. 2006), while increased IFP and vascular collapse reduces intratumoural blood flow, compromising delivery of cytotoxic agents (Jain 1994; Jain 1998).

1.2.3 Angiogenesis Genes

Given the intricate coordination of numerous processes required to establish a new blood supply, it is no surprise that a multiplicity of genes have been implicated in angiogenesis. In this study, a PCR Array consisting of 84 angiogenesis-related genes was used (see Table 2.5 for full list of genes). A description of all the genes is beyond the scope of this work. However, genes encountered in subsequent chapters are discussed below. They fall into 5 broad functional categories: transcription factors, cytokines, growth factors, vascular remodelling factors and matrix remodelling factors. Several of the genes are pleiotropic to varying degrees, falling into more than one functional group.

Transcription Factors

HIF (hypoxia inducible factor) and HAND2 (Heart- and neural crest derivatives-expressed protein 2) are both members of the basic loop-helix-loop (bHLH) family of transcription factors. HIF is the focus of Chapters 3 and 4, and is described in detail below (1.3.1). HAND2 plays an important role in cardiovascular and sympathetic nervous system development (Yamagishi et al. 2000; Voth et al. 2009), and has been reported to regulate ECM remodelling (Yin et al. 2010). Embryonic HAND2 mutations lead to a similar angiogenic phenotype to mutations in VEGF and its receptors, suggesting that HAND2's angiogenic function may be mediated via the VEGF signalling pathway (Yamagishi et al. 2000). It has been reported to be overexpressed in squamous cell carcinoma of the lung (Metodieva et al. 2011).

Cytokines

Chemokines (chemo-attractant cytokines) direct movement of target cells along a concentration gradient, and play an important role in numerous processes including inflammation and angiogenesis. CCL11 (eotaxin), a member of the CC chemokine group (β -chemokines), induces chemotaxis in eosinophils. It stimulates endothelial cell migration, implicating it in angiogenesis (Salcedo et al. 2001). Upregulation of CXCL-3 (GRO3), CXCL-9 and interleukin 8 (IL-8/CXCL-8), all members of the CXC chemokine group, has been reported in CRC tissue (Erreni et al. 2009; Doll et al. 2010). CXCL-3 and IL-8 belong to the angiogenic ELR-positive

subgroup of CXC-chemokines, which as well as attracting and activating neutrophils, can stimulate endothelial cell migration (Vandercappellen et al. 2008). CXCL-9 is a member of the angiostatic ELR-negative subgroup of CXC-chemokines which can be induced by IFN- γ .

The interferons are primarily involved in immune responses to pathogens, and they also play an important role in mediating the immune response to tumour cells (Dunn et al. 2006). They also have angiostatic properties, both by inhibiting angiogenic factors such as VEGF, IL-8, basic fibroblast factor (bFGF) and MMP-9 (von Marschall et al. 2003), and by direct inhibition of endothelial cell function (Indraccolo 2010). IFN- γ is associated with increased CRC risk, and is upregulated in CRC tissue compared to adjacent normal tissue (Slattery et al. 2011; Nam and Park 2012). In a CRC cell-line xenograft model, IFN- γ -stimulated mesenchymal stem cells (MSCs) promote angiogenesis and tumour growth in a VEGF-mediated mechanism (Liu et al. 2011). Interferons have shown significant anti-tumour activity in pre-clinical models of CRC (Ozawa et al. 2001; Choi et al. 2004), and have been used therapeutically in several cancers including Kaposi's sarcoma and malignant melanoma (Dunn et al. 2006). However, while IFN- γ has been shown to improve survival when used as an adjunct in ovarian cancer (Windbichler et al. 2000), a study in CRC showed no benefit (Wiesenfeld et al. 1995).

TNF- α plays a critical role in systemic inflammation, and its role in autoimmune diseases is underlined by the success of anti-TNF therapies such as infliximab in rheumatoid arthritis (Brennan et al. 1989a; Feldmann and Maini 2003). The link between inflammation and cancer is well-established (Karin et al. 2002). Indeed TNF was first identified for its ability to induce haemorrhagic necrosis in cancer cell-lines (Carswell et al. 1975). TNF signalling results in induction of apoptosis and the destruction of tumour-associated blood vessels (Kashii et al. 1999; Prévost-Blondel et al. 2000; Lejeune 2002). However, TNF also promotes the development and progression of cancer (Moore et al. 1999; Balkwill 2006; Egberts et al. 2008; Balkwill 2009). Elevated TNF- α levels have been demonstrated in many cancers including CRC, and are associated with poor prognosis (Naylor et al. 1990; Szlosarek and Balkwill 2003). TNF- α can induce cancer cell production of other angiogenic genes such as VEGF, MMP-9, TGF- β 1 and IL-8 (Nabors et al. 2003; Balkwill 2004; Kulbe et al. 2005; Stuelten et al. 2005). TNF- α has been reported to upregulate VEGF production by mesenchymal stem cells, promoting angiogenesis and tumour growth in CRC cell xenografts (Liu et al. 2011). TGF- β 1 is discussed in the subsequent section, due to its function as both a cytokine and growth factor.

Growth Factors

Growth factors stimulate cellular growth, proliferation and cellular differentiation. Transforming growth factor- β 1 (TGF- β 1) is the founding member of the TGF- β superfamily of

multifunctional cytokines which have a modulatory role on virtually all cell types. It controls processes such as differentiation, proliferation, migration and apoptosis as well as regulating immune responses and the extracellular environment. It has a tumour suppressive effect in early cancer, but paradoxically as tumours progress they can develop the ability to exploit TGF- β signalling pathways to promote tumourigenicity (Massagué 2008). Elevated TGF- β 1 levels have been reported in serum from CRC patients as well as in tumour tissue, and expression correlates with markers of angiogenesis such as VEGF expression, MVD, as well as with metastasis and prognosis (Tsushima et al. 1996; Xiong et al. 2002a; Xiong et al. 2002b; Gulubova et al. 2010).

Ephrin-A3 is a member of the largest family of receptor tyrosine kinase (RTK) systems. The receptors (of which there are 13, designated “Eph”) and the ligands (9, designated “ephrins”) are classified into two groups based on sequence homology and binding preferences. Ephrin A ligands (including ephrin-A3, encoded by the EFNA-3 gene) generally bind EphA receptors, while ephrin B ligands generally bind EphB receptors, but interactions across the classes can occur (Himanen et al. 2004). A large number of receptor-ligand combinations is possible, and Eph-ephrin interactions have pleiotropic functions including axonal guidance, cell migration and angiogenesis (Wilkinson 2000; Adams 2002; Himanen et al. 2004). A unique property of Eph-ephrin interactions is bidirectionality, with signalling being triggered both in the ligand-expressing and the receptor-expressing cell (Mellitzer et al. 1999; Kullander and Klein 2002). Both forward and reverse signalling regulate cell motility, and Eph-ephrin interaction can lead to both cellular attraction and repulsion, depending on the receptor-ligand combination and cell type involved (Davy and Soriano 2005). By modulating changes in attraction/repulsion, migrating cells can be guided along precise paths. Ephrin-A3 is one of the least investigated ephrin ligands.

PDGF (platelet-derived growth factor) is a member of the same growth factor family as VEGF with diverse functions (Yu et al. 2003). It is a potent mitogen for cells of mesenchymal origin, and induces cellular processes including angiogenesis, proliferation, migration and apoptosis (Yu et al. 2003). It is critical in embryonic development, and plays an important role in inflammation and wound healing. Its tumourigenicity was first discovered almost 30 years ago (Deuel et al. 1983), and it has been implicated in several cancers such as gliomas, sarcomas and leukaemias, as well as CRC (Heldin 2012; Peterson et al. 2012).

Vascular Remodelling Factors

The angiopoietin (ANGPT) family of proteins play a central role in angiogenesis. Their functions are mediated through Tie-1 and Tie-2 receptors which are expressed on vascular and lymphatic endothelial cells. Angiopoietin-1 and -2 (ANGPT-1, ANGPT-2) are functionally antagonistic and both signal via the Tie-2 (TEK) receptor. Primarily expressed by mesenchymal

cells, ANGPT-1 recruits mural cells, and promotes assembly and maturation of blood vessels leading to vessel stabilisation and normalisation; conversely, ANGPT-2 is stored by endothelial cells and destabilises vessels, inducing vascular sprouting and branching (Brindle et al. 2006; Fagiani et al. 2011). Overexpression of ANGPT-1 has been reported to suppress angiogenesis and tumour growth in CRC, while ANGPT-2 has the opposite effect (Ahmad et al. 2001b; Stoeltzing et al. 2003). However, in other cancers ANGPT-1 can enhance angiogenesis (Machein et al. 2004), and the aberrant vessels resulting from ANGPT-2 overexpression can lead to reduced tumour growth (Yu and Stamenkovic 2001).

The angiopoietin-like (ANGPTL) proteins, of which there are seven, are structurally similar to the ANGPT family, but do not bind to the Tie receptors, indicating that they may have different functions to the ANGPT proteins (Morisada et al. 2006). Indeed, as well as being involved in angiogenesis, ANGPTL-4 has remarkably diverse functions, and is involved in lipid metabolism, cell-differentiation, vascular permeability and wound healing (Zhu et al. 2012). The ANGPTL-4 protein undergoes cleavage into two fragments which can oligomerize in a number of ways, and can also be subject to further post-translational modification. This structural complexity underlies its multifunctionality (Zhu et al. 2012). The role of ANGPTL-4 in cancer has emerged in recent years, and is summarised in an excellent review by Tan *et al.* (Tan et al. 2012). Of the seven ANGPTLs, ANGPTL-3 is the most closely related to ANGPTL-4 but is much less well understood.

Matrix Remodelling Factors

The matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases which have an important role in the degradation of ECM proteins. As well as removing physical barriers to cell motility and exposing ECM-bound receptor binding sites and regulatory molecules, MMPs can also interact directly with growth factor binding proteins and precursors, cell-adhesion molecules and other proteinases (Egeblad and Werb 2002; Rupp et al. 2008). Their function is critical in normal physiology, for example in embryological development and angiogenesis. They also play a central role in cancer invasion and metastasis. They are upregulated in most types of cancer including CRC, and their expression is associated with poor prognosis (Egeblad and Werb 2002). MMPs are synthesised as inactive zymogens (pro-MMPs) which require proteolytic cleavage either by other activated MMPs or by serine proteases. MMP activity is also tightly regulated by endogenous inhibitors such as α -2-microglobulin and tissue inhibitors of metalloproteinases (TIMPs). MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are both upregulated in CRC and associated with angiogenesis (Kim and Kim 1999).

ANPEP (alanine aminopeptidase) is a zinc-dependent ectopeptidase that is involved in both in normal and malignant development (Wickström et al. 2011). As with the MMPs, its enzymatic activity can both disrupt the ECM structure and modulate function of specific peptide substrates (Bhagwat et al. 2001; Tsujimoto et al. 2007). It promotes human umbilical vein endothelial cell (HUVEC) proliferation, migration and capillary tube formation *in vitro* (Fukasawa et al. 2006). Urokinase-type plasminogen activator (uPA/PLAU) is a component of the urokinase plasminogen activating system (uPAS), which also has both proteolytic and non-proteolytic activity and has been implicated in angiogenesis (Blasi and Carmeliet 2002; Choong and Nadesapillai 2003; Ulisse et al. 2009).

Collagen type IV alpha 3 (α -3(IV)), encoded by the COL4A3 gene, is an isoform of Type IV collagen which is the major structural component of the mammalian basement membrane (BM) (Lemmink et al. 1997). BM disruption is a necessary step in the initiation of invasion of epithelial tumours (Tanjore and Kalluri 2006). α -3(IV) has been shown to inhibit angiogenesis and tumour growth in an *in vivo* melanoma model (Petitclerc et al. 2000). This may be through the action of the angiostatic peptide tumstatin, a product of α -3(IV) cleavage by MMP-9 (Hamano et al. 2003). Another possible mechanism by which α -3(IV) inhibits angiogenesis is through suppression of MMP-2 activation (Martinella-Catusse et al. 2001).

1.2.4 Angiogenesis Inhibition in CRC

There is a multiplicity of angiogenesis inhibiting drugs in current use, and new molecules continue to be developed. In 2009 there were 44 VEGFR inhibitors in development (Ivy et al. 2009), and in 2012 there were 8 novel antiangiogenics in phase III trials (Troiani et al. 2012). Inhibitors are general either anti-VEGF antibodies or inhibitors of receptor tyrosine kinases, but new inhibitors are emerging that have multiple targets, such as regorafenib. They are most commonly used in combination with cytotoxic drugs. This is in part due to a general issue with clinical trials. As ethical considerations preclude the enrolment of patients into a trial where they may be deprived of the best existing therapy (in the “control” arm), the majority of randomised clinical trials (RCTs) have investigated the addition of novel antiangiogenics (and/or placebo) to a standard cytotoxic drug regimen. In some cases, trials have compared antiangiogenic monotherapy with “best supportive therapy” (i.e. no active treatment), but this is generally in patients with advanced disease who have failed treatment with established regimens.

While novel antiangiogenics have shown a great deal of promise in the treatment of advanced disease, their use is hampered by limited response rates, potentially serious side-effects (see below) and by often prohibitive costs. A detailed discussion of all antiangiogenesis therapies

is beyond the scope of this work, and the reader is directed to excellent reviews by Gasparini and by Harrison (Gasparini et al. 2005; Harrison 2012). In this section I outline some general principles of their use in cancer, and then discuss bevacizumab in greater detail (section 1.2.4.1).

An important goal for anti-angiogenic therapy is to reverse the abnormalities in permeability and network structure (Jain 2001). By pruning immature and inefficient blood vessels and eliminating excess endothelial cells, the vasculature is “normalised”, leading to increased vascular patency, improved tumour perfusion and drug delivery, and decreased tumour hypoxia (Dickson et al. 2007; Zhou et al. 2008). In rectal cancer patients treated with bevacizumab for example, a reduction in interstitial hypertension and an increase in pericyte coverage (suggesting normalisation) has been reported (Willett et al. 2004). More direct evidence of normalisation comes from a study in glioblastoma patients, in which angiogenic blockade resulted in a reduction in tumour vessel size and permeability, and a resultant reduction in tissue oedema (Batchelor et al. 2007). However, vessel normalisation is usually transient, and prolonged treatment with neutralising antibodies to VEGF or anti-VEGFR eventually leads to reduced tumour perfusion, impairing oxygenation and drug delivery (Figure 1.8), which suggests that uninterrupted treatment with such drugs may not be optimal for combination chemotherapy with cytotoxic drugs (Ma and Waxman 2008). Intermittent treatment regimes may allow for the recovery of tumour vessels in between periods of anti-angiogenic drug administration, although this introduces the risk of tumour cell recovery from the effects of the cytotoxic drugs.

Interestingly, a rapid decrease in tumour perfusion and drug delivery is seen with receptor tyrosine kinase (RTK) inhibitors (Kim et al. 2005). This may be due to vasoconstriction caused by inhibition of autocrine VEGF-mediated responses which are not seen with neutralising VEGF antibodies (Ma and Waxman 2008). Despite increasing hypoxia and reducing drug delivery, RTKIs can still improve the anti-tumour response when used in combination with cytotoxics (Troiani et al. 2007; Ma and Waxman 2009), particularly when RTKI administration follows a period of cytotoxic monotherapy. This is partly due an increase in drug retention within the tumour, enhancing drug efficacy, which opens up the possibility of treatment modalities such as intratumoural injection of cytotoxics following angiogenesis treatment (Ma et al. 2011; Wang et al. 2011b).

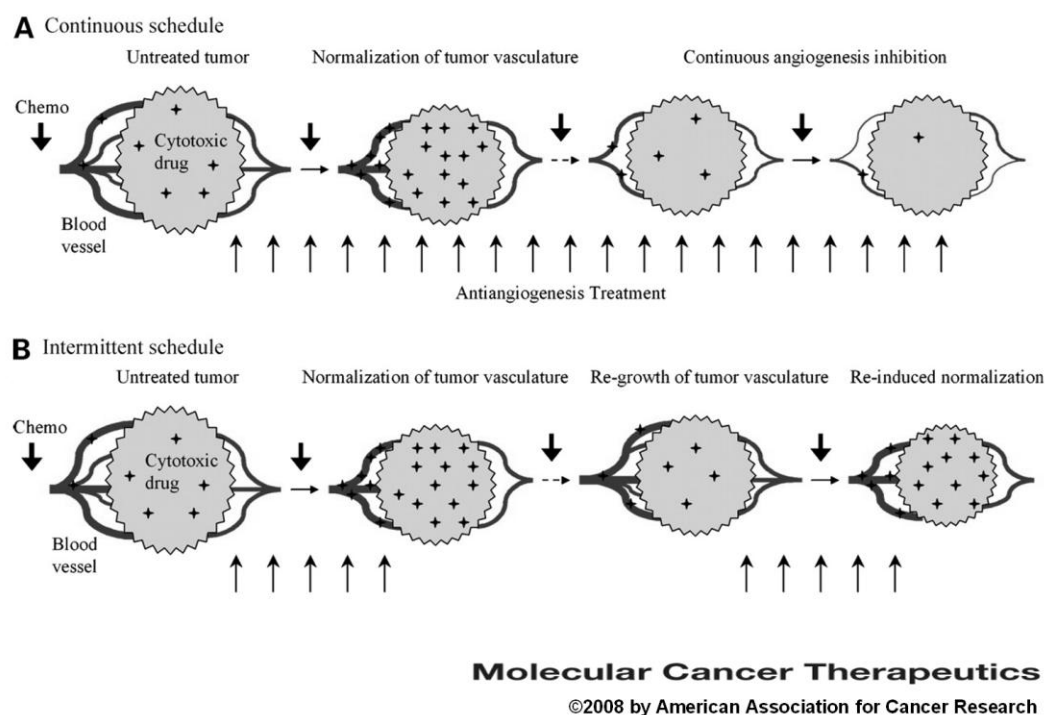


Figure 1.8 Normalisation of tumour vasculature by angiogenesis inhibition

A: Antiangiogenic drugs can induce functional normalization of tumour vasculature, transiently increasing uptake of co-administered cytotoxic drugs. However, continuous treatment ultimately decreases tumour blood flow and uptake of cytotoxics. B: intermittent antiangiogenesis treatment schedules may allow tumour vasculature recovery between drug administration cycles, improving delivery of cytotoxics. From (Ma and Waxman 2008)

1.2.4.1 Bevacizumab

As the first targeted antiangiogenesis therapy to be licensed in cancer, bevacizumab is also the best understood. Its efficacy in clinical trials provided confirmation of the pivotal role of VEGF in cancers in general and CRC in particular. There is a close correlation between VEGF expression and indices of CRC progression. Increased VEGF expression in tumour versus normal tissue was reported almost 20 years ago (Brown et al. 1993). Upregulation is seen in colonic adenomas, with a progressive increase that follows the adenoma-carcinoma sequence (Wong et al. 1999; Kondo et al. 2000). Expression correlates with MVD, a measure of angiogenesis strongly associated with depth of invasion, and lymph node and liver metastasis (Nakasaki et al. 2002). Expression of VEGF and its receptor KDR correlates with tumour proliferation, metastasis and poor prognosis (Takahashi et al. 1995; Ishigami et al. 1998; Lee et al. 2000; Harada et al. 2001).

These observations were supported by evidence from preclinical studies which demonstrated attenuation of tumour growth and metastasis *in vitro* following administration of neutralising VEGF antibodies (Kim et al. 1993; Warren et al. 1995), and the first clinical trials of

bevacizumab began in 1996. In combination with traditional chemotherapy, it was shown to improve survival in late-stage disease (Hurwitz et al. 2004; Kabbinavar et al. 2008). A number of subsequent trials have consistently reported increased response rate (RR), and prolonged progression free survival (PFS) and overall survival (OS). This is seen when used as first-line therapy in addition to the “Saltz Regimen” (irinotecan, FU and LV) (Hurwitz et al. 2004) (Kabbinavar et al. 2005a), or as second-line therapy in combination with newer cytotoxic agents such as in the FOLFOX4 regimen (oxaliplatin, FU and LV) (Giantonio et al. 2007) (Saltz et al. 2008). Two meta-analyses reported consistent improvements in RR (Welch et al. 2010), PFS and OS (Galfrascoli et al. 2010; Welch et al. 2010).

More recent trials have investigated the role of bevacizumab in less advanced disease (Stage II and III CRC), with somewhat unexpected results (Kerr and Young 2011). The NSABP C-08 trial found no benefit of bevacizumab plus FOLFOX over FOLFOX alone (Allegra et al. 2011). In the AVANT trial, the addition of bevacizumab to FOLFOX in fact resulted in a small reduction in overall survival (De Gramont et al. 2011). Interestingly, withdrawal of bevacizumab at the end of the trial period appeared to be associated with an increased rate of relapse (Kerr and Young 2011).

In the context of mCRC where median OS prior to the advent of anti-angiogenic therapy was of the order of 12 months, an improvement of several weeks to months is clinically significant, and there is evidence of improved quality of life during this period (Kabbinavar et al. 2008). Nevertheless, there are significant limitations with regard to response rates, side effects and cost. Although response rates as high as 50% have been reported (Saltz et al. 2008), there remains a significant population of patients who do not benefit from bevacizumab. It has been reported that the development of hypertension or a skin rash after initiation of bevacizumab therapy correlates with better response (Saif et al. 2008; Scartozzi et al. 2009). Similarly, an increase in levels of several circulating biomarkers has been detected in patients after treatment begins, but only one (intercellular adhesion molecule 1, ICAM-1) was found to have any predictive value (Dowlati et al. 2008). However, to date no factors have been identified that can reliably predict response to bevacizumab prior to commencement of therapy (Galfrascoli et al. 2010; Jubb and Harris 2010), and this is currently under investigation in the FOCUS-3 trial in the UK. Even in those patients who do respond to bevacizumab, the demonstrable tumour shrinkage and delayed progression seem to be transient, and are followed by tumour regrowth. It appears that as with most systemic cancer therapies, anti-angiogenic drugs are subject to the development of resistance. Of greater potential concern is the phenomenon of rebound, whereby tumours relapsing after cessation of anti-angiogenic therapy exhibit a more aggressive phenotype, with increased invasiveness and metastasis (Loges et al. 2009). This has been reported in glioblastoma and CRC (Cacheux et al. 2008; Zuniga et al. 2010). Both these clinical studies involved small

numbers, and are contradicted by a pooled analysis of phase III RCTs of bevacizumab in CRC which found no evidence of rebound (Miles et al. 2010). However, rebound has been suggested as a possible explanation for the results from the AVANT trial (see above) (Kerr and Young 2011). A more detailed understanding of the mechanisms underlying treatment failure is clearly necessary, and this is an area of much research and debate (Kerbel et al. 2001; Bergers and Hanahan 2008).

Bevacizumab therapy is associated with adverse events including hypertension, thromboembolic events, bleeding, GI perforation and proteinuria (Ferrara et al. 2005). Two studies have reported a bevacizumab-induced five-fold increase in the incidence of severe hypertension (Hurwitz et al. 2004) (Kabbinavar et al. 2005c). A meta-analysis which included 20 studies and over 12,000 patients with several cancer types found the incidence of all grades of hypertension to be 24% (Ranpura et al. 2010). Hypertension is an expected class effect of anti-angiogenic therapy, since VEGFR activation results in production of the potent vasodilator nitric oxide leading to hypotension, and VEGF infusion has been shown to reduce systolic blood pressure in angina patients (Horowitz et al. 1997; Henry et al. 2003). Bevacizumab has been reported to disrupt the microcirculation in non-tumour tissue *in vivo*, reducing capillary density and endothelial cell vasodilator-response, effects which are reversed after cessation of treatment (Mourad et al. 2008; Steeghs et al. 2010). Patients receiving bevacizumab should have their blood pressure monitored and hypertension treated with oral anti-hypertensives. As there is some evidence that development of severe hypertension is associated with improved response to anti-VEGF therapy, discontinuation of bevacizumab in these cases is controversial (Rixe et al. 2007; Scartozzi et al. 2009).

Mild, asymptomatic proteinuria has further been reported in all studies of bevacizumab (Chen and Cleck 2009). In a meta-analysis, the risk of significant proteinuria was increased by 2.52, although it remains relatively rare, with an incidence of 1.4% (Ranpura et al. 2010). Patients on bevacizumab require monitoring for proteinuria, and cessation of treatment has been recommended if urinary protein excretion exceeds 2g per 24 hours (Chen and Cleck 2009).

VEGF plays an important role in endothelial cell-platelet homeostasis, and bevacizumab has been shown to be internalised by platelets, leading to a profound reduction in platelet-derived VEGF and platelet function (Verheul et al. 2007). Disruption of the VEGF pathway may thus be expected to affect coagulation. Treatment with bevacizumab is associated with a risk of serious arterial thromboembolic events (ATE) (Scappaticci et al. 2007; Schutz et al. 2010). Previous ATE and age over 65 years are significant risk factors (Scappaticci et al. 2007), and aspirin use has a protective effect in these patients. The association between venous thromboembolism (VTE), cancer and cytotoxic chemotherapy is well documented, contributing factors being the pro-

thrombotic state associated both with surgery and with carcinomatosis itself. The evidence for VTE in the context of bevacizumab therapy is less clear cut. Scappaticci *et al* reported no effect on VTE incidence in CRC (Scappaticci *et al.* 2007). However, a meta-analysis has subsequently reported a 33% increase in high-grade (life-threatening) VTE (Nalluri *et al.* 2008). This study analysed 13 trials of bevacizumab in all cancers, and reported an overall 33% increase in all-grades of VTE (relative risk 1.33, 95% CI 1.13-1.56, $p < 0.001$).

Mucocutaneous bleeding and epistaxis are common adverse effects, reported in up to 24% of bevacizumab treated CRC patients (Chen and Cleck 2009), and an increased incidence of severe bleeding requiring transfusion, primarily of the GI tract, has been reported (Giantonio *et al.* 2007). There have also been reports of GI perforation (Hapani *et al.* 2009), and surgical complications including delayed wound healing (Hochster *et al.* 2008), wound failure (dehiscence or incisional hernias) (Allegra *et al.* 2009), and anastomotic leak or breakdown (August *et al.* 2008; Bège *et al.* 2009). Even where these complications are not life-threatening, they can have a major impact on patients' quality of life. The physiological response to injury that is necessary for healing following surgery is dependent on functional platelets and an adequate blood supply. Antiangiogenic therapy, which disrupts both, might therefore be anticipated to adversely affect surgical outcomes. It has been recommended that major surgery be avoided within six weeks of cessation of bevacizumab therapy where possible (Bose *et al.* 2010).

Thus, the efficacy of bevacizumab for mCRC first seen in clinical trials over 10 years ago is balanced by clinical data that has accumulated since, demonstrating limited response rates and significant side-effects. Its failure to increase overall survival in non-metastatic CRC was unexpected. Two recent developments are of significant note. In the US, the FDA announced its withdrawal of approval for bevacizumab in metastatic breast cancer, citing the lack of evidence of sufficient efficacy in the light of significant side-effects (FDA 2010). In the UK, the National Institute for Health and Clinical Excellence announced that bevacizumab is not recommended for metastatic CRC, stating that its efficacy does not justify the high costs (NICE 2010).

The development of bevacizumab was taken to herald a new era in anticancer therapy (Grothey 2005). Some of the initial promise has been fulfilled. Certainly, the concept of angiogenesis blockade as an effective strategy in the management of cancer is proven. As new antiangiogenic agents continue to be developed, the experience from bevacizumab illustrates the need for a deeper understanding of angiogenesis regulation and therapeutic inhibition, particularly with respect to understanding mechanisms of resistance, and the development of predictive biomarkers.

1.3 Hypoxia

Hypoxia plays a pivotal role in cancer, as well as in other diseases such as atherosclerosis and rheumatoid arthritis. The exponential rate of tumour growth typically outstrips the capacity of new blood vessels to develop and infiltrate the expanding tumour mass. The resulting inadequate supply of oxygenated blood leads to hypoxia, which has been demonstrated in cancers of the head and neck, breast, cervix, prostate, skin (melanoma) as well as the colon and rectum (Wouters et al. 2002; Goethals et al. 2006). As discussed in Section 1.2.2, tumours are restricted to 2-3mm³ in size without neo-vascularisation (Folkman and Shing 1992). However, angiogenesis in cancer results in structurally and functionally inept blood vessels (Denekamp 1990; Konerding et al. 1999). The vessel network has a haphazard architecture, and immature vessel walls are “leaky”, resulting in the accumulation of extravascular fluid. This increases interstitial pressure which impedes oxygen diffusion within the tumour microenvironment. Thus, despite the expansion in the tumour vascular network, solid tumours continue to harbour hypoxic regions. Tumour hypoxia has been confirmed indirectly using immunohistochemistry (IHC) to identify exogenously infused imidazole-based markers, or endogenous hypoxia markers such as carbonic anhydrase and other hypoxia-inducible proteins (Wouters et al. 2002; Goethals et al. 2006). Direct measurement using oxygen electrodes shows that intratumoural oxygen tensions are typically below 30 mmHg (approximately 4% O₂) in many solid tumours including CRC (Harris 2002; Goethals et al. 2006).

Tumour hypoxia is an indicator of poor prognosis. It is associated with more advanced stage at time of diagnosis in cervical cancer (Sundfør et al. 1998). In rectal cancer, Mattern *et al.* showed that intratumoural hypoxia correlates with MVD and VEGF expression (Mattern et al. 1996). Prospective studies in several cancers have shown that tumour hypoxia at time of diagnosis predicts increased likelihood of development of metastasis and poorer survival (Brizel et al. 1996; Hockel and Vaupel 2001; Wouters et al. 2002). It is also associated with increased resistance to both chemo- and radiotherapy (Gray et al. 1953; Hockel et al. 1996).

Numerous cellular processes are regulated by hypoxia in tumour cells, and the reader is directed to a comprehensive review by Harris (Harris 2002). These include cell metabolism, proliferation, migration and apoptosis, as well as angiogenesis. Cellular detection of, and adaptation to, decreased oxygen tension is primarily through the action of Hypoxia Inducible Factor (HIF) (see section 1.3.1)

Hypoxia is a highly effective stimulus to angiogenesis through potent HIF-mediated induction of VEGF and other angiogenic factors such as ANGPT-2. Angiogenic responses, albeit markedly attenuated, can also be induced by hypoxia in the absence of HIF, through induction of factors such as IL-8 as well as VEGF (Levy et al. 1996; Ryan et al. 1998; Mizukami et al. 2004;

Mizukami et al. 2005). Hypoxia upregulates VEGF gene transcription through a PI3K/Rho/ROCK-dependent pathway (Mizukami et al. 2006b). In addition to increasing VEGF transcription, hypoxia stabilises VEGF mRNA (Ikeda et al. 1995; Levy et al. 1996).

Hypoxia interferes with normal mechanisms of DNA repair leading to increased genetic instability (Bristow and Hill 2008). It stimulates secretion of MMPs and modulates expression of adhesion molecules, promoting metastasis (Esteban et al. 2006; Wu et al. 2008). In addition, it exerts a strong selection pressure on the heterogeneous population of cancer cells, promoting survival of cells demonstrating more malignant phenotypes (Hockel and Vaupel 2001; Sullivan and Graham 2007). For example, normal responses to hypoxia can lead to apoptosis, which is promoted by the activation of signalling via p53. In a heterogeneous cell population, cells with a wild-type p53 succumb to hypoxia-induced apoptosis, while those with a p53 mutation (common in cancers) are resistant (Graeber et al. 1996).

1.3.1 The HIF Pathway

Hypoxia Inducible Factors (HIFs) are a family of three transcription factors (HIF-1, 2 and 3) which are central to the complex homeostatic mechanisms involved in the cellular response to hypoxic stress. HIF-1 α is the best understood, and has been described as the “Master Regulator” of oxygen homeostasis (Semenza 1998). It has been shown to regulate 70 genes involved in angiogenesis, nutritional stress, tumour metabolism, invasion and cell death (Semenza 2002a; Lee et al. 2004; Wenger et al. 2005). HIF-2 α is less well characterised and very little is known about the role of HIF-3 α . HIF proteins are continuously degraded in normoxia, but become stabilised in hypoxic conditions (generally at below 5% O₂), with increasing activity as oxygen tension decreases (Figure 1.9) (Bracken et al. 2006). This is a consequence of regulation by four enzymes, the three prolyl hydroxylases (PHD-1, -2 and -3) and Factor Inhibiting HIF (FIH-1), all of which require oxygen as well as the cofactors 2-oxoglutarate (2-OG) and iron.

The HIFs are composed of a common constitutively-expressed β -subunit, and three distinct α -subunits (Figure 1.10). Both α and β subunits contain a DNA-binding basic helix-loop-helix motif that, in combination with the adjacent Per-ARNT-Sim (PAS) domain, cause the α - and β -subunits to dimerise. In the α -subunit, an oxygen-dependent degradation (ODD) domain contains proline residues that in normoxia are rapidly hydroxylated by PHDs, leading to proteasomal degradation mediated by binding to the von Hippel–Lindau (vHL) protein (Ivan et al. 2001; Jaakkola et al. 2001; Yu et al. 2001).

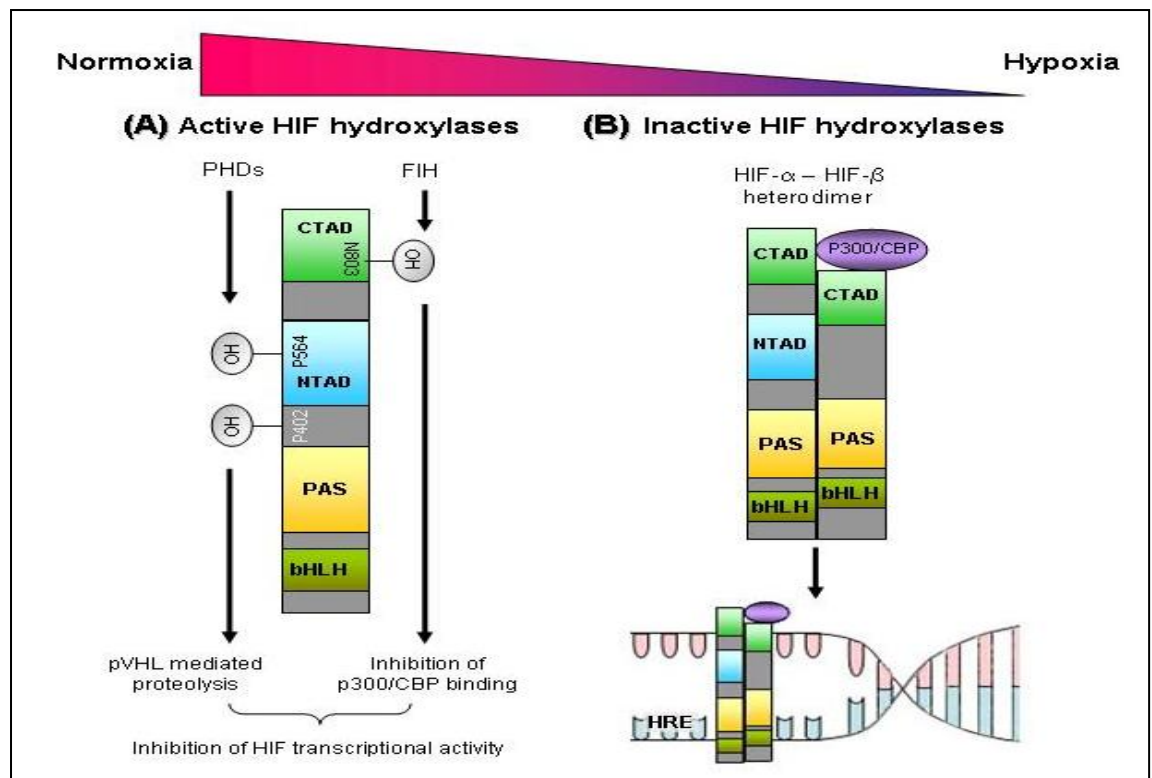


Figure 1.9 Structure and enzyme regulation of HIF-1 α

In the presence of oxygen, hydroxylases are active. Hydroxylation of proline residues P402 and P564 in HIF-1 α (P405 and P530 in HIF-2 α) by PHDs allows interaction with the von Hippel–Lindau protein (pVHL). Complexed with the ubiquitin-activating enzyme (E1), pVHL mediates the ubiquitination (Ub) of HIF1 α , marking it for proteasome degradation before dimerisation with the β -subunit can occur. FIH-1 hydroxylates the asparagine residue N803 (N815 in HIF-2 α) which inhibits binding of the transcription coactivators p300 and CREB-binding protein (CBP). In hypoxia, therefore, HIF-1 α is stabilised by dimerisation with HIF- β , and the heterodimer translocates to the nucleus where transcription of target genes progresses unhindered (Courtesy T. Khong).

vHL is a tumour suppressor gene, a germline mutation of which causes von Hippel–Lindau disease, a heritable cancer syndrome characterised by the formation of multiple, highly angiogenic tumours (Kaelin and Maher 1998). Hydroxylation of specific proline residues in the HIF- α subunit allows binding of the functional vHL gene product pVHL which initiates polyubiquitination (tagging of lysine residues with multiple copies of ubiquitin, a 76 amino-acid basic polypeptide), followed by the recruitment of the E3 ubiquitin ligase complex. This destines α -subunits to proteasomal degradation by 26S proteasome (Salceda and Caro 1997; Maxwell et al. 1999). (In vHL-defective cells such as the renal carcinoma cell-lines RCC and 786-O, HIF- α is constitutively stabilised.) FIH-1 appears to have a role secondary to that of the PHDs, exerting an additional level of negative control on HIF- α protein that has escaped degradation (Stolze et al. 2004). FIH hydroxylates an asparagine residue in the C-terminal activation domain (CTAD) of the α subunit, preventing binding to this region of two co-activators, p300 and CBP, and thus inhibiting HIF transcriptional activity (Lando et al. 2002; McNeill et al. 2002).

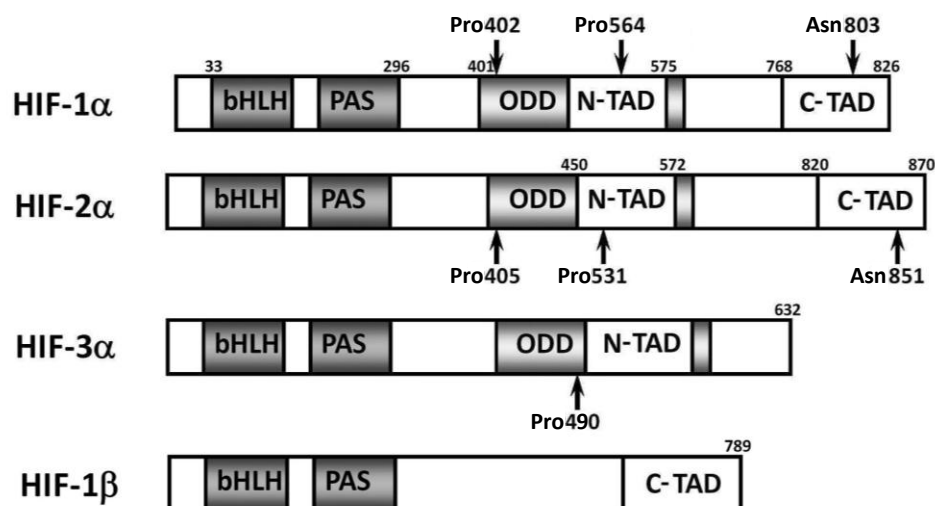


Figure 1.10 Comparative protein structure of HIF- α and β subunits

Both α and β subunits contain a basic helix-loop-helix motif (bHLH) and a Per-ARNT-Sim (PAS) domain, interaction of which allows dimerisation. The α subunits contain an oxygen-dependent degradation (ODD) domain and an N-terminal activation domain (N-TAD). In addition, HIF-1 α and HIF-2 α (but not HIF-3 α) possess a C-terminal activation domain (C-TAD). Oxygen-dependent PHD and FIH enzymes regulate HIF by hydroxylating proline (Pro) and asparagine residues (Asn) respectively (see text for details). Arrows indicate the isoform-specific position of these residues. (Adapted from Smirnova et al. 2012)

The positions of the proline residues that are modified by the PHDs differ for each HIF- α isoform (Figure 1.9). For HIF-1 α the residues are Pro402 and Pro564 (Bruick and McKnight 2001; Epstein et al. 2001; Ivan et al. 2001; Yu et al. 2001); Pro405 and Pro531 for HIF-2 α (Chan et al. 2002); and only one, Pro490, in HIF-3 α (Maynard et al. 2003). Isoform specificity is also seen for FIH, which hydroxylates asparagine residue Asn803 in HIF-1 α , and Asn851 in HIF-2 α (Lando et al. 2002). (HIF-3 α lacks a CTAD, and is therefore not regulated by FIH (Maynard et al. 2003).)

The oxygen dependence kinetics of the PHDs and FIH are such that small changes in oxygen tension cause significant changes in enzymatic activity, and are therefore rapidly transduced to changes in HIF- α protein levels (Epstein et al. 2001; Hirsila et al. 2003). Their catalytic activity falls with oxygen tension, leading to accumulation of the HIF- α subunit, which enters the nucleus to dimerise with HIF-1 β and the transcriptional co-activators CBP/p300. The co-activators remodel the chromatin structure by virtue of their histone acetyltransferase activity, and facilitate recognition by the transcriptional complex of a hypoxia response element (HRE) in the target gene promoter. This directs the expression of HIF-target genes that are central to hypoxia-responses. While this oxygen-dependent post-translational regulation of HIF is the predominant and best understood mechanism of HIF stabilisation, other enzymatic modifications

contribute to HIF regulation, such as acetylation, S-nitrosylation and phosphorylation, all of which determine HIF half-life and/or transcriptional activity (Brahimi-Horn et al. 2005).

The PHDs and FIH-1 are regulated by the availability of co-substrates, including molecular oxygen, HIF and 2-OG, and by the presence of co-factors, such as Fe^{2+} and ascorbate (Ivan et al. 2001; Hewitson et al. 2002). Hydroxylation efficiency is influenced by oxygen availability and affinity of the enzymes for oxygen. The PHDs are more sensitive to hypoxia, and have minimal activity at 1% O_2 , whereas FIH-1 remains active at oxygen tensions as low as 0.2% (Mahon et al. 2001; Lando et al. 2002; Koivunen et al. 2004; Stolze et al. 2004). The hydroxylases bind Fe^{2+} , which keeps the catalytic centre of the enzyme ready for substrate binding. Therefore Fe^{2+} -chelating molecules (iron antagonists), such as desferrioxamine (DFO), CoCl_2 , and Ni^{2+} ions, or reactive oxygen species (ROS) that oxidise Fe^{2+} to Fe^{3+} , act as PHDs inhibitors. 2-OG antagonists also inhibit PHDs and FIH. Dimethyloxalylglycine (DMOG) is one such (exogenous) antagonist, which is a commonly used experimental hypoxia-mimetic. Examples of endogenous antagonists are intermediates of the Krebs cycle (e.g. succinate, fumarate), and by-products of glycolysis (e.g. pyruvate).

Interestingly, while hypoxia decreases the enzymatic activity of PHDs, it induces their transcription and translation (Epstein et al. 2001; Berra et al. 2003; Cioffi et al. 2003; Del Peso et al. 2003; Metzen et al. 2003; Marxsen et al. 2004). Both PHD-2 (Metzen et al. 2005) and PHD-3 (Pescador et al. 2005) gene promoters possess a HRE, and in many cell types an increase in oxygen-mediated HIF- α degradation is seen in chronic hypoxia. The presence of an HRE points to PHD-2 and PHD-3 being HIF targets, and this has been shown demonstrated in a number of cells *in vitro*. PHD-2 is solely regulated by HIF-1 α in hypoxia (Berra et al. 2003; Appelhoff et al. 2004; Aprelikova et al. 2004; Marxsen et al. 2004), whereas PHD-3 is dependent on both HIF-1 α (Aprelikova et al. 2004; Marxsen et al. 2004) and HIF-2 α (Huang et al. 2002; Appelhoff et al. 2004; Aprelikova et al. 2004; Marxsen et al. 2004).

HIF Target Genes

A consensus HRE has been identified in the oxygen-dependent regions of over 70 validated HIF target genes, and microarray experiments suggest that there may be up to 200 HIF targets (Wenger et al. 2005). Four such genes are VEGF, BNIP-3, GLUT-1 and CA-IX. The importance of VEGF in angiogenesis and cancer is described elsewhere (sections 1.1.5.3, 1.3.2.1). BNIP-3 (Bcl2/adenovirus E1B 19d-interacting protein) is a pro-apoptotic gene and is described below (section 1.3.2.2). GLUT-1 (glucose transporter 1) and CA-IX (carbonic anhydrase 9) are both involved in energy metabolism, and have been used as endogenous markers of hypoxia (Airley et al. 2003; Hoskin et al. 2003).

The phenomenon of increased glucose uptake by tumour tissue was first described by Warburg in 1927 (Warburg et al. 1927). The discovery of the glucose transporter GLUT-1 in the hepatoma cell-line HepG2 provided a structural and functional basis of this increased uptake (Mueckler et al. 1985; Airley et al. 2010). GLUT-1 is induced by hypoxia, and indeed is used as an experimental marker of hypoxia (Zhang et al. 1999; Albertella et al. 2008; Chung et al. 2009). It is overexpressed in many cancers including CRC, particularly in peri-necrotic regions (Chung et al. 2009; Airley et al. 2010), and is associated with poor prognosis (Cooper et al. 2003).

The carbonic anhydrases are a family of metalloenzymes that catalyse carbon dioxide hydration. Due to its induction by hypoxia, carbonic anhydrase IX (CA-IX) is commonly used as a marker of cellular hypoxia. CA-IX is overexpressed in many cancers including CRC, and is associated with poor prognosis (Saarnio et al. 1998; Korkeila et al. 2009; McDonald et al. 2012). Tumour cell metabolism is characterised by a high rate of glucose metabolism, the deleterious effects of which are counteracted by CA-IX. It maintains a favourable intracellular pH for tumour growth and survival, as well as facilitating invasiveness by acidifying the extracellular space (Swietach et al. 2010; Parks et al. 2011). It also influences proliferation, migration and apoptosis (Svastová et al. 2003; Cianchi et al. 2010; Shin et al. 2011).

1.3.2 Role Of HIF In Cancer

Due to similarities in structure, regulation and mode of action of the two main HIF- α isoforms they were initially thought to have the same effects. There are, however, significant differences. For example, HIF-1 α is evolutionarily older and is ubiquitously expressed in organisms from *C. elegans* to humans, while HIF-2 α is only expressed in higher vertebrates and in specific tissues (Muz et al. 2009). There is increasing evidence that they play different, even opposing, roles (Seton-Rogers 2007; Loboda et al. 2012). Generally, HIF-1 α is thought to regulate genes involved in metabolism, regulating glycolysis and glucose uptake, as well as angiogenesis, while HIF-2 α has been reported to regulate genes involved in cell-proliferation and tumourigenesis (Franovic et al. 2009). It may be that *in vivo* predominance of HIF-2 α would allow uncontrolled cellular proliferation, with serious consequences in diseases associated with hypoxia, such as cancer. In addition, there is evidence that the relative roles of the two isoforms is tissue-dependent (see below) (Carroll and Ashcroft 2006).

1.3.2.1 Angiogenesis

Tissue hypoxia is a highly potent pro-angiogenic stimulus, acting predominantly through the up-regulation of VEGF, the importance of which has been discussed in Section 1.2. Hypoxia induces transcriptional activation of VEGF mRNA and stabilises VEGF mRNA (Levy et al.

1996). These effects are mediated through both HIF-dependent and HIF-independent pathways (Levy et al. 1996; Mizukami et al. 2006a). Evidence suggests that the relative roles of the HIF- α isoforms in the regulation of VEGF are tissue-specific (

Table 1.2). Interestingly, the cancers in which HIF-2 α is the predominant VEGF regulator are vHL-deficient, suggesting that loss of vHL may lead to a change of predominant isoforms.

HIF induces expression of other genes known to exert angiogenic effects, such as TGF- β_3 , nitric oxide synthase 2, haemoxygenase-1, plasminogen activator inhibitor-1 and endothelin-1 (Semenza 2001). It also promotes transformation of the ECM for sprouting and in-growth of new tumour vessels in CRC by upregulating production of ECM proteases, such as MMP-2, cathepsin D and urokinase plasminogen activator receptor (uPAR) (Krishnamachary et al. 2003). The remodelling of the ECM is also a prerequisite for tumour cell invasion and metastasis. HIF-1 α further enhances the invasive potential of malignant CRC cells by altering expression of integrins on the surface of cancer cells and production of ECM proteins (Krishnamachary et al. 2003).

Table 1.2 VEGF Regulation by HIF- α isoforms in solid tumours

In the studies listed, the relative contribution of HIF-1 α and HIF-2 α to VEGF regulation was investigated. Studies were in human tissue or cell-lines except where specified. All studies compared HIF-1 α to HIF-2 α .

Predominant Isoform		Methodology	Reference
HIF-1 α	HIF-2 α		
Breast		Gene silencing (siRNA)	(Carroll and Ashcroft 2006)
	Renal	Gene silencing (siRNA)	(Raval et al. 2005; Carroll and Ashcroft 2006)
Gastric		immunohistochemistry, Gene silencing (siRNA)	(Song et al. 2009)
Colon		immunohistochemistry	(Imamura et al. 2009)
	Liver (mouse)	Gene knockout	(Rankin et al. 2008)

1.3.2.2 Apoptosis

The term “apoptosis” describes a coordinated program of cell “suicide” by which multicellular organisms can eliminate damaged, infected or redundant cells (Kerr et al. 1972). It is essential for normal development, tissue homeostasis, and defence against pathogens. Evasion of apoptosis is one of the hallmarks of cancer (Hanahan and Weinberg 2011), and hypoxia drives metastasis in part by selection of apoptosis-resistant cells (Graeber et al. 1996; Hockel and Vaupel

2001; Sullivan and Graham 2007). HIF-1 α has been shown to both induce and to prevent hypoxia-induced apoptosis (Carmeliet et al. 1998; Akakura et al. 2001). It stabilises the tumour suppressor p53, and upregulates BNIP-3 in a p53-dependent manner (Fei et al. 2004; Greijer and van der Wall 2004). HIF-2 α is reportedly not involved in hypoxia-induced apoptosis, although it is involved in hypoglycaemia-induced apoptosis (Brusselmans et al. 2001).

The first mammalian regulator of apoptosis to be identified was the anti-apoptotic Bcl-2 (Tsujimoto et al. 1984), and 15 members of the Bcl-2 family have subsequently been identified. Those most closely related to Bcl-2 promote survival by the inhibiting ability of proteases (caspases) to dismantle the cell. Those more distantly related (e.g. BNIP-3, BAX) promote apoptosis through mechanisms which include interference with the activity of anti-apoptotic (Adams and Cory 1998). Opposing members can heterodimerise (e.g. Bcl-2 and BAX) (Oltvai et al. 1993), and appear to titrate each other's function, so that whether activation of apoptosis is dependent on their relative concentrations. HIF-1 α -dependent upregulation of bcl-2 has been reported in embryonic stem-cells (Carmeliet et al. 1998). Interestingly, in breast cancer and melanoma bcl-2 has also been shown to synergise with hypoxia in upregulating VEGF via a HIF-1 α -mediated pathway (Trisciuglio et al. 2010).

BNIP-3 is upregulated in tumour tissue of several cancers including CRC, and is associated with more aggressive disease (Koukourakis et al. 2006; Burton and Gibson 2009). Interestingly, in CRC tumour samples, it has been reported that BNIP-3 expression may be either up- or downregulated (Bacon et al. 2006). HIF-1 α dependent upregulation of BNIP-3 by hypoxia is well-established (Sowter et al. 2001; Greijer and van der Wall 2004), and has been reported in several cancer cell-lines including CRC (Sowter et al. 2001; Bacon et al. 2006). However, not all CRC cell-lines upregulate BNIP-3 in response to hypoxia, and Bacon et al. reported that those which did not were resistant to hypoxia-induced cell death. Preventing hypoxia-induction of BNIP-3 may therefore be a mechanism by which tumours can evade apoptosis. In addition, a direct pro-survival role in the context of hypoxia has been postulated for BNIP-3, through the promotion of autophagy which can prolong survival in the context of defective apoptosis (Degenhardt et al. 2006; Mathew et al. 2007; Bellot et al. 2009).

Survivin, a member of the Inhibitor of Apoptosis Protein (IAP) family, negatively regulates apoptosis. It has been shown to be upregulated in most tumours and in CRC is associated with poorer prognosis (Abd El-Hameed 2005; Sah et al. 2006). In the CRC cell-lines LS174T and SW480, HIF-1 α has been reported to upregulate survivin expression at both mRNA and protein level (Fan et al. 2008; Wu et al. 2010). XIAP (X-linked inhibitor of apoptosis protein), another member of the IAP family, is overexpressed in CRC tissue (Krajewska et al. 2005). It has been shown to protect against hypoxia-induced apoptosis in cholangiocarcinoma

which is particularly resistant to hypoxia, but little is known about its role in CRC (Marienfeld et al. 2004).

1.3.2.3 Cell Adhesion

A critical process in the progression of cancer from *in-situ* through invasive to metastatic disease is the loss of cancer cell adhesion to neighbouring cells and surrounding extracellular matrix, migration from primary to distant sites, and re-establishment of new cell-cell and cell-matrix adhesion at the target organ (Paschos et al. 2009). There is some evidence that hypoxia influences these events, although the mechanisms are poorly understood. It may be expected that hypoxia, which promotes metastasis, would downregulate tumour cell expression of cell-adhesion molecules (CAMs), and this has indeed been reported. For example, E-cadherin, a major component of epithelial cell-cell junctions, has been shown to be downregulated by hypoxia in ovarian and renal cancer (Imai et al. 2003; Esteban et al. 2006), but there is no clear evidence of this in CRC. However, CD151, a transmembrane protein involved in cell-adhesion and migration, is reduced in primary CRC tumour compared to adjacent normal tissue (Chien et al. 2008). The same study reported CD151 downregulation by hypoxia in three CRC cell-lines (SW480, SW620 and HCT116), leading to a reduction in the strength of cell-cell and cell-matrix adhesions.

Vascular-endothelial cadherin (VE-Cadherin; Cadherin 5 (CDH5)) is normally expressed by endothelial rather than epithelial cells. However, epithelial expression of VE-Cadherin is seen in cancers exhibiting “vasculogenic mimicry” (VM), in which tumour cells form capillary-like structures and matrix-rich patterned networks in three-dimensional cultures, imitating the embryonic vasculogenic network (Maniotis et al. 1999). VM has been described in CRC and found to correlate with poor prognosis (Baeten et al. 2009). In a preliminary PCR Array experiment performed in our laboratory prior to the commencement of my study, VE-Cadherin was found to be downregulated by DMOG in Caco-2 cells (T. Khong, unpublished data).

In the same PCR Array experiment, downregulation of Stabilin-1 (STAB-1) was also seen following DMOG stimulation. STAB-1 is a large transmembrane protein that was first described in endothelial cells of hepatic sinusoids. Its multifunctionality is reflected in its numerous aliases which include CLEVER-1 (common lymphatic endothelial and vascular endothelial receptor-1) and FEEL-1 (Fasciclin, EGF-like, laminin-type EGF-like and link domain-containing scavenger receptor 1). It has been implicated in a number of roles related to scavenging, lymphocyte migration, cell-adhesion and angiogenesis (Salmi et al. 2004; Karikoski et al. 2009). Given the versatility of STAB-1 function it is likely that its role is context- and/or cell-specific, and its role in cancer is poorly understood.

1.3.2.4 Inflammation

Virchow first described a tumour-associated inflammatory infiltrate 150 years ago. Macrophages, which can represent up to 50% of tumour mass (Solinas et al. 2009), constitute a highly heterogeneous population, originating from blood monocytes and differentiating into M1 (classically activated) or M2 (alternatively activated) phenotypes. Tumour-associated macrophages (TAMs) which have an M2 phenotype, have been implicated in connecting the innate immune system, chronic inflammation and tumourigenesis (Solinas et al. 2009). Other myeloid-related cells associated with tumours are Tie-2 macrophages and myeloid-derived suppressor cells (MDSCs).

TAMs predominantly infiltrate poorly vascularised, hypoxic regions within solid tumours (Leek and Harris 2002), and enhance angiogenesis, invasion and metastasis through secretion of growth factors and cytokines including VEGF (Condeelis and Pollard 2006; Jedinak et al. 2010). High HIF-2 α expression in TAMs correlates with high tumour grade and poor prognosis in a variety of cancers (Talks et al. 2000). Hypoxia has been reported to induce both HIF-1 α and HIF-2 α in TAMs (Imtiyaz et al. 2010). In mice lacking HIF-2 α in myeloid cells, experimentally-induced hepatic and colonic tumours showed reduced TAM migration and infiltration into tumour tissue, resulting in reduced tumour cell proliferation and progression.

1.3.3 HIFs In CRC

Strong evidence for the critical role played by HIF in CRC is provided by immunohistochemical examination of tumour tissue, and *in vitro* studies. CRC tissue samples consistently coexpress elevated levels of HIF-1 α and VEGF, and these correlate with MVD, local and lymphovascular invasion, and liver metastasis (Kuwai et al. 2003; Simiantonaki et al. 2008). In addition, HIF-1 α activity is induced by genetic mutations that commonly occur in CRC, such as the K-ras oncogene, and the tumour suppressor genes p53, vHL, and PTEN (Ravi et al. 2000; Kuwai et al. 2004; Giles et al. 2006; Greijer et al. 2008; Pencreach et al. 2009). Furthermore, HIF-1 α overexpression promotes tumour growth and angiogenesis in CRC xenograft models (Ravi et al. 2000; Dang et al. 2006).

The relative roles of HIF- α isoforms in CRC have not been extensively studied, and there is controversy in the few published studies in the literature (Table 1.3). Based on immunohistochemical staining in primary rectal carcinoma, Rasheed *et al* have reported that increased expression of both isoforms, but that only HIF-1 α is related to increased vascular invasion, more advanced tumour stage and poorer prognosis (Rasheed et al. 2009). Imamura *et al* report stronger VEGF upregulation and increased MVD with overexpression of HIF-1 α than with HIF-2 α (Imamura et al. 2009). Conversely, both Yoshimura *et al* and Cleven *et al.* found poor prognosis to correlate with increased HIF-2 α staining, but not HIF-1 α (Yoshimura et al. 2004;

Cleven *et al.* 2007). Yoshimura *et al.* also reported HIF-2 α but not HIF-1 α expression to correlate closely with increased MVD.

Table 1.3 Relative Roles of HIF- α isoforms in CRC

Different authors report contradictory roles for HIF-1 α and HIF-2 α in CRC in both *in vivo* and *in vitro* studies. (NB: * Dang *et al.* investigated HIF-1 α only.)

	Predominant Isoform		Methodology	
	HIF-1 α	HIF-2 α		Cell-line
Advanced stage, poor prognosis	Rasheed <i>et al.</i>	Yoshimura <i>et al.</i>	immunohistochemistry	
Angiogenesis	Imamura <i>et al.</i> (VEGF)	Yoshimura <i>et al.</i> (MVD)	immunohistochemistry	
Cell Proliferation	Imamura <i>et al.</i>		gene silencing (siRNA)	SW480
	(Dang <i>et al.</i>)*	Franovic <i>et al.</i>	gene silencing (siRNA)	HCT116

The picture remains unclear with *in vitro* studies. Using gene-silencing in the CRC cell-line SW480 (primary adenocarcinoma, Dukes' B; (Leibovitz *et al.* 1976)), Imamura *et al.* report that HIF-1 α promotes tumour growth whereas HIF-2 α suppresses it. Data from Dang *et al.* supports the pro-proliferative role of HIF-1 α using a different CRC cell-line (HCT116, primary adenocarcinoma; (Brattain *et al.* 1981)), although in their study no comparison was made with HIF-2 α (Dang *et al.* 2006). In contrast, Franovic *et al.* report that in HCT116 cells (and a variety of other cancers), HIF-2 α promotes growth. HIF-2 α knockdown significantly impaired cell proliferation and tumourigenesis, whereas HIF-1 α knockdown had no effect (Franovic *et al.* 2009).

While CRC is generally considered to include rectal as well as colon tumours, there are significant differences between proximal and distal tumours. For example, distant metastases are more common with proximal tumours, and local invasion with distal tumours; prognosis is poorer with proximal tumours (Meguid *et al.* 2008; Benedix *et al.* 2010). While anatomical location in itself contributes to these differences (e.g. distal rectal tumours growing in the relatively confined pelvic space become symptomatic earlier than proximal intra-abdominal tumours), there is evidence that the cellular biology also differs (Fusunyan *et al.* 1998; Bleeker *et al.* 2000). The published literature is often unclear with regard to the anatomical site of the cancers included in the study. For example, while Rasheed *et al.* included only rectal cancers, Imamura *et al.* do not specify the tumour site. Yoshimura *et al.* distinguish between right (proximal) and left (distal)

tumours, noting that the latter stain more strongly for HIF-2 α , but do not state whether rectal cancers are included, or if MVD and prognosis varies independently with anatomical site. (The anatomical site of origin of CRC cell-lines is more difficult to ascertain, many of them having been derived in the 1970s.) This lack of clarity may contribute to the lack of consistency in the data.

1.4 Study Rationale

The potential for HIF inhibition as a therapeutic strategy in CRC has been recognised for some time (Semenza 2002b). There is evidence that HIF-induction enhances resistance to chemotherapy (including novel biologicals) (Rohwer et al. 2010; Zhao et al. 2010) and radiotherapy (Moeller et al. 2007). HIF induction is likely to be a feature of response to antiangiogenic therapy, since effective disruption of tumour blood supply would result in hypoxia, and HIF inhibition may improve the efficacy of antiangiogenic drugs, particularly in tumours overexpressing HIF (Semenza 2012). HIF inhibition is an “off-target” effect of several anti-cancer drugs (e.g. mTOR inhibitors such as temsirolimus; topoisomerase inhibitors such as topotecan) (Semenza 2007). In some cases the inhibition is known to be HIF-1 α specific (Semenza 2012), while for others (including some used in CRC) the effect on HIF-2 α is unknown. Interestingly, a reciprocal relationship between the isoforms, in which inhibition of one leads to induction of the other, has been reported in breast and renal cancer (Raval et al. 2005; Carroll and Ashcroft 2006). If both isoforms have independently deleterious effects, this raises the possibility that selective inhibition of one isoform may in fact be undesirable. Certainly there is evidence that simultaneous inhibition of both isoforms can be beneficial (Burkitt et al. 2009), and drugs that inhibit both isoforms are under development (Bohonowych et al. 2011; Chen and Sang 2011). Despite the evidence that the two isoforms have distinct functions, some publications discussing HIF inhibition continue to consider anti-HIF-1 α activity alone (Wang et al. 2011a; Xia et al. 2012).

Evidently, a deeper understanding of the relative roles of the isoforms (which the evidence suggests are tissue- and disease specific) is required in order to understand the potential consequences of selective HIF-isoform inhibition. This, in addition to the controversy with respect to isoform roles in CRC, points to an unmet need that this study seeks to address.

The main aim of the study was to elucidate the relative roles of HIF-1 α and HIF-2 α in the hypoxia-regulation of genes relevant to CRC pathogenesis in the CRC cell-line Caco-2. Prior to investigation of the role of the HIF isoforms, characterisation of the hypoxia responses of Caco-2 was necessary (Chapter 3). Several genes were selected on the basis of relevance to cancer pathogenesis, and existing evidence of hypoxia-regulation in cancer. In addition to well-described HIF-target genes (VEGF, BNIP-3, GLUT-1 and CA-IX), genes involved in apoptosis (Bcl-2, Survivin, XIAP) and cell adhesion (VE-Cadherin, CD151, STAB-1) were investigated. Given the importance of angiogenesis in the hypoxia-driven pathogenesis of cancer, the study focussed on genes involved in angiogenesis. A PCR Angiogenesis Array was used to identify hypoxia-regulated genes, which were validated by Q-PCR. Following characterisation of Caco-2 responses to hypoxia, the role of the HIF isoforms was investigated by selective knockdown using short interfering RNA (siRNA) (Chapter 4).

The use of an immortalised cell-line such as Caco-2 has several advantages, including the ability to conduct carefully controlled experiments repeatedly, obtaining consistent results, at relatively low cost. However CRC is a highly heterogeneous disease, in which even within a single tumour, a high degree of genotypic and phenotypic diversity is seen (Morel et al. 2008). This is in stark contrast to the clonogenic homogeneity of cell-lines which in itself confers the advantages described above. Cell-lines are therefore far from representative of tumours *in vivo*, and there are significant limitations in translating cell-line-derived data to the clinic or bedside. The use of more than one cell-line in a study can partially address this issue. An alternative that more effectively closes the gap between *in vitro* and *in vivo* milieus is to use cell-lines derived *de novo*, directly from primary tumours. However, this introduces significant technical challenges. Our lab has experience with techniques in which *ex vivo* cultures of cells are derived from fresh tissue (atherosclerotic plaques, rheumatoid joints) (Brennan et al. 1989b; Monaco et al. 2004). Indeed a previous study has succeeded in establishing short-term cultures from CRC tissue which were successfully used in cytokine-stimulation experiments (T. Khong, unpublished data). Therefore, rather than study several CRC cell-lines, this study attempted to develop this technique in order to establish a tumour-derived culture (TDC) model, and to investigate hypoxia-responses in terms of angiogenesis genes, comparing them to Caco-2 responses (Chapter 5).

1.4.1 Objectives

1. To characterise the hypoxia response of Caco-2 cells, in terms of known HIF targets, genes involved in apoptosis and cell-adhesion, and with a particular focus on angiogenesis genes.

(Chapter 3)

2. To investigate the relative contribution of HIF-1 α and HIF-2 α isoforms in this hypoxia response.

(Chapter 4)

3. To establish primary cultures of cells derived directly from CRC tissue and investigate their hypoxia-induced angiogenesis responses, comparing them to those of Caco-2 cells.

(Chapter 5)

1.4.2 Null Hypotheses

1. HIF-1 α and HIF-2 α have identical roles in the hypoxia-regulation of Caco-2 cells.
2. There is no similarity between hypoxia-induced angiogenesis gene expression in TDCs compared to Caco-2 cells.

Chapter 2

2 MATERIALS AND METHODS

2.1 Caco-2 Cells

The Caco-2 cell-line used in the study was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). It is an adherent cell-line (Figure 2.1) that was originally isolated from a primary colonic tumour in a 72-year-old Caucasian male, and expresses characteristics of enterocyte differentiation upon reaching confluence *in vitro* (Fogh et al. 1977). It is widely used to assess intestinal transport of drugs through a cell monolayer absorption model. Tumourigenicity of the cell-line is demonstrated by the development of moderately-well differentiated adenocarcinoma following inoculation into nude mice, although low “take” rates have been reported, limiting its use in xenograft models (de Bruijne et al. 1993; Liu et al. 2007).

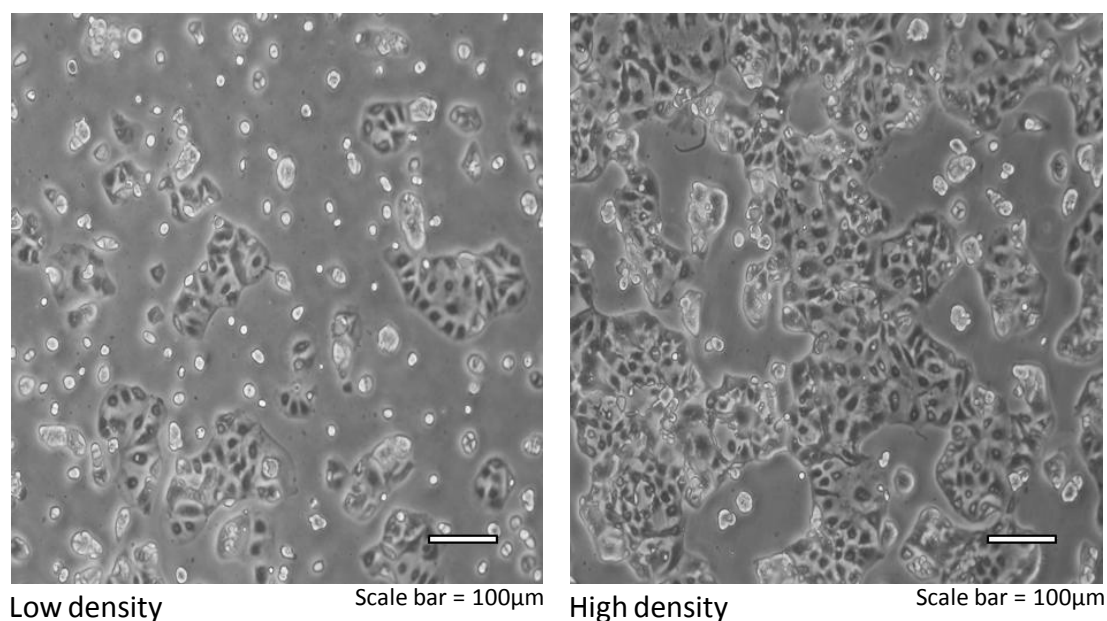


Figure 2.1 Photomicrograph of Caco-2 cells

Figure shows adherent Caco-2 cells at low (left) and high (right) density (confluence).

2.1.1 Cell Culture

The cells were thawed on receipt according to supplier's instructions in the Microbiological Safety Cabinet (Napflow 1200, Napco, Thermo Scientific, Waltham, MA, USA). The thawed cells were resuspended in 10mL of culture medium and plated in a 75cm² flask (Falcon™ T75, Becton Dickinson Labware, Bedford, MA, USA). With the lid loosened to allow free exchange of gases, the cells were incubated in a humidified atmosphere at 37°C in 21% O₂ and 5% CO₂ (Napco 6500 Incubator, Napco, Thermo Scientific, Waltham, MA, USA). The base medium used was Eagle's Minimum Essential Medium (EMEM) (Biowhittaker, Lonza,

Switzerland) containing non-essential amino acids (NEAA) and sodium pyruvate. The culture medium was completed by supplementation with 10% Foetal Bovine Serum (FBS; Biowest, Nuaille, France), 2mM Glutamine, and 1.1 U/mL of streptomycin (100 U/mL) and 1.1 µg/mL penicillin (PAA Laboratories GmbH, Pasching). This is referred to as “complete culture medium” hereafter. Medium was changed every 2 days.

Caco-2 cells undergo enterocytic differentiation upon reaching full confluence, therefore cells were passaged at 80% confluence. Cells were trypsinised with 4 mL of 0.25% Trypsin/EDTA to release the cells into suspension, with the duration of trypsinisation varying from 3 to 10 minutes depending on the passage number and confluence of the cell culture. The proteolytic activity of trypsin was neutralised by the addition of 10 mL of complete culture medium (with 10% FBS). An 80% confluent cell culture within a T75 flask yielded 1×10^7 cells. Cells were generally passaged every 5 days to a subcultivation ratio of 1:3.

In order to maintain the stock of Caco-2 cells, at the first passage after thawing frozen cells, one third of the cells yielded was re-frozen in liquid nitrogen (freezing medium was 10% DMSO in FBS). Passage number can influence protein expression and functional characteristics of cell-lines (Wenger et al. 2004; Hughes et al. 2007). This has been reported in several studies of Caco-2 (Briske-Anderson et al. 1997; Yu et al. 1997; Sambuy et al. 2005). During early experiments I observed that with each passage it took longer for cells to detach when trypsinised. Significantly greater time was required by passage 6 or 7, although further passages remained possible. Indeed studies investigating the effect of passage number on cell-line function generally compared passage numbers below 30 (“low”) and above 80 (“high”). Given these relatively high numbers, it was felt that potential variability in my results would adequately minimised by discarding cells after 5 passages. To ensure this, flasks were routinely labelled with passage number, but passage numbers were not recorded for data analysis purposes.

Loss of adhesion in adherent epithelial cells can occur in response to infection or exposure to toxins (Zodl et al. 2003; Minnaard et al. 2004). It is a feature of epithelial cell apoptosis (Frisch and Francis 1994), and detachment has been shown to precede and induce apoptosis in Caco-2 cells (Giovannini et al. 1999; Kozlova et al. 2001). Cells were therefore discarded if significant detachment was seen.

2.1.2 Stimulation

Caco-2 cells were seeded in 960mm² trays with a seeding cell population of approximately 2×10^5 per well (density approximately 2×10^4 cells per cm²). The cell cultures were then allowed to settle for 48 hours in complete culture medium prior to commencement of experiments. The medium was changed prior to stimulation with DMOG or hypoxia. Cells were

incubated for 24 hours before removal of supernatants and, following cell lysis in the appropriate buffer, cells were harvested and extraction of protein and/or RNA was performed (see below).

The Galaxy 'R' carbon dioxide incubator (Wolf, York, England) allows levels of oxygen, carbon dioxide and nitrogen to be varied independently. In these experiments, carbon dioxide saturation was kept constant at 5%, while the oxygen saturation was initially varied between 1% and 10%. Care was taken to minimise re-oxygenation of cells during harvesting of cells. Since the unstable HIF-1 α protein has a half-life of 5 minutes, the time interval between transferring cells out of the incubator to time of cell lysis was kept to a minimum. As well as being oxygen-dependent, the HIF regulating enzymes (PHDs and FIH-1) also require 2-OG as substrate. Dimethyloxallylglycine (DMOG) is a competitive 2-OG analogue, and is therefore commonly used as a hypoxia-mimetic. DMOG (Biomol, Plymouth Meeting, PA, USA) was initially used at concentrations of 0.5 and 1 mM.

2.1.3 Transfection

Gene-silencing using RNA interference is an invaluable method of studying the role of a specific gene. One available technique is the use of short interfering RNAs (siRNAs). These are short (21-23nt) anti-sense RNA sequences which, on binding the target mRNA, lead to its cleavage. They are highly specific, with a single nucleotide mis-match often being sufficient to prevent cleavage of the target mRNA (Tuschl 2001). Knockdown of the target genes is achieved by lipid-mediated transfection. A lipophilic transfection agent (Lipofectamine or Oligofectamine) is mixed with siRNA in aqueous medium Opti-MEM Reduced Serum Medium (all three reagents from Invitrogen). After a brief period of incubation, the lipids form liposomes containing siRNA. When these are added to the cells, they merge with phospholipid bilayer of the cell membrane, releasing the siRNA into the cell.

Caco-2 cells were seeded in 48cm² plates at a density of 1.5×10^5 cells/cm². 50% confluence was achieved after 48 hours, at which time transfection was performed. Since serum and antibiotics reduce the efficiency of transfection, the medium was changed to serum- and antibiotic-free medium at the start of transfection. Prior to formation of the liposome complexes, the transfection agent was diluted in Opti-MEM (1:50 dilution for lipofectamine, 1:7.5 for oligofectamine). The siRNA was diluted in Opti-MEM separately. After 5 minutes the two were mixed and incubated for 20 minutes at room temperature to allow complex formation. This was then added to the cells gently to ensure that the complexes were not disrupted. Cells were then incubated for 6 hours at 37°C, after which antibiotics and FBS were added to the final concentrations of complete culture medium. This protocol was developed following optimisation experiments in which variations of transfection agent dilution, siRNA concentration, cell confluence and serum starvation were used (see Chapter 4 Results). Hypoxia and/or DMOG

stimulation experiments were commenced 24 hours after the transfection period was complete. siRNA sequences were purchased from MWG (Heidelberg, Germany) and details are shown in Table 2.1. siLuc from Dharmacon (Thermo Fisher Scientific, Cramlington, UK) was used as an irrelevant control.

Table 2.1 siRNA Sequences

A list of siRNA sense sequences. siRNA were purchased from MWG (Heidelberg, Germany), apart from siLuc purchased from Dharmacon (Thermo Fisher Scientific, Cramlington, UK)

siRNA	Sense Sequence
siHIF-1 α	5' – [agcaguaggaauggaacauu] _{RNA} [tt] _{DNA} 3'
siHIF-2 α	5' – [gcgacagcuggaguaugaaau] _{RNA} [tt] _{DNA} -3'
siFIH-1	5' [caguugcgcaguuauagcuuc] _{RNA} [tt] _{DNA} 3'
siLuc	5' – [cguacgcggaaucucga] _{RNA} [tt] _{DNA} 3'

2.2 Primary Colorectal Cancer Cells

Ethical approval was granted for the project (Local Ethics Research Committee Ref: 07/Q0407/6). All patients included were attending Charing Cross Hospital for bowel resection as part of their treatment for CRC. The tissue used for this study was obtained from the resection specimen at the time of operation, with no additional procedure required for the patient, with written, informed consent obtained pre-operatively. Demographic and pathological data were obtained from medical records and histopathology reports.

Immediately after surgical resection of the specimen comprising of tumour with adequate margins of normal tissue, a small piece of tissue (approximately 1cm³) was taken from the edge of the tumour. This was placed in a sample pot containing 20mL of transport medium (RPMI 1640 containing amphotericin 2.5 μ g/mL, gentamicin 100 μ g/mL. Penicillin 200U/mL and streptomycin 200 μ g/mL (PAA Laboratories GmbH, Pasching, Germany)) and transferred to the laboratory (or stored at 4°C where this was not possible immediately). Tissue was washed by repeatedly agitating in 10mL of fresh transport medium 5 times, and then mechanically digested using surgical forceps, a disposable scalpel and scissors, taking care to discard first any necrotic areas, connective tissue or blood clots. The tissue was then subjected to enzymatic digestion in Collagenase A (Roche, Mannheim, Germany; 1mg/mL in RPMI) for 1 hour at 37°C. The resulting cell suspension was diluted with Hank's Buffered Salt Solution (HBSS; Biowhittaker) and sieved through a nylon filter (70 μ m pore size; BD Biosciences, Franklin Lakes, USA) to remove debris and disaggregate the cells. The resulting cell suspension was then subjected to a

differential density-gradient separation. It was carefully layered onto an equal volume of Histopaque®-1077 (Sigma-Aldrich, St Louis, MO, US) in a 15mL conical tube (Falcon), and centrifuged at 400G for 30 minutes. Cells at the interface between Histopaque and HBSS were extracted and washed by further centrifugation (150G, 10 minutes) in HBSS. The resulting pellet was resuspended in 1mL primary cell culture medium (transport medium supplemented with 20% FBS) and the cells counted by the trypan-blue exclusion method. Cells were plated on duplicate 600mm² tissue culture plates at a density of 5×10^4 cells/cm² (in duplicate or triplicate wells where cell yield allowed), and incubated at 37°C. They were allowed to settle for 1 hour before commencing hypoxia experiments, with one plate being transferred to the hypoxia incubator (1% O₂). After 24 hours supernatants were removed for protein analysis by ELISA, and RNA extracted as for Caco-2 cells. These cultures are referred to as tumour-derived cultures (TDCs) hereafter.

There was considerable morphological heterogeneity seen in the TDCs (Figure 5.1, Figure 5.2). Since the only “purifying” step (density gradient separation) was designed to reduce red cell numbers, it was anticipated that the methodology would yield populations of mixed cells. In addition to CRC tumour cells, these might include fibroblasts, tumour-associated macrophages and other immune cells (Franks 1976; Park et al. 2004). In order to confirm the presence of colonic epithelial cells, expression of epithelial cell markers Ep-CAM and VE-Cadherin was evaluated by Q-PCR (section 2.3.3), and the CRC tumour marker carcinoembryonic antigen (CEA) protein by ELISA (section 2.4.1). Due to technical and time restraints, more detailed characterisation (or further purification) of the TDCs was not performed in this study (see Future Work, section 6.2.2).

2.3 Analysis of gene expression by Polymerase Chain Reaction (PCR)

2.3.1 RNA Extraction

Caco-2 cells remained firmly adherent throughout the experiment, while TDCs were partly in suspension. Initial steps for cell harvest were different for each cell-type. For Caco-2 cells the supernatant was removed and stored at -20°C for protein analysis. Each well was washed with 1mL of cold (4°C) phosphate buffered saline (PBS (Merck)) before the addition of 250µL RNA-Bee™ (AMS Biotechnology, Abingdon, U.K). (As RNABee contains phenol which is toxic and volatile, the rest of the extraction was performed in a fume cupboard.) Cells were scraped from the bottom of the well with a cell scraper, and the cell/RNABee mixture was transferred to separate 1.5mL Eppendorf tubes.

For the TDCs, from each well the medium containing suspended cells was transferred to separate 2mL Eppendorfs. 250 μ L of RNABee was added to the adherent cells in each well. The suspended cells were centrifuged for 3 minutes at 1500rpm, at the end of which the pellet was preserved, and the supernatant was removed and stored at -20°C for protein analysis. The adherent cells were scraped from the bottom of each well, and the resulting cell/RNABee mixture from each well was used to dissolve the corresponding pellet from the centrifuged cells that had been in suspension, before transfer to a clean 1.5mL Eppendorf. Particular care was taken to ensure that adherent and suspended cells from the same well were mixed together. Therefore, the sample for each well contained RNA from both the adherent and the suspended cells. Extraction then proceeded in the same way for both Caco-2 cells and TDCs.

50 μ L of chloroform was added and the sample vortexed for 30-60 seconds, and kept on ice for 5 minutes. The samples were then centrifuged at 12,000G at 4°C for 15mins, resulting in a biphasic mixture. The top, clear aqueous layer containing RNA (approximately 100 μ L) was transferred to a fresh Eppendorf, and 125 μ L isopropan-2-ol added to precipitate the RNA. After gentle agitation of the tubes to ensure thorough mixing, the sample was left at room temp for 10 minutes, after which centrifugation (12,000G, 4°C, 5 minutes) revealed a cream/yellow-coloured pellet of RNA. (Where RNA concentration is low, samples require longer to precipitate, and in practice samples were usually stored in isopropan-2-ol at -80°C overnight before proceeding to centrifugation). The isopropan-2-ol was discarded taking care not to lose the pellet which was then washed with 500 μ L of 70% ethanol. After further centrifugation (7,500G, 4°C, 5 minutes) the ethanol was discarded leaving the RNA pellet which was then allowed to air-dry (the ethanol is more volatile than isopropan-2-ol, speeding up the air-drying process) before being dissolved in 30 μ L of RNase-free water (treated with diethylpyrocarbonate (DEPC)).

In order to remove DNA contamination, RNA was treated with DNase (TurboDNase™, Ambion, Applied Biosystems, Warrington, UK). For each 30 μ L RNA sample, a reaction mix of RNasin (1 μ L), TurboDNase (2 μ L) and buffer (4 μ L) was added. After 30 minutes incubation at 37°C, a further 1 μ L TurboDNase was added. Following incubation for another 30 minutes, DNase inactivation agent (8 μ L) was added, before centrifugation for 2 minutes at 10,000g. The aqueous phase was then transferred to a fresh Eppendorf, and the pellet containing DNA contaminant was discarded.

RNA concentration and purity was then measured by spectrophotometry (Nanodrop ND 1000, Thermo Fisher Scientific, Cramlington, UK). Absorbance (Abs) was measured at 260nm and 280nm wavelengths, and RNA concentration and purity were calculated using the following formulae:

$$\text{RNA concentration } (\mu\text{g/mL}) = \text{Abs}_{260} \times 40 \times \text{dilution factor} *$$

$$\text{RNA purity} = \text{Abs}_{260} / \text{Abs}_{280}$$

(*40 is a conversion factor for RNA.)

The ratio of absorbance at 260nm and 280nm provides an estimation of contaminants that absorb the ultraviolet light, such as protein. The RNA sample was considered pure when the ratio was between 1.8 and 2.

2.3.2 *cDNA Synthesis*

Direct amplification of RNA is limited due to the inability of the Taq polymerase to recognise RNA as a template. To overcome this limitation the retroviral reverse transcriptase enzyme M-MLV reverse transcriptase from the Moloney murine leukaemia virus (Roth et al. 1985) was used to produce a complementary strand of DNA (cDNA) from a RNA template. The reaction mixture comprises of an RNA template, an oligoDT primer, triphosphate deoxynucleotides, reverse transcriptase enzyme and a reaction medium.

Following RNA quantitation by spectrophotometry, the volume of RNA to be included in the reaction was determined in order to achieve a total quantity of 500ng of RNA. DEPC water was then added to the RNA sample to achieve a total volume of 11 μL and 1 μL of OligoDT primer was added. The specimen was incubated in the PCR machine at 70°C for 10 minutes to unravel any secondary structures within the template and then rapidly cooled for 5 minutes to prevent these secondary structures from reforming. A prepared reaction mix comprising of the reagents listed in Table 2.2 was then added to each specimen. The M-MLV reverse transcriptase, RNase H Minus, Point Mutant (M-MLV RT (H-)) was obtained from Promega (Promega, Madison, USA). The specimen was then returned to the PCR machine and incubated at 21°C for 10 minutes to allow annealing between primer and RNA template. The temperature was subsequently raised to 42°C and maintained for an hour to provide optimal conditions for the reverse transcriptase enzyme to catalyse cDNA synthesis. At the end of the reaction, the enzyme was inactivated by heating the specimen for 15 minutes at 70°C. The final product was diluted in double distilled water and stored at -20°C.

Table 2.2 Reverse Transcription Reagents and Volumes

Components of RT-PCR master mix. 8 μ L was added to each sample. RNasin (Ribonuclease Inhibitor to preserve RNA integrity); dNTPs (deoxyribonucleotide triphosphates) including a mixture of dATP, dGTP, dCTP, dTTP; M-MLV Reverse Transcriptase (all purchased from Promega, Southampton, UK). DEPC (diethylpyrocarbonate).

Reagents	Volume (μ L)
DEPC water	1.5
dNTP	1.0
M-MLV enzyme	0.5
RNasin	1.0
Buffer	4.0

2.3.3 Quantitative PCR (Q-PCR)

PCR allows amplification of a short, well defined part of a DNA strand which acts as a template. In order to amplify a specific gene of interest, primers consisting of short artificial strands of DNA designed to complement the beginning and end of the DNA fragment is used. Sequences for the primers used are listed in Table 2.3.

Table 2.3 Sequences of primers used in gene expression experiments and expected product sizes.

Primer	Primer sequence		Gene Product Size (bp)
	Forward	Reverse	
HIF-1 α	cacctctggactgcctttc	ggctgcatctcgagactttt	194
HIF-2 α	ccttcaagacaaggtctgca	ttcatccgtttccacatcaa	443
VEGF	cttgcttgctgctctacct	ctgcatggatgatgttgact	282
BNIP-3	ctgctgctctctcatttgct	accccaggatctaacagctc	168
GLUT-1	tggcatggcgggttgt	ccagggtagctgctccagc	63
ANGPTL-4	ccactgggaccaggatcac	cggaagtactggccgttgag	115
CA-IX	gctgtcaccagggtggggt	ccagtctcggctacctgtct	100
Survivin	agccagatgacgacccata	caagggttaattcttcaaactgctt	92
BAX	ctgacggcaacttcaactgg	tcttgatccagcccaacag	159
XIAP	caatatggagactcagcagttgga	gcactattttcaagataaaagccgtt	85
Bcl-2	tcgccctgtggatgactga	cagagacagccaggagaaatca	133
PHD-2	gcacgacaccgggaagtt	ccagctcccgttacagt	178
PHD-3	ttgggatgccaagctaca	cgtgtgggttctctacgatct	125
FIH-1	caatgtactggtggcatcacatag	ggccactttctgatgagctt	131

Ep-CAM	cgcagctcaggaagaatgtg	tgaagtacactggcattgacg	87
CD151	gctggagatcatcgctggtatc	ggtaggtagcgctggtcat	100
VE-Cadherin	gccaggtatgagatcggtgt	gtgtcttcaggcagcagacaaa	151
STAB-1	actcttcgtccctgtcaatg	tcactgatgatgaggctgag	155
18S	gtaacccgttgaaccccat	ccatccaatcggtagtagcg	152
β -actin	cccagagcaagagagg	gtccagacgcaggatg	364
TBP	tgctccagaatatgcctct	caatggttttcaagctttcca	203

The PCR reaction requires a mixture of specific concentrations of deoxynucleotide triphosphates (dNTPs), PCR buffer and polymerase enzyme (all of which are contained in SYBR Green Jumpstart™ Taq ReadyMix™ (Sigma, St Louis, MO, USA) in appropriate proportions), in addition to forward and reverse primers, purified water, and the cDNA template (Table 2.4).

Table 2.4 Constituents of Q-PCR reaction mix

Reagent	Concentration (μ M)	Volume (μ L)
SYBR Green Jumpstart™ Taq ReadyMix™	2×	7.5
Primer (Forward & Reverse)	6.25	0.6
Template DNA	1:5 dilution with H ₂ O	6.0
Purified water		0.9

The Rotor-Gene 6000, a 72 closed-tube centrifugal thermal cycler (Corbett Research, Mortlake, Sydney, Australia) was used to amplify, detect and quantify the gene products. Each sample was analysed in triplicate. The Rotor-Gene was programmed to perform 40 amplification cycles, each comprising of 95°C for 10 seconds (DNA denaturation), followed by 60°C for 15 seconds (annealing) and finally 72°C for 20 seconds (extension).

SYBR Green, used as the detection agent, emits minimal fluorescence in its unbound state. During the PCR reaction, increasing amounts are bound within the newly-synthesised double-stranded DNA causing an increase in fluorescence emission which is detected and recorded in real-time. The number of amplification cycles required to reach an arbitrarily chosen level (threshold) of fluorescence emission (termed the C_t value) is directly proportional to the starting amount of the target cDNA. Relative quantification, or the comparative C_t method, was used to determine changes in the steady-state transcription of a gene following stimulation. To minimise errors, for example due to inefficiencies in reverse transcription, C_t values for the target

gene in each sample were normalised to the ubiquitously expressed “housekeeping gene” 18S (the small sub-unit of ribosomal RNA) in the same sample, using the equation:

$$\Delta C_t \text{ TARGET} = \Delta C_t \text{ TARGET} - \Delta C_t 18S$$

The C_t value for the target gene in the stimulated sample is then compared to that in the control or calibrator sample. The relative expression (fold-change) is given by $2^{-\Delta\Delta C_t}$ where:

$$\Delta\Delta C_t = \Delta C_t \text{ TARGET} - \Delta C_t \text{ CALIBRATOR}$$

Down-regulation of gene expression may be expressed in terms of fold-change, in which case it is a number between zero and 1, or fold-regulation which allows downregulation to be expressed as (the negative of) a number greater than one, simplifying comparisons of up- and downregulation. Fold-regulation is calculated as follows:

$$\text{Fold regulation} = -1/(\text{fold-change})^\dagger$$

[†] (where fold-change is less than 1)

For example, a fold-change of 0.5 equals a fold-regulation of -2, or a 2-fold downregulation. (Where genes are upregulated, fold-regulation is taken to mean the same as fold-change, and the above calculation is not performed.)

2.3.4 PCR Array

The PCR array system enables analysis of the expression of a focused panel of genes. Each PCR array profiles simultaneously a collection of disease- or pathway-specific genes, generating single, gene-specific amplicons and preventing the co-amplification of non-specific products.

To profile the expression of 84 genes involved in angiogenesis, the Human Angiogenesis RT² Profiler™ PCR Array (SABiosciences, West Sussex, UK) was used. A list of the genes in the array is shown in Table 2.5. The genes include growth factors and their receptors, chemokines and cytokines, matrix molecules (proteases and their inhibitors), adhesion molecules and transcription factors. cDNA prepared as described in 2.3.2 was mixed with nuclease-free water and RT² PCR SYBR® Green Master Mix (SABiosciences), and aliquoted to duplicate 96-well plates coated with a set of optimised primers. The reaction was performed using the ABI Prism 7700 Sequence Detector and the real-time PCR cycling programme (Software Detection System 1.9.1) from Applied Biosystems (Foster City, CA, USA). The arrays were initially heated to 95°C for 10 minutes to activate the polymerase, and this was followed by 40 repeated cycles comprising of 15

seconds at 95°C for melting, 40 seconds for annealing at 55°C and 20 seconds for extension at 78°C. C_t values were obtained using the pre-installed ABI Prism® 7700 Sequence Detection System software by setting the threshold at the exponential phase of gene amplification. Relative gene expression was calculated by $2^{-\Delta\Delta C_t}$ comparative method using PCR array data analysis software provided by the manufacturer. Since one of the five housekeeping genes on the Array, GAPDH (glyceraldehyde-3-phosphate dehydrogenase) is known to be hypoxia regulated, gene expression was normalised to the other four (B2M: Beta-2-microglobulin, HPRT1: Hypoxanthine phosphoribosyltransferase 1, RPL13A: Ribosomal protein L13a, ACTB: Actin beta). The relative levels of genomic DNA contamination were evaluated by the built-in RNA quality controls.

Table 2.5 Genes in the Human Angiogenesis RT² Profiler™ PCR Array

A list of the 84 Genes included in the Angiogenesis RT² Profiler™ PCR Array (Catalogue PAHS-024A).

Refseq	Symbol	Gname	Description	Function
NM_005163	AKT1	AKT/MGC99656/PKB/PKB-ALPHA/PRKBA/RAC/RAC-ALPHA	V-akt murine thymoma viral oncogene homolog 1	Transcription factor
NM_001146	ANGPT1	AGP1/AGPT/ANG1	Angiopoietin 1	Vascular remodelling
NM_001147	ANGPT2	AGPT2/ANG2	Angiopoietin 2	Vascular remodelling
NM_014495	ANGPTL3	ANGPT5/FHBL2	Angiopoietin-like 3	Adhesion molecule
NM_001039667	ANGPTL4	ANGPTL2/ARP4/FI AF/HFARP/NL2/PGAR/pp1158	Angiopoietin-like 4	Cellular metabolism & vascular remodelling
NM_001150	ANPEP	APN/CD13/GP150/LAP1/P150/PEPN	Alanyl (membrane) aminopeptidase	Extracellular matrix remodelling
NM_001702	BAI1	FLJ41988/GDAIF	Brain-specific angiogenesis inhibitor 1	Adhesion molecule
NM_002986	CCL11	MGC22554/SCYA11	Chemokine (C-C motif) ligand 11	Chemokine
NM_002982	CCL2	GDCF-2/HC11/HSMCR30/MCAF/MCP-1/MCP1/MGC9434/SCYA2/SMC-CF	Chemokine (C-C motif) ligand 2	Chemokine
NM_001795	CDH5	7B4/CD144/FLJ17376	Cadherin 5, type 2 (vascular endothelium), VE-Cadherin	Adhesion molecule
NM_030582	COL18A1	FLJ27325/FLJ34914/KNO/KNO1/KS/MGC74745	Collagen, type XVIII, alpha 1	Adhesion molecule
NM_000091	COL4A3	-	Collagen, type IV, alpha 3 (Goodpasture antigen)	Adhesion molecule
NM_001511	CXCL1	FSP/GRO1/GROa/MGSA/MGSA-a/NAP-3/SCYB1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	Chemokine
NM_001565	CXCL10	C7/IFI10/INP10/IP-10/SCYB10/crg-2/gIP-10/mob-1	Chemokine (C-X-C motif) ligand 10	Chemokine

NM_002090	CXCL3	CINC-2b/GRO3/GROg/MI P-2b/MIP2B/SCYB3	Chemokine (C-X-C motif) ligand 3	Chemokine
NM_002994	CXCL5	ENA-78/SCYB5	Chemokine (C-X-C motif) ligand 5	Chemokine
NM_002993	CXCL6	CKA-3/GCP-2/GCP2/SCYB6	Chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	Chemokine
NM_002416	CXCL9	CMK/Humig/MIG/S CYB9/crg-10	Chemokine (C-X-C motif) ligand 9	Chemokine
NM_001953	TYMP	ECGF/ECGF1/MED PS1/MNGIE/MTDP S1/PDECGF/TP/hP D-ECGF	Thymidine phosphorylase	Growth Factor
NM_001400	S1PR1	CHEDG1/D1S3362/ ECGF1/EDG-1/EDG1/FLJ58121/S 1P1	Sphingosine-1-phosphate receptor 1	Adhesion molecule
NM_182685	EFNA1	B61/ECKLG/EFL1/ EPLG1/LERK1/TN FAIP4	Ephrin-A1	Growth factor
NM_004952	EFNA3	EFL2/EPLG3/Ehk1- L/LERK3	Ephrin-A3	Growth factor
NM_004093	EFNB2	EPLG5/HTKL/Htk- L/LERK5/MGC126 226/MGC126227/M GC126228	Ephrin-B2	Growth factor
NM_001963	EGF	HOMG4/URG	Epidermal growth factor	Growth factor
NM_000118	ENG	CD105/END/FLJ41 744/HHT1/ORW/O RW1	Endoglin	Adhesion molecule
NM_004444	EPHB4	HTK/MYK1/TYRO 11	EPH receptor B4	Growth factor receptor
NM_001432	EREG	ER	Epiregulin	Growth factor
NM_000800	FGF1	AFGF/ECGF/ECGF - beta/ECGFA/ECGF B/FGF- alpha/FGFA/GLIO7 03/HBGF1	Fibroblast growth factor 1 (acidic)	Growth factor
NM_002006	FGF2	BFGF/FGFB/HBGF -2	Fibroblast growth factor 2 (basic)	Growth factor
NM_000142	FGFR3	ACH/CD333/CEK2/ HSFGFR3EX/JTK4	Fibroblast growth factor receptor 3	Growth factor receptor
NM_004469	FIGF	VEGF-D/VEGFD	C-fos induced growth factor (vascular endothelial growth factor D)	Growth factor
NM_002019	FLT1	FLT/VEGFR1	Fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)	Growth factor receptor
NM_021973	HAND2	DHAND2/FLJ16260 /Hed/MGC125303/ MGC125304/Thing2 /bHLHa26/dHand	Heart and neural crest derivatives expressed 2	Transcription factor
NM_000601	HGF	DFNB39/F- TCF/HGFB/HPTA/S	Hepatocyte growth factor (hepapoietin A; scatter	Growth factor

		F	factor)	
NM_001530	HIF1A	HIF-1alpha/HIF-1/HIF-1-ALPHA/MOP1/PAS D8/bHLHe78	Hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	Transcription factor
NM_006665	HPSE	HPA/HPA1/HPR1/H PSE1/HSE1	Heparanase	Transcription factor
NM_002165	ID1	ID/bHLHb24	Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	Transcription factor
NM_002167	ID3	HEIR-1/bHLHb25	Inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	Transcription factor
NM_024013	IFNA1	IFL/IFN/IFN-ALPHA/IFN-alphaD/IFNA13/IFN A@/MGC138207/M GC138505/MGC138 507	Interferon, alpha 1	Cytokine
NM_002176	IFNB1	IFB/IFF/IFNB/MGC 96956	Interferon, beta 1, fibroblast	Cytokine
NM_000619	IFNG	IFG/IFI	Interferon, gamma	Cytokine
NM_000618	IGF1	IGF-I/IGF1A/IGFI	Insulin-like growth factor 1 (somatomedin C)	Growth factor
NM_000576	IL1B	IL-1/IL1-BETA/IL1F2	Interleukin 1, beta	Cytokine
NM_000600	IL6	BSF2/HGF/HSF/IF NB2/IL-6	Interleukin 6 (interferon, beta 2)	Cytokine
NM_000584	IL8	CXCL8/GCP-1/GCP1/LECT/LUC T/LYNAP/MDNCF/MONAP/NAF/NAP-1/NAP1	Interleukin 8	Adhesion molecule
NM_002210	ITGAV	CD51/DKFZp686A0 8142/MSK8/VNRA	Integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)	Adhesion molecule
NM_000212	ITGB3	CD61/GP3A/GPIIIa	Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	Adhesion molecule
NM_000214	JAG1	AGS/AHD/AWS/C D339/HJ1/JAGL1/M GC104644	Jagged 1	Cell differentiation
NM_002253	KDR	CD309/FLK1/VEGF R/VEGFR2	Kinase insert domain receptor (a type III receptor tyrosine kinase)	Growth factor receptor
NM_005560	LAMA5	KIAA1907	Laminin, alpha 5	Matrix
NM_007015	LECT1	BRICD3/CHM-I/CHM1/MYETS1	Leukocyte cell derived chemotaxin 1	Chemokine
NM_000230	LEP	FLJ94114/OB/OBS	Leptin	Cytokine
NM_002391	MDK	FLJ27379/MK/NEG F2	Midkine (neurite growth-promoting factor 2)	Growth factor
NM_004530	MMP2	CLG4/CLG4A/MM P-II/MONA/TBE-1	Matrix metalloproteinase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)	Matrix
NM_004994	MMP9	CLG4B/GELB/MA NDP2/MMP-9	Matrix metalloproteinase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	Matrix

NM_004557	NOTCH4	FLJ16302/INT3/MG C74442/NOTCH3	Notch 4	Transcription factor
NM_003873	NRP1	BDCA4/CD304/DK FZp686A03134/DK FZp781F1414/NP1/ NRP/VEGF165R	Neuropilin 1	Angiogenesis receptor
NM_003872	NRP2	MGC126574/NP2/N PN2/PRO2714/VEG F165R2	Neuropilin 2	Angiogenesis receptor
NM_002607	PDGFA	PDGF-A/PDGF1	Platelet-derived growth factor alpha polypeptide	Growth factor
NM_000442	PECAM1	CD31/FLJ34100/FL J58394/PECAM-1	Platelet/endothelial cell adhesion molecule	Adhesion molecule
NM_002619	PF4	CXCL4/MGC13829 8/SCYB4	Platelet factor 4	Chemokine
NM_002632	PGF	D12S1900/PGFL/PL GF/PlGF-2/SHGC- 10760	Placental growth factor	Growth factor
NM_002658	PLAU	ATF/UPA/URK/u- PA	Plasminogen activator, urokinase	Matrix
NM_000301	PLG	DKFZp779M0222	Plasminogen	Growth factor
NM_020405	PLXDC1	DKFZp686F0937/F LJ36270/FLJ45632/ TEM3/TEM7	Plexin domain containing 1	Angiogenesis
NM_021935	PROK2	BV8/KAL4/MIT1/P K2	Prokineticin 2	Growth factor
NM_000962	PTGS1	COX1/COX3/PCOX 1/PGG/HS/PGHS- 1/PGHS1/PHS1/PT GHS	Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	Transcription factor
NM_002615	SERPINF1	EPC-1/PEDF	Serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1	Growth factor
NM_021972	SPHK1	SPHK	Sphingosine kinase 1	Transcription factor
NM_015136	STAB1	CLEVER-1/FEEL- 1/FELE- 1/FEX1/KIAA0246/ STAB-1	Stabilin 1	Endosome trafficking
NM_000459	TEK	CD202B/TIE- 2/TIE2/VMCM/VM CM1	TEK tyrosine kinase, endothelial	Growth factor receptor
NM_003236	TGFA	TFGA	Transforming growth factor, alpha	Growth factor
NM_000660	TGFB1	CED/DPD1/LAP/T GFB/TGFbeta	Transforming growth factor, beta 1	Growth factor
NM_003238	TGFB2	MGC116892/TGF- beta2	Transforming growth factor, beta 2	Growth factor
NM_004612	TGFBR1	AAT5/ACVRLK4/A LK- 5/ALK5/LDS1A/LD S2A/SKR4/TGFR-1	Transforming growth factor, beta receptor 1	Growth factor receptor
NM_003246	THBS1	THBS/THBS- 1/TSP/TSP-1/TSP1	Thrombospondin 1	Adhesion molecule
NM_003247	THBS2	TSP2	Thrombospondin 2	Adhesion molecule
NM_003254	TIMP1	CLGI/EPA/EPO/FLJ	TIMP metalloproteinase	Matrix

		90373/HCI/TIMP	inhibitor 1	
NM_003255	TIMP2	CSC-21K	TIMP metalloproteinase inhibitor 2	Matrix
NM_000362	TIMP3	HSMRK222/K222/K222TA2/SFD	TIMP metalloproteinase inhibitor 3	Matrix
NM_000594	TNF	DIF/TNF-alpha/TNFA/TNFSF 2	Tumour necrosis factor	Cytokine
NM_006291	TNFAIP2	B94/EXOC3L3	Tumour necrosis factor, alpha-induced protein 2	Cytokine
NM_003376	VEGFA	MGC70609/MVCD 1/VEGF/VPF	Vascular endothelial growth factor A	Growth factor
NM_005429	VEGFC	Flt4-L/VRP	Vascular endothelial growth factor C	Growth factor
NM_004048	B2M	-	Beta-2-microglobulin	Housekeeping
NM_000194	HPRT1	HGPRT/HPRT	Hypoxanthine phosphoribosyltransferase 1	Housekeeping
NM_012423	RPL13A	L13A/TSTA1	Ribosomal protein L13a	Housekeeping
NM_002046	GAPDH	G3PD/GAPD/MGC 88685	Glyceraldehyde-3-phosphate dehydrogenase	Housekeeping
NM_001101	ACTB	PS1TP5BP1	Actin, beta	Housekeeping

2.4 Analysis of protein expression

Quantification was performed by Enzyme-linked Immunoassay (secreted protein, in supernatants), and by Western Blot (intra-cellular protein from lysed cells).

2.4.1 ELISA

At the end of each experiment, supernatants were collected and stored at -20°C. Extraction of intra-cellular protein was performed as detailed below (Section 2.4.2.1). Supernatants were then thawed at room temperature before measurement of proteins of interest using the ELISA sandwich technique. In brief, a capturing antibody to the antigen of interest is bound to a polystyrene well in a 96-well plate, and the protein to be measured is then added. An immune complex consisting of capturing antibody and protein is then recognised by detecting antibody conjugated to biotin with a strong affinity for streptavidin-conjugated to horseradish peroxidase enzyme (HRP). A colourimetric reaction with a stable substrate (e.g. tetramethylbenzidine (TMB)) enables visualisation of the captured protein by measuring using spectrophotometer.

VEGF

The wells of a 96-well polystyrene microtitre plate (NUNC, Roskilde, Denmark) was coated with 100µL of mouse anti-human capture antibody (R&D Systems, Minneapolis, USA) diluted to a working concentration of 1µg/mL in 1% PBS and left to incubate overnight at 4°C.

The wells were emptied and after 3 washes with PBS/Tween (0.01% solution), the plate was blocked with 250µL of 2% bovine serum albumin (BSA) to reduce non-specific protein binding, and left to agitate at room temperature for 2 hours. The plate was washed before 100µL of sample and standards were pipetted into the wells, and a seven point standard curve was generated using 3-fold serial dilutions from the highest standard of 10000pg/mL. The plate was left to incubate overnight at 4°C to optimise capture, and unbound antigens were subsequently washed away with wash buffer. 100µL of biotinylated goat antibody specific for VEGF (R&D Systems, Minneapolis, USA) diluted to a concentration of 200ng/mL was added to each well and incubated for 2 hours at room temperature. The detection antibody binds specifically to the primary antigen-antibody complex and subsequent addition of 100µL of diluted streptavidin-horseradish peroxidase (HRP) (R&D Systems, Minneapolis, USA) reagent results in stable, high affinity binding between the biotin and streptavidin components. HRP catalyses a colorimetric change of a colourless substrate solution of hydrogen peroxide and TMB (R&D Systems, Minneapolis, USA) to turquoise-blue. When the lowest standard concentration changed colour, sulphuric acid (R&D Systems, Minneapolis, USA) was added to each well to halt the chromogenic reaction, resulting in a change of colour from turquoise blue to bright yellow. Since the colour formed is proportional to the amount of antigen in the test solution, the optical density of each well was determined using a microplate reader set to a wavelength of 450nm and the protein concentrations were determined using software from Ascent (Version 2.6, Thermo Labsystems).

Carcinoembryonic Antigen (CEA)

Measurement of CEA was performed using an ELISA kit from BQ Kits (San Diego, USA) comprising of a total of 96 microtitre wells (in 12 strips of 8) pre-coated with monoclonal anti-CEA antibody. In this solid-phase ELISA, the test sample is allowed to react simultaneously with the primary antibody coating the wells and a soluble secondary HRP-conjugated antibody. The kit contains 5 pre-diluted CEA standards (2-fold dilutions from a maximum concentration of 50ng/ml), enzyme conjugate reagent (goat anti-CEA antibody conjugated to HRP), TMB reagent and stop solution (hydrochloric acid). Having placed the desired number of pre-coated well strips into the 96-well holder, 50µL of samples (diluted in the appropriate culture medium where necessary) and standards were added. 100µL of enzyme conjugate was then added, ensuring thorough mixing (1200 rpm on rotating platform for 30 seconds). Following incubation for 60 minutes at room temperature, the incubation mixture was discarded, and the wells washed with distilled water. 100µL/well of TMB solution was added, and the plate was incubated at room temperature for 20 minutes or until the lowest standard had changed from colourless to blue (whichever was sooner). The reaction was stopped with 100µL/well stop solution. Absorbance was read at 450nm, and protein concentrations were determined using Ascent software.

2.4.2 Western Blotting

2.4.2.1 Protein Extraction and Measurement

At the end of each experiment, once the supernatant had been removed, cells were lysed with 100 μ L of protein lysis buffer, the components of which are detailed in Table 2.6. After scraping adherent cells from the well, the sample was transferred to a 2mL conical Eppendorf. The sample at this stage is very viscous, and requires homogenisation. This was performed using a hand-held homogeniser (IKA-Ultra-Turrax® T8 homogeniser, IKA®-Werke GMBH & CO. KG, Staufen, Germany). A Bicinchoninic assay (BCA) (Pierce, Rockford, USA) was performed to quantify the total protein in each sample. This was necessary to ensure equal total protein loading for Western Blot analysis. The BCA test was performed in a 96-well polystyrene plate (NUNC) and duplicates of 10 μ L volumes of each standard or unknown sample were pipetted into the wells. 200 μ L of the working reagent, comprising of a 50:1 mixture of BCA Reagent A:B were added to individual wells and reagents were mixed on a plate agitator for 30 seconds. The plate was covered and incubated at 37°C for 30 minutes and then cooled to room temperature prior to absorbance measurement at 540nm using a plate reader. The concentration of protein within individual wells was determined by Ascent software (Version 2.6, Thermo Labsystems) by referring to the 7-point standard curve.

Table 2.6 Constituents of protein lysis buffer

*Sodium dodecyl sulphate (SDS); **Dithiothreitol (DTT); ***Protease inhibitor cocktail P-8340 (Sigma, St Louis, MO, USA) which comprises of 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), pepstatin A, E-64, bestatin, leupeptin, and aprotinin; [†]PMSF: phenylmethylsulfonyl fluoride.

Reagent	Concentration/ μ M	Volume
Urea	10M	7.79mL
Glycerol		1mL
SDS*	10%	1mL
TRIS pH 6.8	1M	100 μ L
DTT**	1M	10 μ L
Protease Inhibitor Cocktail ***	50mM	100 μ L
PMSF [†]	100mM	50 μ L
Total		10mL

2.4.2.2 Protein Separation, Transfer and Detection

Gel electrophoresis was performed using the NuPAGE Novex System (Invitrogen, Carlsbad, CA, USA) of pre-casted Bis-Tris-HCl buffered polyacrylamide (4-12%) gels to separate the denatured proteins according to molecular weight, with a protein “ladder”, consisting of a mixture of proteins with defined molecular weights, used to calibrate the gel. To ensure loading of equal amounts of protein (30-40µg), lysates were diluted with the appropriate volume of distilled H₂O. Lanes were each loaded with 25µL of sample (denatured at 95°C for 5 minutes) plus 5µL of loading buffer. The gel was then immersed within an electrophoresis tub containing running buffer (1g/L SDS (Merck, AnalaR, Germany), 3g/L Tris base (Merck) and 14.4g/L Glycine (Merck)) and a current of was applied across the gel for 1 hour (140V). The proteins, now separated according to size, were blotted onto a polyvinylidene difluoride (PVDF) membrane (Merck). The gel and membrane were sandwiched together between blotting paper and sponge layers held in a cassette and placed in transfer buffer (10mM Tris base, 100mM glycine, 10% methanol, 0.005% SDS). Electroelution of the protein from the gel onto the membrane was performed by application of a current at 25V overnight followed by 100V for 90 minutes (at 4°C).

To reduce non-specific protein binding, the membrane was soaked in blocking buffer (1% PBS, 5% fat-free milk powder (Premier Brands, UK), 1% BSA (Sigma, St Louis, MO, USA) and 0.1% Tween (Sigma, St Louis, MO, USA) for 1 hour on a rotating platform. The membrane was then incubated a primary antibody directed at the target protein, followed by a biotinylated secondary antibody. The blot was developed with ECL-Plus chemiluminescence detection kit (GE Healthcare Lifesciences, Piscataway, NJ, USA) and exposed to photographic film. α -tubulin, a structural protein, was used as a control to ensure equal total protein loading. Details of the antibodies used are shown in Table 2.7.

Table 2.7 Antibodies used for Western Blotting

Abbreviations: 1°Ab – primary antibody, 2°Ab – secondary antibody, Mo – monoclonal, Po – polyclonal, RAM – rabbit-anti mouse, SAR - swine anti-rabbit antibody (Dako, Glostrup, Denmark).

1°Ab	Company	1°Ab dilution	2°Ab	2°Ab dilution	Size (kDa)
MoAb anti-HIF-1 α	Becton Dickinson, Oxford, UK	1:250	RAM	1:3000	120
MoAb anti- HIF-2 α	Santa Cruz Biotechnology, Heidelberg, Germany	1:250	RAM	1:3000	115
PoAb anti-FIH-1	Abcam, Cambridge, UK	1:1000	SAR	1:5000	40.6
PoAb anti-PHD-2	Abcam, Cambridge, UK	1:1000	SAR	1:5000	46
MoAb anti-PHD-3	Millipore, Billerica, USA	1:25	RAM	1:3000	27

MoAb anti-BNIP-3	Abcam, Cambridge, UK	1:1000	RAM	1:4000	30
PoAb anti-GLUT-1	Alpha Diagnostic Intl, San Antonio, USA	1:750	SAR	1:3000	40 - 80
PoAb anti-CA-IX	Abcam, Cambridge, UK	1:1000	SAR	1:3000	54, 58
MoAb anti- α-tubulin	Sigma-Aldrich, Poole, UK	1:3000	RAM	1:3000	50

2.5 Statistical analysis

Graph Pad Prism 5.04 software package (Graph Pad Software, La Jolla, CA, USA) was used in the analyses of the data. To evaluate the effects of the stimuli (hypoxia and DMOG) and siRNA transfection on gene expression, analysis of variance (ANOVA), followed by a Bonferroni's Multiple Comparison Test was used to compare more than two groups, or a student *t*-test if two groups were analysed. Results are expressed as the mean $\pm$ standard error of the mean (SEM) or standard deviation (SD) as indicated. *P* values less than 0.05 were considered significant (**p* < 0.05, ***p* < 0.01, ****p* $\leq$ 0.001).

Chapter 3

3 CHARACTERISATION OF CACO-2 CELL RESPONSES TO HYPOXIA

3.1 Introduction

The Caco-2 cell-line was originally isolated from a male patient with colorectal adenocarcinoma, and it is widely used as an *in vitro* model of CRC (Fogh et al. 1977). While it has been reported to have low malignant potential in comparison with other cell-lines, invasive behaviour has been demonstrated in certain conditions (Caro et al. 1995; Kermorgant et al. 2001). Furthermore, subcutaneous injection of Caco-2 cells leads to tumour formation in SCID mice (Oikonomou et al. 2007). Tumourigenicity of the cell-line is demonstrated by the development of moderately-well differentiated adenocarcinoma following inoculation into nude mice, although low “take” rates have been reported, limiting its use in xenograft models (de Bruïne et al. 1993; Liu et al. 2007). Other adherent CRC cell-lines such as SW480, SW620 and HT-29 are also derived from adenocarcinoma, and have been reported to exhibit more aggressive behaviour than Caco-2 cells (Both et al. 1999).

In common with approximately 50% of colorectal tumours, Caco-2 cells have a mutant p53 oncogene, which is known to be associated with increased VEGF production (Olofsson et al. 1998; Liu and Bodmer 2006). Accordingly, the cell-line is known to express VEGF, which is upregulated by a number of stimuli including hypoxia (Morote-Garcia et al. 2008; Gentile et al. 2011). p53 has an important role in the regulation of apoptosis through its interaction with BNIP-3 (BCL2/adenovirus E1B 19kDa interacting protein 3), a member of the Bcl-2 family. HIF-1 α has been shown to stabilise p53, and hypoxia upregulates BNIP-3 in a p53-dependent manner in various cell-lines (Fei et al. 2004; Greijer and van der Wall 2004). Two other important oncogenes in CRC are K-ras and BRAF, mutations of which are present in 45% and 15% of colorectal tumours respectively (Kikuchi et al. 2009). Both oncogenes can modulate the HIF pathway. K-ras mutation has been shown to enhance HIF-1 α protein synthesis, while BRAF mutation enhances both HIF-1 α and HIF-2 α synthesis. Both mutations are associated with resistance to the anti-EGFR antibody cetuximab and with poorer prognosis. Caco-2 cells contain the wild-type of both oncogenes (Brink et al. 2003; Kikuchi et al. 2009), and have been reported to express both HIF-1 α and HIF-2 α , although weak HIF-2 α expression (with minimal induction by hypoxia) has been reported by some groups (Mizukami et al. 2004; Bruning et al. 2011).

3.2 Objectives

The first part of this study aimed to characterise the responses to hypoxia and the hypoxia mimetic and HIF hydroxylase inhibitor Dimethyloxalylglycine (DMOG) of Caco-2 cells.

Expression of HIF- α isoforms, known HIF targets, and genes involved in apoptosis and cell adhesion was investigated. In addition, a PCR array was used to characterise the response of 84 angiogenesis genes to hypoxia and DMOG. The cells used in these studies were purchased from ATCC and frozen in liquid nitrogen before thawing for use. It has been estimated that up to 35% of cell-lines may be cross-contaminated or mis-identified (Hughes et al. 2007). As well as standardising experimental procedures for subsequent experiments, characterisation of Caco-2 responses had the additional benefit of addressing these risks.

3.3 Results

3.3.1 Expression of HIF- α Isoforms and Known HIF Targets in Caco-2 Cells

Initial experiments sought to establish the presence of the HIF- α isoforms in Caco-2 cells, and to investigate the effect of hypoxia on HIF- α protein expression. Figure 3.1 shows expression of HIF-1 α and HIF-2 α protein following 24 hours stimulation with DMOG (1mM) or exposure to hypoxia (1% O₂).

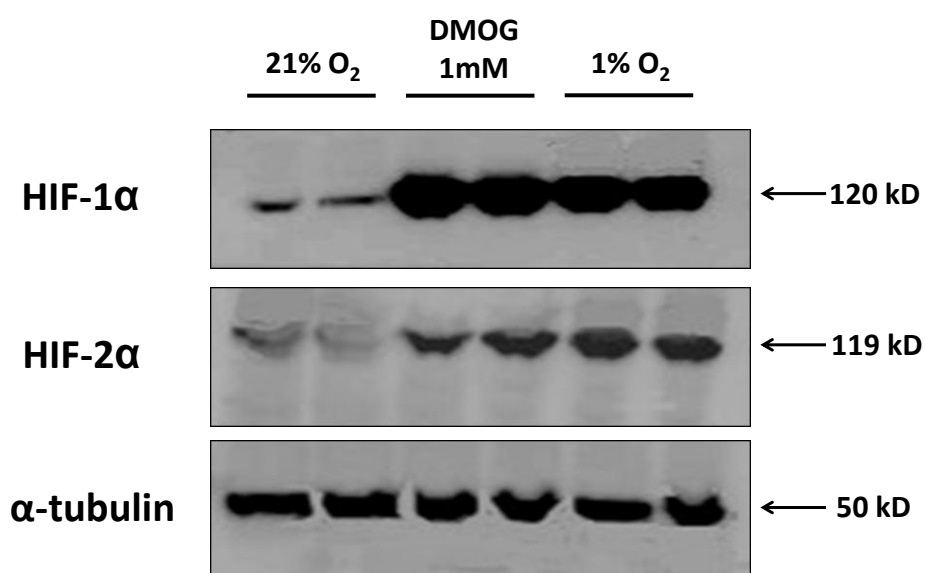


Figure 3.1 HIF-1 α and HIF-2 α protein stabilisation by DMOG and hypoxia

Caco-2 cells were exposed to hypoxia (1% O₂) or DMOG (1mM) for 24 hours, in duplicate, with normoxia (21% O₂) control. Figure shows HIF-1 α (120kD) and HIF-2 α (119kD) protein expression from a representative experiment performed in duplicate wells, evaluated by Western Blot. α -tubulin (50kD) is shown as loading control.

Both isoforms are expressed weakly in normoxia, with HIF-2 α being barely detectable. However, both isoforms show strong induction by both DMOG and hypoxia. The effect of hypoxia on known HIF targets was then investigated.

Figure 3.2 shows expression of VEGF, BNIP-3, GLUT-1 and CA-IX mRNA following stimulation with DMOG or hypoxia. These are considered to be standard levels of stimulation in

in vivo hypoxia studies and are generally taken to be comparable in terms of induction of HIF-dependent responses.

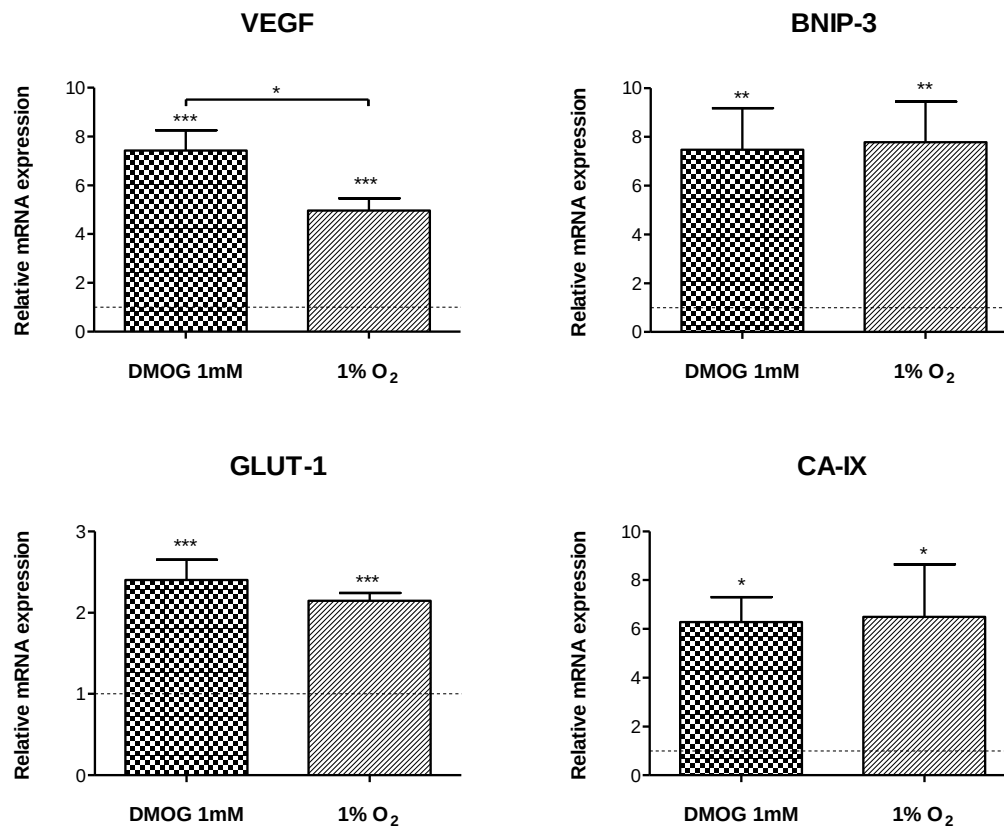


Figure 3.2 Upregulation of known HIF target genes by DMOG and hypoxia

Caco-2 cells were treated with DMOG (1mM) or exposed to hypoxia (1% O₂) for 24 hours with untreated normoxia control. mRNA expression was evaluated by Q-PCR using the $2^{-\Delta\Delta C_t}$ method. Graphs show VEGF, BNIP-3, GLUT-1 and CA-IX expression normalised to untreated normoxia (dashed line). Data are mean $\pm$ SEM from 3 experiments performed in triplicate wells, analysed by 1-way ANOVA (ΔC_t versus normoxia, non-significant unless stated; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

VEGF mRNA was upregulated more strongly by DMOG (7.43 ± 0.82) than by hypoxia (4.96 ± 0.50) ($p < 0.001$ versus normoxia for both; $p < 0.05$ DMOG versus hypoxia). There was no significant difference in upregulation by DMOG and hypoxia for BNIP-3 (7.47 ± 1.71 versus 7.78 ± 0.06 ; both $p < 0.01$ versus normoxia), GLUT-1 (2.40 ± 0.24 versus 2.15 ± 0.10 ; both $p < 0.001$ versus normoxia) or CA-IX (6.29 ± 2.26 versus 6.49 ± 2.15 ; both $p < 0.05$ versus normoxia).

The effect of hypoxia and DMOG stimulation on these HIF targets at the protein level is shown in Figure 3.3. After stimulation with either DMOG or hypoxia, supernatants were

collected, cells lysed and protein extracted. Secreted VEGF protein in the supernatants was measured by ELISA, and other proteins measured by Western Blotting of cell lysates.

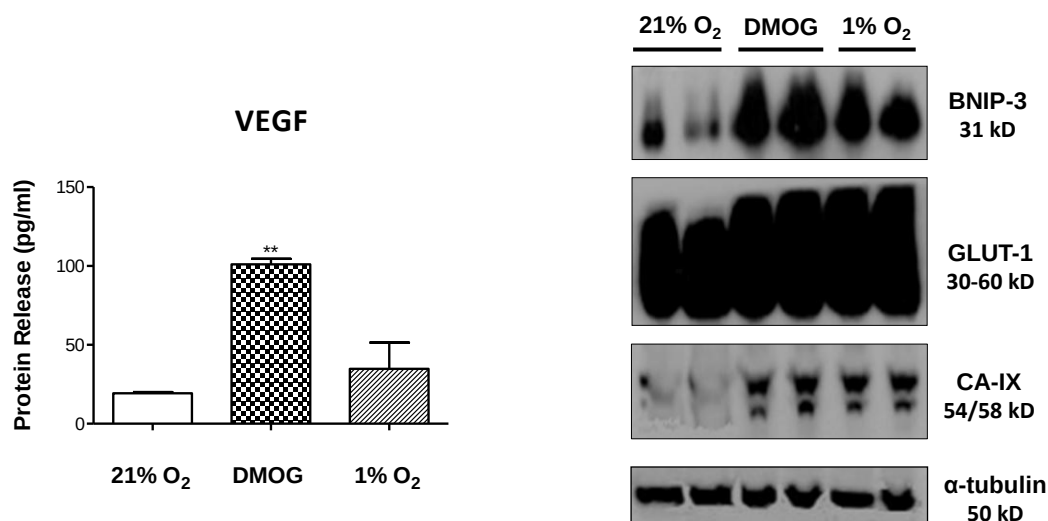


Figure 3.3 Protein expression of HIF targets in normoxia, DMOG and hypoxia

Caco-2 cells were treated with DMOG (1mM) or exposed to hypoxia (1% O₂) for 24 hours in duplicate wells, with untreated normoxia (21% O₂) control. The left panel shows VEGF protein expression in supernatants as evaluated by ELISA. Data are mean $\pm$ SD from a representative experiment performed in duplicate wells (**p<0.01 versus normoxia). The right panel shows Western Blots from the same experiment for BNIP-3 (31kD), GLUT-1 (30-60kD) and CA-IX (54/58kD) protein expression in cell lysates, with α -tubulin (50kD) loading control.

From a basal level of 19.29 ± 0.56 pg/mL in control (normoxic) samples, VEGF protein was upregulated more than 5-fold to 101.1 ± 2.45 pg/mL ($p < 0.01$) by DMOG, with a more modest 1.8-fold to 34.79 ± 11.76 pg/mL by hypoxia (not statistically significant). Both BNIP-3 and CA-IX were expressed weakly in normoxia, while GLUT-1 which characteristically appears as a smear rather than a discrete band, was abundantly expressed. DMOG appeared to be marginally more potent in stimulating BNIP-3 than hypoxia, while CA-IX and GLUT-1 were upregulated to a similar extent by both stimuli.

The response of these four genes to hypoxia was examined further, firstly investigating the effect of increasing durations of hypoxia exposure in a time-course experiment with 2, 6 and 24 hour time points (Figure 3.4).

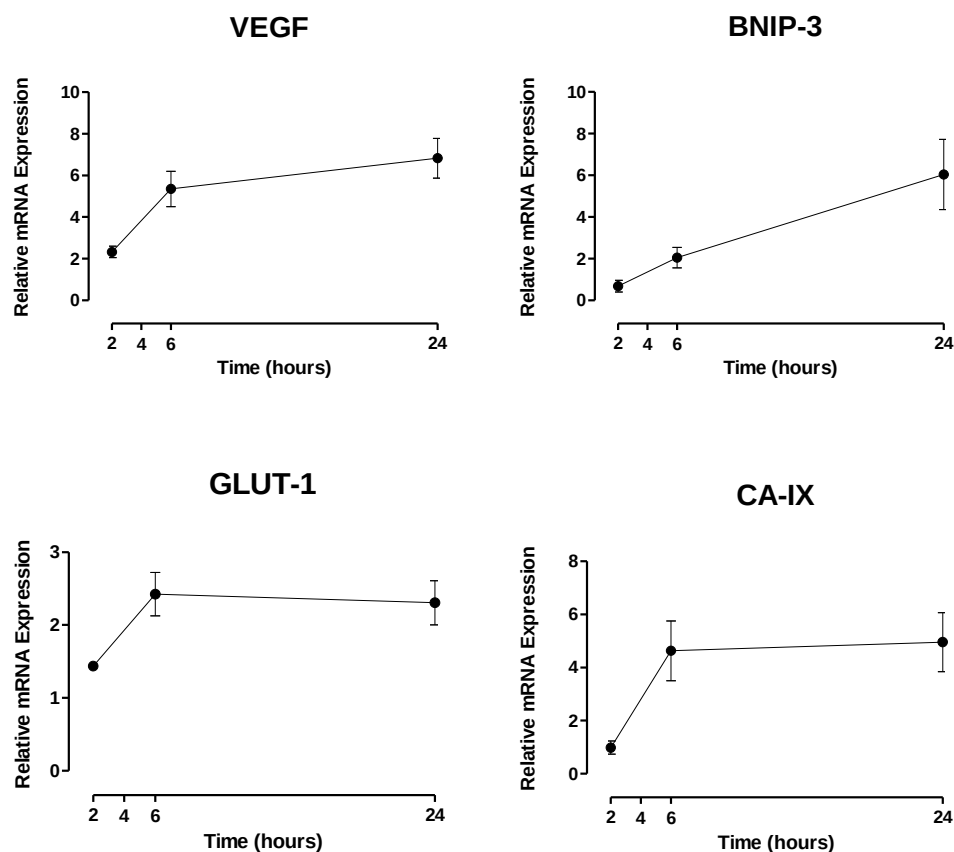


Figure 3.4 Time course of HIF target mRNA expression in hypoxia

Caco-2 cells were exposed to hypoxia (1% O₂) for 2, 6 or 24 hours. mRNA expression was evaluated by Q-PCR, normalised to normoxia at 2 hours. Graphs show VEGF, BNIP-3, GLUT-1 and CA-IX expression. Data are mean ± SD from a representative experiment performed in triplicate wells.

Overall, mRNA expression of all four genes was maximal at 24 hours. VEGF was upregulated earliest, with a fold-change of 2.33 ± 0.11 at 2 hours, rising to 5.35 ± 0.35 at 6 hours. A small further increase to 6.83 ± 0.39 was seen at 24 hours. A more linear time-course was seen with BNIP-3, which was initially downregulated (0.69 ± 0.11) at 2 hours, then upregulated to 2.05 ± 0.20 at 6 hours, with a further rise to 6.04 ± 0.76 at 24 hours. GLUT-1 and CA-IX both reached a plateau between 6 and 24 hours. GLUT-1 was modestly upregulated at 2 hours (1.44 ± 0.02), increasing to 2.42 ± 0.12 at 6 hours followed by a modest drop to 2.31 ± 0.12 at 24 hours. CA-IX was essentially unchanged at 2 hours (0.99 ± 0.1), reaching 4.63 ± 0.46 and 4.96 ± 0.46 at 6 and 24 hours respectively.

The effect of increasing levels of hypoxia was investigated in subsequent experiments (Figure 3.5).

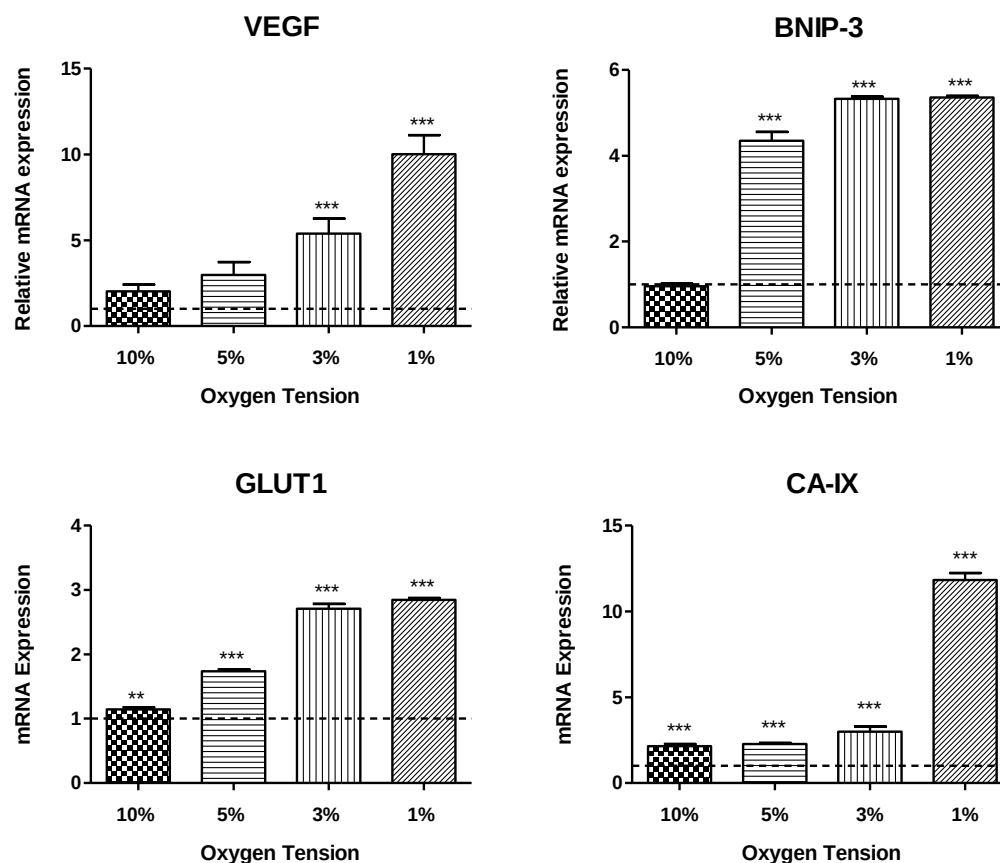


Figure 3.5 HIF target mRNA expression in decreasing oxygen tensions

Caco-2 cells were exposed to 10%, 5%, 3% or 1% O₂ for 24 hours. mRNA expression was evaluated by Q-PCR using the $2^{-\Delta\Delta C_t}$ method. Graphs show VEGF, BNIP-3, GLUT-1 and CA-IX expression normalised to normoxia (dashed line). Data are mean $\pm$ SEM from 2 experiments performed in triplicate wells, analysed by 1-way ANOVA (ΔC_t versus normoxia; ** $p < 0.01$, *** $p < 0.001$).

Upregulation of VEGF mRNA expression was seen at 10% O₂ and 5% O₂ (2.02 ± 0.39 and 2.99 ± 0.74 respectively) without reaching statistical significance. Further upregulation was seen at 3% O₂ (5.40 ± 0.87), almost doubling at 1% O₂ (10.02 ± 1.11) ($p < 0.001$ versus normoxia for both oxygen tensions). BNIP-3 expression was unchanged at 10% O₂ (0.96 ± 0.06), but increased sharply to 4.36 ± 0.2 ($p < 0.001$) at 5% O₂, with only modest further increases at 3% O₂ and 1% O₂ (5.33 ± 0.05 and 5.36 ± 0.03 ; $p < 0.001$ for both). GLUT-1 was marginally increased at 10% O₂ (1.15 ± 0.03 ; $p < 0.01$), increasing to 1.74 ± 0.03 at 5% O₂, 2.71 ± 0.08 at 3% O₂, with a modest further increase to 2.85 ± 0.03 at 1% O₂ ($p < 0.001$ for all three). CA-IX was modestly and increasingly upregulated at 10%, 5% and 3% O₂ (2.15 ± 0.12 , 2.26 ± 0.08 and 2.99 ± 0.30 respectively), with a much greater increase at 1% O₂ (11.84 ± 0.41) ($p < 0.001$ for all four oxygen tensions). Of note, VEGF and CA-IX expression at 1% O₂ (10.02 and 11.84 respectively) was significantly greater in this experiment than in Figure 3.2 (4.96 and 6.49). This discrepancy may be due to a difference in passage number of cells used in the two experiments (see Discussion, section 3.4).

Variations in the time course and oxygen sensitivity of these HIF-dependent genes suggest that they may not be regulated by identical mechanisms. In the next experiment, the effect of stimulation with different DMOG concentrations was investigated (Figure 3.6).

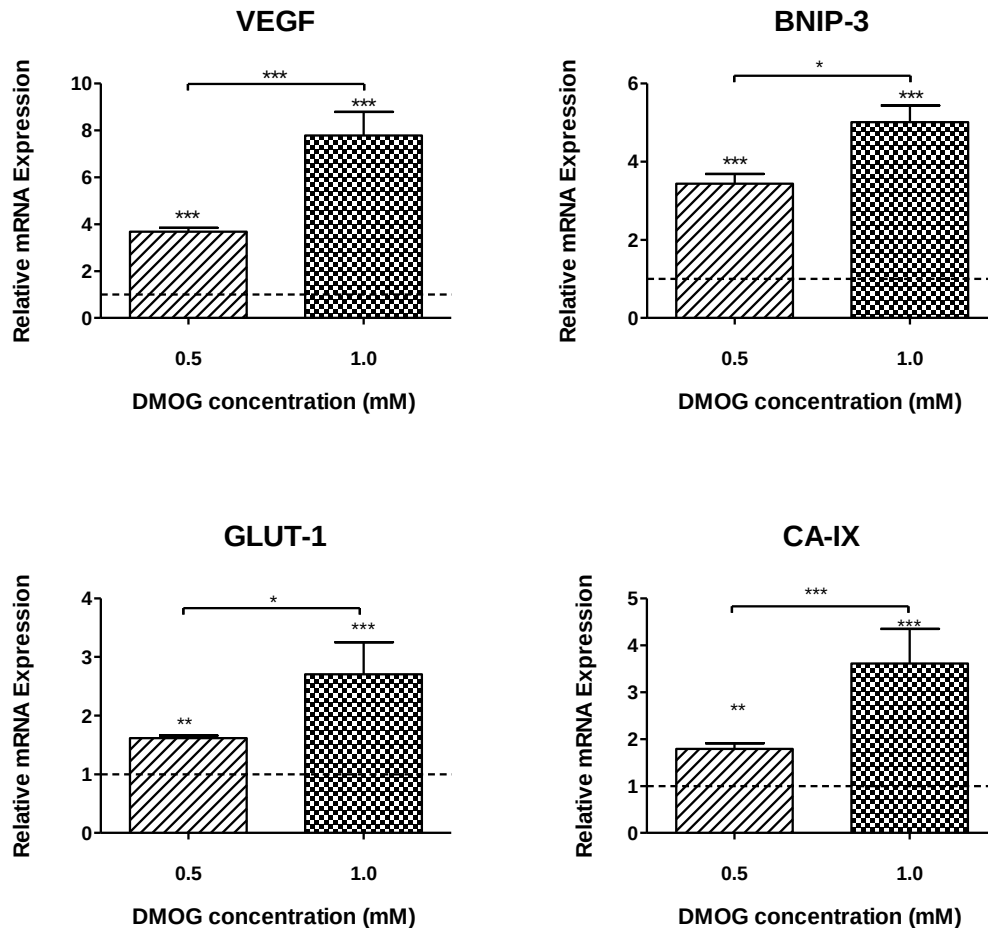


Figure 3.6 HIF target mRNA expression in different DMOG concentrations

Caco-2 cells were treated with 0.5mM or 1mM DMOG for 24 hours with untreated control. mRNA expression was evaluated by Q-PCR. Graphs show VEGF, BNIP-3, GLUT-1 or CA-IX expression normalised to control (dashed line). Data are mean $\pm$ SEM from replicate experiments performed in triplicate wells, analysed by 1-way ANOVA (ΔC_t versus untreated; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

A dose-dependent relationship between DMOG concentration and mRNA expression was seen for all four genes. Fold-change for VEGF expression was 3.67 ± 0.16 at 0.5mM DMOG and 7.79 ± 0.10 at 1.0mM ($p < 0.001$ for both). For BNIP-3, fold-changes were 3.44 ± 0.25 and 5.01 ± 0.43 respectively ($p < 0.001$ for both). GLUT-1 expression was 1.62 ± 0.04 ($p < 0.01$) for 0.5mM and 2.71 ± 0.54 ($p < 0.001$) for 1.0mM DMOG. For CA-IX fold-changes were 1.79 ± 0.12 ($p < 0.01$) for 0.5mM and 3.62 ± 0.74 ($p < 0.001$) for 1.0mM DMOG.

3.3.2 Hypoxia-Induced Expression of Apoptosis and Cell-Adhesion Genes

Having established the effect of DMOG and hypoxia on known HIF targets, genes involved in apoptosis (Figure 3.7) and cell-adhesion (Figure 3.8) were studied next.

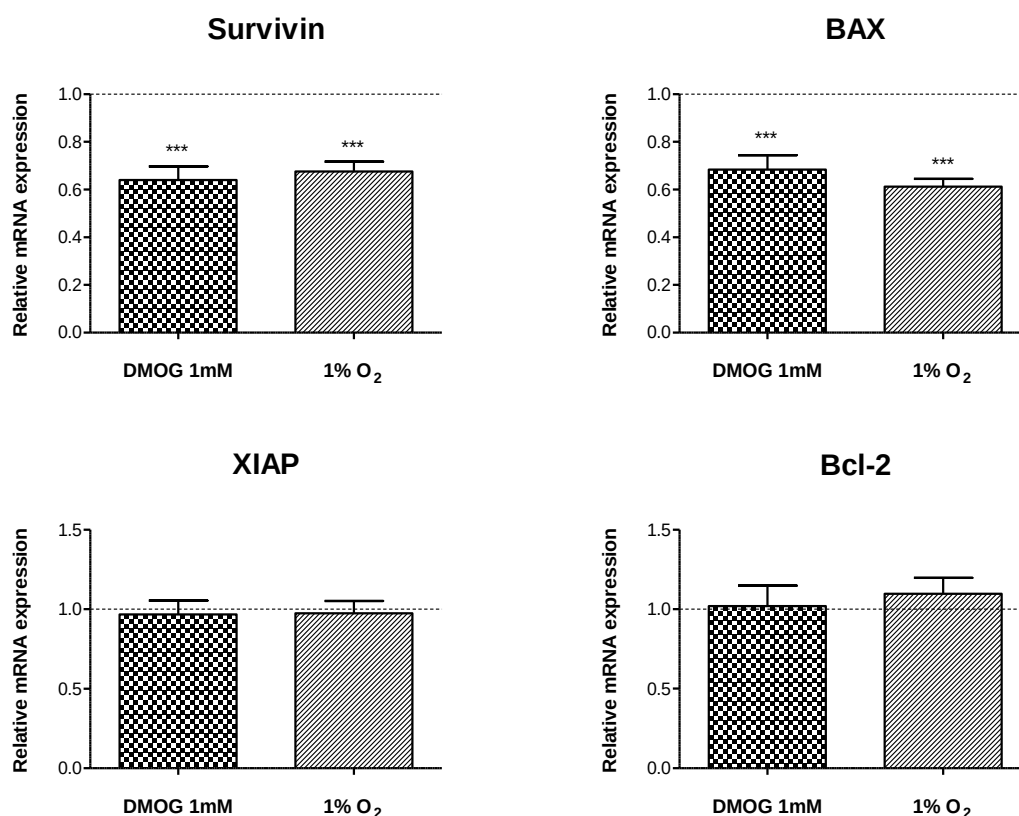


Figure 3.7 Effect of hypoxia and DMOG on apoptosis genes

Caco-2 cells were treated with DMOG or exposed to hypoxia (1% O₂) for 24 hours with untreated normoxia control. mRNA expression was evaluated by Q-PCR. Graphs show survivin, BAX, XIAP and Bcl-2 expression normalised to untreated normoxia (dashed line). Data are mean $\pm$ SEM from 3 experiments performed in triplicate wells, analysed by 1-way ANOVA (Δ Ct versus normoxia; *** p<0.001).

Survivin and BAX mRNA were both down-regulated by DMOG and hypoxia, to a similar degree. Survivin expression was 0.64 ± 0.06 relative to untreated cells in response to DMOG and 0.67 ± 0.04 in response to hypoxia ($p < 0.001$ for both). BAX expression in DMOG- and hypoxia-treated cells was 0.68 ± 0.06 and 0.61 ± 0.03 respectively ($p < 0.001$). Expression of XIAP (0.97 ± 0.09 DMOG, 0.97 ± 0.08 hypoxia) and Bcl-2 (1.02 ± 0.13 DMOG, 1.10 ± 0.10 hypoxia) were unaffected by either stimulus.

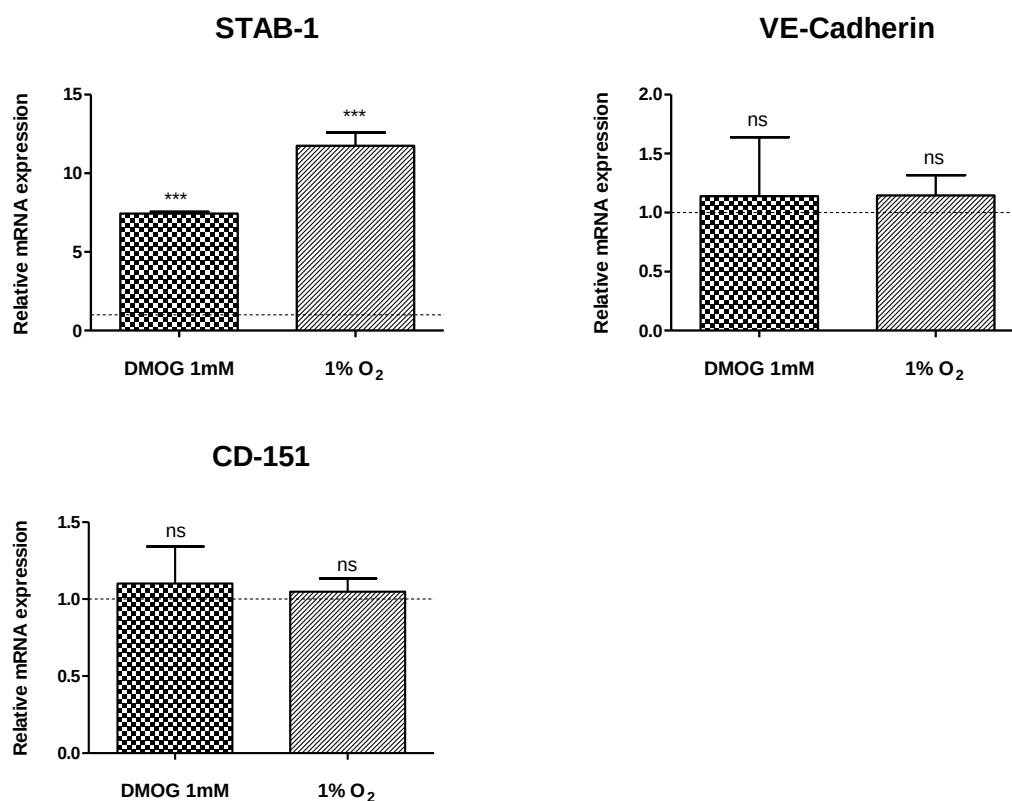


Figure 3.8 Effect of hypoxia and DMOG on cell-adhesion molecule genes

Caco-2 cells were treated with DMOG or exposed to hypoxia (1% O₂) for 24 hours with untreated or normoxia control. mRNA expression was evaluated by Q-PCR. Graphs show STAB-1, VE-Cadherin and CD-151 expression normalised to untreated normoxia (dashed line). Data are mean $\pm$ SEM from replicate experiments performed in triplicate wells, analysed by 1-way ANOVA (Δ Ct versus normoxia; *** $p < 0.001$, ns = not significant).

STAB-1 was strongly upregulated by DMOG stimulation, with a fold-change of 7.49 ± 0.06 ($p < 0.001$). Greater upregulation was seen with hypoxia (11.75 ± 0.83 ; $p < 0.001$). Both VE-Cadherin and CD-151 were unchanged. VE-Cadherin expression was 1.19 ± 0.19 in DMOG-treated cells and 1.17 ± 0.17 in hypoxia-treated cells, while CD-151 expression was 1.10 ± 0.09 and 1.05 ± 0.09 respectively.

3.3.3 Hypoxia-Induced Angiogenesis Response: Identification of Novel Hypoxia-Regulated Genes

The primary focus of this study was to explore Caco-2 angiogenesis responses. To this end, the RT²Profiler™ PCR Angiogenesis Array (SA Biosciences) was used to analyse mRNA extracted from cells stimulated with DMOG (Figure 3.9) or hypoxia (Figure 3.10) and compared with normoxia controls. A full list of the genes included in the array is presented in

Table 2.5. The manufacturer's software was used to calculate fold-changes by the $2^{-\Delta\Delta C_t}$ method, with greater than two-fold up- or down-regulation taken as a meaningful change. The results are displayed in a scatter plot, as in Figure 3.9, in which $2^{-\Delta C_t}$ values in hypoxia/DMOG (y-axis) are plotted against normoxia controls (x-axis). The solid line represents equal expression, and the upper and lower dashed lines represent two-fold up- or downregulation respectively. Genes lying towards the top right of the graph are more abundantly expressed than those to the bottom left. 31 of the 84 genes were not detected in Caco-2 cells ($C_t > 30$) (Table 5.7 final column, and Table 7.1).

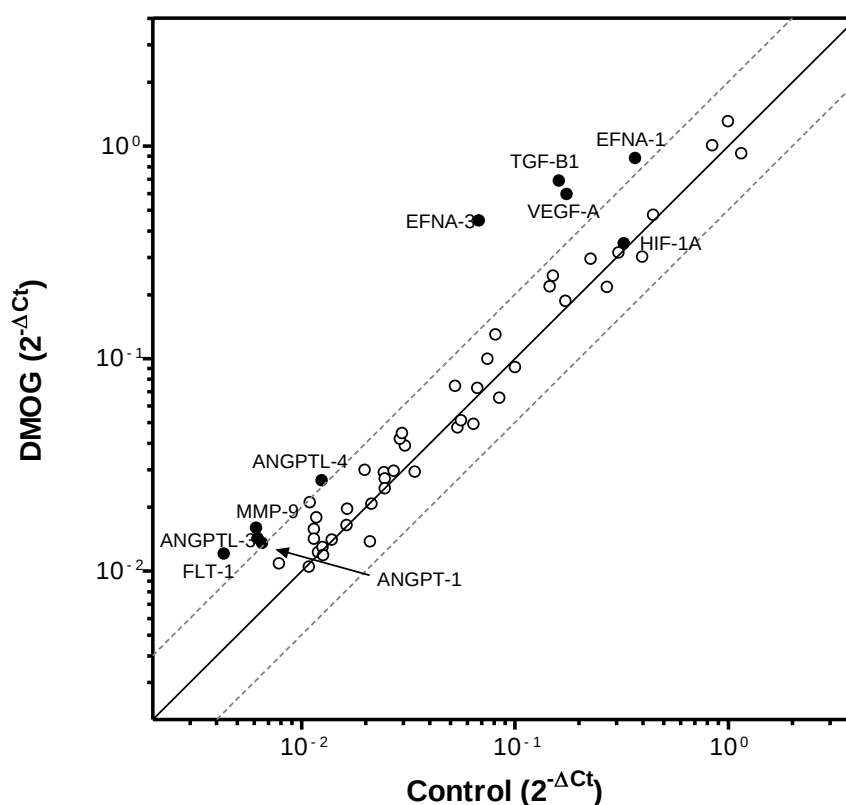


Figure 3.9 Expression pattern of angiogenesis genes following DMOG exposure

Caco-2 cells were treated with DMOG 1mM for 24 hours. mRNA expression was evaluated in duplicate by RT² Profiler Angiogenesis PCR Array and $2^{-\Delta C_t}$ values plotted against untreated control. Figure shows a scatter plot from a representative experiment. Solid line indicates no change; dashed lines indicate two-fold increase (upper) and decrease (lower). Genes upregulated more than two-fold are labelled and shown in black. HIF-1 α is also shown in black (HIF-1A).

Following DMOG stimulation, 9 genes were upregulated greater than two-fold. These were ANGPT-1 (fold-change 2.09), ANGPTL-3 (2.31), ANGPTL-4 (2.61), EFNA-1 (2.41), EFNA-3 (6.63), FLT-1 (2.81), MMP-9 (2.42), TGF- β 1 (TGF-B1, 4.30), and VEGF-A (3.42). HIF-1 α expression was unchanged (1.08). No gene was downregulated more than two-fold.

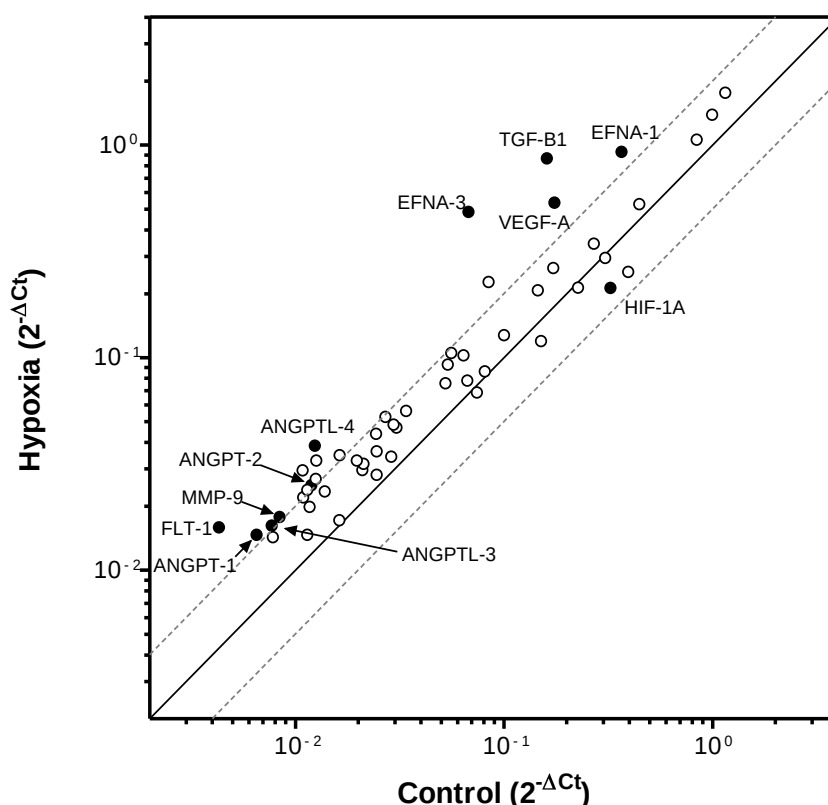


Figure 3.10 Expression pattern of angiogenesis genes following exposure to hypoxia

Caco-2 cells were treated exposed to hypoxia (1% O₂) for 24 hours with normoxia control. mRNA expression was evaluated in duplicate by RT² Profiler Angiogenesis PCR Array and 2^{-ΔCt} values in hypoxia plotted against normoxia control. Figure shows a scatter plot from a representative experiment. Solid line indicates no change; dashed lines indicate two-fold increase and two-fold decrease. Selected genes that were upregulated more than two-fold are labelled and shown in black. HIF-1α is also shown in black (HIF-1A).

A similar pattern was seen following hypoxia stimulation. The same 9 genes that were upregulated by DMOG were also induced by hypoxia. ANGPT-1 was upregulated by 2.26, ANGPTL-3 by 2.10, ANGPTL-4 by 3.11, EFNA-1 by 2.55, EFNA-3 by 7.19, FLT-1 by 3.70, MMP-9 by 2.11, TGF-β1 by 5.39, and VEGF-A by 3.07. A further 8 genes were upregulated by hypoxia between 2 to 3-fold: ANGPT-2 (2.18), EGF (2.69), Heparanase (HPSE, 2.02), Insulin-like growth factor 1 (IGF, 2.10), interleukin-1 beta (IL1-β, 2.13), plexin-domain-containing-1 (PLXDC1, 2.15), prokineticin-2 (PROK-2, 2.73), and VEGF-C (2.60). HIF-1α was downregulated (fold-change 0.66; fold-regulation -1.52).

Both hypoxia and DMOG are known to induce genes in a non-HIF-dependent manner (Wood et al. 1996; Elvidge et al. 2006). From the above experiments, the 9 genes that were upregulated by both hypoxia and DMOG and are thus likely to be HIF-dependent, are represented in Figure 3.11.

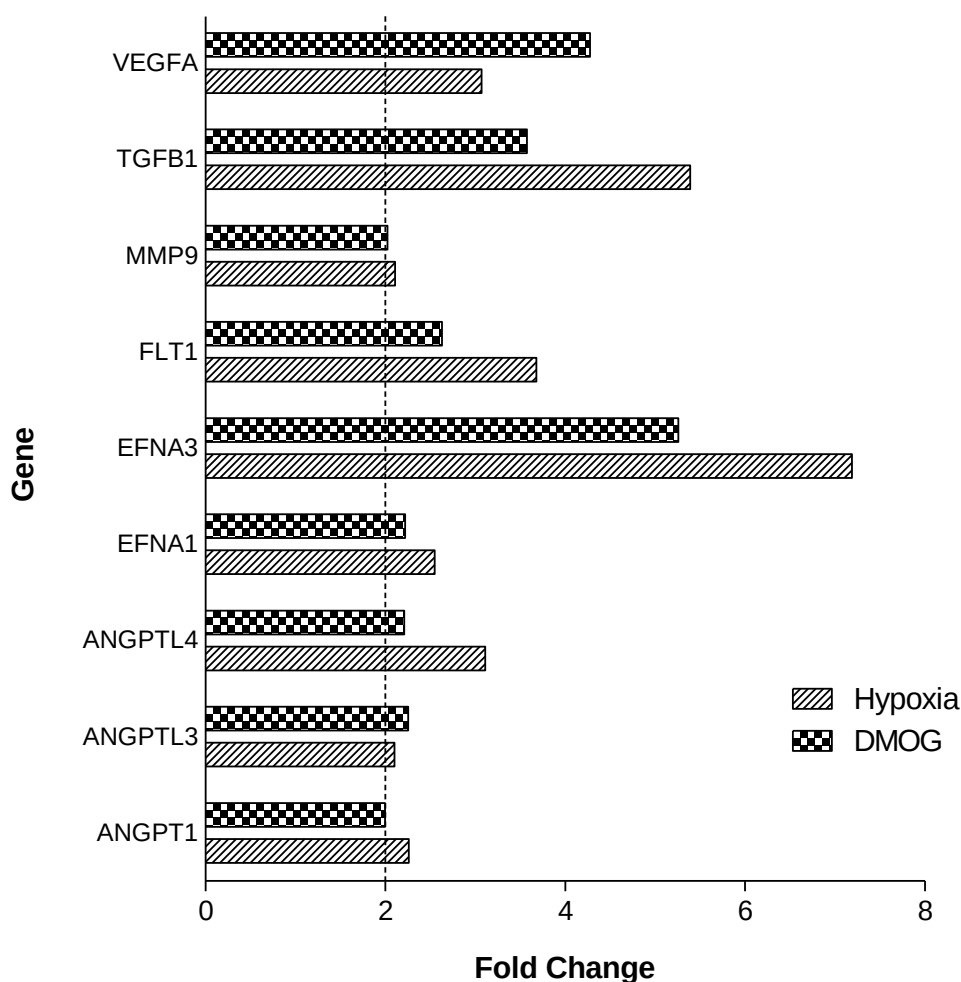


Figure 3.11 Angiogenesis genes upregulated by both DMOG and hypoxia

Caco-2 cells were treated with DMOG or exposed to hypoxia (1% O₂) for 24 hours with untreated normoxia control. mRNA expression was evaluated by RT² Profiler Angiogenesis PCR Array and fold-changes *versus* normoxia calculated. Figure shows genes upregulated at least two-fold by both DMOG and hypoxia. This figure is derived from the data shown in Figure 3.9 and Figure 3.10.

The hypoxic regulation of VEGF in CRC angiogenesis and its importance is well described. Although it has been reported that ANGPTL-4 is hypoxia-dependent in other CRC cell-lines, this is a novel finding in Caco-2 cells (Imamura et al. 2009). Two recent studies report hypoxic induction of TGF- β 1 protein in SW480 (Xian et al. 2011) and Caco-2 cells (Abajo et al. 2012). Neither study investigated mRNA levels. The hypoxia-regulation of ephrin-A3 has not been previously described in cancer CRC. Further investigation of angiogenesis responses focused on these three novel genes.

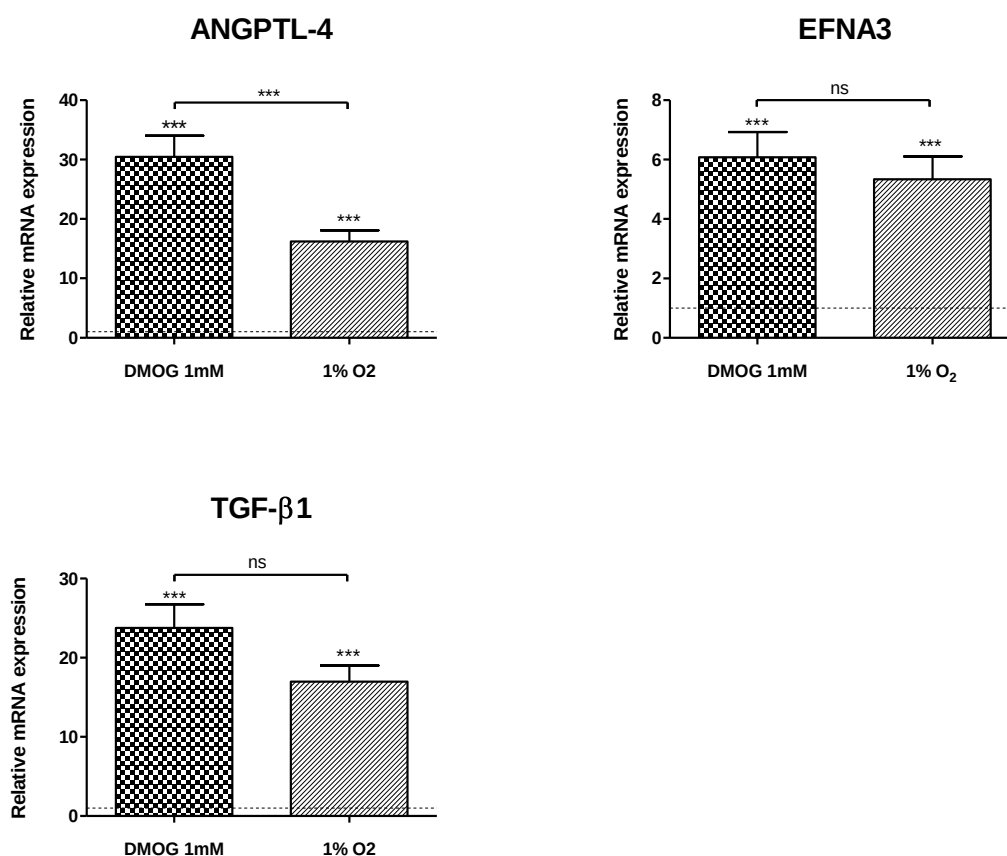


Figure 3.12 Q-PCR validation of selected hypoxia-induced angiogenesis genes

Caco-2 cells were treated with DMOG or exposed to hypoxia (1% O₂) for 24 hours with untreated normoxia control. mRNA expression was evaluated by Q-PCR. Graphs show ANGPTL-4, EFNA-3 and TGF-β1 expression normalised to normoxia (dashed line). Data are mean ± SEM from at least 3 experiments performed in triplicate, analysed by 1-way ANOVA (***) $p < 0.001$ versus normoxia, ns = not significant).

Firstly, the array results were validated by Q-PCR (Figure 3.12). All three genes were upregulated by both DMOG and hypoxia. For ANGPTL-4 and TGF-β1, DMOG was a more potent stimulus than hypoxia. ANGPTL-4 upregulation was 30.45 ± 3.59 ($p < 0.001$) for DMOG and 16.22 ± 1.87 ($p < 0.001$) for hypoxia ($p < 0.001$ for DMOG versus hypoxia). Fold-change for TGF-β1 expression was 23.76 ± 2.98 ($p < 0.001$) by DMOG and 16.96 ± 2.05 ($p < 0.001$) by hypoxia, although this difference did not reach statistical significance. Ephrin-A3 expression was similar following DMOG (6.08 ± 0.85 ; $p < 0.001$) and hypoxia (5.34 ± 0.77 ; $p < 0.001$).

Response to DMOG stimulation was dose-dependent as shown in Figure 3.13.

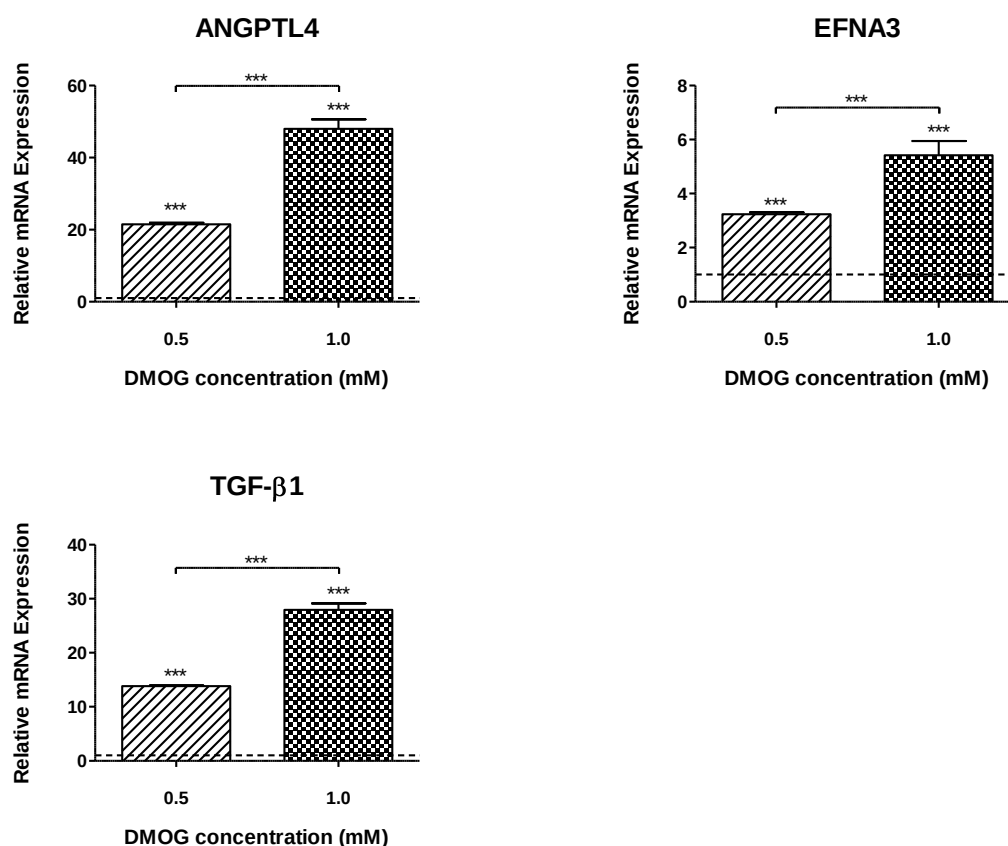


Figure 3.13 Novel angiogenesis genes –effect of DMOG concentration

Caco-2 cells were treated with 0.5mM or 1mM DMOG for 24 hours with untreated control. mRNA expression was evaluated by Q-PCR. Graphs show ANGPTL-4, TGF-β1 and EFNA-3 expression normalised to untreated control (dashed line). Data are mean $\pm$ SEM from replicate experiments performed in triplicate, analysed by 1-way ANOVA (Δ Ct versus untreated; *** p<0.001).

Fold-change for ANGPTL-4 expression was 21.48 ± 0.45 at 0.5mM DMOG and 47.99 ± 2.61 at 1.0mM (both p<0.001). For EFNA-3, fold-changes were 3.28 ± 0.07 and 5.41 ± 0.53 respectively (both p<0.001). TGF-β1 was upregulated by 13.82 ± 0.14 for 0.5mM and 27.94 ± 1.23 for 1.0mM (both p<0.001).

The experiments described thus far sought to characterise the response of downstream HIF targets to both DMOG and hypoxia. The results are summarised in Table 3.1. To complete characterisation of Caco-2 hypoxia-mediated responses, the expression of the HIF-regulating enzymes was investigated (next section).

Table 3.1 Characterisation of Caco-2 mRNA Response to Hypoxia

Table shows Caco-2 mRNA responses to hypoxia (1% O₂) and DMOG 1mM stimulation of genes involved in apoptosis, cell-adhesion and angiogenesis. Only genes that were evaluated by Q-PCR are shown. ↑ = increase, ↓ = decrease, ↔ = no change

	Gene	DMOG	Hypoxia
Known HIF Targets	VEGF	↑	↑
	GLUT-1	↑	↑
	CA-IX	↑	↑
	BNIP-3	↑	↑
Apoptosis	Survivin	↓	↓
	BAX	↓	↓
	XIAP	↔	↔
	Bcl-2	↔	↔
Cell-adhesion	STAB-1	↑	↑
	VE-Cad	↔	↔
	CD-151	↔	↔
Angiogenesis	ANGPTL-4	↑	↑
	EFNA-3	↑	↑
	TGF-β1	↑	↑

3.3.4 Expression of HIF-Regulatory Enzymes

The expression of the HIF-regulating enzymes PHD-2, PHD-3 and FIH-1 was investigated at mRNA (Figure 3.14) and protein level (Figure 3.15).

There was a trend towards greater mRNA upregulation with hypoxia stimulation than with DMOG, but this did not reach statistical significance. PHD-3 mRNA was more strongly upregulated than PHD-2, with fold-changes of 3.75 ± 0.12 after DMOG and 4.52 ± 0.43 after hypoxia stimulation ($p < 0.001$ versus normoxia for both; DMOG versus hypoxia not significant). There was a small downregulation of FIH-1 mRNA after DMOG stimulation (0.82 ± 0.02) and mild upregulation after hypoxia (1.12 ± 0.22), but these changes were not statistically significant.

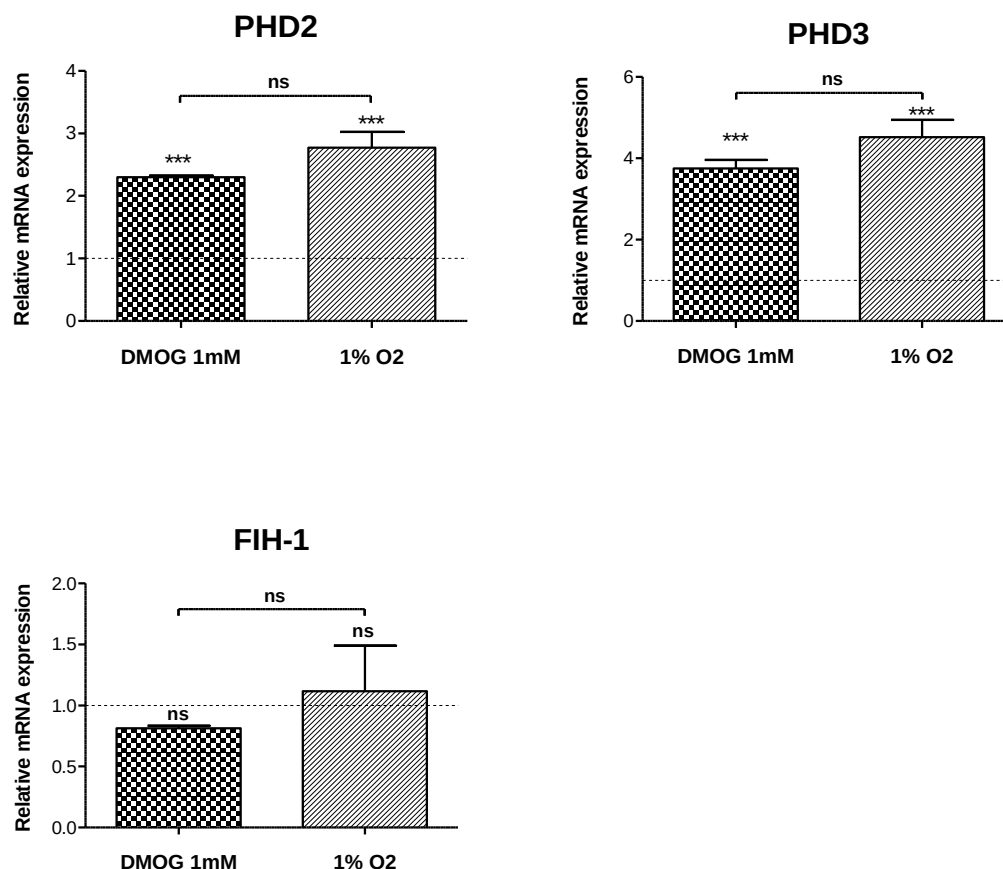


Figure 3.14 HIF hydroxylase mRNA expression in DMOG and hypoxia

Caco-2 cells were exposed to DMOG or hypoxia (1% O₂) for 24 hours with untreated normoxia control. mRNA expression was evaluated by Q-PCR. Graphs show PHD-2, PHD-3 and FIH-1 expression normalised to normoxia (dashed line). Data are mean $\pm$ SD from a representative experiment, analysed by 1-way ANOVA (Δ Ct versus normoxia; * p<0.05, ** p<0.01, *** p<0.001, ns = not significant).

At the protein level, while PHD-2 was expressed in normoxia, PHD-3 was barely detected by Western Blot (Figure 3.15). There was strong induction of both PHDs with DMOG and hypoxia. In contrast to mRNA levels, DMOG appeared to be a more potent inducer of the PHD proteins than hypoxia. Normoxic FIH-1 protein expression was not significantly altered by either DMOG or hypoxia stimulation.

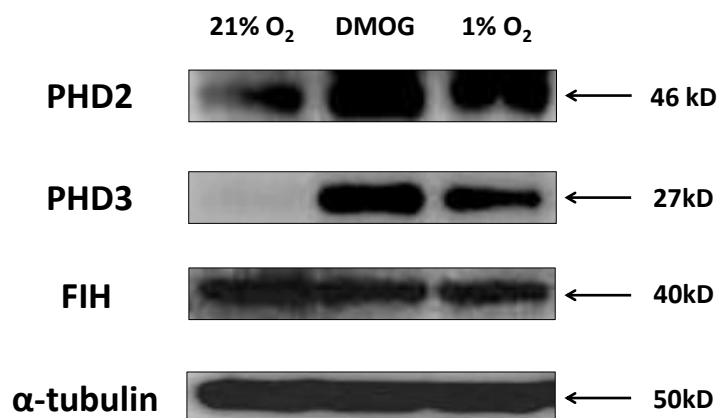


Figure 3.15 HIF hydroxylase protein expression in DMOG and hypoxia

Caco-2 cells were treated with DMOG or exposed to hypoxia for 24 hours with untreated normoxia control. Figure shows PHD-2 (46kD), PHD-3 (27kD) and FIH-1 (40kD) protein expression from a representative experiment, evaluated by Western Blot. α -tubulin (50kD) is shown as loading control.

3.4 Discussion

The objective of this part of my study was to characterise the response of Caco-2 CRC cells to hypoxia and to the hypoxia mimetic DMOG. Initial experiments sought to confirm HIF- α isoform expression in these cells. Expression of well-known HIF targets was examined in some detail, evaluating dose response and kinetics. Results indicated that at 24 hours, the HIF-pathway was maximally activated by 1% O₂ or DMOG 1mM. Subsequent experiments evaluated the hypoxia- and DMOG-induced expression of genes involved in apoptosis, cell-adhesion and angiogenesis, as well as the HIF regulatory enzymes. Genes that have been previously reported to be hypoxia regulated, particularly those for which there is evidence of involvement in CRC pathogenesis, were selected for evaluation. In addition, an angiogenesis PCR array identified three novel hypoxia-regulated angiogenesis which were subsequently evaluated by Q-PCR. A summary of these results is provided in Table 3.1.

A general observation of note is that there was some inconsistency between experiments in terms of the degree of induction of certain genes. For example in Figure 3.2, expression of VEGF and CA-IX at 1% O₂ (4.96 and 6.49 respectively) was approximately half of that in Figure 3.5 (10.02 and 11.84). Similarly, ANGPTL-4 expression at 1mM DMOG was 30.45 in Figure 3.12 and 47.99 in Figure 3.13. This discrepancy may be due to passage number, which has been shown to influence functional characteristics of cell-lines (Wenger et al. 2004; Hughes et al. 2007). This phenomenon may contribute to variations across different studies, as passage number is not commonly referred to (Sambuy et al. 2005). In Caco-2 cells, passage-dependent variations in mRNA expression of glucose transporter protein GLUT-3 (2.4-fold) and the enzyme sucrose-isomaltase (7- to 10-fold) have been reported (Chantret et al. 1994; Anderle et al. 2003). The range of passage number in my experiments was small (1 to 5) in comparison to these studies (30 to 200), and unfortunately passage number was not routinely recorded for data analysis purposes. Data from 2 to 3 separate experiments were commonly pooled. To require that data pooling be restricted to experiments using cells of identical passage number would have required a significant increase in experiment number or size (more replicates per experiment). In the analysis and discussion of these results, the emphasis is on differences between experimental conditions *within* an experiment, rather than differences *between* experiments which may be confounded by passage number.

It was clearly demonstrated in my study that in Caco-2 cells both HIF- α isoforms are expressed, and strongly upregulated by 24 hours stimulation with both DMOG 1mM and hypoxia (1% O₂) (Figure 3.1). This is in contrast to Mizukami *et al.* (Mizukami et al. 2004), who described weak normoxic expression with minimal hypoxia (1% O₂) induction of HIF-2 α in Caco-2 at 12 hours, and concluded that this isoform was unlikely to play a role in hypoxia-regulation in this

cell-line. Bracken *et al*, using the same antibody as Mizukami *et al* but a lower oxygen tension (0.5% O₂), showed moderate but clear hypoxia-induction of HIF-2 α at 16 hours (Bracken *et al*. 2006). Both these studies used a different antibody to that used in my study, which in the case of both 1% O₂ and 24 hour stimulation produced a consistently strong HIF-2 α response. Passage number, which is not quoted in these studies, might also contribute to these discrepancies. In contrast to HIF- α protein, mRNA levels were downregulated by hypoxia. This data is presented and discussed in Chapter 4.

My data show that normoxic Caco-2 cells express PHD-2 protein while PHD-3 is barely detected. PHD-2 is the dominant HIF-regulating enzyme in normoxia in most cell types (Berra *et al*. 2003). In CRC, while PHD-2 expression is equal in tumour and adjacent normal tissue, downregulation of PHD-3 in the tumour has been reported, and correlates with increased tumourigenesis and metastasis (Xue *et al*. 2010). PHD-3 knockdown in CRC cell-lines resulted in cytokine resistance and enhanced tumourigenesis, leading the authors to conclude that PHD-3 may have a tumour suppressor role. Of note, these experiments were conducted in normoxic conditions, and the mechanism involved inhibition of the transcription factor nuclear factor kappa-B (NF- κ B) rather than HIF hydroxylation. The significance of this effect in the context of hypoxia is unclear. In Caco-2 cells PHD-2 and PHD-3 were induced by both hypoxia and DMOG at mRNA and protein level. Hypoxic induction of PHD-2 and PHD-3 has been previously reported in several cancer cell-lines, including CRC (Berchner-Pfannschmidt *et al*. 2008; Yan *et al*. 2011), and an HRE has been identified in both gene promoters (Metzen *et al*. 2005; Pescador *et al*. 2005). In Caco-2 cells, upregulation of PHD-2 mRNA by hypoxia (Bruning *et al*. 2011), and of PHD-2 and PHD-3 mRNA by iron-deprivation, which has a hypoxia-mimetic effect, has been reported (Hu *et al*. 2010), but neither study examined protein expression. Since the PHDs are inactivated by hypoxia, their upregulation leads to rapid clearance of accumulated HIF once normal oxygen levels are restored (D'Angelo *et al*. 2003; Marxsen *et al*. 2004). However, it has also been reported that in prolonged hypoxia (1% O₂, 24-48 hours), PHD-2 and PHD-3 can reduce HIF protein levels in glioblastoma cells (Henze *et al*. 2010). It therefore seems likely that they are not completely inactive at 1% O₂, and that their upregulation by hypoxia serves to protect cells from HIF accumulation in chronic hypoxia.

FIH-1 expression in Caco-2 cells has been previously reported (Bracken *et al*. 2006), although the effect of hypoxia was not investigated as in my study. FIH-1 expression has also been demonstrated in several cancer cell-lines from different tissues including CRC, and, in support of my data, was not found to be induced by hypoxia even at 0.2% O₂ (Stolze *et al*. 2004). In lung cancer cells, however, Ke *et al*. reported downregulation of FIH-1 mRNA by the hypoxia-mimetic cobalt chloride (Ke *et al*. 2005), which is known to inhibit FIH-1 hydroxylation of HIF- α

(Hewitson et al. 2002), suggesting that there may be HIF-1 α -dependent hypoxic regulation of FIH in these cells.

As might be anticipated based on the expression of HIF- α proteins, the known HIF targets VEGF, BNIP-3 and CA-IX were all upregulated at both the mRNA and protein levels by DMOG and hypoxia stimulation. For VEGF (and to a lesser extent BNIP-3), DMOG was a more potent stimulus than hypoxia (Figure 3.3). This may be explained by the relative oxygen sensitivities of the HIF regulating enzymes (Koivunen et al. 2004; Tian et al. 2011). Using siRNA techniques, Stolze *et al.* reported that PHD-2 retains full activity at 2% O₂, but this was barely discernible at 0.5% O₂ (Stolze et al. 2004). In contrast, FIH-1 retained significant activity even at 0.2% O₂. Tian *et al.* investigated enzyme activity using antibodies to the specific proline and asparagine residues hydroxylated by the PHDs and FIH respectively. They showed that prolyl hydroxylation occurs even at 0.25% O₂, albeit markedly attenuated, whereas significant asparagine hydroxylation persisted at 0.1% O₂. By contrast, DMOG 1mM inhibited both proline and asparagine hydroxylation completely (Tian et al. 2011). The fact that CA-IX and GLUT-1 were equally stimulated by both DMOG and hypoxia suggests that the action of FIH-1 may be less important than that of the PHDs, whereas for VEGF (and to a lesser extent BNIP-3), FIH-1 may play an important role. Further information may be gleaned by co-stimulation experiments, to see if the addition of DMOG to hypoxic cells increases target gene expression.

FLT-1 (FMS-like tyrosine kinase, also known as VEGFR-1) is one of three known VEGF receptor tyrosine kinases (VEGFR-1, -2 and -3). Most cellular responses to VEGF are mediated by VEGFR-2 (KDR/Flk-1) (Ferrara et al. 2003). FLT-1 has complex, context-dependent functions (Chung and Ferrara 2011). It can negatively regulate VEGFR-2 by sequestering VEGF, thus acting as a “decoy receptor” (Kendall and Thomas 1993; Park et al. 1994; Roberts et al. 2004). It can regulate monocyte migration as well as endothelial cell production of growth factors and proteases (Barleon et al. 1996; Hiratsuka et al. 2002; LeCouter et al. 2003). FLT-1 is expressed in cancer cell-lines and tissues including CRC (Masood et al. 2001; Fan et al. 2005) and its activation leads to increased migration and invasion in CRC cell-lines (Fan et al. 2005; Lesslie et al. 2006). In agreement with my data, hypoxia has been shown to induce FLT-1 expression both *in vitro* and *in vivo*, and an HRE has been identified in the FLT-1 promoter (Tuder et al. 1995; Gerber et al. 1997).

Upregulation of BNIP-3 by hypoxia is well-established (Mellor and Harris 2007). It has been described in Caco-2 cells (Bacon et al. 2006), reflecting the data from the present study, as well as in several other cancer cell-lines (Sowter et al. 2001). BNIP-3 upregulation in tumour tissue is associated with several cancers including, and correlates with more aggressive tumours (Burton and Gibson 2009). In CRC, BNIP-3 expression is associated with local invasion and

lymph node metastasis (Koukourakis et al. 2006). However, Bacon *et al.* reported reduced BNIP-3 expression in 64% of CRC tumour samples, with increased expression in 12% (Bacon et al. 2006). A non-significant trend towards increasing tumour grade with decreasing BNIP-3 expression was seen. In eight CRC cell-lines investigated, only three (Caco-2, LS14T and Colo-741) showed hypoxic BNIP-3 induction (Bacon et al. 2006). In hypoxia-responsive LS14T cells, silencing of BNIP-3 reduced hypoxia-induced apoptosis, indicating that BNIP-3 does indeed have a pro-apoptotic role in these cells. Cell-lines in which BNIP-3 was unresponsive to hypoxia were resistant to hypoxia-induced cell death. Thus it may be that down-regulation of BNIP-3, and/or preventing its hypoxia-induction, is a mechanism by which tumours can evade apoptosis. Other mechanisms of bypassing BNIP-3-induced apoptosis have been suggested, such as nuclear sequestration of BNIP-3 in lung cancer (Giatromanolaki et al. 2004) and inhibition of BNIP-3 function in endothelial cells by growth factors such as EGF and IGF (Kothari et al. 2003).

In addition, a direct pro-survival role in the context of hypoxia has been postulated for BNIP-3, through the promotion of autophagy (Mathew et al. 2007). This intracellular degradation process clears damaged proteins or organelles, resulting in the recycling of intracellular constituents which can serve as an alternative energy source for metabolically stressed cells, for example in hypoxia. In the context of defective apoptosis, autophagy can prolong survival by mitigating metabolic stress and genome damage (Degenhardt et al. 2006; Karantza-Wadsworth and White 2007). Hypoxia can induce autophagy, and BNIP-3 plays an important role in this mechanism (Bellot et al. 2009).

My data showed BAX suppression by hypoxia in Caco-2 cells, while Bcl-2 was unaffected. This is in agreement with Erler *et al.* who found that hypoxia downregulated BAX in three CRC cell-lines (SW480, HT29, and HCT116), and had no effect on Bcl-2 expression in two of the cell-lines (Bcl-2 was moderately increased by hypoxia in HCT116) (Erler et al. 2004). Survivin, a member of the Inhibitor of Apoptosis Protein (IAP) family, negatively regulates apoptosis. It has been shown to be upregulated in most tumours and in CRC is associated with poorer prognosis (Abd El-Hameed 2005; Sah et al. 2006). In the CRC cell-lines LS174T and SW480, HIF-1 α has been reported to upregulate survivin expression at both mRNA and protein level (Fan et al. 2008; Wu et al. 2010). This is at odds with the results presented here, in which survivin mRNA was downregulated in Caco-2 cells. XIAP (X-linked inhibitor of apoptosis protein), another member of the IAP family, is overexpressed in CRC tissue (Krajewska et al. 2005). It has been shown to protect against hypoxia-induced apoptosis in cholangiocarcinoma which is particularly resistant to hypoxia, but little is known about its role in CRC (Marienfeld et al. 2004). In Caco-2 cells, neither hypoxia nor DMOG affected XIAP mRNA levels. Thus, hypoxia had opposing effects on the pro-apoptotic genes BNIP-3 (induced) and BAX (suppressed), while anti-apoptotic genes were either suppressed (survivin) or unchanged (Bcl-2, XIAP).

Discrepancies between my data and the literature suggests cell-specificity in the hypoxia response of apoptosis genes. Functional studies are indicated in order to elucidate the consequence of these alterations.

Due to their induction in hypoxic tissue and limited expression in normal tissue, both CA-IX and GLUT-1 are used as experimental markers of hypoxia (Swinson et al. 2003; Chung et al. 2009). Overexpression of both is seen in CRC, and is associated with poor prognosis (Cooper et al. 2003; Korkeila et al. 2009). CA-IX induction by hypoxia has been reported to enhance invasiveness and resistance to hypoxia-induced cell death in HT-29 cells (Sansone et al. 2009). GLUT-1 hypoxia-induction in Caco-2 cells, as seen in my data, has been previously reported at protein level following 48 hours of hypoxia or cobalt chloride stimulation (Carrière et al. 1998).

Alterations in cancer cell adhesion to neighbouring cells and surrounding extracellular matrix are necessary in order for invasion and metastasis to occur, and there is evidence that this is influenced by hypoxia (Paschos et al. 2009). Hypoxia has also been shown to downregulate CD151 protein and mRNA in CRC cell-lines derived from both primary (SW480 and HCT116) and metastatic tumours (SW620), leading to a reduction in the strength of cell-cell and cell-matrix adhesion (Chien et al. 2008). However my study found no change in CD151 expression in Caco-2 cells.

Upregulation of CAMs by hypoxia in cancer has also been reported, as in the case of VE-Cadherin in melanoma cells (Zhao et al. 2012). VE-Cadherin is normally expressed by endothelial rather than epithelial cells, but its expression in cancers of epithelial origin a feature of vasculogenic mimicry (VM) (section 1.3.2.3) (Maniotis et al. 1999). While VM has been described in CRC and found to correlate with poor prognosis, the role of VE-Cadherin and hypoxia in CRC is not known (Baeten et al. 2009). According to my data, as with CD151, VE-Cadherin does not appear to be affected by hypoxia in Caco-2 cells.

My Q-PCR data showed significant upregulation of stabilin-1 (STAB-1) by both hypoxia and DMOG. The array data differed in that no change was seen in DMOG treated cells (fold-change 1.10), although less than 2-fold upregulation was seen in hypoxia (1.94). STAB-1 is a large multifunctional protein, with roles related to scavenging, lymphocyte migration, cell-adhesion and angiogenesis (Salmi et al. 2004; Karikoski et al. 2009). Its role in cancer is poorly understood. It has been found to be upregulated in lymphatic vessels in breast cancer tissue, where it was associated with increased lymph node metastasis (Ammar et al. 2011). VEGF has been found to induce STAB-1 expression in endothelial cells, while in breast cancer hypoxia has been reported to upregulate STAB-1 in tumour associated macrophages (Wary et al. 2003; Movahedi et al. 2010). Conversely, in other cell types such as monocytes and bone-marrow-

derived stem-cells, hypoxia has been shown to downregulate STAB-1 expression (Bosco et al. 2006; Ong et al. 2010). To my knowledge, expression of STAB-1 has not been previously reported in CRC. Of note, previous work in our lab using the same angiogenesis array had shown downregulation of STAB-1 by DMOG in Caco-2 (hypoxia was not investigated). Expression of cell adhesion molecules can be influenced by cell-cell contact (Kobayashi et al. 1992; Kunz et al. 1996), so that confluence levels in adherent cells such as Caco-2 may be expected to affect expression. It was noted that previous experiments had been conducted with cells at 30% confluence, whereas cells in my experiments were at 50%. In an experiment with cells exposed to hypoxia at 15%, 30% and 60% confluence, STAB-1 expression relative to normoxia was reduced in the first two conditions, but was increased at 60% (data not shown). This suggests that cell-cell contact can alter the direction of hypoxia response. Further experiments are required to confirm this finding, and it would be interesting to explore the underlying mechanisms.

Angiopoietin-1 and -2 (ANGPT-1, ANGPT-2), which are functionally antagonistic, and play a central role in angiogenesis. ANGPT-1 recruits mural cells, and promotes assembly and maturation of blood vessels leading to vessel stabilisation and normalisation; conversely, ANGPT-2 is stored by endothelial cells and destabilises vessels, inducing vascular sprouting and branching (Brindle et al. 2006; Fagiani et al. 2011). Overexpression of ANGPT-1 suppresses angiogenesis and tumour growth in CRC, while ANGPT-2 has the opposite effect (Ahmad et al. 2001b; Stoeltzing et al. 2003). However, in other cancers ANGPT-1 can enhance angiogenesis (Machein et al. 2004), and the aberrant vessels resulting from ANGPT-2 overexpression can lead to reduced tumour growth (Yu and Stamenkovic 2001). Hypoxic induction of both ANGPT-1 and ANGPT-2 has been reported in endothelial cells (Skuli et al. 2009), and of ANGPT-2 in CRC cell-lines (Gu et al. 2006). Interestingly, marked hypoxic downregulation of ANGPT-1 has been reported in rat glioma cells (Enholm et al. 1997; Abdulmalek et al. 2001). In my array data, ANGPT-1 was upregulated by both hypoxia and DMOG in Caco-2 cells. ANGPT-2 was also upregulated by hypoxia, but not by DMOG. This suggests a HIF-independent component to hypoxic ANGPT induction, although it is interesting to note that an HRE has been identified in the ANGPT-2 promoter (Simon et al. 2008).

ANGPTL-4 has remarkably diverse functions, and in addition to angiogenesis, it is involved in lipid metabolism, cell-differentiation, vascular permeability and wound healing (Zhu et al. 2012). The structural complexity of the ANGPTL-4 protein underlies this multifunctionality. Its role in cancer is reviewed by Tan *et al.* (Tan et al. 2012). Elevated expression levels have been detected in several cancers, including CRC, in which expression correlates with venous invasion and metastasis (Nakayama et al. 2011). ANGPTL-4 is induced by fasting and can be upregulated by TGF- β 1 (see below) (Padua et al. 2008). There is evidence that ANGPTL-4 can be both pro- and anti-tumourigenic. It enhances tumour proliferation, inhibits apoptosis, and promotes

metastasis (Padua et al. 2008; Zhang et al. 2008; Huang et al. 2011; Kim et al. 2011). However, it has also been reported to inhibit angiogenesis and metastasis (Ito et al. 2003; Galaup et al. 2006). The controversy as to its roles may be due in part to context or tissue specificity, and to the complexity of its structure (it is often unclear which isomer is under investigation). HIF-dependent hypoxia upregulation is seen in breast cancer cells, and an HRE has been identified in the ANGPTL-4 promoter (Hu et al. 2009; Zhang et al. 2012). Upregulation by hypoxia has not been previously reported in Caco-2, although it has been seen in other CRC cell-lines (Kim et al. 2011). The role of HIF in regulating ANGPTL-4 is investigated in Chapter 4.

ANGPTL-3, which is closely related to ANGPTL-4 but is much less well understood. There are differences in expression patterns, regulation and function (Li 2006). As well as being involved in lipid metabolism, ANGPTL-3 has been reported to stimulate endothelial cell adhesion and migration with similar potency to VEGF (Camenisch et al. 2002). Hypoxic induction of ANGPTL-3, as shown here in Caco-2 cells, has not been previously reported.

As described in the Chapter 1 (section 1.2.3), the Eph/ephrin family is the largest family of receptor tyrosine kinase (RTK) systems. The 13 receptors (Eph) and 9 ligands (ephrins) are classified as either class A or B based on sequence homology and binding preferences. Ligands generally bind receptors in the same class, but interactions across the classes can occur (Himanen et al. 2004), thus a large number of receptor-ligand combinations is thus possible. In addition to the unique bidirectionality of Eph-ephrin interactions, this contributes to the ability of the Eph/ephrin family to regulate numerous diverse functions including axonal guidance, cell migration and angiogenesis. Aberrant Eph/ephrin expression is seen in several cancers including CRC, and correlates with stage and prognosis (Miyazaki et al. 2003; Fox and Kandpal 2004; Jubb et al. 2005; Herath et al. 2006; Herath et al. 2009). The precise role of Eph-ephrin interactions in CRC is complex, and is reviewed by Herath and Boyd (Herath and Boyd 2010). In general terms, several Eph receptors which may play an oncogenic role are upregulated in early CRC. Subsequent gene silencing is seen in more advanced tumours, which may play a role in invasion and metastasis. Ephrin-A3 is one of the least investigated ephrin ligands. In normal tissues, it is most abundantly expressed in the brain (Davis et al. 1994). Low levels are seen in normal colon, with equal expression in colon tumours (Hafner et al. 2004). In the lung, marked upregulation is seen in tumour versus normal tissue. Hypoxic induction of ephrin-A3 mRNA has been reported in endothelial cells, although interestingly ephrin-A3 protein was downregulated (Fasanaro et al. 2008). In our laboratory, hypoxic induction of ephrin-A3 mRNA has been shown in rheumatoid fibroblasts (Larsen et al. 2012). Upregulation of several Eph receptors and ephrins by hypoxia has been reported in hepatoma and prostate cancer cell-lines (Vihanto et al. 2005), although this study did not investigate ephrin-A3. To my knowledge, the hypoxic induction of ephrin-A3 seen in Caco-2 has not been previously reported in CRC, and is further investigated in Chapter 4.

As well as degrading of ECM proteins (exposing ECM-bound receptor binding sites and regulatory molecules), MMPs can interact directly with growth factor binding proteins, cell-adhesion molecules and other proteinases (Egeblad and Werb 2002; Rupp et al. 2008). As well as having critical functions in normal physiology, they play a central role in cancer invasion and metastasis. Upregulation of MMPs is seen in most types of cancer, including CRC, and is associated with poor prognosis (Egeblad and Werb 2002). Both MMP-2 and MMP-9, which are produced by stromal cells as well as cancer cells, are upregulated in CRC and associated with angiogenesis, advanced tumour stage, increased metastasis and poorer survival (Grigioni et al. 1994; Zeng and Guillem 1996; Zeng et al. 1996; Kim and Kim 1999; Kikuchi et al. 2000). Conversely, decreased TIMP-3 expression in CRC tissue has been reported to be associated with advanced stage, poorer differentiation and increased metastasis (Bai et al. 2007). Hypoxia induction of both MMP-2 and MMP-9 has been previously reported in the CRC cell-line LoVo (Wu et al. 2008). However in this current study, only MMP-9 was induced by hypoxia in Caco-2 cells.

TGF- β 1 is a multifunctional cytokine which plays an important role in regulating immune responses to cancer, as well as controlling numerous cellular processes in virtually all cell types. It has a tumour suppressive effect in the early stages of cancer, but promotes tumourigenicity as cancers progress (Massagué 2008). CRC patients have elevated serum TGF- β 1 levels, and the overexpression seen in tumour tissue correlates with VEGF expression, MVD and metastasis (Tsushima et al. 1996; Xiong et al. 2002a; Xiong et al. 2002b). TGF- β 1 overexpression is also related to poor prognosis, but the correlation is seen in early rather than late stage CRC (Gulubova et al. 2010). Lower TGF- β 1 expression is seen in high grade tumours and those with lymph node metastases than in early tumours (Bacman et al. 2007). Similarly, Abajo *et al.* have reported that cell-lines derived from CRC metastases show lower TGF- β 1 expression than those from primary tumours such as Caco-2 (Abajo et al. 2012). There is little published data on the effect of hypoxia on TGF- β 1. Correlation of TGF- β 1 and HIF-1 α expression has been reported in CRC (Wincewicz et al. 2010). Direct evidence of hypoxic TGF- β 1 induction is scarce, with a single study using SW480 CRC cells reporting upregulation of TGF- β 1 protein (Xian et al. 2011). Hypoxic upregulation of TGF- β 1 mRNA, as shown in Caco-2 cells herein, has not previously been reported. The role of HIF in this induction is investigated in Chapter 4.

Summary

In this chapter, the responses of Caco-2 cells to hypoxia and DMOG were characterised. Expression of both HIF isoforms, and their upregulation at the protein level by both hypoxia and DMOG was confirmed. This was associated with upregulation of known HIF targets. Hypoxia responses of genes involved in apoptosis and cell-adhesion were also characterised. Using a gene array, a group of 9 angiogenesis genes that were upregulated by both hypoxia and DMOG were identified. In this cell-line, hypoxia-dependence of ANGPTL-4 and TGF- β 1 has not been previously described. To my knowledge, this is the first report of regulation of EFNA-3 by hypoxia in a cancer cell-line. In the subsequent chapter, the role of the HIF- α isoforms in the regulation of some of these genes was investigated further.

Chapter 4

4 ROLE OF HIF- α ISOFORMS IN THE HYPOXIA-MEDIATED RESPONSE OF CACO-2 CELLS

4.1 Introduction

While the two main HIF- α isoforms were initially thought to be functionally redundant, there is increasing evidence that they play different roles in cellular responses to changes in oxygen tension (Esteban et al. 2006). Generally, HIF-1 α is thought to regulate genes involved in metabolism, regulating glycolysis and glucose uptake, as well as angiogenesis, while HIF-2 α has been reported to regulate genes involved in cell proliferation and tumourigenesis (Muz et al. 2009). The controversy in the published literature regarding the role of HIF- α isoforms in CRC has been reviewed in Chapter 1. While some studies suggest that HIF-1 α but not HIF-2 α expression is associated with markers of angiogenesis and poor prognosis, others propound the opposite view (Yoshimura et al. 2004; Rasheed et al. 2009). *In vitro* studies of cell proliferation are similarly contradictory (Franovic et al. 2009; Imamura et al. 2009).

My results described in Chapter 3 demonstrate that hypoxia and DMOG upregulate HIF- α protein and induce expression of genes involved in angiogenesis in Caco-2 cells. Studies examining the relative role of HIF- α isoforms in Caco-2 hypoxia responses are limited. Bruning *et al.* found that HIF-1 α but not HIF-2 α suppressed hypoxic upregulation of micro-RNAs, showing that this interaction is important in the regulation of HIF-1 α levels in prolonged hypoxia (Bruning et al. 2011). Kikuchi *et al.* used Caco-2 cells in a study showing that K-ras and BRAF mutations can affect HIF-1 α and HIF-2 α expression differentially, but this required induction of ectopic expression of the mutations, since these cells have wild-type forms of both genes (Kikuchi et al. 2009). With regard to angiogenesis, Mizukami *et al.* demonstrated that hypoxic induction of VEGF in Caco-2 cells was only partly due to HIF-1 α activity, but in this study significant levels of HIF-2 α were not detected (Mizukami et al. 2004). A study by Zgouras *et al.* showing that HIF-1 α regulates butyrate-induced normoxic VEGF expression in Caco-2 cells did not investigate the possible involvement of HIF-2 α (Baba et al. 2010). Moreover, while studies have linked HIF-1 α expression with apoptosis in Caco-2, none examined the role of HIF-2 α (Yoshimura et al. 2004; Franovic et al. 2009).

4.2 Objectives

The discovery of the gene-regulating properties of dsRNA by Fire and Mello (Fire et al. 1998) led to the award of the Nobel Prize for Medicine in 2006. RNA interference (RNAi) has since become a valuable tool for studying the function of specific genes (Cleven et al. 2008). Introducing short-interfering RNA (siRNA) sequences identical to a specific mRNA allows the

silencing of the target gene. This technique was selected to investigate the effect of selectively silencing either HIF-1 α or HIF-2 α on the Caco-2 responses mediated by hypoxia and DMOG described in Chapter 3.

4.3 Results

4.3.1 Optimisation Of Transfection Protocol

Gene silencing using siRNA transfection was utilised to investigate the relative contributions of the HIF- α isoforms to the Caco-2 hypoxia responses described in Chapter 3. A series of optimisation experiments were first carried out using siHIF-1 α , varying siRNA concentrations, transfection agents, cell density and serum starvation. Transfection efficiency, expressed as percent knockdown (% KD) was calculated as follows:

$$\% \text{ KD} = \left\{ 1 - \left(\frac{[\text{HIF-1}\alpha]_{\text{siHIF-1}\alpha}}{[\text{HIF-1}\alpha]_{\text{siControl}}} \right) \right\} \times 100$$

where $[\text{HIF-1}\alpha]_{\text{siHIF-1}\alpha}$ is HIF-1 α mRNA expression after siHIF-1 α treatment, and $[\text{HIF-1}\alpha]_{\text{siControl}}$ is HIF-1 α mRNA expression after siControl treatment at the same concentration.

In the first experiment, two different concentrations of siRNA and three different volumes of lipofectamine were used (Figure 4.1). As recommended by the manufacturer, a lipofectamine volume of 1 μ L (in a final transfection volume of 600 μ L) was used, as well as 0.5 μ L and 1.5 μ L. Cells were at 50% confluence at time of transfection, and were serum-starved for 4 hours prior to addition of the serum- and antibiotic-free transfection mix. After 6 hours transfection, medium was supplemented with serum and antibiotics. Experiments were carried out in 24-well plates.

Using 50nM siRNA, HIF-1 α knockdown was 35% with 0.5 μ L lipofectamine (siHIF-1 α expression 0.67 ± 0.06 versus 1.00 ± 0.09 siControl; $p < 0.01$), 47% with 1.0 μ L (0.56 ± 0.06 versus 1.05 ± 0.11 ; $p < 0.001$), and 39% with 1.5 μ L (0.63 ± 0.05 versus 1.02 ± 0.08 ; $p < 0.01$). At 100nM siRNA, differences between siHIF-1 α and siControl did not reach statistical significance. With 0.5 μ L lipofectamine HIF-1 α expression was slightly increased by transfection (1.17 ± 0.20 versus 1.01 ± 0.02 , -16% KD), and was weakly knocked down with 1.0 μ L (0.39 ± 0.01 versus 0.51 ± 0.09 , 23% KD; not significant), and 1.5 μ L (0.65 ± 0.04 versus 0.82 ± 0.01 , 21% KD; not significant). The greatest transfection efficiency was seen using 50nM siRNA and 1.0 μ L lipofectamine (47% KD), and these conditions were used in subsequent experiments. This same siRNA against HIF-1 α consistently resulted in >85% KD in other cell types in our laboratory.

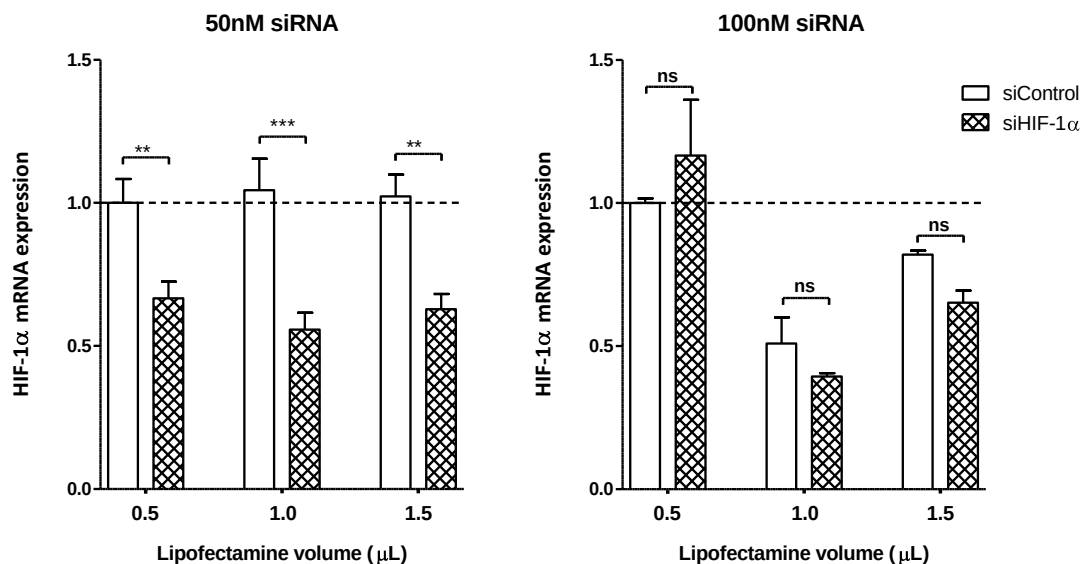


Figure 4.1 Transfection with different siRNA concentrations and lipofectamine volumes
Caco-2 cells were seeded at a density of 1.5×10^5 cells/cm². On reaching 50% confluence, cells were serum-starved for 4 hours before transfection with siHIF-1 α or siControl at 50 or 100nM, using 0.5, 1.0 or 1.5 μ L of lipofectamine transfection agent, in serum- and antibiotic-free medium. After 6 hours, medium was supplemented with serum and antibiotics. 48 hours from the start of transfection, RNA was extracted and mRNA expression evaluated by Q-PCR. Cells were incubated in normoxia throughout the experiment. Graphs show HIF-1 α expression at 50nM (left) and 100nM (right) siRNA, normalised to siControl at 0.5 μ L lipofectamine (dashed line). Data are mean $\pm$ SEM from 3 replicate experiments, with ΔC_t analysed by 1-way ANOVA *versus* siControl (** $p < 0.01$, *** $p < 0.001$, ns = not significant).

Next, the effect of confluence at the time of transfection and of prolonged serum starvation was investigated. Cells were seeded in order to reach 25% or 50% confluence at the time of transfection. They were serum-starved for a period of either 4 or 20 hours before transfection with 50nM siRNA with 1.0 μ L lipofectamine for 6 hours (Figure 4.2). mRNA was extracted after 48 hours.

The greatest transfection efficiency, resulting in 71% KD, was seen in cells transfected at 50% confluence and serum-starved for 4 hours (0.29 ± 0.03 for siHIF-1 α *versus* 1.0 for siControl; $p < 0.001$). These were the same conditions that achieved 47% KD in the first experiment (Figure 4.1). 20 hours of serum-starvation resulted in 41% KD (0.28 ± 0.02 *versus* 0.48 ± 0.03 ; $p < 0.01$). For cells transfected at 25% confluence, 4 hour starvation resulted in 29% KD (0.49 ± 0.01 *versus* 0.69 ± 0.06 ; not significant), while 20 hour starvation led to 56% KD (0.26 ± 0.03 *versus* 0.58 ± 0.01 ; $p < 0.01$). However, it appears that longer serum starvation and transfection at lower confluence also reduces HIF-1 α mRNA levels in cells transfected with siControl.

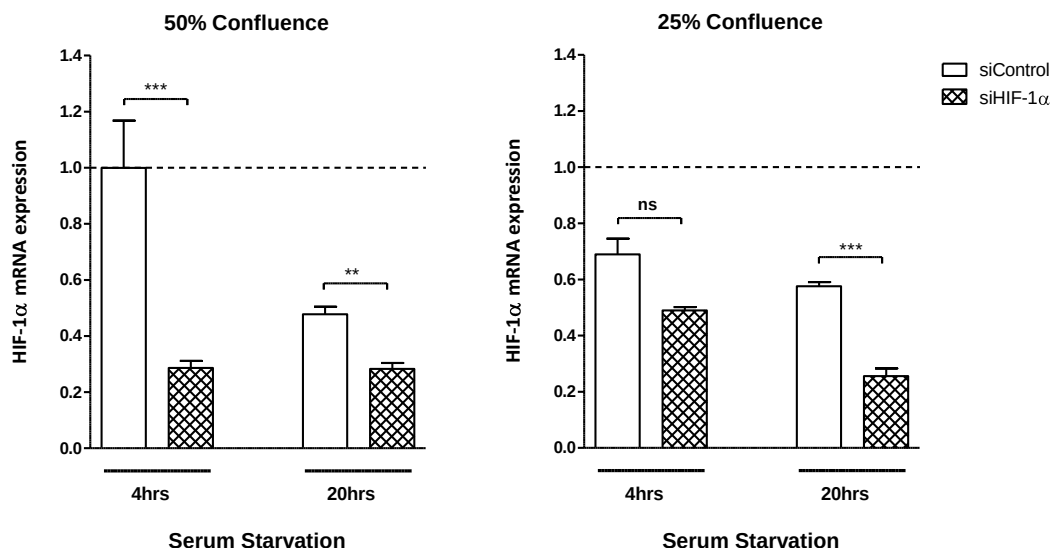


Figure 4.2 Effect of confluence and serum starvation using lipofectamine

Cells were seeded at a density of 1.5 or 0.75×10^5 cells/cm², in order to reach 50% or 25% confluence respectively at the time of transfection. Medium was replaced with serum-free medium 4 or 20 hours before the start of transfection with 50nM siHIF-1α or siControl, using 1.0μL lipofectamine in serum- and antibiotic-free medium. After 6 hours, medium was supplemented with serum and antibiotics. 48 hours from the start of transfection, RNA was extracted and mRNA expression evaluated by Q-PCR. Cells were incubated in normoxia throughout the experiment. Graphs show HIF-1α expression in cells transfected at 50% (left) and 25% (right) confluence, normalised to siControl 50% confluence with 4 hour serum starvation (dashed line). Data are mean $\pm$ SEM from 2 replicate experiments, with ΔC_t analysed by 1-way ANOVA *versus* siControl (** $p < 0.01$, *** $p < 0.001$, ns = not significant).

To determine the effect of transfection agent, the same experiment was repeated using oligofectamine (Figure 4.3). Following manufacturer's instructions, 200nM siRNA was used with 1.0μL oligofectamine in a final volume of 250μL (1:250 dilution). Conditions were otherwise identical to Figure 4.2. In contrast to lipofectamine, the greatest transfection efficiency was seen with 20 hours serum-starvation at 50% confluence, where 77% KD was achieved (0.30 ± 0.02 *versus* 1.30 ± 0.07 ; $p < 0.001$). 4 hours starvation at 50% confluence resulted in 47% KD (0.53 ± 0.04 *versus* 1.0 ; $p < 0.001$). At 25% confluence, 54% KD was seen after 20 hours serum-starvation (0.57 ± 0.05 *versus* 1.22 ± 0.02 ; $p < 0.001$), while at 4 hours, there was no significant knockdown (0.57 ± 0.04 *versus* 0.60 ± 0.13 , 5% KD; not significant).

To ensure that the lack of greater transfection efficiency was not due to a mechanistic effect of silencing the HIF-1α gene, the effect of using a different siRNA was then investigated. After 4 hours serum-starvation, cells were transfected at 50% confluence with siFIH-1 at 50, 100 and 200nM using oligofectamine (Figure 4.4). At 50nM siFIH-1, FIH-1 expression was knocked down by 36% relative to control (0.64 ± 0.03 *versus* 1.0 ± 0.10 ; $p < 0.001$). At 100nM knockdown was 44% (0.59 ± 0.03 *versus* 1.01 ± 0.08 ; $p < 0.001$) and at 200nM it was also 44% (0.69 ± 1.21 *versus* 1.21 ± 0.07 ; $p < 0.001$).

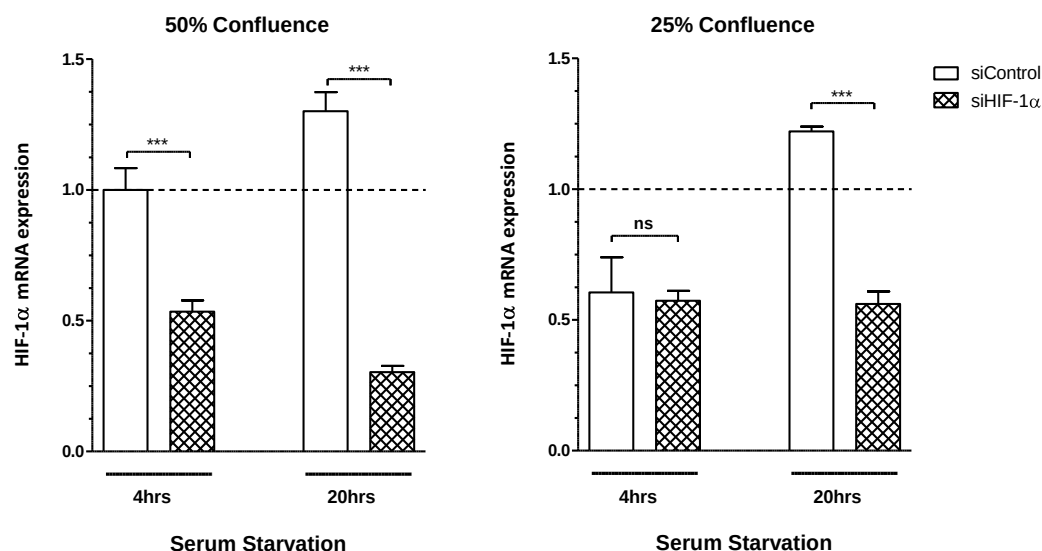


Figure 4.3 Effect of confluence and serum starvation using oligofectamine

Cells were seeded at a density of 1.5 or 0.75×10^5 cells/cm², in order to reach 50% or 25% confluence respectively at the time of transfection. Medium was replaced with serum-free medium 4 or 20 hours before the start of transfection with 200nM siHIF-1α or siControl, using 1.0μL oligofectamine in 250μL serum- and antibiotic-free medium. After 6 hours, 2mL of full medium was added. 48 hours from the start of transfection, RNA was extracted and mRNA expression evaluated by Q-PCR. Cells were incubated in normoxia throughout the experiment. Graphs show HIF-1α expression in cells transfected at 50% (left) and 25% (right) confluence, normalised to siControl 50% confluence with 4 hour serum starvation (dashed line). Data are mean $\pm$ SEM from 2 replicate experiments, with ΔC_t analysed by 1-way ANOVA *versus* siControl (***) $p < 0.001$, ns = not significant).

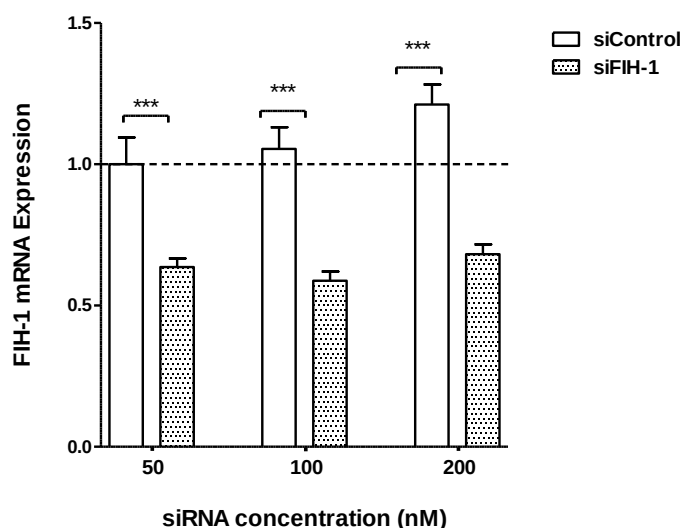


Figure 4.4 Transfection efficiency with siFIH-1 using oligofectamine

Cells were seeded in order to reach 50% confluence at the time of transfection. Medium was replaced with serum-free medium 4 hours before the start of transfection with 50, 100 or 200nM siFIH-1 or siControl, using 1.0μL oligofectamine in 250μL serum- and antibiotic-free medium. After 6 hours, 2mL full medium was added. 48 hours from the start of transfection, RNA was extracted and mRNA expression evaluated by Q-PCR. Cells were incubated in normoxia throughout the experiment. Graphs show FIH-1 expression normalised to 50nM siControl (dashed line). Data are mean $\pm$ SEM from 3 replicate experiments, with ΔC_t analysed by 1-way ANOVA *versus* siControl (***) $p < 0.001$).

In these preliminary experiments, the greatest transfection efficiency (77%) was seen using oligofectamine with cells serum-starved for 20 hours. However, serum-starvation had a profound effect on cell adherence (Figure 4.5), which was taken as a surrogate for viability (section 2.1.1). Cell detachment was seen after 4 hours, which significantly increased with prolonged serum-starvation. In addition, the 77% KD was achieved using the manufacturer's recommended siRNA concentration of 200nM. This was not dissimilar to the 71% KD achieved with 50nM siRNA and 1.0 μ L lipofectamine (Figure 4.2), a level of transfection that compares favourably with the literature (Mizukami et al. 2004; Morel et al. 2008). Therefore the use of oligofectamine was abandoned for subsequent experiments, and cells were not serum-starved prior to transfection, to preserve viability.

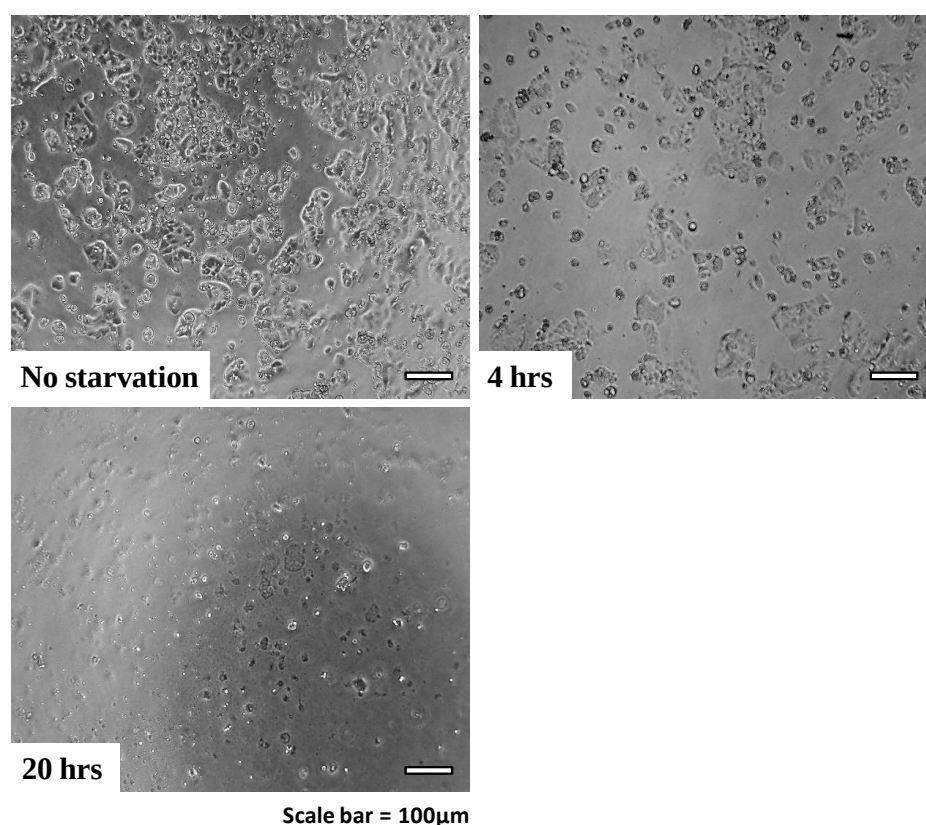


Figure 4.5 Effect of serum starvation on cell attachment

Cells were seeded at 1.5×10^5 cells/cm² in order to reach 50% confluence at transfection. At 4 or 20 hours prior to the start of transfection using oligofectamine, medium was replaced with serum-free medium (or full-medium in the no starvation control, top left). Figure shows photomicrographs taken immediately before the start of transfection. There were progressively fewer adherent cells with increasing duration of starvation. Scale bar represents 100 μ m.

The next step was to scale up the experiments in order to ensure adequate mRNA and protein quantities for hypoxia experiments (Figure 4.6, Figure 4.7). Initially, cells were plated in 6-well plates and were transfected at 50% confluence with no serum starvation. Transfection with 50nM siHIF-1 α , siHIF-2 α or siControl was carried out using 6.0 μ L lipofectamine in a final volume of 2mL (1:300 dilution) of serum- and antibiotic-free medium. After 6 hours, medium

was supplemented with serum and antibiotics. After 24 hours, cells were exposed to hypoxia for a further 24 hours before supernatants were collected, cells lysed and mRNA and protein extracted.

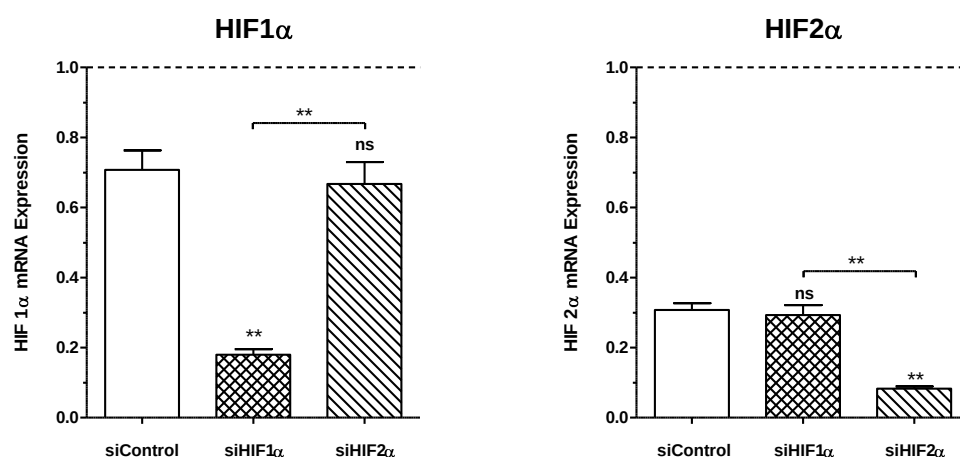


Figure 4.6 Hypoxic HIF- α mRNA expression following selective knockdown

Cells were transfected in triplicate at 50% confluence for 6 hours with 50nM siHIF-1 α , siHIF-2 α or siControl using 1.0 μ L lipofectamine. 24 hours from the start of transfection, cells were placed in hypoxia (1% O₂) for 24 hours with untransfected normoxia (21% O₂) controls. Cells were then lysed, RNA was extracted, and expression evaluated by Q-PCR. Graph shows HIF-1 α (left) and HIF-2 α (right) mRNA expression normalised to untransfected normoxia (dashed line). Data are mean $\pm$ SEM from 3 experiments, with ΔC_t analysed by 1-way ANOVA *versus* siControl unless otherwise shown (** $p < 0.01$, ns = not significant).

Figure 4.6 shows HIF- α mRNA expression. With siHIF-1 α treatment, HIF-1 α expression was knocked down by 75% relative to control (fold-change 0.18 ± 0.02 *versus* 0.71 ± 0.06 ; $p < 0.01$) while HIF-2 α expression was unchanged (0.29 ± 0.03 *versus* 0.31 ± 0.02 , 5% KD; not significant). With siHIF-2 α treatment, HIF-1 α was unchanged (0.67 ± 0.06 *versus* 0.71 ± 0.06 , 6% KD; not significant) while HIF-2 α was knocked down by 72% (0.08 ± 0.01 *versus* 0.31 ± 0.02 ; $p < 0.01$).

Protein expression was then analysed by Western Blot (a representative experiment is shown in Figure 4.7). siHIF-1 α suppressed HIF-1 α protein expression almost completely but did not affect HIF-2 α expression. Conversely, siHIF-2 α significantly reduced HIF-2 α protein but did not affect HIF-1 α . This did not significantly affect cell attachment (Figure 4.8), suggesting that the cells remained viable. This transfection protocol gave a transfection efficiency of greater than 70%, despite the lack of serum starvation, and therefore it was maintained throughout remaining transfection experiments.

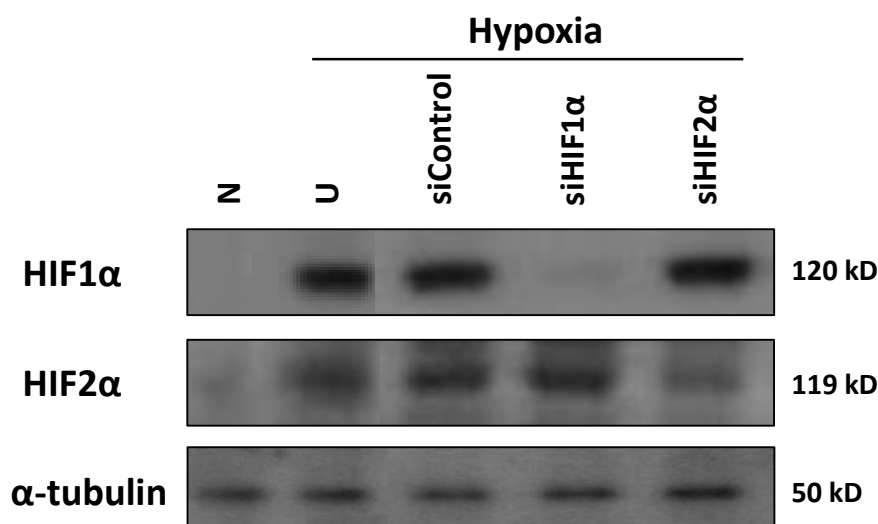


Figure 4.7 Hypoxic HIF- α protein expression following selective HIF knockdown

Cells were transfected at 50% confluence for 6 hours with 50nM siHIF-1 α , siHIF-2 α or siControl using 1.0 μ l lipofectamine, with untransfected control (U). After incubation in normoxia for 24 hours from the start of transfection, cells were placed in hypoxia (1% O₂) for 24 hours, with untreated normoxia (N) control. Cells were then lysed, protein extracted, and expression evaluated by Western Blot. Figure shows HIF-1 α (120kD) and HIF-2 α (119kD) protein expression from a representative experiment, with α -tubulin (50kD) loading control. The blot was cut between N and U to remove an irrelevant lane.

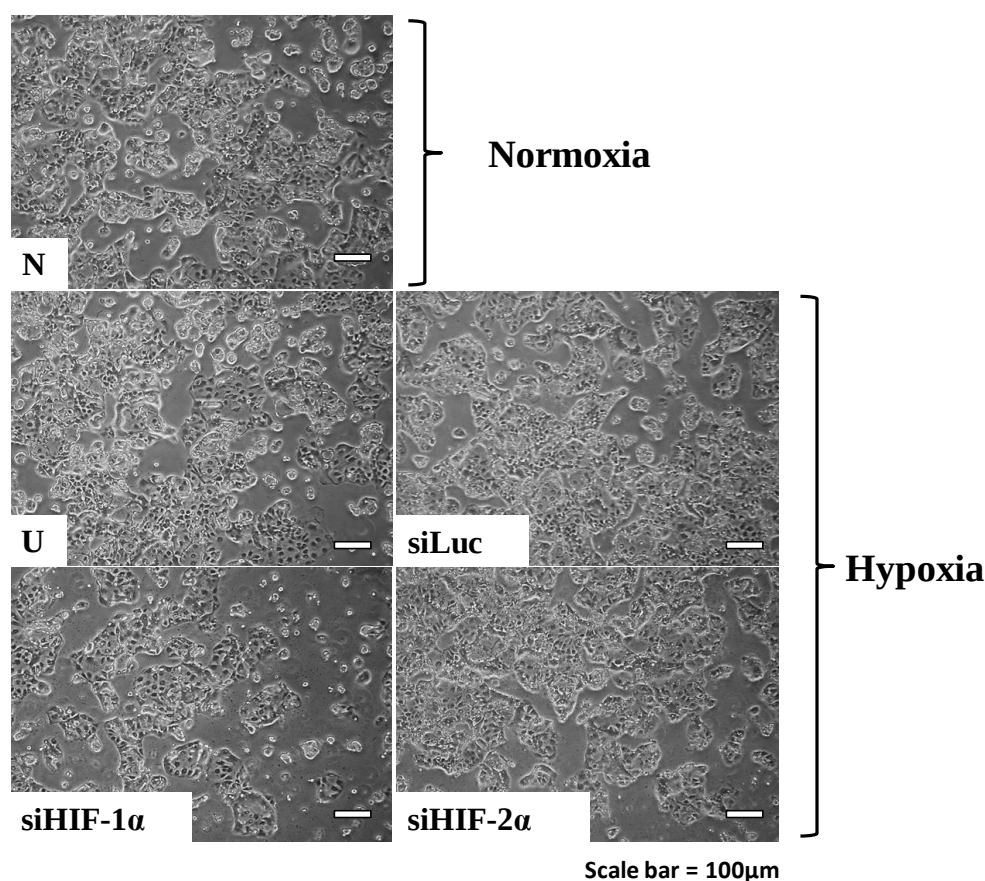


Figure 4.8 Transfected cells remain adherent after 24hours hypoxia

Cells were transfected at 50% confluence for 6 hours with 50nM siHIF-1 α , siHIF-2 α or siControl using 1.0 μ L lipofectamine with untransfected control (U). After incubation in normoxia for 24 hours from the start of transfection, cells were placed in hypoxia (1% O₂) for 24 hours with normoxia (21% O₂) control (N). Figure shows photomicrographs taken at 100x magnification. Scale bar represents 100 μ m.

4.3.2 HIF- α Isoform Knockdown In Hypoxia-Treated Caco-2 Cells

Having established a reliable transfection protocol, the effect of HIF- α isoform knockdown on target genes was investigated.

4.3.2.1 Known HIF Targets

Following transfection with siHIF-1 α or siHIF-2 α , expression of VEGF and BNIP-3 mRNA were evaluated (Figure 4.9). In cells treated with siControl, VEGF mRNA was upregulated by a fold-change of 9.50 ± 1.54 in hypoxia after 24 hours. Expression after siHIF-1 α treatment was significantly reduced to 5.80 ± 0.76 ($p < 0.01$), while there was a more modest reduction with siHIF-2 α , at 7.36 ± 0.97 (not significant). BNIP-3 mRNA expression was markedly reduced by siHIF-1 α compared to siControl (1.83 ± 0.40 versus 5.81 ± 0.82 ; $p < 0.05$), but unchanged by siHIF-2 α (4.94 ± 0.92 ; not significant).

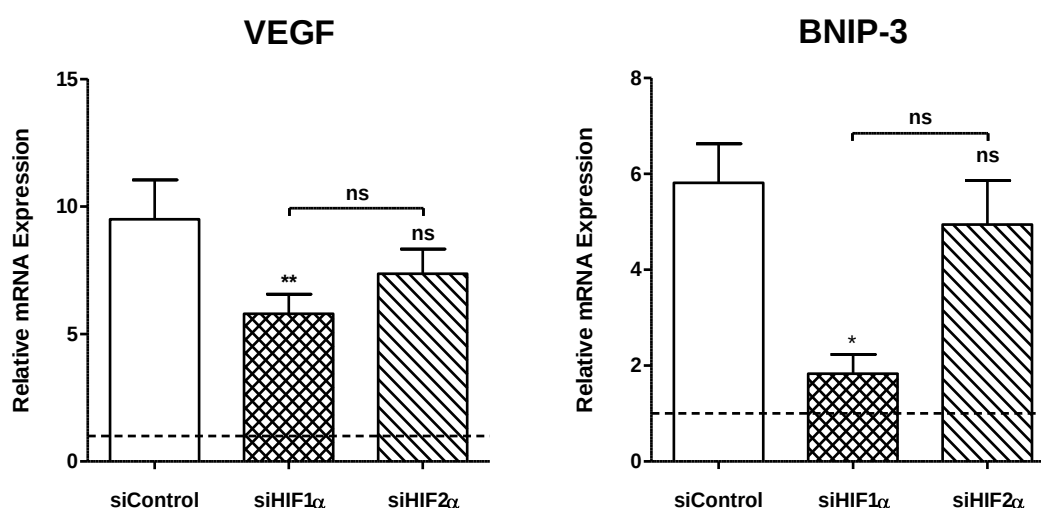


Figure 4.9 HIF- α knockdown effect on hypoxia-induction of known HIF target mRNA

Cells were transfected at 50% confluence for 6 hours with siHIF-1 α , siHIF-2 α or siControl. After 24 hours of incubation, cells were placed in hypoxia (1% O₂) for 24 hours with normoxia controls. RNA was extracted and expression evaluated by Q-PCR. Graph shows VEGF and BNIP-3 mRNA expression normalised to untransfected normoxia (21% O₂, dashed line). Data are mean $\pm$ SEM from at least 3 replicate experiments, analysed by 1-way ANOVA *versus* siControl unless otherwise shown (* $p < 0.05$, ** $p < 0.01$, ns = not significant).

4.3.2.2 Novel Angiogenesis Genes

The role of HIF- α isoforms in the hypoxia-induced upregulation of the novel genes ANGPTL-4, EFNA-3 and TGF- β 1 was next examined (Figure 4.10). Hypoxia-induced ANGPTL-4 mRNA expression in siControl-treated Caco-2 cells (fold-change 12.19 ± 1.01 relative to untreated normoxic control) was attenuated by siHIF-1 α (5.80 ± 0.48 ; $p < 0.001$). Expression was increased slightly by siHIF-2 α (14.38 ± 0.56), but this was not statistically significant. For EFNA-3, mRNA expression was again attenuated by siHIF-1 α relative to siControl (3.65 ± 0.42 *versus*

5.50±0.81 respectively; $p<0.05$), and to a lesser, statistically non-significant extent, by siHIF-2 α (4.28±0.60). Hypoxic TGF- β 1 expression was attenuated both by siHIF-1 α (2.38±0.17 *versus* 3.37±0.19; $p<0.01$), and by siHIF-2 α (2.69±0.17; $p<0.05$).

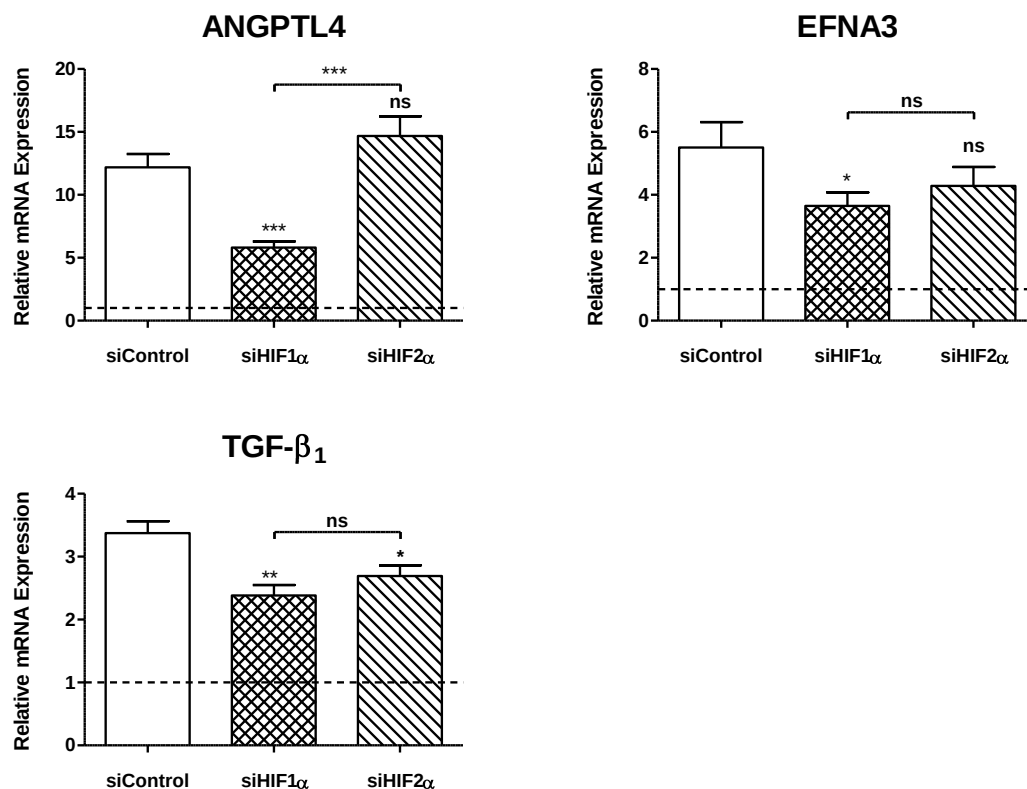


Figure 4.10 Effect of HIF- α isoform knockdown on novel angiogenesis genes

Cells were transfected siHIF-1 α , siHIF-2 α or siControl. After 24 hours of incubation, cells were placed in hypoxia (1% O₂) for 24 hours with normoxia controls. RNA was extracted and expression evaluated by Q-PCR. Graph shows ANGPTL-4, EFNA-3 and TGF- β 1 mRNA expression normalised to untransfected normoxia (21% O₂, dashed line). Data are mean $\pm$ SEM from 5 experiments conducted in triplicate, analysed by 1-way ANOVA *versus* siControl unless otherwise shown (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, ns = not significant).

4.3.2.3 Apoptosis Genes

The effect of HIF- α isoform knockdown on hypoxia-induced apoptosis gene expression was analysed next. As previously shown (Figure 3.7), survivin mRNA expression was reduced in hypoxia (siControl fold-change 0.70±0.05 *versus* untreated normoxic control), and this was unaffected by either siHIF-1 α or siHIF-2 α (0.68±0.07 and 0.70±0.12 respectively; both non-significant). Similarly for BAX, a more modest reduction by hypoxia (0.84±0.11 relative to normoxia; see also Figure 3.7) was unaffected by siHIF-1 α and siHIF-2 α (0.85±0.07 and 0.74±0.08 respectively; both non-significant). XIAP expression was unaffected by hypoxia (siControl 0.95±0.13 *versus* normoxia; see also Figure 3.7), siHIF-1 α (1.16±0.18) or siHIF-2 α (0.91±0.24; all non-significant). Bcl-2 expression was similarly unaffected by hypoxia

(0.99 ± 0.08 ; see also Figure 3.7) or siHIF-1 α (1.03 ± 0.11). A modest reduction in the presence of siHIF-2 α was not statistically significant (0.81 ± 0.12).

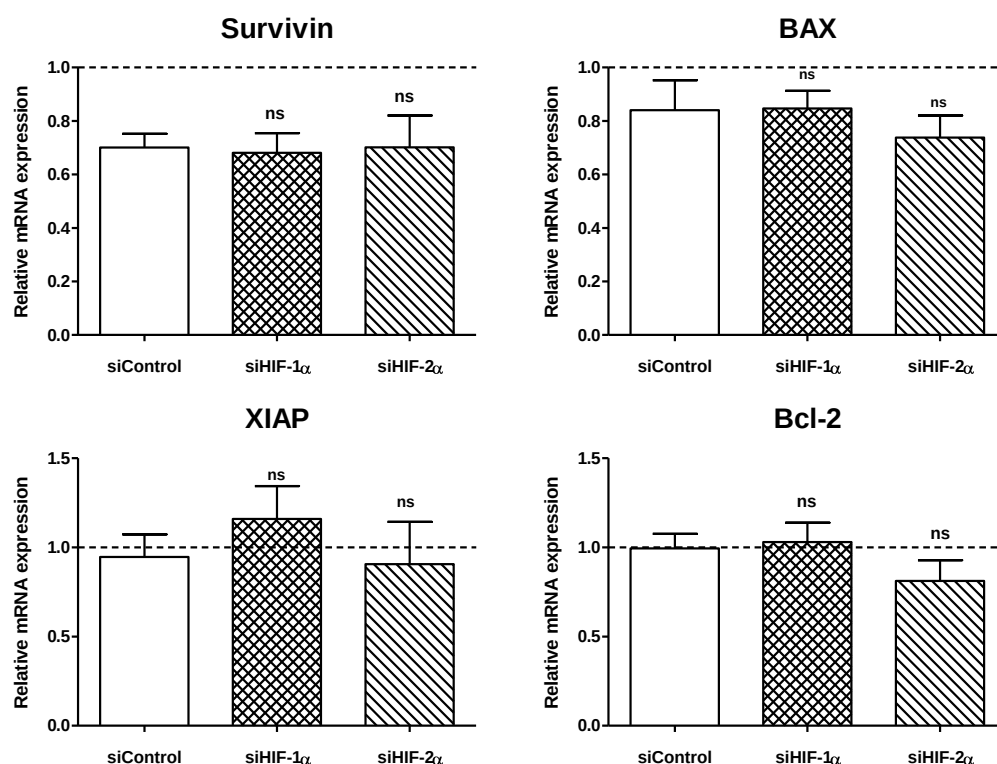


Figure 4.11 Effect of HIF- α isoform knockdown on apoptosis gene expression

Cells were transfected siHIF-1 α , siHIF-2 α or siControl. After 24 hours of incubation, cells were placed in hypoxia (1% O₂) for 24 hours with normoxia controls. RNA was extracted and expression evaluated by Q-PCR. Graph shows BAX, Bcl-2, Survivin and XIAP mRNA expression normalised to untransfected normoxia (21% O₂, dashed line). Data are mean $\pm$ SEM from 2 experiments conducted in triplicate, analysed by 1-way ANOVA *versus* siControl (ns = not significant).

4.3.3 HIF- α isoform knockdown in DMOG-Treated Caco-2 Cells

The experiments in the previous section were repeated using DMOG stimulation rather than hypoxia. Cells were transfected using the protocol developed in Section 4.3.2. After 24 hours, cells were treated with DMOG 1mM for 24 hours before supernatants were collected, cells lysed and mRNA and protein extracted. Figure 4.12 shows HIF- α mRNA expression.

With siHIF-1 α treatment, HIF-1 α expression was knocked down by 81% relative to control (fold-change 0.22 ± 0.11 *versus* 1.11 ± 0.09 ; $p < 0.05$) while HIF-2 α expression was unchanged (1.00 ± 0.21 *versus* 1.11 ± 0.09 , -10% KD; not significant). With siHIF-2 α treatment, HIF-1 α was unchanged (0.99 ± 0.31 *versus* 0.86 ± 0.14 , -15% KD; not significant) while HIF-2 α was knocked down by 92% (0.065 ± 0.02 *versus* 0.86 ± 0.14 ; $p < 0.05$).

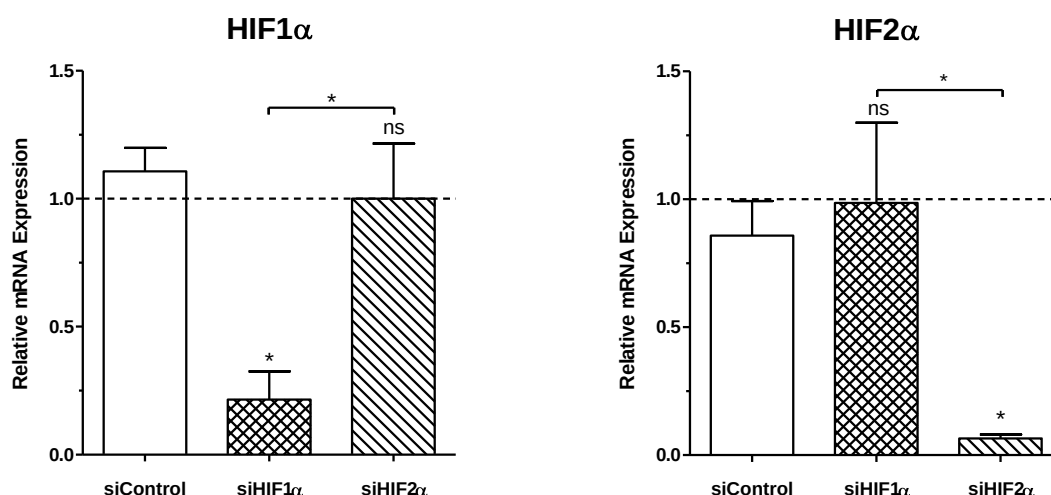


Figure 4.12 DMOG-induced HIF- α mRNA expression following selective knockdown

Cells were transfected in duplicate at 50% confluence for 6 hours with 50nM siHIF-1 α , siHIF-2 α or siControl using lipofectamine. 24 hours from the start of transfection, cells were treated with DMOG 1mM for 24 hours. Cells were then lysed, RNA was extracted, and expression evaluated by Q-PCR. Graph shows HIF-1 α (left) and HIF-2 α (right) mRNA expression normalised to untransfected untreated control (dashed line). Data are mean $\pm$ SEM from 1 experiment conducted in triplicate, with ΔC_t analysed by 1-way ANOVA *versus* siControl unless otherwise shown (* $p < 0.05$, ns = not significant).

Protein expression was analysed by Western Blot (a representative experiment is shown in Figure 4.13). siHIF-1 α suppressed HIF-1 α protein expression markedly. HIF-2 α expression was perhaps marginally enhanced. Conversely, siHIF-2 α significantly reduced HIF-2 α protein while slightly increasing HIF-1 α expression.

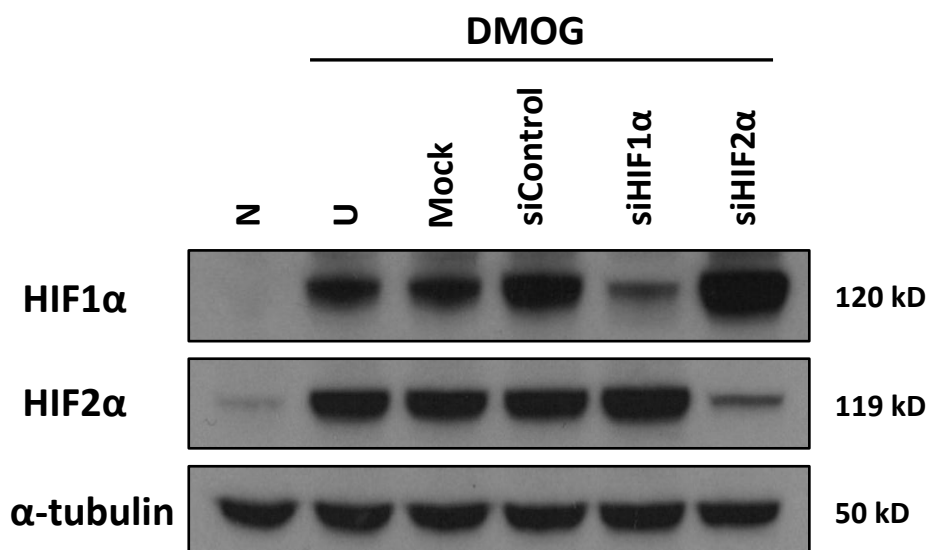


Figure 4.13 DMOG-induced HIF- α protein expression following transfection

Cells were transfected at 50% confluence for 6 hours using lipofectamine only (mock), or with 50nM siHIF-1 α , siHIF-2 α or siControl, or left untransfected (U). 24 hours from the start of transfection, cells were treated with 1mM DMOG for a further 24 hours with untreated “normoxia” (N) controls. Cells were then lysed, protein extracted, and expression evaluated by Western Blot. Figure shows HIF-1 α (120kD) and HIF-2 α (119kD) protein expression from a representative experiment, with α -tubulin (50kD) loading control.

4.3.3.1 Known HIF Targets

Following transfection with siHIF-1 α or siHIF-2 α , expression of VEGF and BNIP-3 were evaluated at mRNA (Figure 4.14) and protein level (Figure 4.15). In cells treated with siControl, VEGF mRNA was upregulated by a fold-change of 8.83 ± 0.94 in hypoxia. Expression after siHIF-1 α treatment was significantly reduced to 4.02 ± 1.18 ($p < 0.05$), while there was no reduction with siHIF-2 α , at 10.59 ± 0.75 (not significant). BNIP-3 mRNA expression was markedly reduced by siHIF-1 α compared to siControl (0.95 ± 0.24 versus 7.32 ± 1.46 ; $p < 0.01$), but unchanged by siHIF-2 α (9.65 ± 2.39 ; not significant).

To determine whether changes in mRNA were also reflected at the protein levels, supernatants were assayed for VEGF, and cell lysates for BNIP-3 (Figure 4.15). VEGF protein was undetected in normoxia. In DMOG-treated cells, expression was upregulated to a similar extent for untransfected, mock and siControl, at 25.71 ± 0.96 , 24.41 ± 3.1 and 26.35 ± 3.89 pg/mL respectively. siHIF-1 α resulted in a 0.46-fold reduction of VEGF expression to 14.1 ± 0.53 pg/mL ($p < 0.05$), while siHIF-2 α increased expression 1.44-fold to 37.94 ± 3.89 pg/mL, although this was not statistically significant. Low BNIP-3 expression in normoxia was markedly and equally upregulated for untransfected, mock and siControl. This was substantially reduced by siHIF-1 α , but unaffected by siHIF-2 α .

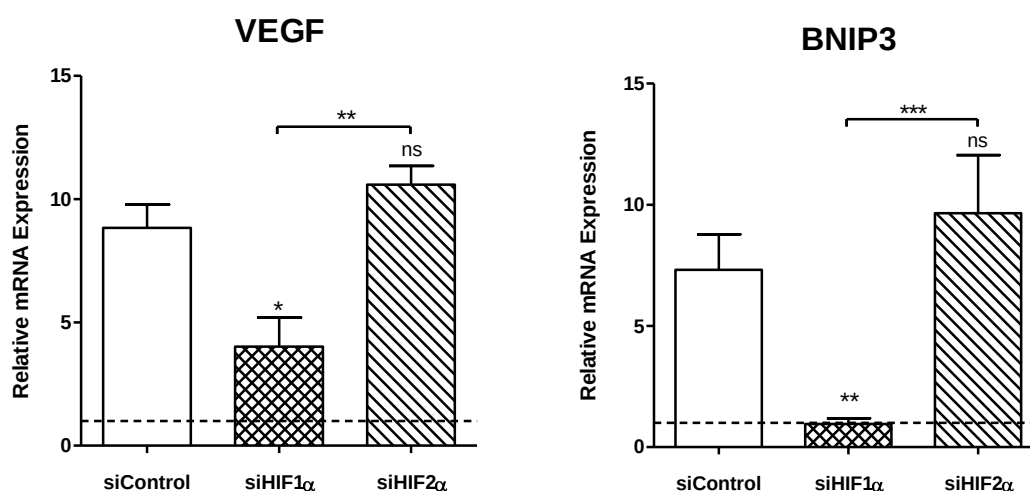


Figure 4.14 DMOG-induced HIF target mRNA expression

Cells were transfected at 50% confluence for 6 hours with 50nM siHIF-1 α , siHIF-2 α or siControl. After incubation in normoxia for 24 hours from the start of transfection, cells treated with 1mM DMOG for 24 hours with normoxia (untreated) controls. Cells were then lysed, mRNA extracted, and expression evaluated by Q-PCR. Graph shows VEGF (left) and BNIP-3 (right) mRNA expression normalised to untransfected normoxia (dashed line). Data are mean $\pm$ SEM from 1 experiment conducted in triplicate, with ΔC_t analysed by 1-way ANOVA (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant, versus siControl unless otherwise shown).

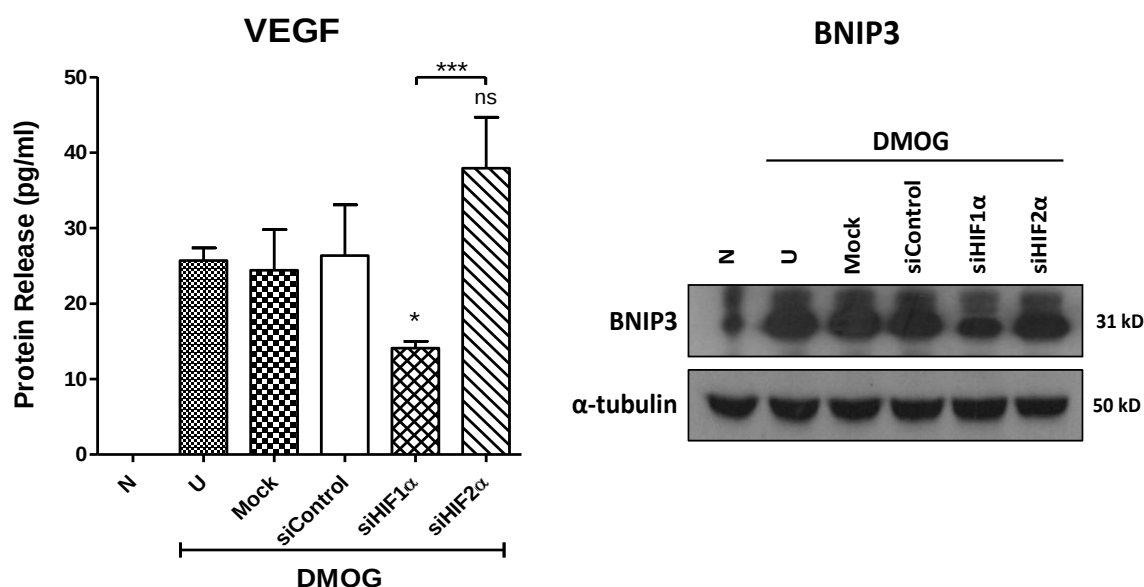


Figure 4.15 HIF- α knockdown effect on known HIF target proteins

Cells were transfected for 6 hours with lipofectamine only (mock) or with 50nM siHIF-1 α , siHIF-2 α or siControl and incubated in normoxia (N). 24 hours from the start of transfection, cells treated with 1mM DMOG for 24 hours with untreated controls. Supernatants were collected, cells lysed and protein extracted, and protein expression was evaluated. VEGF protein in the supernatant was measured by ELISA (left). Data are mean $\pm$ SD from 1 experiment, with ΔC_t analysed by 1-way ANOVA (* $p < 0.05$, *** $p < 0.001$, ns = not significant, *versus* siControl unless otherwise shown). BNIP-3 protein expression (31kD) was evaluated by Western Blot (right), with α -tubulin (50kD) loading control. U = untransfected cells exposed to DMOG.

4.3.3.2 Novel Angiogenesis Genes

The HIF- α isoform dependence of novel genes identified in Chapter 3 is shown in Figure 4.16. DMOG was a potent inducer of ANGPTL-4 mRNA, with a fold-change of 31.82 ± 2.23 for siControl relative to untreated control. siHIF-1 α reduced this substantially to 5.74 ± 1.07 , while siHIF-2 α increased it to 52.37 ± 3.52 ($p < 0.001$ for both). A fold-change in EFNA-3 expression of 7.35 ± 0.76 for siControl was reduced by siHIF-1 α (4.36 ± 0.49), and to a lesser extent by siHIF-2 α (5.20 ± 0.51), but did not reach statistical significance in either case. TGF- β 1 expression was 21.48 ± 0.52 for siControl, which was reduced to 2.46 ± 0.74 ($p < 0.001$) by siHIF-1 α . siHIF-2 α had little effect (14.73 ± 2.38 , non-significant).

Data from Chapter 3 showed that PHD-2 and PHD-3 were upregulated by hypoxia and DMOG. Differential regulation of these enzymes by HIF-1 α and HIF-2 α has been reported in glioblastoma (Henze et al. 2010). The effect of selective HIF- α knockdown in Caco-2 cells was investigated next.

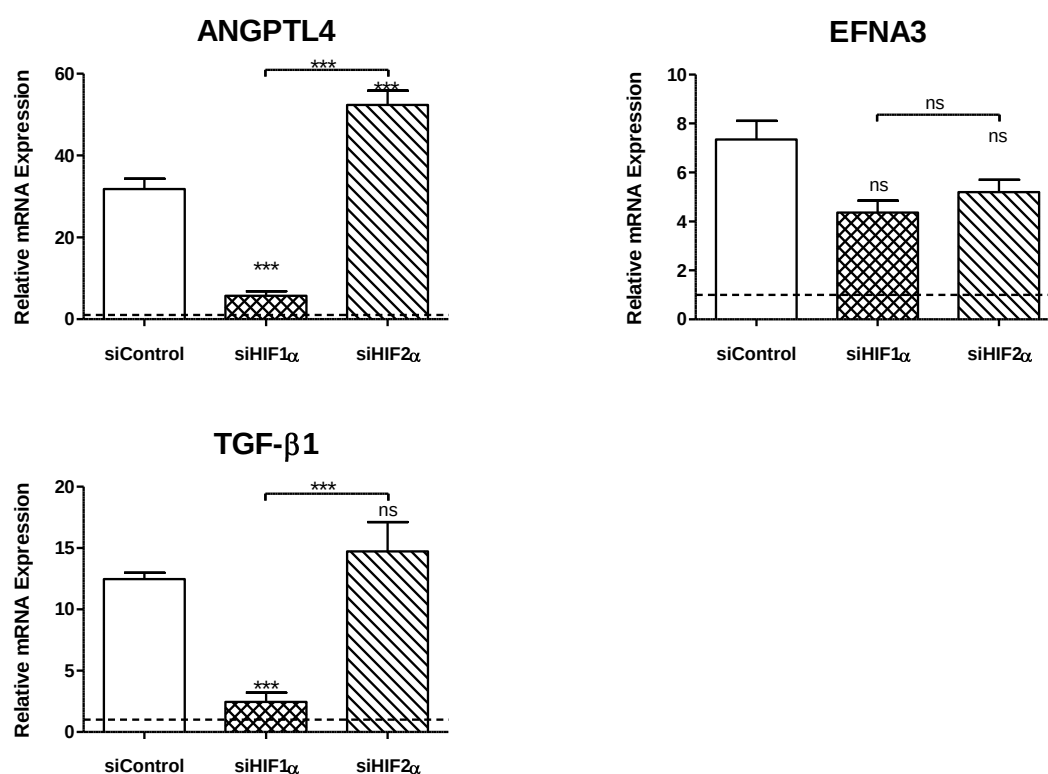


Figure 4.16 Effect of HIF- α isoform knockdown on novel angiogenesis genes in DMOG

Cells were transfected at 50% confluence for 6 hours with 50nM siHIF-1 α , siHIF-2 α or siControl. 24 hours from the start of transfection, cells treated with 1mM DMOG for 24 hours with normoxia (untreated) controls. Cells were then lysed, mRNA extracted, and expression evaluated by Q-PCR. Graph shows ANGPTL-4 (top left) EFNA-3 (top right) and TGF- β 1 (bottom) mRNA expression normalised to untransfected normoxia (dashed line). Data are mean $\pm$ SEM from 1 experiment conducted in triplicate, with ΔC_t analysed by 1-way ANOVA (***) $p < 0.001$, ns = not significant, *versus* siControl unless otherwise shown).

The results of these experiments, which sought to characterise the relative roles of HIF-1 α and HIF-2 α in the regulation of downstream HIF targets, are summarised in Table 4.1 below. Isoform-specificity in the HIF-dependent regulation of HIF regulatory enzymes was also investigated (next section).

Table 4.1 Isoform dependence of HIF-targets in Caco-2 cells

Table summarises HIF- α isoform dependence at mRNA level in Caco-2 cells stimulated with either hypoxia or DMOG. ND = not determined. (Brackets indicate statistically non-significant; * also seen at protein level.)

GENE	Hypoxia	DMOG
ANGPTL-4	HIF-1 α (HIF-2 α negative role)	HIF-1 α HIF-2 α negative role
EFNA-3	HIF-1 α (HIF-2 α)	(HIF-1 α , HIF-2 α)
TGF-β1	HIF-1 α HIF-2 α	HIF-1 α (HIF-2 α negative role)
VEGF	HIF-1 α (HIF-2 α)	HIF-1* (HIF-2 α negative role)*
BNIP-3	HIF-1 α	HIF-1 α *

4.3.3.3 HIF Regulatory Enzymes

DMOG-induced expression of PHD-2 and PHD-3 mRNA following HIF- α knockdown is shown in Figure 4.17. PHD-2 expression for siControl was 3.07 ± 0.34 , and was markedly reduced to 0.80 ± 0.11 ($p < 0.001$) by siHIF-1 α but unaffected by siHIF-2 α at 3.15 ± 0.54 (not significant). PHD-3 was strongly upregulated in siControl at 7.96 ± 1.29 . This was markedly suppressed by siHIF-2 α at 3.24 ± 0.31 ($p < 0.01$). A more modest effect was seen with siHIF-1 α at 4.81 ± 0.96 , which did not reach statistical significance.

It has been previously reported that PHD-2 is exclusively HIF-1 α dependent, as shown here, while PHD-3 is a target for both HIF- α isoforms (Aprelikova et al. 2004). Since HIF-1 α suppression of PHD-3 was not statistically significant at mRNA level, expression at the protein level was evaluated by Western Blot (Figure 4.18). The modest reduction of PHD-3 mRNA by siHIF-1 α was not seen at protein level, but there was significant protein downregulation following siHIF-2 α treatment.

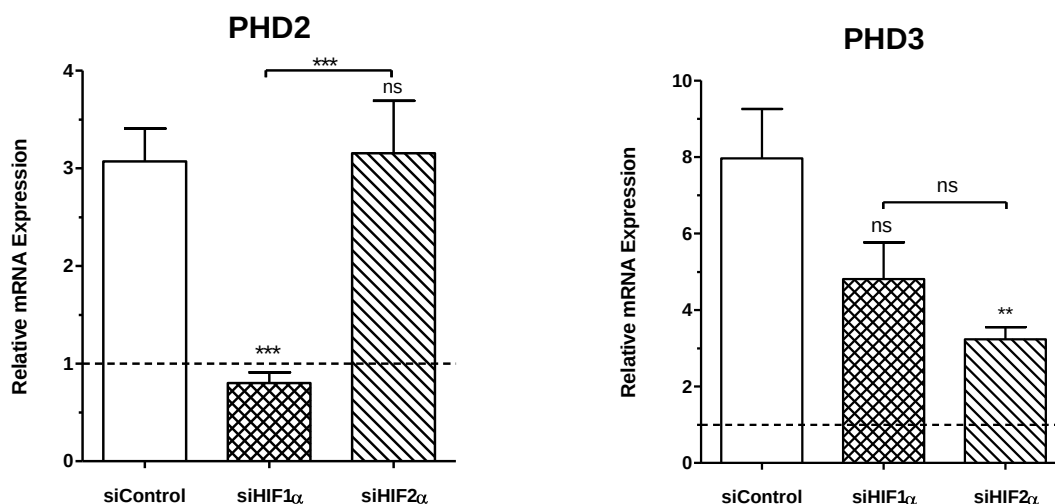


Figure 4.17 Effect of HIF- α isoform knockdown on DMOG-induced HIF regulatory enzymes at mRNA level

Cells were transfected at 50% confluence for 6 hours with 50nM siHIF-1 α , siHIF-2 α or siControl. 24 hours from the start of transfection, cells treated with 1mM DMOG for 24 hours with normoxia (untreated) controls. Cells were then lysed, mRNA extracted, and expression evaluated by Q-PCR. Graph shows PHD2 (left) and PHD3 (right) mRNA expression normalised to untransfected normoxia (dashed line). Data are mean $\pm$ SEM from 1 experiment conducted in triplicate, with ΔC_t analysed by 1-way ANOVA (** $p < 0.01$, *** $p < 0.001$, ns = not significant, versus siControl unless otherwise shown).

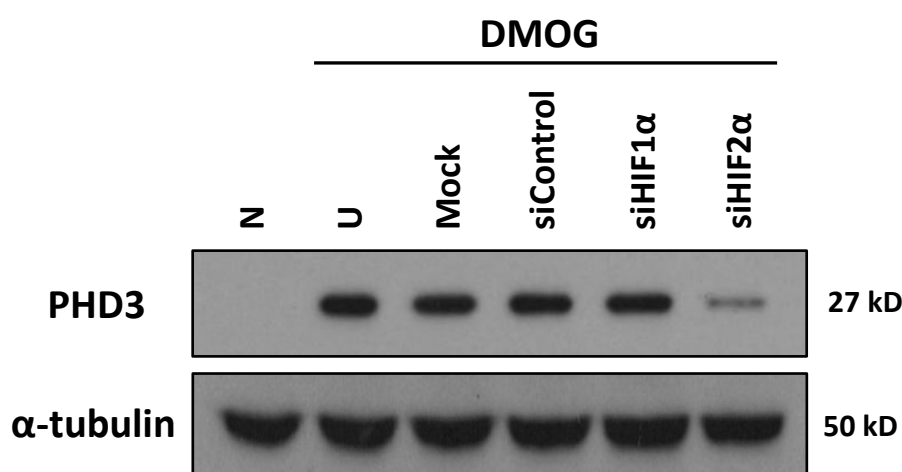


Figure 4.18 Effect of HIF- α isoform knockdown on DMOG-induced PHD-3 protein

Cells were transfected at 50% confluence for 6 hours using lipofectamine only (mock) or with 50nM siHIF-1 α , siHIF-2 α or siControl, or left untransfected (U). 24 hours from the start of transfection, cells were treated with 1mM DMOG for 24 hours with untreated “normoxia” (N) controls. Cells were then lysed, protein extracted, and PHD-3 (27kD) expression was evaluated by Western Blot, with α -tubulin (50kD) loading control.

4.4 Discussion

In contrast to other cell-types with which our lab has experience (e.g. synovial fibroblasts, skin fibroblasts, endothelial cells, smooth muscle cells), Caco-2 cells initially proved to be difficult to transfect. The manufacturer's literature provided only general guidelines for optimising transfection, while the published literature was often unhelpful, in that transfection protocols lacked sufficient detail. Despite variation in siRNA concentration, transfection agent type and concentration, cell-confluence and serum-starvation, mRNA transfection efficiencies remained initially between 50 and 70%. This is comparable to some of the literature. For example Mizukami *et al.* and Morel *et al.* reported transfection efficiencies of 50-60% (Mizukami *et al.* 2004; Morel *et al.* 2008). However, as the experiments were scaled up, and perhaps as proficiency with the technique improved, knockdown efficiencies of 70-90% were achieved. Of note, at these levels, HIF- α protein expression was markedly reduced.

It is interesting to note that although HIF-1 protein levels were upregulated by hypoxia, mRNA expression was significantly downregulated. This supports predominance of post-translational regulation of HIF- α (by PHD and FIH-1) under hypoxic conditions. Hypoxic mRNA suppression was more pronounced for HIF-2 α than for HIF-1 α , but was not seen with DMOG stimulation. This is in contrast to a study by Bruning *et al.*, who reported HIF-1 α suppression, but no change in HIF-2 α in Caco-2 cells. In this study, cells were fed with preconditioned media to achieve instantaneous hypoxia, whereas in my study, hypoxia was achieved gradually – whether this explains the discrepancy is unclear. Hypoxic suppression of HIF α mRNA has been described previously in mouse liver (Wenger *et al.* 1998) and for both HIF-1 α (Larsen *et al.* 2012) and HIF-2 α in rheumatoid synoviocytes in our own laboratory (Dr. Barbara Muz, unpublished data) (Table 4.2). Interestingly, in lung tissue while HIF-1 α was downregulated, HIF-2 α was in fact upregulated (Uchida *et al.* 2004; Chen *et al.* 2006) by hypoxia. Further studies reveal variation in HIF- α isoform responses to chemical inhibition of PHD and FIH-1. Differential regulation was observed in DMOG-stimulated synoviocytes by Muz, and in hepatoma cells stimulated with 2,2 dipyridyl (DP, a PHD- and FIH-1-inhibiting hypoxia-mimetic) by Warnecke *et al.* (Warnecke *et al.* 2008).

The downregulation of HIF-1 α mRNA may be explained by negative feedback involving the naturally-occurring antisense transcript (aHIF), which destabilises HIF-1 α mRNA by recognising homologous sequences (Thrash-Bingham and Tartof 1999). Our group has shown that hypoxia, and more potently by DMOG, upregulate aHIF in synoviocytes, subsequently decreasing HIF-1 α mRNA (Dr Helene Larsen, unpublished data). Moreover, aHIF contains an HRE and is HIF-1 α -dependent, implicating it in a negative feedback loop with HIF-1 α . A further mechanism by which HIF-1 α mRNA may be downregulated by hypoxia involves a so-called reverse HRE

(rHRE) sequence reported in the HIF-1 α promoter region (Xu et al. 2012). rHREs are conventional HRE sequences found on the minus DNA strand, and are associated with transcriptional repression by hypoxia (Naravula and Colgan 2001; Mazure et al. 2002). Xu *et al.* reported that both HIF-1 α and HIF-2 α protein can bind the rHRE in the HIF-1 α promoter, resulting in epigenetic modifications that suppress HIF-1 α transcription. This was observed in renal cancer cells that are deficient in vHL, and resulted in a shift from HIF-1 α to HIF-2 α dominance, resulting in tumour progression. Whether a similar mechanism exists in vHL-competent cells such as Caco-2 is unknown.

Table 4.2 Regulation of HIF- α mRNA by hypoxia and enzyme inhibitors

There is variation in HIF-1 α and HIF-2 α mRNA expression in response to hypoxia and/or hypoxia-mimetic drugs in different tissues. (DMOG: Dimethyloxalylglycine; DP: 2,2dipyridyl. ND = not determined.)

Tissue	HIF-1 α		HIF-2 α		Reference
	Hypoxia	PHD/FIH-1 inhibition	Hypoxia	PHD/FIH-1 inhibition	
Synoviocytes	↓	↓↓	↓	↑	Muz 2011 (DMOG)
Liver	↓ ^a	→ ^b	ND	↑ ^b	^a Wenger 1998 ^b Warnecke 2008 (DP)
Lung	↓	ND	↑	ND	Uchida 2004, Chen 2006
Colon	↓	ND	→	ND	Bruning 2011

Further insight into the complex regulation of HIF-1 α comes from the hypoxic induction of microRNAs (miRNAs) which has been reported in breast and colon cancer cell-lines (Camps et al. 2008; Bruning et al. 2011). miRNAs are short non-coding RNA sequences that bind complementary sequences on target mRNA resulting in translational repression and/or target degradation. Bruning *et al.* described the HIF-1 α -dependence of miRNA-155 in Caco-2 cells, concluding that it is involved in a negative feedback loop that suppresses HIF-1 α activity in cells exposed to prolonged hypoxia.

The experiments described herein show greater suppression of HIF-2 α than HIF-1 α mRNA by hypoxia. It seems likely that there is a negative feedback mechanism that regulates HIF-2 α in a similar manner to HIF-1 α , but neither an antisense transcript, an rHRE nor an miRNA has been described.

Of the PHD enzymes, PHD-1 and PHD-3 are distinct from PHD-2. The latter, which is older in evolutionary terms (Rytönen et al. 2011), is more involved in the regulation of HIF-1 α (also more “primitive” than HIF-2 α), and is itself a HIF-1 α rather than a HIF-2 α target (Aprelikova et al. 2004), as supported by the data in the current study. Aprelikova *et al.* also reported that regulation of PHD-2 levels was biphasic, with HIF-independent mechanisms responsible for initial induction (up to 4 hours), and HIF-1 α coming into play in prolonged hypoxia. By contrast PHD-1 and PHD-3 are involved in HIF-2 α regulation (Takeda et al. 2007; Aragonés et al. 2008; Takeda et al. 2008). However, as in the current study, PHD-3 can be regulated by both HIF-1 α and HIF-2 α (Aprelikova et al. 2004), although HIF-2 α is the more potent.

HIF- α isoform dependence of HIF target genes is shown in Table 4.1. In experiments conducted in hypoxia, HIF-1 α knockdown was consistently found to reduce hypoxic VEGF expression at the mRNA level. HIF-2 α knockdown had a similar but weaker effect, which did not reach statistical significance. However following DMOG stimulation, while HIF-1 α knockdown again downregulated VEGF, there was a trend towards upregulation of VEGF following HIF-2 α knockdown. This was more pronounced at mRNA than at protein level (although again did not reach statistical significance). The results in hypoxia are supported by data from Imamura *et al.* (Imamura et al. 2009). Using the CRC cell-line SW480, HIF-1 α or HIF-2 α were stably knocked down and the cells introduced into immunodeficient mice. VEGF protein expression in the resulting tumours was 50% lower than controls in HIF-1 α deficient tumours, and 20% lower in HIF-2 α deficient tumours. In the breast cancer cell-line MCF-7, however, while HIF-1 α knockdown suppressed VEGF protein expression, HIF-2 α knockdown in fact increased it, reflecting my findings in DMOG stimulated Caco-2 (Carroll and Ashcroft 2006).

This reciprocal relationship between the HIF- α isoforms has also been reported in 786-O cells (Raval et al. 2005). Carroll and Ashcroft also found VEGF to be almost exclusively HIF-2 α dependent in the renal carcinoma cell-lines RCC4 and 786-O. Both of these cell-lines lack a functioning vHL, and HIF-2 α exclusivity of VEGF regulation in vHL deficiency has also been reported in liver tissue (Rankin et al. 2008). In the canonical HIF pathway, vHL-mediated ubiquitination leads to degradation of both HIF- α isoforms. However, that vHL function is closely related to HIF- α isoform selectivity is supported by data from Koh *et al.* who described a novel E3-ubiquitin ligase, the hypoxia-associated factor (HAF) (Shahrzad et al. 2010). HAF selectively targets HIF-1 α for vHL-independent degradation, while promoting HIF-2 α activity (Dang et al. 2006). In addition, p53 is a negative regulator of HIF-1 α (Ravi et al. 2000). It may be that suppression of HIF-1 α in these tumours promotes a switch to HIF-2 α -dependent hypoxia responses (Koh et al. 2011; Seton-Rogers 2011).

Regulation of TGF- β 1 closely resembled that of VEGF, with both HIF- α isoforms regulating it in hypoxia (HIF-1 α slightly predominating), and a trend towards a suppressive role for HIF-2 α following DMOG stimulation. This is supported by a study in lung epithelial cells which showed that hypoxic induction of TGF- β 1 is dependent on both HIF- α isoforms (DMOG not investigated) (Zhou et al. 2009). The interaction between TGF- β 1, hypoxia and HIF is complex. In normoxic conditions, TGF- β 1 can induce both HIF- α isoforms (Chae et al. 2011) and VEGF (Sánchez-Elsner et al. 2001). TGF- β 1 signalling is mediated by the Smad family of transcriptional co-modulators, one of which (Smad3) can cooperate with both HIF-1 α and HIF-2 α . However, the interactions have opposing effects, with HIF-2 α interaction increasing TGF- β 1-induced VEGF, and HIF-1 α suppressing it.

Of the 5 apoptosis genes investigated, only BNIP-3 showed HIF-dependence. The hypoxic induction of BNIP-3 is well-described in the literature. As in the current study, BNIP-3 has been widely described as a HIF-1 α target (Sowter et al. 2001; Greijer and van der Wall 2004). In the presence of acidosis, such as is seen in prolonged hypoxia, BNIP-3 leads to the elimination by apoptosis of stressed cells (Wang 2012). This requires the presence of wild-type p53, which is itself stabilised by HIF-1 α . The pro-apoptotic effect of BNIP-3 is abrogated if p53 is mutated, as in Caco-2 cells and in many cancers, protecting the cells from hypoxia-induced cell-death (Fei et al. 2004). As discussed in Chapter 3, both up- and downregulation of BNIP-3 expression has been described in CRC (Bacon et al. 2006). In this study, BNIP-3 was induced by hypoxia in some CRC cell-lines but not others. Cell-lines that did not upregulate BNIP-3 were resistant to hypoxia-induced apoptosis. However, Bacon *et al.* found no correlation between BNIP-3 hypoxia response and p53 status. Of note, in the vHL-deficient renal cancer cell-line RCC4, which constitutively overexpresses HIF-1 α and HIF-2 α , BNIP-3 is unresponsive to HIF-1 α but strongly down-regulated by HIF-2 α (Raval et al. 2005).

The three novel hypoxia-induced angiogenesis genes identified by PCR array in Chapter 3 were all found to be HIF targets. My data show clear HIF-1 α regulation of ANGPTL-4 in Caco-2. In addition, a reciprocal effect was seen with HIF-2 α (as with VEGF). This was seen in both hypoxia and DMOG stimulation, while for VEGF it was seen following DMOG only. HIF-1 α dependence of ANGPTL-4 has been reported in endothelial cells and cardiomyocytes (Belanger et al. 2002; Manalo et al. 2005). These studies did not investigate HIF-2 α . Two studies have shown downregulation of ANGPTL-4 following inhibition of either HIF- α isoform, in breast cancer (Zhang et al. 2012) and CRC (Burkitt et al. 2009).

The function of Ephrin-A3 has been discussed in detail in Chapter 3. Bidirectional signalling of the ephrin ligand-receptor system regulate cell motility by modulating changes in attraction and repulsion between cells, guiding migrating cells along precise paths. Aberrant

expression of ephrin receptors in CRC correlates with prognosis (Jubb et al. 2005; Herath et al. 2009). Hypoxia induction of ephrin-A3 has been described in prostate and hepatoma cell-lines (Vihanto et al. 2005) but not in CRC. The involvement of HIF in ephrin-A3 regulation has not previously been investigated in cancer. Recent work in our laboratory identified ephrin-A3 as a HIF-1 α target in rheumatoid fibroblasts, with no involvement of HIF-2 α (Larsen et al. 2012). While my experiments confirm that ephrin-A3 is HIF regulated in Caco-2 cells, the data suggests that both isoforms may play a role, although the effect of HIF-2 α did not reach statistical significance.

The difference between HIF- α isoform specificity in hypoxia versus DMOG stimulated cells, such as is most clearly seen with VEGF and TGF- β 1, indicates that there may be HIF-independent mechanisms involved in the regulation of these genes. HIF-independent mechanisms of hypoxic VEGF regulation have been postulated (Mizukami et al. 2007). This may be through HIF-independent hypoxic induction of other transcription factors such as NF- κ B and AP-1 (Schmidt et al. 2007), or through miRNAs (Hua et al. 2006). HIF-independence of VEGF and TGF- β 1 hypoxia induction in Caco-2 could be confirmed by double knockdown of HIF-1 α and HIF-2 α , or knockdown of HIF- β .

Preliminary experiments to investigate effects of HIF knockdown on Caco-2 cell function were performed (section 0). Firstly, Caco-2 cells transfected with siHIF-1 α or siHIF-2 α were incubated in normoxia for 5 days (photographed daily, Figure 7.1), and trypsinised and counted at the end of the experiment. Cell counts were reduced approximately equally in siHIF-1 α and siHIF-2 α treated cells (by 34% and 40% respectively; Figure 7.2). This may have been due to reduced proliferation or increased cell-death/apoptosis. Therefore a BrdU (bromodeoxyuridine) proliferation assay was used, in which the labelled thymidine analog (BrdU) is incorporated into cellular DNA in proportion to proliferation. Proliferation was measured at 24 and 48 hours in cells transfected with siHIF-1 α or siHIF-2 α (Figure 7.3). At both time-points, siHIF-2 α reduced proliferation (by 26.9% at 24 hours, and 32% at 48 hours; $p < 0.01$ *versus* siControl for both), while small changes for siHIF-1 α were statistically not significant (9% reduction at 24 hours, 14% increase at 48hrs). These experiments need to be repeated in hypoxia. Cautious interpretation of these preliminary results suggests that HIF-2 α may have an anti-proliferative role in Caco-2 cells, as has been reported in HCT116 cells (Franovic et al. 2009). Attempts to examine HIF isoform roles in angiogenic function, by investigating the effect of conditioned media on endothelial cell migration, encountered technical difficulties, and further work is required to optimise the assay.

Summary

The objective of this chapter was to investigate the relative contributions of the HIF- α isoforms in CRC tumourigenesis. Some studies point to the primacy of HIF-1 α regulation, while others suggest that HIF-2 α is dominant. The data from my experiments demonstrate that of angiogenesis-related genes investigated, all were subject to HIF-1 α -mediated upregulation when stimulated by either hypoxia or DMOG. For some genes, namely ephrin-A3, TGF- β 1 and PHD-3, HIF-2 α also contributed to hypoxia induction. An interesting finding was a possible reciprocal role of the isoforms, particularly in DMOG stimulated cells, which has been previously suggested in breast and renal cancer. The importance of the HIF pathway in cancer makes it an attractive target for therapeutic inhibition. This data suggests that selective HIF-1 α inhibition may be undesirable. Further work, including simultaneous knockdown of HIF- α isoforms and functional studies, is required to further examine the significance of these results.

As with other cancers, it is clear that CRC is a highly heterogeneous disease, and that tumour cells from different patients, and within a single tumour, differ significantly in genotype and phenotype. Translation of results derived from a monoclonal cell-line is therefore potentially problematic. This can be partly addressed by the use of multiple cell-lines. In order to address this issue, I adopted an alternative strategy. The experiments in Chapter 5 sought to establish primary cultures derived directly from tumour tissue, in order to compare hypoxia-responses with those of Caco-2 cells. Concordance between results from the two cell-types would lend support to the validity of the results in Chapter 3 and Chapter 4.

Chapter 5

5 TUMOUR-DERIVED CULTURES OF PRIMARY CRC CELLS: CHARACTERISATION OF RESPONSES TO HYPOXIA

5.1 Introduction

In the experiments described in Chapter 3 and Chapter 4, the roles of the HIF- α isoforms in the regulation of angiogenic genes in CRC were explored using the Caco-2 cell-line. Cell-lines are invaluable in furthering our understanding of tumour biology and elucidating potential targets for therapies. Their ubiquity in cell biology research stems from a number of factors, including ease of use, ready availability and relatively low financial cost. They allow the focussed investigation of dynamic cell responses to myriad stimuli in carefully controlled experimental conditions. Perhaps their most valuable feature is their clonogenic capability, which allows them to consistently reproduce cell populations with identical properties. The homogeneity and stability of these cell-systems allows consistent experimental observations, strengthening the validity of comparisons between different studies. In addition, the biology of commonly-used lines is well-characterised, and careful selection of the appropriate cell-line allows isolation of certain factors for investigation, such as the presence or absence of particular mutations.

However, cell-lines suffer considerable limitations. Most importantly, the homogeneity that yields the benefits described above means that the *in vitro* experimental environment is, by necessity, far from representative of the *in vivo* milieu. The tumour microenvironment consists of a wide variety of both cellular and non-cellular elements, all of which interact in a dynamic and highly complex manner. It has a significant contributory role in the transformation, survival, proliferation, invasion and metastasis of cancer cells. The ability to translate findings from cell-line experiments is therefore significantly limited by the very lack of complexity that makes cell-line cultures so useful. Furthermore, a fundamental concept in the use of such models is that the cell-line selected represents its originating cell-type. However, the genetic stability of cell-lines is by no means absolute. Over time, cell-lines may acquire multiple genetic mutations, losing important characteristics of the original cell and gaining novel ones. Since genetic instability is one of the hallmarks of cancer, this “genotypic drift” is of particular concern in lines derived from cancer cells. In addition to this, cross-contamination and misidentification has been reported to affect up to a third of cell-lines held in major repositories (Hughes et al. 2007). Finally, in recent years it has become clear that CRC is a highly heterogeneous disease, with a great degree of variation between tumours from different patients, and even between synchronous tumours in the same patient (Mizukami et al. 2004). Indeed, even within a single tumour, a high degree of genotypic and phenotypic diversity is seen (Morel et al. 2008). This further limits the ability of a homogenous cell-line to model the behaviour of “real” cancer cells. The use of several cell-lines

can only partially mitigate this deficiency. Taken together, these factors may call into question the validity of data obtained from cell-line work.

One way to address some of these limitations is to establish *de novo* cell cultures directly from patient tissue. Such cultures retain differentiated features of the originating tissue, allowing more robust translation of experimental data. To this end, a variety of methods have been employed to establish CRC from ascitic effusions, metastatic tissues (regional lymph nodes and distant metastatic sites), and primary tumours. While cell-lines are more readily established from ascitic fluid than from primary tumours, these usually contain non-epithelial cells (e.g. mesothelial cells, lymphocytes) which, although more readily propagated, do not originate from the site of primary tumour, and thus are not representative of the primary cancer. Alternative methods of establishing cell cultures directly from tumour tissue include explantation techniques, or dissociation of cancer cells from primary tissue either by enzymatic digestion or mechanical spill-out technique. The procedure of disaggregating solid colorectal tumours to produce cell suspensions by enzymatic digestion, typically with collagenase, has previously been described (Thrash-Bingham and Tartof 1999; Aragonés et al. 2008; Camps et al. 2008; Rankin et al. 2008; Rytönen et al. 2011). A similar technique has been successfully employed in our laboratory to culture cells from human atherosclerotic plaques and rheumatoid arthritic (RA) tissue, allowing the study of pathways underlying their pro-inflammatory components (Brennan et al. 1989a; Feldmann and Maini 2003; Monaco et al. 2004). Significantly, the technique employed in our laboratory did not aim to establish *de novo* cell-lines, but used short-term cultures of less than 7 days. Also significant is that these cultures are heterogeneous, since they are not purified or enriched to isolate a particular cell-type. Therefore they more accurately model *in vivo* inter-cellular interactions.

The establishment of CRC cell cultures from primary tissue is challenging. Problems encountered include microbial infection, contamination and overgrowth of fibroblasts, selective isolation of viable clusters of cancer cells from a milieu of dead and dying cells, and the inability of cell-lines to grow and propagate (Franks 1976; Park et al. 2004). The difficulty in overcoming these challenges in order to sustain such cultures beyond a few days is borne out by previous work in our laboratory (Mr. T. Khong, MD(Res) thesis, Imperial College London, 2009; unpublished data).

In this study, the decision was taken to bypass the difficulties of attempting to maintain tumour-derived cultures (TDCs) indefinitely, but instead to use the cultures while they remained viable (within 48 hours of being isolated), to investigate hypoxia-responses. In addition to the TDCs more closely resembling *in vivo* behaviour than Caco-2 cells, the use of the same hypoxia-

stimulation system in both cell-types would allow more robust comparisons to be drawn, providing a degree of validation for the results presented in Chapters 3 and 4.

5.2 Objectives

The objective of this part of my study was to establish short-term *ex-vivo* cultures of a heterogeneous cell population isolated from CRC tissue following surgical resection of the tumour. These tumour-derived cultures (TDCs) would be characterised by assessing morphology, as well evaluating expression of epithelial cell markers Ep-CAM (Epithelial Cell Adhesion Molecule) and VE-Cadherin, and the CRC tumour marker carcinoembryonic antigen (CEA). Subsequently, TDCs would be maintained for less than 48 hours during which time hypoxia-stimulation studies would be performed, with a focus on angiogenic gene expression. Results would then be compared with those of Caco-2 cells described in Chapter 3.

5.3 Results

5.3.1 Demographics of CRC patients in study

Tissue was obtained from a total of 34 patients, of which 15 were male and 19 were female (Table 5.1). The male to female ratio was 0.79:1, which is lower than the national figure of 1.23:1, but this difference was not statistically significant ($p=0.19$, Chi-squared test). The median age overall was 70.5 (range 51-91, mean $\pm$ SEM 69.9 $\pm$ 1.7 years) which is close to the national median age at diagnosis of 71. There was no difference in mean age between genders (male 69.9 $\pm$ 2.4 years, female 69.1 $\pm$ 2.4 years; $p=0.68$, unpaired t-test).

Table 5.1 Patient demographics for tumour samples

Table shows age and sex information for patients from whom tumour samples were obtained.

Parameter	All	Male	Female
n	34	15	19
%	-	44.1	55.9
Mean Age, years $\pm$ SEM (range)	69.9 $\pm$ 1.7 (51-91)	69.1 $\pm$ 2.4 (56-85)	70.5 $\pm$ 2.4 (51-91)

Pathological characteristics obtained from the histopathology reports for all 34 patients are shown in Table 5.2. A total of 17 (50%) of the tumours were located in the right colon (proximal to splenic flexure), 8 (23.5%) were in the left colon (between splenic flexure and recto-sigmoid junction), and 9 (26.5%) were rectal (distal to recto-sigmoid). National figures from 2007-2009 show a more even distribution than in this cohort (right 32.9%, left 35.2% and rectum 31.9%) (Cancer Research U.K. 2012).

There were 4 Dukes' A (12.1%), 12 Dukes' B (36.4%), 13 Dukes' C (39.4%) and 4 Dukes' D tumours (12.1%). One tumour was a mucinous cystadenoma, a benign tumour of the appendix, and therefore the Dukes' staging was not applicable. National figures for Dukes' Stage distribution are incomplete, with 34% of cases being unclassified (National Cancer Intelligence Network 2009). When unknown cases are excluded, the distribution of Dukes' Stage in national data is very similar to this cohort (Table 5.2).

Table 5.2 Pathological characteristics of tumours

Table shows pathological data obtained from histopathological reports for all 34 tumours (n/a = not applicable). Data in the literature is shown for comparison: ^a(Cancer Research U.K. 2012), ^b(National Cancer Intelligence Network 2009), ^c(Athas 2004).

Parameter	n	%	Literature (%)
Site			
Right (Proximal) colon	17	50.0	32.9 ^a
Left (Distal) colon	8	23.5	31.9 ^a
Rectum	9	26.5	35.2 ^a
Dukes' Stage			
A	4	12.1	13.8 ^b
B	12	36.4	36.9 ^b
C	13	39.4	35.4 ^b
D	4	12.1	13.8 ^b
n/a	1		
Differentiation			
well	0	0	12.4 ^c
moderate	21	63.6	60.5 ^c
poor	11	33.3	25.9 ^c
undifferentiated	1	3.0	1.3 ^c

In this cohort 21 tumours (63.6%) were moderately differentiated, 11 were poorly differentiated (33.3%) and 1 (3.0%) was undifferentiated. There were no well-differentiated tumours. National data on differentiation for the UK is not available. A British study by Roxburgh

et al. found a lower rate of higher grade tumours (13% poor or undifferentiated) than in the current cohort (Roxburgh et al. 2010). However Dukes' D cancers were excluded in that study. An American study using national cancer registry data found comparable rates, the main difference being the absence of any well-differentiated tumours in the current cohort (Athas 2004).

5.3.2 Characterisation Of TDCs

Following a process of mechanical and enzymatic digestion, cells were separated from undigested tissue by filtration. After density-gradient separation to remove red blood cells, the remaining cells were counted. Cell yields varied considerably, from 2.5×10^5 to 1×10^7 cells/ml. The amount of starting material also varied considerably (from 0.08 to 2.62g), and was correlated to the total cell yield ($R^2 = 0.35$, $p < 0.01$; data not shown). Cells were then seeded, in duplicate or triplicate wells where possible, aiming for a cell density of 5×10^4 cells/cm², and incubated in normoxia. In subsequent hypoxia experiments, a second plate was seeded. Because of the variation in total cell yields and preference for replicate wells, the actual cell density ranged from 2.0×10^4 to 7.5×10^4 cells/cm² (mean 4.6×10^4 cells/cm²). Each TDC comprised cells from an individual patient.

The cell population was of mixed morphology and size, with some single cells as well as many forming clumps, as illustrated in Figure 5.1. While some cells were adherent, many of the smaller cells remained in suspension. At low magnification, the smaller cells resembled adherent Caco-2 cells at low confluence. At higher magnification, some of the smaller cells appeared to be anucleate, suggesting that some RBCs remained despite the density-gradient separation step.

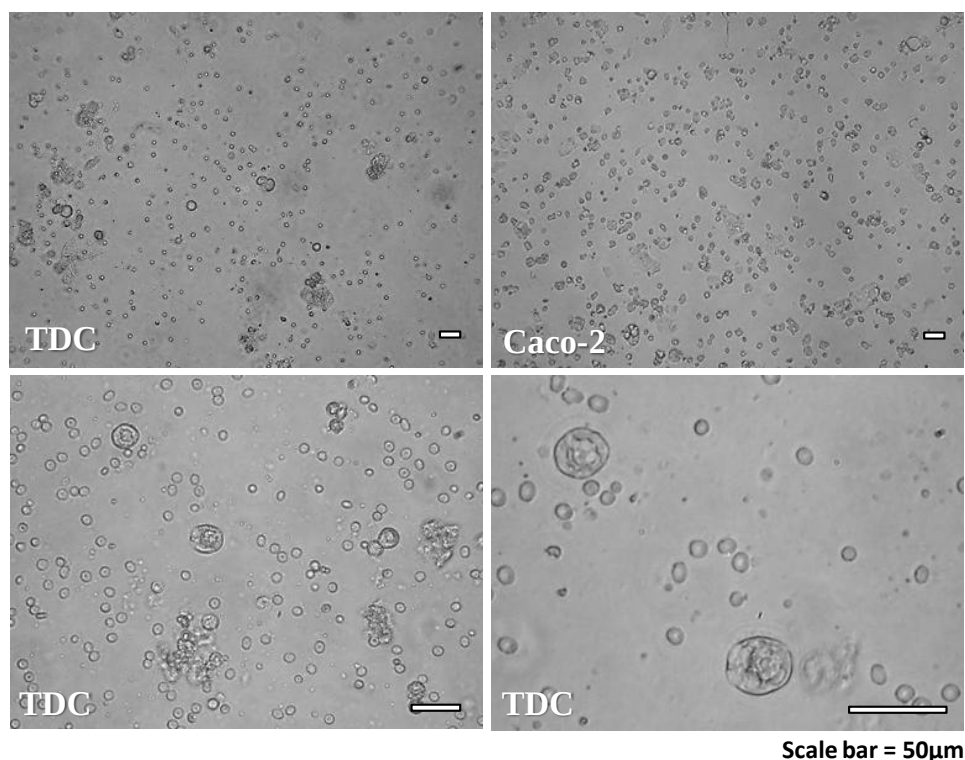


Figure 5.1 Phase-contrast microscopy of tumour-derived cell culture after 24 hours

Cells obtained following mechanical and enzymatic digestion of tissue from a single CRC specimen were seeded at a cell density of 5×10^4 cells/cm² and incubated in normoxia. Photos taken at 24 hours at 40x (top left), 100x (bottom left) and 200x (bottom right) magnification show a heterogeneous population of cells. Caco-2 cells at low confluence (40x magnification) are shown in the top right panel for comparison. Scale bar represents 50µm.

When the cultures were maintained for 7 days, this heterogeneity persisted. There appeared to be some proliferation, particularly of the larger adherent cells, with some cells aggregating into islands (Figure 5.2). Caco-2 proliferated at a much higher rate, and by this time-point were fully confluent. There was an increased amount of acellular debris, indicating significant cell-death. This was similar to previous experience in our laboratory in a study by Mr. T. Khong, who found that despite using various specialised culture media, these cells were difficult to maintain beyond a few days (unpublished data), with infection being common problem. This experience contributed to the decision to limit the objectives of this study to the maintenance of cultures for less than 48 hours, which was sufficient time for hypoxia-stimulation experiments.

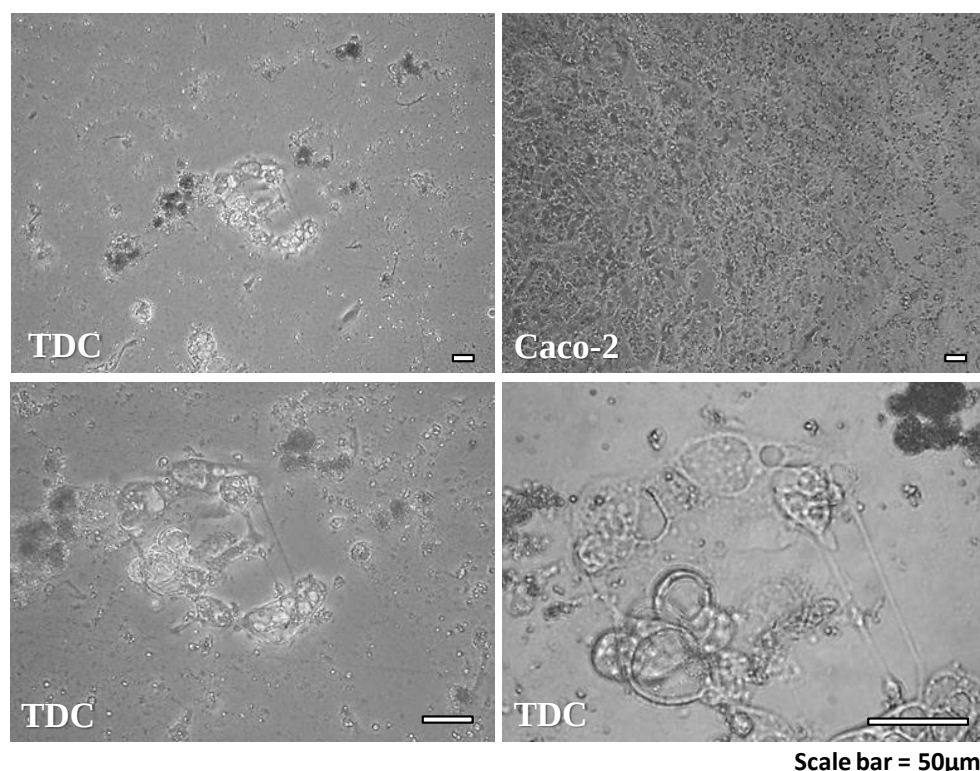


Figure 5.2 Phase contrast microscopy of TDCs after 7 days

Cells obtained following mechanical and enzymatic digestion of tissue from a CRC specimen were seeded at a cell density of 5×10^4 cells/cm² and incubated in normoxia, with medium refreshed every 48 hours. Photos are of the same culture as in Figure 5.1, taken after 7 days at 40x (top left), 100x (bottom left) and 200x (bottom right) magnification. Some aggregation of large polygonal cells into islands is seen, notably in the centre of the image, with a small number of isolated cells peripherally, some with spindle-like morphology. Caco-2 cells close to confluence (40x magnification) are shown in the top right panel for comparison. Scale bar represents 50µm.

In order to further characterise these mixed cultures, expression of epithelial cell-markers Ep-Cam and VE-Cadherin, and the CRC tumour marker CEA, were evaluated. After overnight incubation in normoxia, supernatants were collected and RNA extracted. RNA was obtained for 22 of the 34 TDCs, with RNA yields per condition ranging from 297ng (9.89ng/µL) to 6.62mg (220ng/µL). Of these, 9 yielded less than the minimum RNA concentration required to make cDNA (22.72ng/µL for one RT-PCR reaction). RNA yield and cell yield showed a linear correlation ($R^2 = 0.55$, $p < 0.001$; data not shown)

As shown in Figure 5.3, Ep-CAM was expressed in all TDCs tested, with significant variation in expression levels between different donors ($2^{-\Delta Ct}$ range 1.22×10^{-6} to 6.75×10^{-4}). The expression level in TDCs was very similar to that in Caco-2 cells, the mean $2^{-\Delta Ct}$ values being $1.01 \pm 0.41 \times 10^{-4}$ and $0.84 \pm 0.17 \times 10^{-4}$ respectively (not significant, unpaired t-test). VE-Cadherin was more weakly expressed (assuming comparable primer efficiencies) than Ep-CAM both in both Caco-2 cells and TDCs. It was expressed in all TDCs, with greater donor variation than for

Ep-CAM ($2^{-\Delta Ct}$ value range 2.02×10^{-8} to 3.71×10^{-6}). Caco-2 cells expressed higher levels of VE-Cadherin than TDCs (mean $3.29 \pm 0.41 \times 10^{-5}$ versus $2.04 \pm 0.37 \times 10^{-6}$; $p < 0.001$, unpaired t-test).

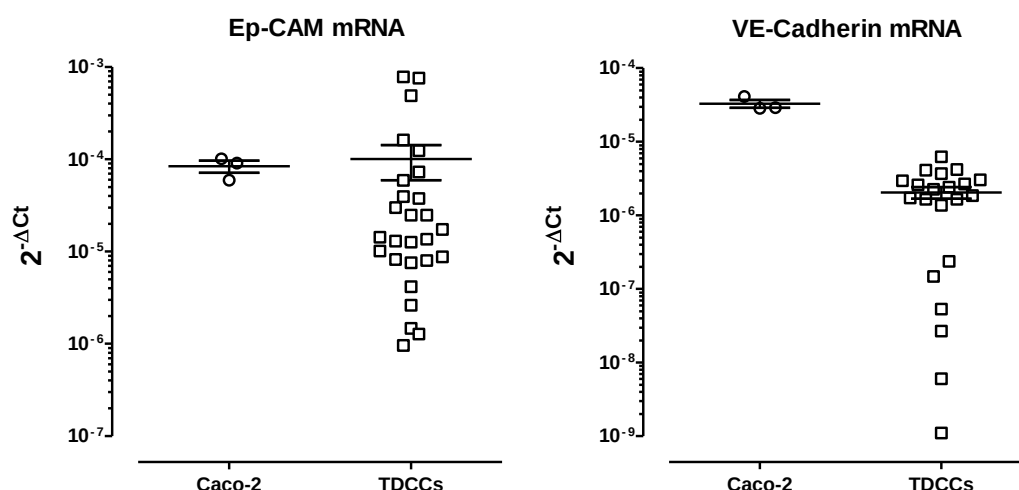


Figure 5.3 Expression of Epithelial Markers in TDCs

TDCs from 5 donors were incubated in normoxia for 24 hours, in duplicate wells where cell yields allowed. RNA was extracted, and mRNA expression was evaluated by Q-PCR. $2^{-\Delta Ct}$ values, which correspond to gene abundance, were calculated normalised to 18S. The graphs show $2^{-\Delta Ct}$ values for Ep-CAM (left) and VE-Cadherin (right), with values for Caco-2 shown as reference. Each data point represents a single well, and the solid line and error bars show the overall mean $\pm$ SEM.

Because of the relatively low cell yields, it was not possible to extract both RNA and protein from cell lysates. Therefore CEA protein was measured in the supernatants by ELISA (Figure 5.4). CEA was detected in all TDCs tested, and as with Ep-CAM and VE-Cadherin, levels were highly donor dependent (mean 550 ± 157 ng/mL, range 8.14 ± 1.27 to 1870 ± 130 ng/mL). CEA secretion was unaffected by hypoxia. CEA expression was not detected in supernatants from Caco-2 cells (data not shown).

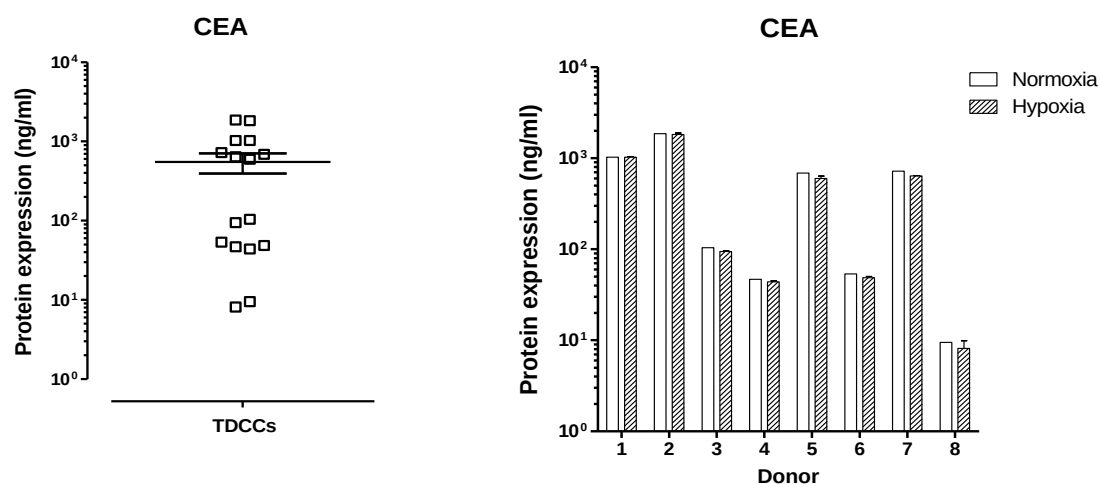


Figure 5.4 CEA secretion by TDCs

TDCs were exposed to hypoxia for 24 hours with normoxia controls, and supernatants were collected. CEA expression was evaluated in duplicates by ELISA. Left: CEA concentration in all conditions is plotted to illustrate the range of expression levels. Each point represents mean concentration from duplicate measurements, and solid lines show the overall mean $\pm$ SEM ($n = 8$). Right: CEA concentration in normoxia and hypoxia for individual donors is plotted (data are mean $\pm$ SEM); analysis by 2-way ANOVA showed the difference between donors to be statistically significant ($p < 0.001$), but the difference between normoxia and hypoxia for each donor was not.

These characterisation experiments demonstrated that the protocol employed allowed the establishment of TDCs from individual donor tumours, and that these cultures contained cells of epithelial origin. Furthermore, for 50% of the cohort, the cultures yielded RNA of sufficient amount and quality to perform Q-PCR.

5.3.3 Hypoxia-Induced Angiogenesis Genes

5.3.3.1 Preliminary Q-PCR Data

The next objective was to examine the effect of hypoxia on the expression of angiogenesis genes. Due to the limited amount of RNA, it was not possible to perform both hypoxia- and DMOG-stimulation experiments. Initially, Q-PCR was used to evaluate expression of four angiogenesis genes (VEGF, ANGPTL-4, EFNA-3 and TGF- β 1) for two donors (Figure 5.5, Figure 5.6).

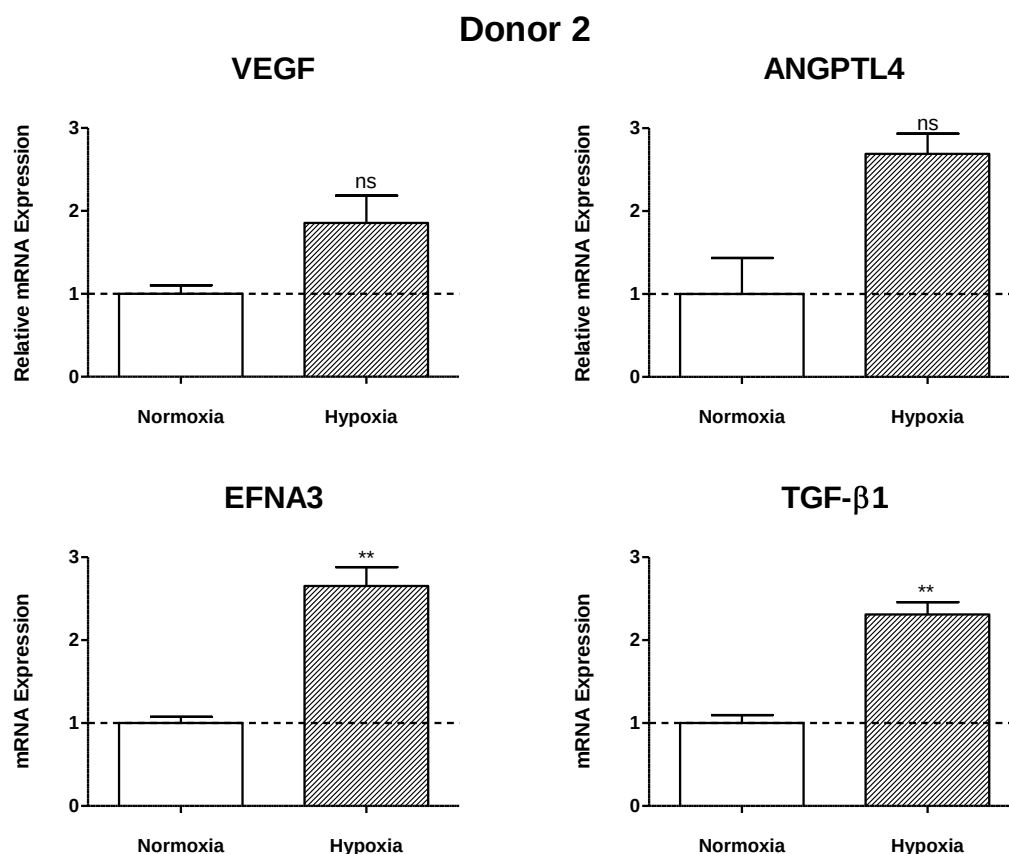


Figure 5.5 Effect of hypoxia on angiogenesis gene expression in TDC from a single donor (Donor 2)

TDC from Donor 2 was exposed to hypoxia for 24 hours with normoxia control. mRNA expression was evaluated by Q-PCR. Figure shows VEGF, ANGPTL-4, EFNA-3 and TGF-β1 expression normalised to normoxia (dashed line). Data are mean ± SD from 1 experiment (Δ Ct versus normoxia; ** $p < 0.01$, ns = not significant; unpaired t-test).

For Donor 2, all four genes were upregulated by hypoxia, although this was only statistically significant for EFNA-3 and TGF-β1. VEGF was increased by a fold-change of 1.86 ± 0.57 , ANGPTL-4 by 2.69 ± 0.42 , EFNA-3 by 2.65 ± 0.39 ($p < 0.01$) and TGF-β1 by 2.31 ± 0.26 ($p < 0.01$). The hypoxia response was weaker for Donor 3. Expression was 1.26 ± 0.27 for VEGF, 1.74 ± 1.24 for ANGPTL-4, 1.38 ± 0.10 ($p < 0.05$) for EFNA-3 and 0.95 ± 0.34 for TGF-β1 (not significant unless otherwise stated).

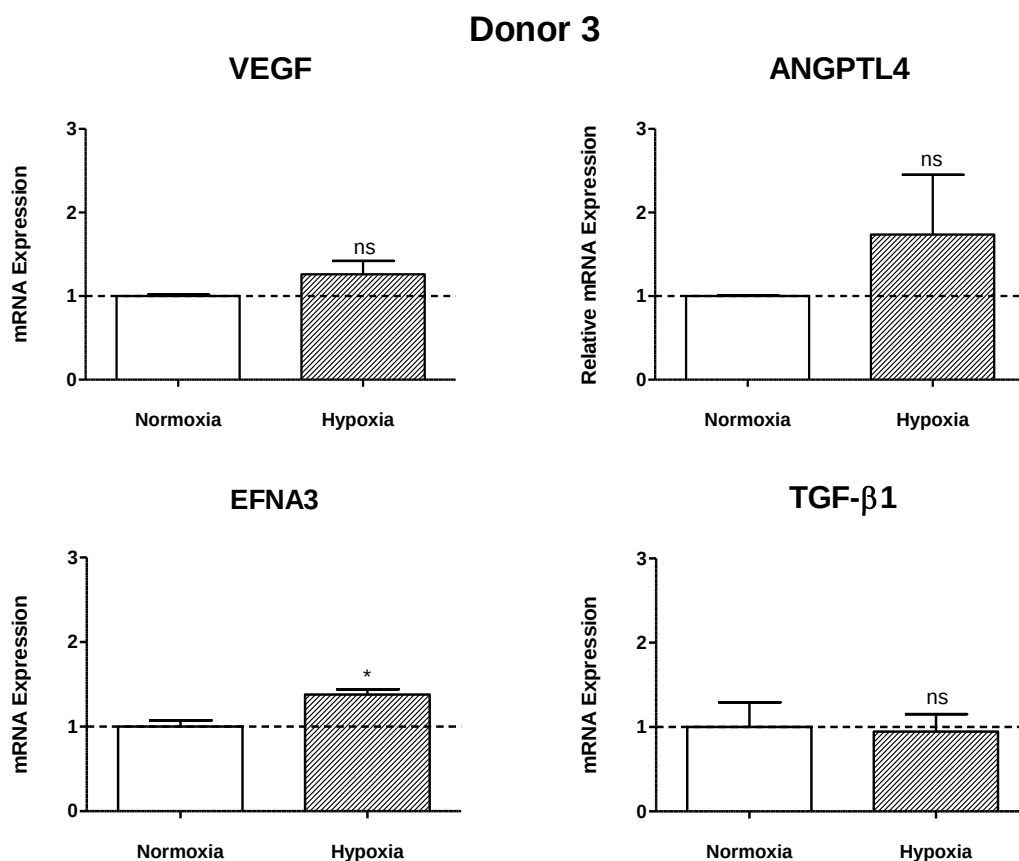


Figure 5.6 Effect of hypoxia on angiogenesis gene expression in TDC from a single donor (Donor 3)

TDC from Donor 3 was exposed to hypoxia for 24 hours with normoxia control. mRNA expression was evaluated by Q-PCR. Figure shows VEGF, ANGPTL-4, EFNA-3 and TGF-β1 expression normalised to normoxia (dashed line). Data are mean $\pm$ SD from 1 experiment (Δ Ct *versus* normoxia; * $p < 0.05$, ns = not significant; unpaired t-test).

These initial data showed that the method allowed the evaluation of hypoxia-responses for TDCs at mRNA level. They also suggested that there was a variation in hypoxia response between TDCs from different donors. In order to investigate this further, the PCR Angiogenesis Array was employed.

5.3.3.2 Array Data

As with Caco-2 cells, the RT² Profiler™ PCR Angiogenesis Array (SABiosciences, Frederick, MD, USA) was used to analyse mRNA extracted from hypoxia-stimulated TDCs and compared with normoxia controls. The array required enough RNA for a minimum of 3 RT-PCR reactions, which limited the number TDCs that were suitable. TDCs from 6 consecutive donors that yielded sufficient RNA were selected.

5.3.3.2.1 Array Donor Demographics

The donor and tumour characteristics for the 6 samples used in the array are shown in Table 5.3. There were 4 male and 2 female patients, with an overall mean age of 64.2 ± 6.4 years (male 66.0 ± 5.9 , female 60.5 ± 7.8 ; $p = 0.38$ by unpaired t-test) (Table 5.4).

Table 5.3 Demographic and pathology characteristics of donors used in array

Table shows age, gender, site (proximal or distal colon, or rectum) and differentiation grade for the 6 donors analysed by Angiogenesis Array.

	Donor					
	1	2	3	4	5	6
Age (yrs)	66	73	68	64	59	55
Gender	F	M	M	M	M	F
Site	Rectum	Proximal	Distal	Rectum	Distal	Distal
Dukes' Stage	A	B	A	C	C	C
Differentiation	moderate	moderate	moderate	moderate	moderate	moderate

Table 5.4 Array patients demographics statistics

Table shows age and sex statistics for donors analysed by Angiogenesis Array.

Parameter	Male	Female	All
n	4	2	6
%	66.7	33.3	100
Mean Age, years $\pm$ SEM	66.0 ± 3.0	60.5 ± 5.5	64.2 ± 2.6

As shown in Table 5.5 and Table 5.6 the donors were representative of the full cohort of 34 patients. Due to the low number of donors, they were grouped into colon *versus* rectum, and Dukes' A and B *versus* Dukes' C and D for statistical analysis. Compared to the full cohort, p values for male *versus* female distribution and age were 0.18 and 0.40 respectively. In the array group, there was 1 proximal colon tumour, 3 distal colon and 2 rectal tumours. P value for colon *versus* rectum compared to the full cohort was 1.00. 2 array group tumours were Dukes' A, 1 was Dukes' B and 3 were Dukes' D ($p=1.00$ for ratio of (Dukes' A and B) to (Dukes' C and D); array group *versus* full cohort). All array group tumours were moderately differentiated ($p=0.15$ for moderate *versus* poor/undifferentiated compared to full cohort).

Table 5.5 Array patients pathology statistics

Table shows pathology statistics for donors analysed by array.

Parameter	n	%
Site		
Proximal colon	1	16.7
Distal colon	3	50.0
Rectum	2	33.3
Dukes' Stage		
A	2	33.3
B	1	16.7
C	3	50.0
D	0	0
Differentiation		
well	0	0
moderate	6	100
poor	0	0
undifferentiated	0	0

Table 5.6 Demographics and pathology statistics – Array Donors versus Full CohortCharacteristics of 6 donors from whom TDCs were analysed by array are compared to the full cohort of 34 patients. [†]Fisher's exact test.

Parameter	Array	Full Cohort	P value [†]
Age (years± SD)	64.2± 6.4	69.9 ± 9.7	0.18
Gender: male <i>versus</i> female	4:2 (2:1)	15:19 (0.8:1)	0.40
Site: colon <i>versus</i> rectum	4:2 (2:1)	25:9 (2.8:1)	1.00
Dukes' Stage: (A & B) <i>versus</i> (C & D)	3:3 (1:1)	16:17 (0.9:1)	1.00
Differentiation: moderate <i>versus</i> poor/undifferentiated	6:0	21:12	0.15

5.3.3.2.2 Array Results

mRNA extracted from hypoxia-stimulated TDCs was compared with normoxia controls. A full list of the genes included in the array, and their functions, is presented in Table 2.5. The manufacturer's software was used to calculate fold-regulation by the $2^{-\Delta\Delta C_t}$ method, with greater than two-fold up- or down-regulation taken as a meaningful change. Fold-regulations for all genes and donors are shown in Table 5.7 (changes that were less than two-fold are represented thus: $\leftrightarrow$). Gene expression for hypoxia-stimulated Caco-2 cells is also shown in the same table. Individual scatter plots for each donor are shown subsequently in Figure 5.7 to Figure 5.12.

Table 5.7 Hypoxia-induced changes in expression of genes in the Human Angiogenesis RT² Profiler™ PCR Array in TDCs from 6 donors and in Caco-2 cell-line

See Table 2.5 for full list and function of genes in the Angiogenesis RT² Profiler™ PCR Array. Data are fold up- or down-regulation. $\leftrightarrow$ = change > -2.0 and < 2.0 . ND = not detected. (* = upregulation was also seen in DMOG-stimulated Caco-2 cells.)

Symbol	Donor Number						Caco-2
	1	2	3	4	5	6	
AKT1	-13.69	2.60	$\leftrightarrow$	$\leftrightarrow$	-2.57	5.13	$\leftrightarrow$
ANGPT-1	ND	ND	ND	ND	8.43	ND	2.26*
ANGPT-2	$\leftrightarrow$	ND	-5.02	$\leftrightarrow$	$\leftrightarrow$	29.99	2.10
ANGPTL3	3.72	4.10	4.50	$\leftrightarrow$	$\leftrightarrow$	2.31	2.10*
ANGPTL-4	7.19	2.39	$\leftrightarrow$	7.19	3.40	3.45	3.11*
ANPEP	4.76	4.63	$\leftrightarrow$	2.06	$\leftrightarrow$	2.62	$\leftrightarrow$
BAI1	ND	ND	ND	ND	-2.58	-3.40	ND
CCL11	9.38	ND	ND	5.28	$\leftrightarrow$	5.41	ND
CCL2	2.18	ND	ND	2.33	$\leftrightarrow$	-2.02	$\leftrightarrow$
CDH5	75.06	-43.68	15.24	$\leftrightarrow$	$\leftrightarrow$	ND	ND
COL18A1	$\leftrightarrow$	ND	$\leftrightarrow$	2.35	$\leftrightarrow$	-2.91	$\leftrightarrow$
COL4A3	-9.48	ND	ND	$\leftrightarrow$	-4.97	ND	$\leftrightarrow$
CXCL1	2.27	$\leftrightarrow$	$\leftrightarrow$	2.08	$\leftrightarrow$	$\leftrightarrow$	ND
CXCL10	3.82	ND	$\leftrightarrow$	$\leftrightarrow$	$\leftrightarrow$	$\leftrightarrow$	ND
CXCL3	9.03	ND	14.23	6.39	-4.11	$\leftrightarrow$	ND
CXCL5	11.24	ND	ND	2.06	$\leftrightarrow$	$\leftrightarrow$	ND
CXCL6	11.96	ND	$\leftrightarrow$	2.17	$\leftrightarrow$	ND	ND

CXCL9	16.56	19.60	↔	2.42	↔	13.34	ND
TYMP	↔	-2.04	↔	↔	↔	↔	ND
S1PR1	2.25	↔	-2.66	↔	↔	-3.10	↔
EFNA1	6.99	↔	-2.18	↔	↔	↔	2.55*
EFNA-3	10.63	2.00	↔	↔	↔	3.21	7.19*
EFNB2	2.27	↔	-2.43	↔	↔	↔	↔
EGF	ND	ND	ND	3.42	ND	ND	2.69
ENG	2.91	↔	↔	↔	↔	↔	↔
EPHB4	2.97	↔	↔	2.59	↔	↔	↔
EREG	2.43	ND	↔	↔	↔	↔	↔
FGF1	15.14	ND	ND	↔	↔	ND	ND
FGF2	3.53	ND	↔	↔	↔	↔	ND
FGFR3	↔	↔	2.10	↔	-6.06	↔	↔
FIGF	16.97	ND	18.27	ND	-2.27	↔	ND
FLT1	10.70	62.57	14.63	2.06	↔	3.26	3.68*
HAND2	18.83	-48.21	ND	-4.89	-11.82	↔	ND
HGF	6.13	15.68	32.47	↔	-2.45	13.34	ND
HIF-1A	22.09	5.00	2.03	↔	↔	↔	↔
HPSE	4.96	ND	↔	↔	-3.40	ND	2.01
ID1	↔	↔	↔	↔	↔	↔	↔
ID3	↔	3.21	↔	↔	↔	↔	↔
IFNA1	4.36	ND	20.34	-19.16	-2.07	ND	ND
IFNB1	13.78	75.65	46.89	-18.90	-2.25	ND	ND
IFNG	4.91	46.09	13.37	-6.70	-3.74	ND	ND
IGF1	6.80	ND	9.89	↔	-2.01	ND	2.08
IL1B	↔	-14.86	2.24	-2.02	↔	↔	2.14
IL6	4.14	62.57	3.14	↔	-2.65	7.00	ND
IL8	4.81	3.66	↔	4.39	↔	↔	↔
ITGAV	3.25	↔	↔	2.23	↔	2.47	↔

ITGB3	21.19	39.84	15.41	2.11	-3.18	2.34	↔
JAG1	13.09	2.11	↔	2.94	↔	↔	↔
KDR	7.46	79.69	25.39	↔	-2.81	-2.49	↔
LAMA5	3.47	ND	↔	2.44	↔	↔	↔
LECT1	20.53	227.15	78.05	ND	-2.94	5.41	ND
LEP	580.04	27.71	4.42	ND	26.84	↔	ND
MDK	8.31	↔	2.29	↔	↔	2.49	ND
MMP2	4.39	7.14	↔	3.51	↔	2.63	↔
MMP-9	12.51	5.72	8.20	↔	↔	↔	2.11*
NOTCH4	3.94	2.89	↔	2.35	↔	ND	ND
NRP1	2.66	36.03	5.34	↔	↔	↔	↔
NRP2	3.82	ND	4.76	↔	↔	↔	↔
PDGFA	3.52	ND	3.89	↔	↔	↔	↔
PECAM1	↔	ND	ND	↔	-2.24	↔	↔
PF4	9.71	15.74	32.14	-8.28	↔	-2.47	ND
PGF	81.01	146.14	21.20	-17.69	2.51	↔	ND
PLAU	22.39	28.27	6.73	2.17	↔	↔	↔
PLG	92.41	137.31	ND	-191.34	-66.26	ND	ND
PLXDC1	-4.72	ND	2.64	2.31	↔	-2.12	2.14
PROK2	ND	ND	ND	ND	↔	ND	2.74
PTGS1	↔	3.53	2.26	-15.14	↔	↔	ND
SERPINF1	-2.85	ND	-3.72	2.56	↔	-2.52	↔
SPHK1	↔	↔	↔	↔	↔	↔	↔
STAB1	-4.92	ND	↔	↔	↔	↔	↔
TEK	-2.53	ND	ND	-2.87	↔	ND	ND
TGFA	↔	↔	↔	↔	↔	↔	↔
TGFB1	-2.21	↔	2.17	↔	↔	↔	5.39*
TGFB2	↔	ND	-2.07	↔	↔	10.48	↔
TGFBR1	↔	ND	↔	↔	↔	8.38	↔

THBS1	↔	-8.45	↔	-5.96	↔	↔	↔
THBS2	↔	ND	-3.63	-5.94	↔	-2.44	↔
TIMP1	↔	↔	↔	↔	↔	↔	↔
TIMP2	-2.11	↔	2.31	-2.96	↔	↔	↔
TIMP3	-3.03	ND	↔	-39.40	↔	↔	ND
TNF	-9.55	ND	ND	-108.76	↔	↔	ND
TNFAIP2	-4.11	↔	↔	-4.04	↔	↔	ND
VEGFA	↔	↔	↔	↔	2.32	2.38	3.07*
VEGFC	-4.00	ND	6.50	↔	↔	3.90	2.60

The results for each donor are displayed in scatter plots in which $2^{-\Delta C_t}$ values in hypoxia (y-axis) are plotted against normoxia controls (x-axis). The solid line represents equal expression, and the upper and lower dashed lines represent two-fold up- or downregulation respectively. Genes lying towards the top right of the graph are more abundantly expressed than those to the bottom left. In order to simplify comparison of TDCs and Caco-2 responses, the expression of the 9 genes that were upregulated by both hypoxia and DMOG in Caco-2 is highlighted (ANGPT-1, ANGPTL-3, ANGPTL-4, EFNA-1, EFNA-3, FLT-1, MMP-9, TGF- β 1 and VEGF-A; Figure 3.11). In each scatter plot, these Caco-2 signature genes (CSGs) are labelled and shown in black.

For donor 1 (Figure 5.7), the expression of 63 genes was changed in response to hypoxia, compared to 17 for Caco-2. Of these 63 genes, 51 were upregulated and 12 were downregulated, while in Caco-2, none were downregulated. One of the 9 CSGs was downregulated (TGF- β 1, fold-regulation -2.21), one was unchanged (VEGF-A), and 6 were upregulated (fold-regulation 3.72 for ANGPTL-3, 7.19 for ANGPTL-4, 6.99 for EFNA-1, 10.63 for EFNA-3, 10.70 for FLT-1, 12.51 and for MMP-9). ANGPT-1 was not detected ($C_t > 30$). Fold-regulations were generally higher than for Caco-2. The relative abundance of these 9 genes was similar in both cell-types, with VEGF, TGF- β 1 and the ephrins being more abundant than MMP-9, FLT-1 and the angiopoietins. Overall, only 4 genes were undetected in donor 1.

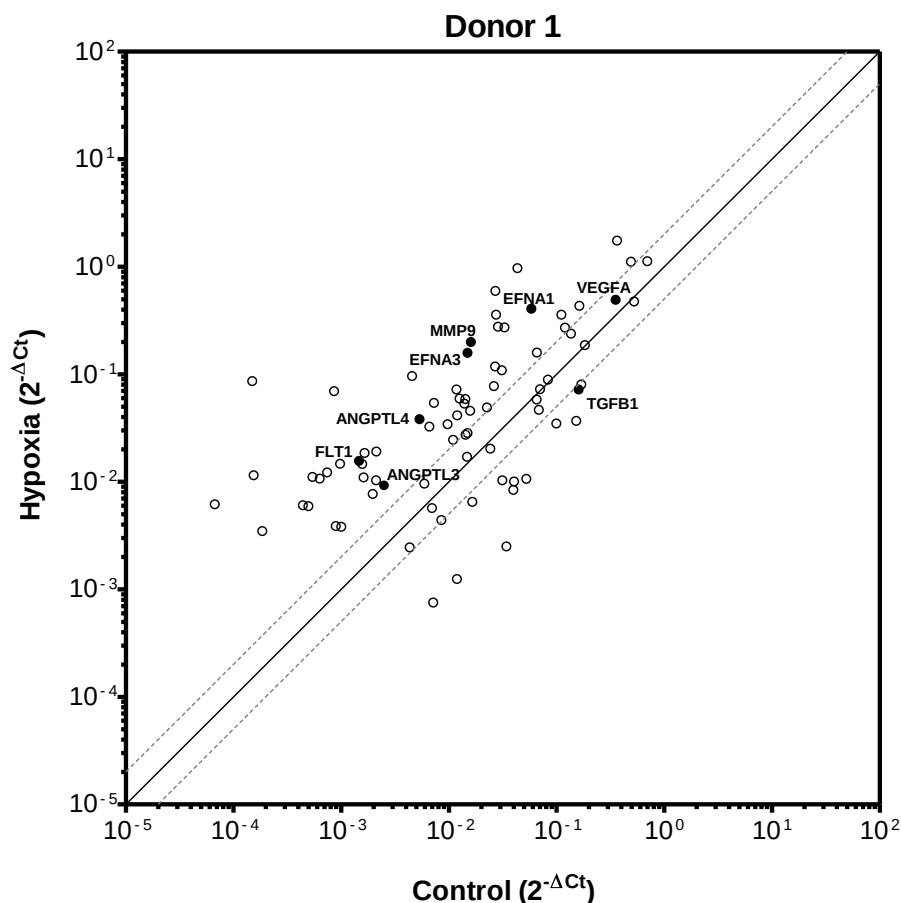


Figure 5.7 Hypoxia-induced angiogenesis gene expression Donor 1

The TDC from donor 1 was exposed to hypoxia (1% O₂) for 24 hours. mRNA was extracted and expression evaluated in duplicate by RT² Profiler Angiogenesis PCR Array. Figure shows the scatter plot with 2^{-ΔCt} values plotted against untreated control. Solid line indicates no change; dashed lines indicate two-fold increase (upper) and decrease (lower). Genes that were upregulated more than two-fold in Caco-2 cells (CSGs) are labelled and shown in black.

For donor 2 (Figure 5.8), fewer genes were detected, with 34 having Ct values greater than 30. Of the remaining 50, 28 were upregulated, 5 were downregulated and 17 unchanged. Of the CSGs, 5 were upregulated (fold-regulation 4.10 for ANGPTL-3, 2.39 for ANGPTL-4, 2.00 for EFNA-3, 62.57 for FLT-1 and 5.72 for MMP-9). No change was seen for EFNA-1, TGF-β1 and VEGF-A, and ANGPT-1 was again undetected.

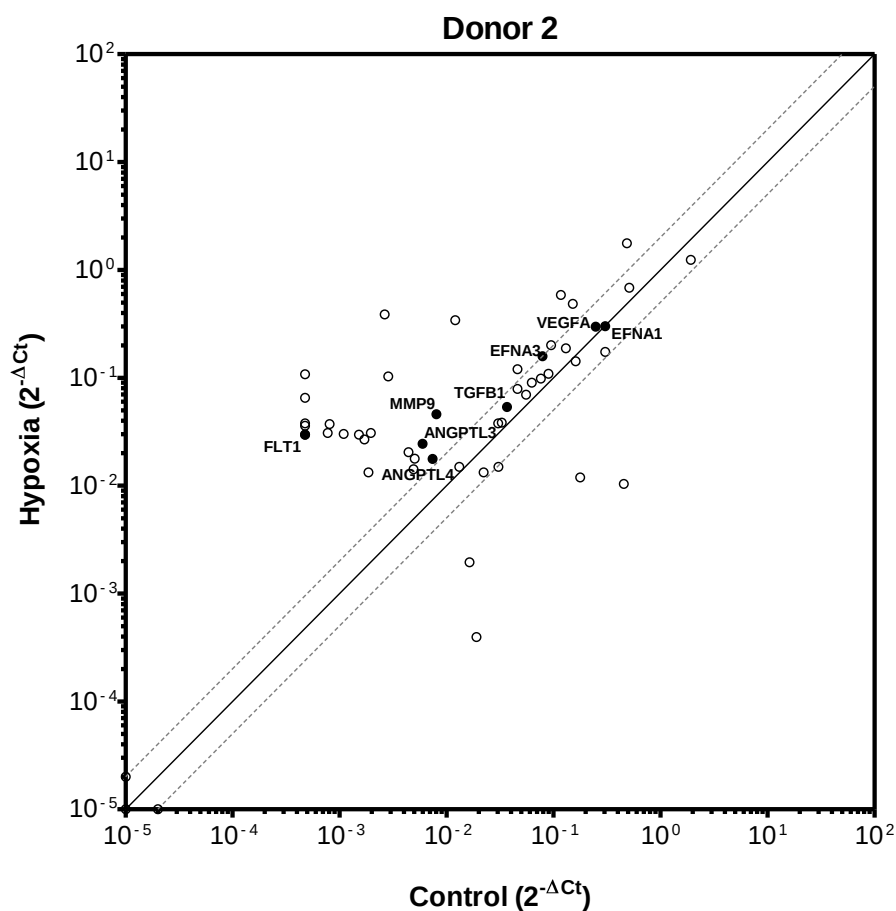


Figure 5.8 Hypoxia-induced angiogenesis gene expression Donor 2

The TDC from donor 2 was exposed to hypoxia (1% O₂) for 24 hours. mRNA was extracted and expression evaluated in duplicate by RT² Profiler Angiogenesis PCR Array. Figure shows the scatter plot with 2^{-ΔCt} values plotted against untreated control. Solid line indicates no change; dashed lines indicate two-fold increase (upper) and decrease (lower). Genes that were upregulated more than two-fold in Caco-2 cells (CSGs) are labelled and shown in black.

For donor 3 (Figure 5.9), while 70 genes were detected, 32 of these were unchanged. Of the remaining 38, 31 were upregulated and 7 were downregulated. Of the CSGs, 4 were upregulated (fold-regulation 4.50 for ANGPTL-3, 14.63 for FLT-1, 8.20 for MMP-9 and 2.17 for TGF-β1) and one was downregulated (fold-regulation -2.18 for EFNA-1). ANGPTL-4, EFNA-3 and VEGF-A were unchanged, while ANGPT-1 was not detected.

For donor 4 (Figure 5.10), only 6 genes were not detected. Half of the 78 detected genes (39) were unchanged. Of the remainder, 23 were upregulated, and 16 were downregulated. Only 2 of the CSGs were upregulated (fold-regulation 7.19 for ANGPTL-4 and 2.06 for FLT-1). 6 were unchanged (ANGPTL-3, EFNA-1, EFNA-3, MMP-9, TGF-β1 and VEGF-A) while ANGPT-1 was again undetected.

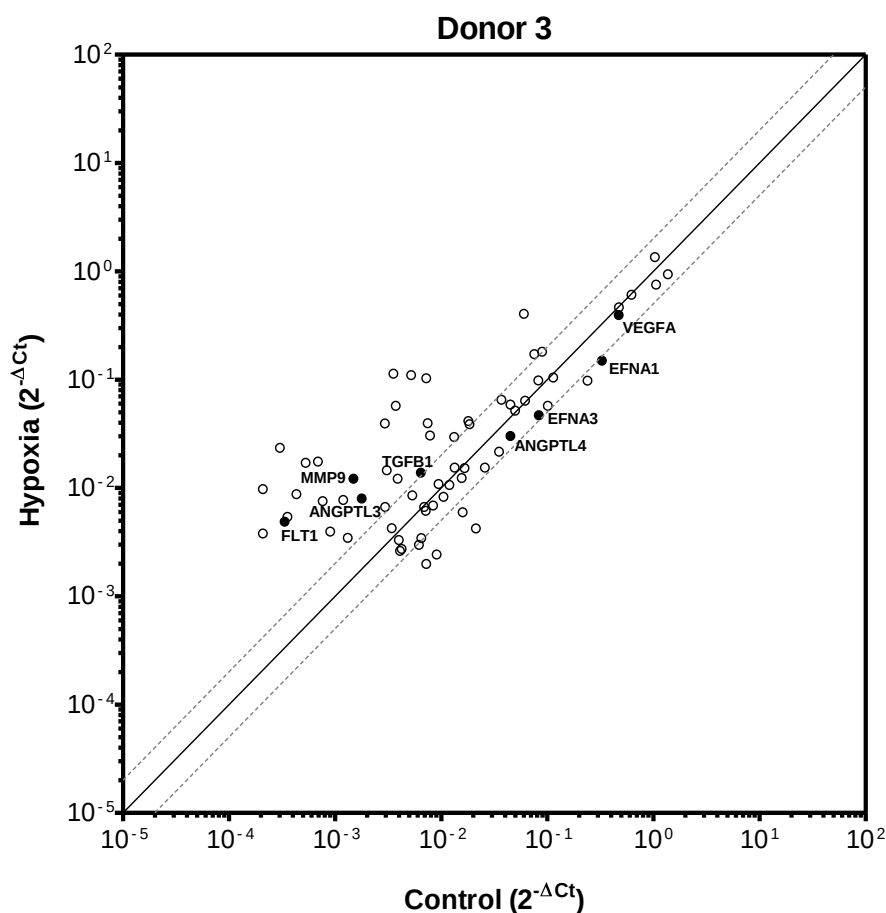


Figure 5.9 Hypoxia-induced angiogenesis gene expression Donor 3

The TDC from donor 3 was exposed to hypoxia (1% O_2) for 24 hours. mRNA was extracted and expression evaluated in duplicate by RT² Profiler Angiogenesis PCR Array. Figure shows the scatter plot with $2^{-\Delta Ct}$ values plotted against untreated control. Solid line indicates no change; dashed lines indicate two-fold increase (upper) and decrease (lower). Genes that were upregulated more than two-fold in Caco-2 cells are labelled and shown in black.

Only one gene was undetected in donor 5 (Figure 5.11). 24 genes changed, with 5 upregulated and 19 downregulated. Unlike the other donors, all CSGs including ANGPT-1 were detected. Of the 9, ANGPT-1, ANGPTL-4 and VEGF-A were upregulated (fold-regulation 8.43, 34.0 and 2.32 respectively) and the remaining 6 were unchanged.

There were 31 undetected genes for donor 6 (Figure 5.12). Of the remaining 53, 17 were upregulated and 36 were unchanged, while none were downregulated. 5 of the CSGs were upregulated (fold-regulation 2.31 for ANGPTL-3, 3.45 for ANGPTL-4, 3.21 for EFNA-3, 3.26 for FLT-1 and 2.38 for VEGF-A). No change was seen for EFNA-1, MMP-9 and TGF-β1, while ANGPT-1 was undetected.

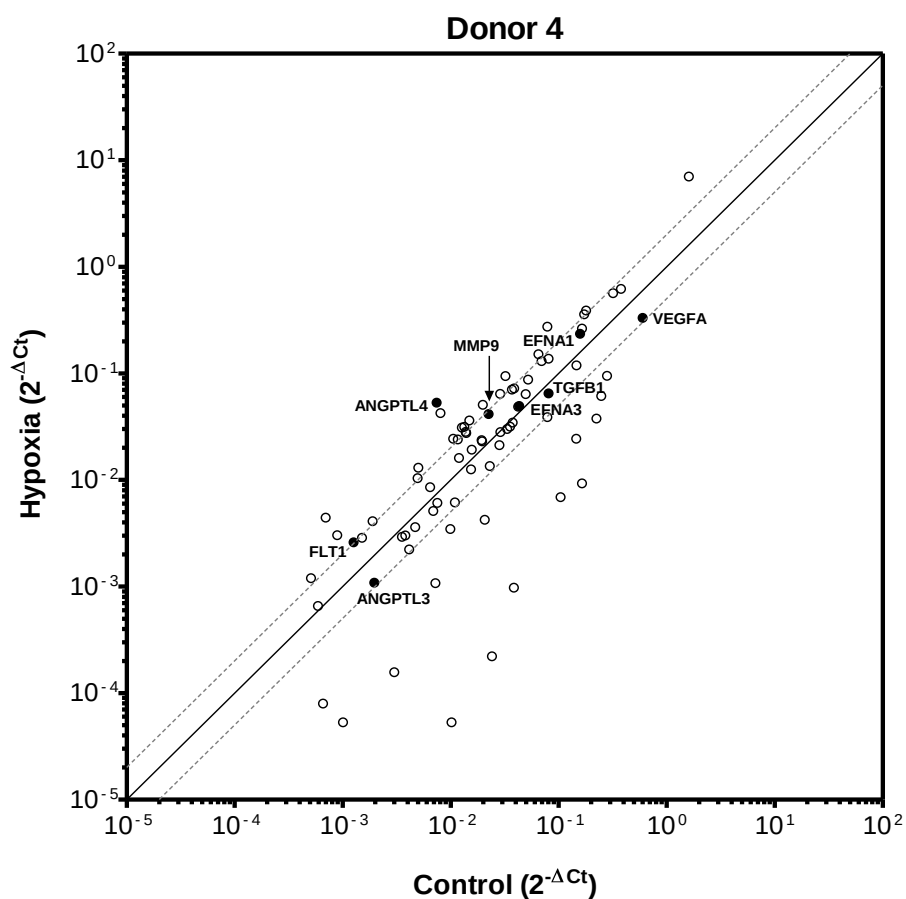


Figure 5.10 Hypoxia-induced angiogenesis gene expression Donor 4

The TDC from donor 4 was exposed to hypoxia (1% O₂) for 24 hours. mRNA was extracted and expression evaluated in duplicate by RT² Profiler Angiogenesis PCR Array. Figure shows the scatter plot with $2^{-\Delta C_t}$ values plotted against untreated control. Solid line indicates no change; dashed lines indicate two-fold increase (upper) and decrease (lower). Genes that were upregulated more than two-fold in Caco-2 cells (CSGs) are labelled and shown in black.

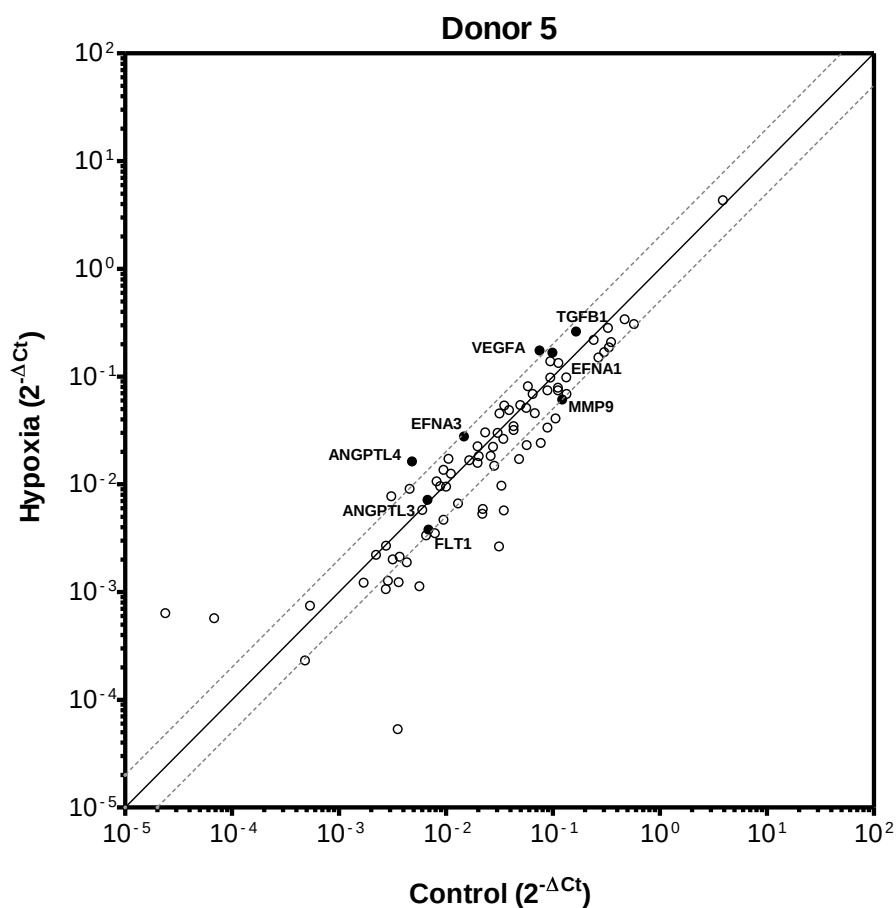


Figure 5.11 Hypoxia-induced angiogenesis gene expression Donor 5

The TDC from donor 5 was exposed to hypoxia (1% O₂) for 24 hours. mRNA was extracted and expression evaluated in duplicate by RT² Profiler Angiogenesis PCR Array. Figure shows the scatter plot with 2^{-ΔCt} values plotted against untreated control. Solid line indicates no change; dashed lines indicate two-fold increase (upper) and decrease (lower). Genes that were upregulated more than two-fold in Caco-2 cells (CSGs) are labelled and shown in black.

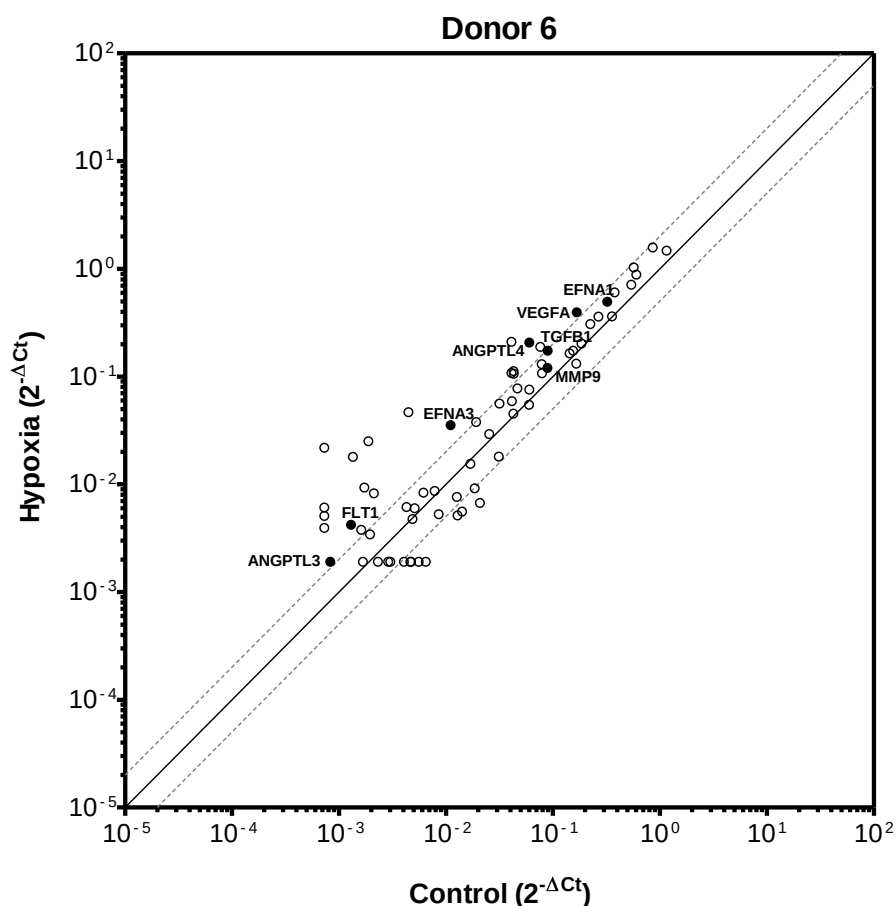


Figure 5.12 Hypoxia-induced angiogenesis gene expression Donor 6

The TDC from donor 6 was exposed to hypoxia (1% O₂) for 24 hours. mRNA was extracted and expression evaluated in duplicate by RT² Profiler Angiogenesis PCR Array. Figure shows the scatter plot with 2^{-ΔCt} values plotted against untreated control. Solid line indicates no change; dashed lines indicate two-fold increase (upper) and decrease (lower). Genes that were upregulated more than two-fold in Caco-2 cells (CSGs) are labelled and shown in black.

As illustrated in Figure 5.7 to Figure 5.12, there was significant heterogeneity in the pattern of hypoxia-induced angiogenic gene expression between donors. Data from the six scatter plots are presented in Table 5.8. For each donor, the number of genes detected (“Detected Genes”), and the number whose expression changed by greater than ±2-fold (“Altered genes”) was tabulated. There was significant variation in the total number of genes detected per donor, ranging from 83 in donor 5 (99% of the 84 genes on the array) to 50 in donor 2 (60%). “Altered genes” ranged from 63 (75% of the 84 genes) for donor 1, to 24 (29%) for donor 5. Of the “Altered genes”, the proportion that were upregulated varied from 85% in donor 2 (28 of 33 genes) to 21% for donor 5 (5 of 19 genes).

Table 5.8 Patterns of Hypoxia-Induced Angiogenesis Gene expression

Table summarises hypoxia-induced changes in expression of the 84 genes on the Angiogenesis Array. For the 6 donors analysed, the number of genes that were detected, upregulated or downregulated is shown. (*Genes that were not altered by ± 2 -fold are excluded.) Figures for Caco-2 are included for comparison.

	Donor						Caco2
	1	2	3	4	5	6	
Detected Genes	80	50	70	78	83	69	53
% of total (n=84)	95	60	83	93	99	82	63
*Altered Genes	63	33	38	39	24	29	17
% of total (n=84)	75	39	45	46	29	35	20
*Upregulated	51	28	31	23	5	20	17
% of altered genes	81	85	82	59	21	69	100
*Downregulated	12	5	7	16	19	9	0
% of altered genes	19	15	18	41	79	31	0

The pooled expression of CSGs in all six donors is shown in Figure 5.13. 3 genes were upregulated greater than two-fold in the TDCs, namely ANGPTL-3 (fold-regulation 2.63), ANGPTL-4 (2.79) and FLT-1 (5.22). EFNA-3 and MMP-9 had fold-regulations close to 2 (1.94 and 1.86 respectively). The remaining three had minimal fold-regulations (1.39 for EFNA-1, 1.19 for TGF- β 1, and 1.09 for VEGF-A). ANGPT-1 was only detected in one donor.

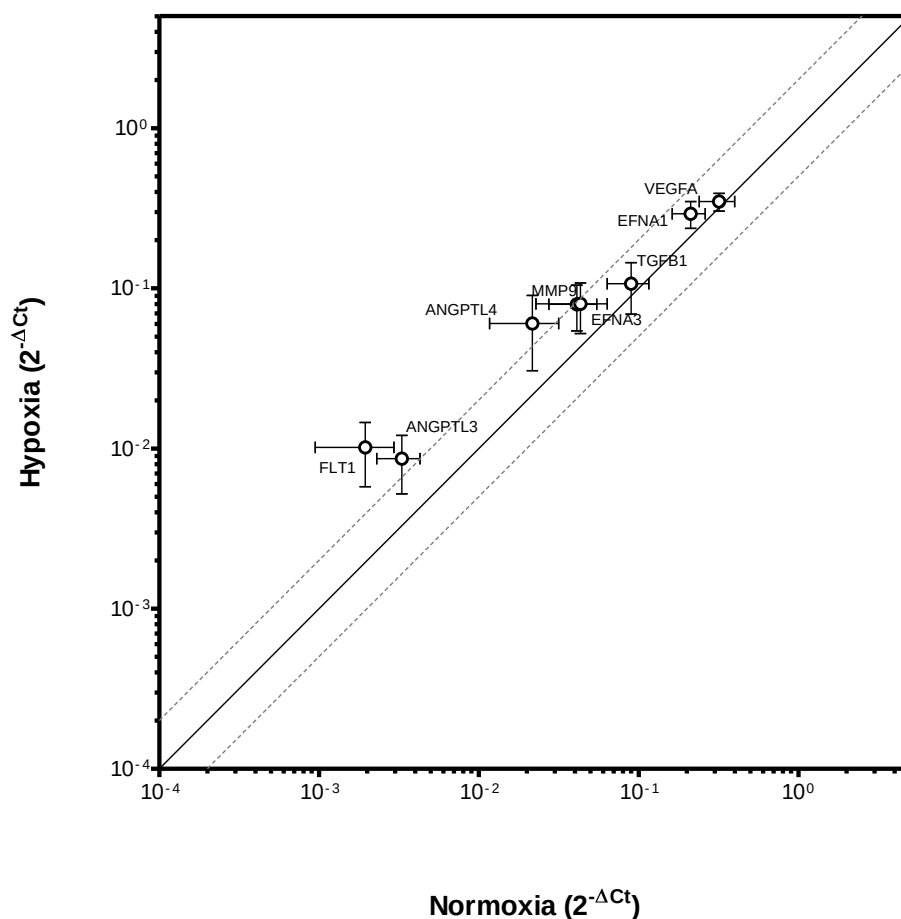


Figure 5.13 TDC Expression of Caco-2 Signature Genes – Pooled data

TDC were exposed to hypoxia (1% O₂) for 24 hours. mRNA was extracted, and following cDNA amplification by RT-PCR, gene expression was evaluated by RT² Profiler Angiogenesis PCR Array in duplicate. Figure shows the scatter plot with 2^{-ΔCt} values plotted against untreated control. Only genes upregulated greater than two-fold by hypoxia in Caco-2 are shown (CSGs). Data are mean ± SEM from 6 donors. Solid line indicates no change; dashed lines indicate two-fold increase (upper) and decrease (lower).

Figure 5.14 shows genes that were up- or down-regulated greater than two-fold in 3 or more donors. To exclude genes where expression levels varied greatly between donors, only those for which the SEM of 2^{-ΔCt} values was less than half of the mean were included. In addition to the 3 genes shared with Caco-2, a further 15 novel genes were identified, giving a total of 18 putative TDC Signature Genes (TSGs). 5 were downregulated (fold-regulations: COL4A3 -4.12, HAND2 -5.81, TEK -2.47, TIMP-3 -3.89 and TNF -8.81). The remaining 13 genes were upregulated (ANPEP 2.06, CCL11 2.61, CXCL3 2.95, CXCL9 3.24, HIF1A 2.51, IFNG 2.67, IL8 2.15, MMP2 2.44, PDGFA 2.13 and PLAU 3.22).

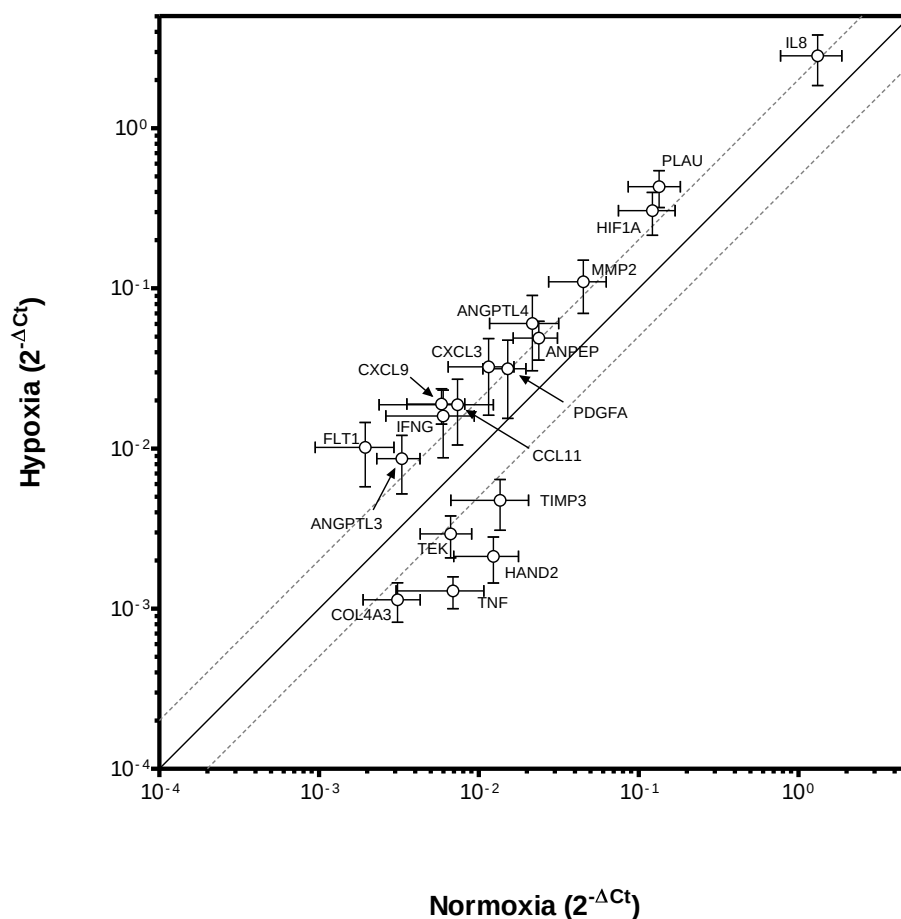


Figure 5.14 Hypoxia-responsive genes in TDCs - pooled data

TDC were exposed to hypoxia (1% O₂) for 24 hours. mRNA was extracted and expression evaluated in duplicate by RT² Profiler Angiogenesis PCR Array. Figure shows the scatter plot with 2^{-ΔCt} values plotted against untreated control. Data are mean ± SEM from 6 donors. Solid line indicates no change; dashed lines indicate two-fold increase (upper) and decrease (lower).

5.3.3.2.3 Q-PCR Validation

In order to validate the array data, it was necessary to independently evaluate gene expression by Q-PCR. Of the 34 donors in the full cohort, sufficient RNA for cDNA amplification was obtained in 13. Yields for several of these donors were only sufficient for one RT-PCR reaction, which limited the number of genes that could be tested. Therefore, in order to maximise use of limited cDNA, and to allow meaningful comparisons to be made across all 13 patients, four of the nine CSGs were selected for which validated primers were available, namely ANGPTL-4, EFNA-3, TGF-β1 and VEGF. Demographic and pathological data for all 13 patients is shown in the APPENDIX (Table 7.2 and Table 7.3).

Using the same primers that were used in Caco-2 cells, Q-PCR was performed on the same cDNA that was used in the arrays. The results for these 6 donors were pooled, and are

shown in Figure 5.15. The Q-PCR data paralleled that from the array in Figure 5.13, with mean ANGPTL-4 expression being greater than two-fold, while EFNA-3, TGF- β_1 and VEGF expression was unchanged. Of note, for donors 5 and 6, VEGF was not detected by Q-PCR in either normoxia or hypoxia. Fold-change was 2.52 ± 0.55 for ANGPTL-4 (mean $\pm$ SEM, range 0.19 to 3.89), 1.43 ± 0.32 for EFNA-3 (range 0.43 to 2.63), 1.06 ± 0.28 for TGF- β_1 (range 0.39 to 2.30) and 1.08 ± 0.32 for VEGF (range 0.23 to 1.79).

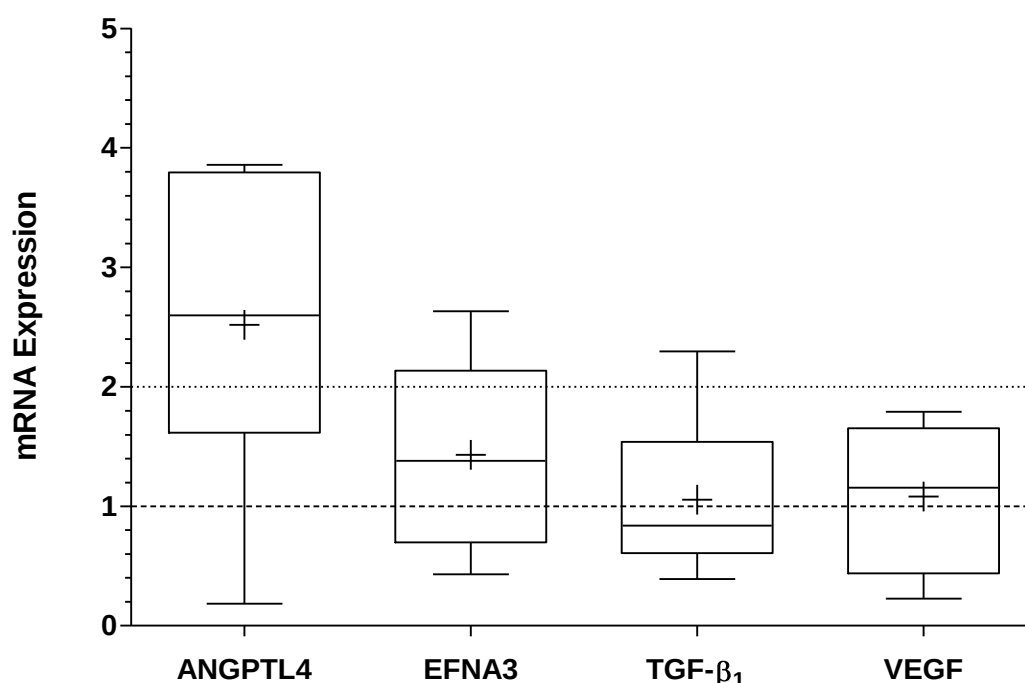


Figure 5.15 Q-PCR Validation of Array data

mRNA used in the preceding array experiments was analysed by Q-PCR. Figure shows a box-whisker plot of ANGPTL-4, EFNA-3, TGF- β_1 and VEGF fold-changes for all 6 patients normalised to normoxia (dashed line). The dotted line represents a fold-change of 2, greater than which upregulation was taken to be meaningful. Data are 25%, 50% (median) and 75% percentiles (box), with + representing the mean, and whiskers representing maximum and minimum values.

Q-PCR analysis of these four genes was also performed for the 7 donors that were not analysed by array. The individual fold-changes for all 13 donors are presented in Figure 5.16. Each data point corresponds to the fold-change for an individual donor, with open triangles representing the donors analysed by array. A similar pattern was seen for ANGPTL-4, EFNA-3 and TGF- β_1 , with mean upregulation the latter two genes being below two-fold. However, 4 of the additional donors showed higher upregulation of VEGF, with one reaching a fold-change of 12.1, leading to an overall mean that was greater than 2-fold. Fold-change was 2.84 ± 0.53 for ANGPTL-

4 (mean $\pm$ SEM, range 0.13 to 6.58), 1.65 ± 0.28 for EFNA-3 (range 0.43 to 3.27), 0.91 ± 0.13 for TGF- β 1 (range 0.39 to 2.30) and 2.59 ± 1.03 for VEGF (range 0.23 to 12.07). There was no correlation between gene induction and clinical parameters.

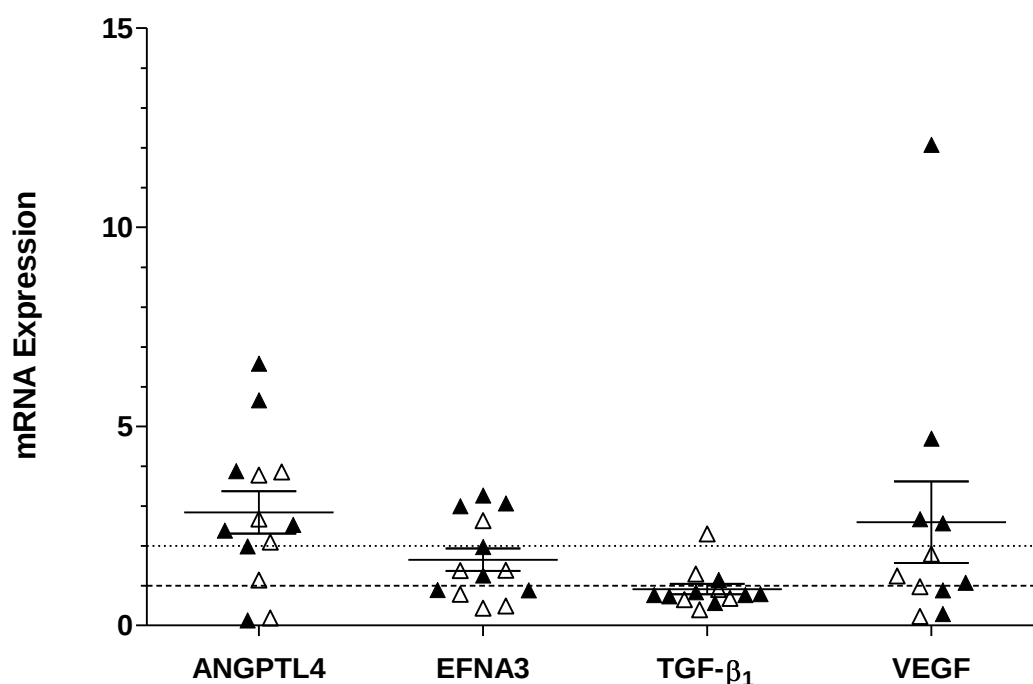


Figure 5.16 Q-PCR data for all donors

For all 13 donors that yielded sufficient RNA, angiogenesis gene expression was analysed by Q-PCR. Figure shows a scatter plot of ANGPTL-4, EFNA-3, TGF- β 1 and VEGF fold-changes normalised to normoxia (dashed line). Each data point represents mean fold-change for a single donor, with open triangles representing donors which were also analysed by array. The dotted line represents a fold-change of two, greater than which upregulation was taken to be meaningful. Solid lines and error bars represent the grand mean $\pm$ SEM for each gene.

The array data demonstrated that in response to hypoxia, TDCs upregulated a number of angiogenesis genes. While there was significant variation between donors, a total of 18 TDC “signature genes” (TSGs) were consistently altered (Figure 5.17). 3 of these genes were also upregulated in Caco-2 cells. Thus a total of 24 genes were found to be hypoxia-regulated in CRC. The functions of these genes broadly fall into 5 groups, namely transcription factors, cytokines, growth factors, vascular remodelling factors and matrix remodelling/adhesion factors (Figure 5.18).

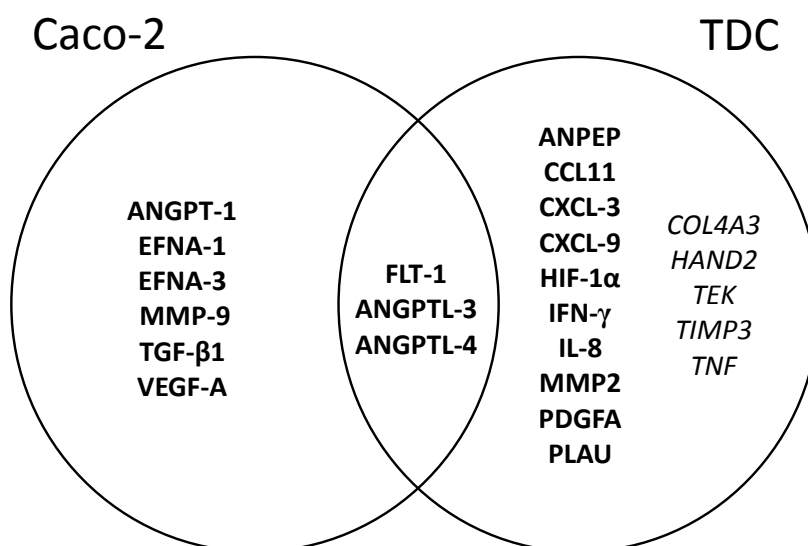


Figure 5.17 Angiogenesis genes induced by hypoxia in Caco-2 cells and TDCs

Figure shows genes that were up- or downregulated by more than 2-fold (downregulated genes are shown in italics). 3 genes featured in the response signature of both cell-types, while 6 were only upregulated in Caco-2 cells, and 15 were exclusive to TDCs.

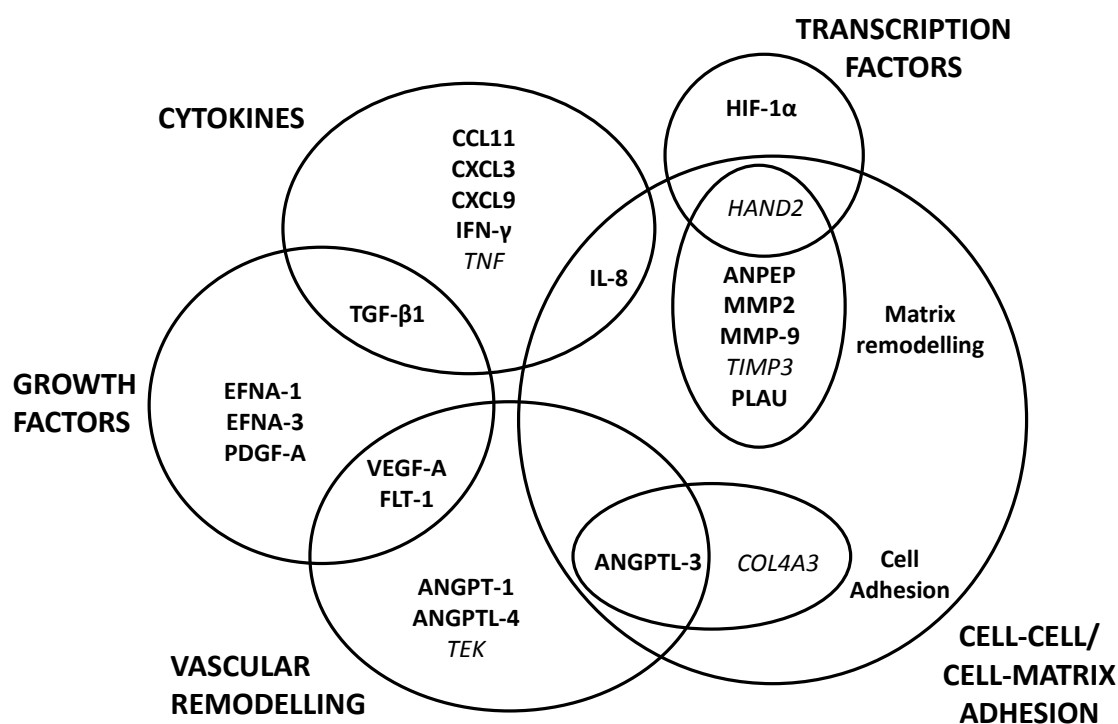


Figure 5.18 Functions of hypoxia-induced genes

Angiogenesis genes that changed greater than two-fold following hypoxia stimulation in both Caco-2 and TDCs fall into 5 groups in terms of function. Genes in italics were downregulated.

5.4 Discussion

The use of *ex vivo* short-term cultures of mixed cell populations derived from whole diseased tissue has been used successfully in our laboratory to study rheumatoid arthritis and atherosclerosis (Brennan et al. 1989b; Monaco et al. 2004). Following enzymatic digestion of synovial membranes and disaggregation of cell clusters by filtration, cultures are maintained for up to 7 days. A major benefit of this technique is that it generates a heterogeneous cell population which models cellular interactions that take place *in vivo* significantly more accurately than homogenous cell-lines. The potential therapeutic benefit of TNF- α blockade in rheumatoid arthritis was first identified in such cultures, derived from synovial membranes of rheumatoid arthritis patients (Brennan et al. 1989a). This discovery heralded a new era of targeted and highly effective therapeutics for rheumatoid arthritis and other chronic inflammatory diseases (Feldmann 2002; Feldmann and Maini 2003). Subsequent adaptation of the technique to establish cultures derived from atherosclerotic plaques has been used to elucidate the role of NF- κ B (Monaco et al. 2004) and toll-like receptors in the pathophysiology of atherosclerosis (Monaco et al. 2009).

As described in the introduction to this chapter, the use of *de novo* cell-cultures from primary tumour tissue addresses some of the limitations of established cell-lines such as homogeneity and “genotypic drift”. Adaption of the methodology used in rheumatoid and atherosclerotic tissue to CRC tumour samples has previously been attempted in our laboratory (T. Khong, unpublished data). While isolating cells from CRC tissue was shown to be feasible, the objective of that study was to establish and propagate long-term cultures, which was thwarted by significant technical challenges. However, success was achieved with stimulation experiments performed immediately after isolation of the cells. For my study, therefore, I chose to follow more closely the rheumatoid/atherosclerosis model and restrict efforts to establishing short-term cultures. Similarities between the responses of tumour-derived cultures and those of Caco-2 cells would serve to validate the cell-line data obtained using the same experimental hypoxia model, as presented in Chapter 3. Furthermore, evaluation of the angiogenesis response to hypoxia of TDCs might give an insight into the angiogenic phenotype of the individual donor tumour. In view of the paucity of predictive markers for CRC, particularly with regard to response to angiogenic therapy, this model might provide data to address this.

It is important to note that the aim of the technique is to investigate the ability of the cultures to respond to hypoxia, rather than to assess the hypoxic status of the original tumour. The latter objective would require immediate fixation once the sample is obtained, and would result in an analysis that provided a “snapshot” of the level of hypoxic activation at the time of fixation. A significant limitation of this approach is that tumour oxygen levels are highly dynamic *in vivo*. While the integrity of the blood supply to the tumour determines the maximum oxygen delivery

that can be achieved, the actual oxygen tension at any given timepoint is dependent on local blood flow and arterial oxygen saturation. Hypotension and hypoxaemia are not uncommon during surgery (Bijker et al. 2007; Ehrenfeld et al. 2010). Furthermore, the surgical technique requires ligation of the blood supply to the tumour-bearing segment of colon prior to its resection. Depending on the technical difficulty of the operation, there may be up to 30 minutes (or more) between interruption of the blood supply and removal of the specimen. Even at the earliest time that the sample could be taken from the excised specimen and fixed, the oxygen tension bears little relation to “normal” levels, and the significant variation in resection time confounds comparisons between samples obtained from different operations. More biologically relevant is the way the cancer cells respond to hypoxic episodes. The manner in which the extracted cells respond to hypoxia (or other stimuli), provided they remain viable, is likely to remain unchanged within the lifespan of these short-term cultures. It is this capacity that this model aims to evaluate.

Previous experience with establishing mixed cultures from rheumatoid and atherosclerotic tissue has demonstrated that a delay of up to 72 hours from surgery to enzymatic digestion does not significantly affect the yield of viable cells. For colonic tissue, however, I observed a drop-off in yields if digestion was delayed for more than 48 hours, with bacterial overgrowth being common beyond 72 hours. Therefore enzymatic digestion was commenced within 24 hours of surgery wherever possible. Once established, although there were still some viable cells after 7 days, cell viability was observed to decrease significantly after 48 hours of incubation in normoxia. In addition to observations from rheumatoid and atherosclerotic cultures that cytokine expression peaks between 24 and 48 hours (Brennan et al. 1989b; Monaco et al. 2004), this prompted the decision to perform experiments immediately after isolation of the TDCs for a period 24 hours. As well as minimising loss of cell viability, and investigating the cells when they are at their most active, 24 hour stimulation also allowed direct comparison with the Caco-2 data.

Attempts were made to establish TDCs using tissue from 34 patients. Comparison of demographic and pathological data with national statistics showed that this cohort was representative of the general UK population of CRC patients and tumours. Despite the restriction of the technique to establishing short-term (as opposed to long-term) cultures, significant technical challenges were encountered, particularly with the earliest samples. This may be attributable in part to an initial learning curve. One of the main problems encountered, particularly with earlier samples, was that of insufficient cell yields. A number of factors were observed to influence this. The first was insufficient tissue, and there was, as might be anticipated, a correlation between sample mass and cell yield. In addition, tissue taken from the leading edge of the tumour yielded more cells than that taken from the centre, which tended to be necrotic. Another observation was that samples with a firm texture, which was evident when dicing the

sample with a scalpel and possibly indicated fibrosis and/or necrosis, yielded fewer cells. A high proportion of fatty tissue, manifested as a layer of stringy, yellow material which persisted after filtration to remove acellular debris following collagenase treatment, also tended to lower cell yields. Factors affecting cell yield also influenced RNA yield, but the correlation was less clear-cut. Some small samples, for example, yielded higher RNA concentrations than much larger ones. There was, however, a linear correlation between cell yield and RNA yield (data not shown).

Density-gradient separation was introduced into the protocol in a previous study in our laboratory in which the aim was to establish long-term CRC cultures. The effect of RBC “contamination” was thought to have potential adverse effects on the propagation of these cultures. Nevertheless, the depletion of otherwise large numbers of RBCs appeared to enrich the epithelial cell fraction. Certainly, my observation was that omission of density-gradient separation resulted in very large numbers of anuclear RBCs, which when plated at the target cell density led to much lower RNA yields. A drawback of gradient separation is that it increases the time required to complete the cell dissociation process by almost an hour. Attempts were made to achieve RBC depletion using a RBC lysis buffer, but this proved a far less efficient method and was not pursued further.

Of the total 34 patients, the evaluation of angiogenesis response to hypoxia was possible in 13 (38%). The major limitation was the lack of sufficient RNA for cDNA synthesis. None of the first 9 TDCs yielded sufficient RNA. If one assumes that this was due to a learning-curve effect, excluding these 9 patients gives an improved rate of 52%. This compares favourably to success rates for the establishment of long-term cultures from primary tissue, which range from 10% to 45% (Leibovitz et al. 1976; McBain et al. 1984; Kirkland and Bailey 1986).

Initial characterisation of the TDCs was performed by observing cell morphology. Microscopy revealed significant morphological heterogeneity, which became more apparent when the cultures were maintained for several days. Some cells with similar morphology to Caco-2 cells were seen. Despite density-gradient separation it was observed that the cultures contained some RBC-like cells. In addition to colonic tumour cells that are exclusively of epithelial origin, the methodology employed to establish these cultures would be anticipated to generate a variety of other cell types, such as fibroblasts, tumour-associated macrophages and other immune cells. Further characterisation experiments therefore aimed to identify epithelial cell markers. Q-PCR was used to evaluate the expression of the epithelial cell markers Ep-CAM and VE-Cadherin. The former is exclusively expressed by epithelial cells, and expression of the latter, although predominantly found in vascular endothelium, has also been reported in tumours of epithelial origin (Labelle et al. 2008). VE-Cadherin expression in Caco-2 cells was demonstrated in my earlier experiments (Figure 3.8). Both markers were expressed in TDCs, indicating the presence

of epithelial cells. Mean Ep-Cam expression was similar in TDCs and Caco-2 cells, while mean VE-Cadherin was much lower in TDCs. There was significant variation between donors, which may reflect variation in epithelial cell fraction.

Expression of carcinoembryonic antigen (CEA), a tumour marker used in the follow-up of post-surgical CRC patients, was also evaluated. CEA expression has been reported in normal epithelial cells outside of the GI tract at low levels (e.g. uterine cervix and sweat glands), and can be elevated in tumours arising from these tissues (Metze et al. 1996; Ikeda et al. 2012). In the colon CEA is exclusively found in columnar epithelial and goblet cells (Hammarström 1999), and expression has been reported in Caco-2 cell, in which it was found to be hypoxia regulated and HIF-1 α dependent (Kokkonen et al. 2007). CEA was readily detected in TDC supernatants at high concentrations, indicating the presence of tumour cells. Expression was unaffected by hypoxia. In addition CEA was not detected in Caco-2 cell supernatants. While this is contrary to Kokkonen *et al.*, that study detected CEA by Western Blot using whole cell lysates and did not investigate secreted CEA in supernatants.

These characterisation experiments indicated the presence of cells of epithelial origin in the TDCs. Accurate cell-type identification was not performed, and this is an important next step in the development of this model.

Angiogenic responses of the TDCs were then investigated. Due to the limited RNA yields, it was not possible to evaluate responses to both hypoxia and DMOG. The Caco-2 array data indicated that hypoxia stimulated more pronounced responses than DMOG, both in terms of fold-changes and number of altered genes, and the decision was taken to use hypoxia for the TDC experiments. Before utilising the array, preliminary experiments used Q-PCR to evaluate hypoxia-induced expression of four genes (VEGF, ANGPTL-4, EFNA-3 and TGF- β 1) in two donors. Results were similar, but not identical, to the Caco-2 data. A significant benefit of the PCR array is the ability to rapidly generate large amounts of data. However, its utility is somewhat limited by high costs (over £1000 per donor assayed), and the complexity of the data generated can be difficult to interpret. It was therefore used to evaluate angiogenic gene responses in a limited number of patients. Of the 13 TDCs from which sufficient RNA was obtained, 6 consecutive samples were assayed. Within the limitations of the small sample size, statistical analysis indicated that these 6 were representative of the whole cohort of 34 in terms of demographics and pathology. In order to validate the array results, Q-PCR was used to analyse expression of VEGF, ANGPTL-4, EFNA-3 and TGF- β 1. Pooled analysis of Q-PCR data for the 6 donors reflected the array results, with greater than 2-fold upregulation seen for ANGPTL-4 only (Figure 5.13 and Figure 5.15).

Overall it was clear that there was marked heterogeneity in the pattern of hypoxia-induced angiogenic gene expression between donors. A simple illustration of this can be seen in the number of genes detected, and of genes altered greater than ± 2 -fold for each donor (Table 5.8). Genes detected varied from 83 (99%) to 50 (60%), and the number of genes altered ranged from 63 (75%) to 24 (29%). When altered genes were considered alone, upregulation predominated in response to hypoxia stimulation some donors (e.g. 28 of 33 genes in donor 2), while in others genes were mainly downregulated (14 of 19 genes for donor 5).

Variability of cell composition between individual TDCs would limit the validity of any direct comparisons. If the variations in hypoxia response were entirely due to cell-type heterogeneity, one would anticipate little or no consistency in either gene abundance or fold-changes between the donors. Furthermore, there would be little or no similarity between TDC gene expression patterns and that of the CRC monoculture of Caco-2 cells. Array analysis of Caco-2 cells identified 9 genes that were consistently upregulated (“Caco-2 signature” genes) (Figure 3.11). As illustrated in Figure 5.13, when the data for all 6 donors was pooled, there was consistency within the cohort in terms of both abundance (based on $2^{-\Delta C_t}$ values) and fold-changes (based on $2^{-\Delta\Delta C_t}$ values) for 8 of the 9 CSGs. In addition, gene abundance was comparable to that in Caco-2 cells. While further work is necessary to more accurately characterise the TDCs, this consistency suggests that the data from Caco-2 cells and TDCs are mutually supportive to a degree.

Since there was significant inter-donor expression of the 84 genes in the array, an attempt was made to identify genes for which there were consistent changes. Results for all 6 donors were pooled, and only genes that were detected in 3 or more donors were included (Figure 5.14). To eliminate genes for which abundance varied greatly between donors, an arbitrary cut-off was set. Genes were excluded if the SEM of $2^{-\Delta C_t}$ values was greater than half of the mean. This analysis resulted in a “TDC signature” of 18 genes which were up- or downregulated by greater than 2-fold. This was distinct from the Caco-2 signature, although there was some overlap, with 3 genes being upregulated in both cell types (Figure 5.17). The Caco-2 signature included only genes induced by both hypoxia and DMOG, since these are most likely to be HIF-dependent (8 genes that were exclusively hypoxia-regulated were excluded). Only hypoxia-stimulation was used for the TDC array, and it may be that several of the 18 genes identified are not induced by DMOG, and hence likely to be HIF-independent. (None of the 8 genes that were only hypoxia-regulated in Caco-2 cells featured in the TDC signature.) A further distinction between the two cell types was that 5 genes were downregulated in TDCs, whereas none were in Caco-2.

The wide variation in gene expression patterns revealed by the array data may reflect heterogeneity in the proportions of mixed cell-types in each TDC. However, as described in the

introduction to this chapter, it is clear that CRC is a highly heterogeneous disease, and these variations may reflect real differences in the hypoxic response of tumour cells from different donors. Clearly, identification of correlations between expression patterns and clinical or demographic data would address this question, but a meaningful analysis was not possible due to the low number of donors assayed by PCR Array. Due to the prohibitive cost, subsequent TDCs were analysed by Q-PCR only, focusing on VEGF, ANGPTL-4, EFNA-3 and TGF- β 1 expression. These genes were chosen for several reasons. Having been identified as part of the Caco-2 signature in Chapter 3, their regulation by HIF was shown in Chapter 4. The importance of VEGF in CRC is well established. The role of ANGPTL-4 in angiogenesis and cancer has emerged over the last decade, and although its expression in CRC has been reported to correlate to poor prognosis (Nakayama et al. 2011; Tan et al. 2012), there is some controversy as to whether it promotes or enhances angiogenesis and metastasis (Galaup et al. 2006; Kim et al. 2011). TGF- β 1 is known to be upregulated in early cancer, with subsequent downregulation as invasion and metastasis develop (Massagué 2008). Recent evidence suggests a role for hypoxic regulation of TGF- β 1 (Wincewicz et al. 2010; Xian et al. 2011). Hypoxic regulation of ephrin-A3 has recently been reported in rheumatoid arthritis in our laboratory, but its role in cancer is not known.

Q-PCR analysis was performed for a further 7 patients. The expression pattern of ANGPTL-4 (upregulated), EFNA-3 and TGF- β 1 (unchanged) that was seen in the 6 array donors persisted when Q-PCR data for all 13 patients was analysed (Figure 5.16). However, some of the additional patients showed much higher VEGF upregulation. No correlation with clinical data was seen, but this may be a function of the low sample size. Whether the ability of TDCs to upregulate these four genes (or other genes) is related to clinical parameters is an interesting question. Clearly greater numbers are necessary, and this is the subject of on-going work in our laboratory.

Thus, the Caco-2 and TDC signatures included a total of 24 hypoxia-induced angiogenesis genes. These fall into 5 broad functional groups, namely transcription factors, cytokines, growth factors, vascular remodelling factors and matrix remodelling/adhesion factors (Figure 5.18). These genes are discussed below.

Transcription Factors

The transcription factors altered in Caco-2 and TDC were HIF-1 α and HAND2. The function of HIF-1 α has been extensively described in earlier chapters. As shown in Chapter 4, HIF-1 α mRNA levels in Caco-2 are suppressed by hypoxia, due to a negative feedback mechanisms including aHIF (Thrash-Bingham and Tartof 1999; Bruning et al. 2011; Xu et al. 2012). Moderate downregulation was also seen in the array data (Figure 3.10). HIF-1 α was

upregulated in TDCs, which suggests that this negative feedback loop may be suppressed or absent in these cultures.

HAND2 overexpression in squamous cell carcinoma of the lung has been reported (Metodieva et al. 2011). While it has an important role in cardiovascular and sympathetic nervous system development, but its role in cancer is poorly understood. Its angiogenic function may be mediated via the VEGF signalling pathway (Yamagishi et al. 2000), and it has been reported to regulate ECM remodelling (Yin et al. 2010). The significance of hypoxic downregulation of HAND2 seen here is unclear, and requires further investigation.

Cytokines

A number of cytokines and chemokines relevant to angiogenesis were altered by hypoxia in both cell types. CCL11 (eotaxin) stimulates endothelial cell migration, implicating it in angiogenesis (Salcedo et al. 2001). In lymphoma and ovarian cancer, it can promote proliferation and inhibit apoptosis (Levina et al. 2009; Miyagaki et al. 2011). In CRC, increased eosinophil infiltration correlates to better prognosis (Pretlow et al. 1983; Fernández-Aceñero et al. 2000). CRC patients have decreased plasma concentrations of CCL11, with lower levels associated with later stage, while expression is increased in tumour versus normal tissue (Wågsäter et al. 2007). Hypoxic suppression of CCL11 expression by adipocytes has been reported (Famulla et al. 2012), in contrast to the upregulation seen in TDCs in my study.

Upregulation of CXCL-3, CXCL-9 and IL-8 has been reported in CRC tissue (Erreni et al. 2009; Doll et al. 2010). CXCL-3 and IL-8 are angiogenic, stimulating endothelial cell migration (Vandercappellen et al. 2008). CXCL-3 is also associated with metastasis in several cancers, and is upregulated by prolonged hypoxia in myeloid dendritic cells, but little is known about hypoxic-induction of CXCL-3 in CRC (Blengio et al. 2012). In the absence of functional HIF-1 α , IL-8 is upregulated by hypoxia and can mediate VEGF-induced tumour angiogenesis (Mizukami et al. 2005). Increased expression of CXCL9 (which is angiostatic unlike CXCL-3 and IL-8) has been reported in CRC tissue (Erreni et al. 2009), and exogenous CXCL9 has been shown to have tumour-suppressive effects in both *in vitro* and mouse models of CRC (Ruehlmann et al. 2001). Although downregulation of CXCL9 by hypoxia has been reported in glioma (Marotta et al. 2011), there are no reports of hypoxia-regulation in CRC.

The interferons exert their angiostatic effects both directly by inhibiting endothelial cell function (Indraccolo 2010), and indirectly, by inhibiting angiogenic factors such as VEGF, IL-8, and MMP-9 (von Marschall et al. 2003). IFN- γ is upregulated in CRC tissue and is associated with increased CRC risk (Slattery et al. 2011; Nam and Park 2012). IFN- γ -stimulated

mesenchymal stem cells (MSCs) promote angiogenesis and tumour growth in a VEGF-mediated, HIF-1 α -dependent mechanism in a CRC cell-line xenograft model (Liu et al. 2011).

The importance of TNF- α in systemic inflammation is evidenced by the success of anti-TNF therapies in rheumatoid arthritis (Brennan et al. 1989a; Feldmann and Maini 2003). Septic shock is the clinical end-point of the TNF- α -mediated response to bacterial endotoxins (Tracey et al. 1986), in which HIF-1 α has been shown to play a role (Peyssonnaud et al. 2007). The link between inflammation and cancer is well-established (see Chapter 1). While TNF can have anti-tumour effects (Kashii et al. 1999) it also promotes the development and progression of cancer (Balkwill 2006; Balkwill 2009). Elevated TNF- α levels in CRC is associated with poor prognosis (Naylor et al. 1990; Szlosarek and Balkwill 2003). TNF- α can induce cancer cell production of IL-8, MMP-9, TGF- β 1 and VEGF (Nabors et al. 2003; Balkwill 2004; Kulbe et al. 2005; Stuelten et al. 2005). Hypoxia induces TNF- α expression in macrophages (Liu et al. 2008), but reduces adipocyte expression of TNF- α -induced cytokines by downregulating the TNF- α receptor TNF-R1 (Famulla et al. 2012). TNF- α has been reported to upregulate VEGF production by mesenchymal stem cells in a HIF-1 α -dependent manner, promoting angiogenesis and tumour growth in CRC cell xenografts (Liu et al. 2011), but it can also inhibit HIF-1 α activity in osteoblasts (Mendonça et al. 2011). In view of its complex, context-specific regulation and function, it is difficult to interpret the hypoxic downregulation of TNF- α seen in my TDC array data. The therapeutic targeting of TNF- α activity in cancer has been the focus of much research (Szlosarek and Balkwill 2003). A key question is whether therapies should aim to stimulate or inhibit TNF- α (Balkwill 2009). A systematic review of clinical trials found no increased risk of malignancy in rheumatoid arthritis patients receiving anti-TNF therapy (Setoguchi et al. 2006). Early clinical trials of TNF antagonists in advanced cancer have demonstrated some efficacy in a limited proportion of patients (Harrison et al. 2007; Brown et al. 2008). TGF- β 1 is discussed in the subsequent section, due to its function as both a cytokine and growth factor.

Growth Factors

Growth factors stimulate cellular growth, proliferation and cellular differentiation, and a total of 6 growth factors/growth factor receptors (TGF- β 1, ephrin-A1 and -A3, PDGF-A, VEGF and its receptor FLT1) were hypoxia-regulated. Transforming growth factor- β 1 (TGF- β 1) and its interactions with the HIF pathway has been discussed earlier (sections **Error! Reference source not found.** and 3.4). In CRC, elevated TGF- β 1 levels are associated with increased angiogenesis and poor prognosis (Gulubova et al. 2010), and correlation of TGF- β 1 and HIF-1 α expression has been reported in CRC (Wincewicz et al. 2010). In a study of a variety of 50 cancer cell-lines derived from various primary tissues, a trend towards downregulation of TGF- β 1 protein

expression in response to hypoxia was seen (Heinzman et al. 2008), which is reflected at the mRNA level in my TDC data.

The ephrins have been discussed in Chapter 1 and Chapter 3. Aberrant Eph/ephrin expression is seen in several cancers including CRC, and correlates with stage and prognosis (Miyazaki et al. 2003; Fox and Kandpal 2004; Jubb et al. 2005; Herath et al. 2006; Herath et al. 2009). The precise role of ephrin ligand-receptor interactions in CRC is complex. A oncogenic role has been proposed for several Eph receptors which are upregulated in early CRC, and their subsequent silencing in more advanced tumours is thought to play a role in invasion and metastasis (Herath and Boyd 2010). Marked upregulation of ephrin-A3 is seen in lung tumours, but equal expression in normal colon and CRC has been reported (Hafner et al. 2004). Upregulation of several Eph receptors and ephrins by hypoxia has been reported in hepatoma and prostate cancer cell-lines (Vihanto et al. 2005), but the hypoxic induction of ephrin-A3 seen in Caco-2 has not been previously reported in CRC.

PDGF (platelet-derived growth factor) is a member of the same growth factor family as VEGF with diverse functions (Yu et al. 2003). It is a potent mitogen for cells of mesenchymal origin, and induces cellular processes including angiogenesis, proliferation, migration and apoptosis (Yu et al. 2003). It is critical in embryonic development, and plays an important role in inflammation and wound healing. Its tumourigenicity was first discovered almost 30 years ago (Deuel et al. 1983), and it has been implicated in several cancers such as gliomas, sarcomas and leukaemias (Heldin 2012). PDGF has been detected in CRC tissue, and elevated levels have been reported in platelets from CRC patients (Peterson et al. 2012). PDGF can promote tumourigenesis through autocrine (Uhrbom et al. 2000) and paracrine mechanisms (Sundberg et al. 1997). Hypoxia has been shown to enhance PDGF signalling via a HIF-1 α -mediated mechanisms in pulmonary arterial smooth muscle cells (ten Freyhaus et al. 2011), and breast cancer (Schito et al. 2012) leading to enhanced metastasis. Therapeutic targeting of the PDGF pathway has shown promise in cancers such as chronic myeloid leukaemia and gastro-intestinal stromal tumours. Imatinib (Gleevec ©) a potent PDGF receptor inhibitor, has FDA approval for the treatment of both cancers (Cohen et al. 2005; Cohen et al. 2012), and in CRC has been shown to inhibit CRC cell growth and stromal fibroblast activity *in vitro* (Stahtea et al. 2007).

Vascular Remodelling Factors

The angiopoietins (ANGPT) and angiopoietin-like (ANGPTL) proteins have been discussed in Chapter 1 and Chapter 3. Overexpression of ANGPT-1 has been reported to suppress angiogenesis and tumour growth in CRC, while ANGPT-2 has the opposite effect (Ahmad et al. 2001b; Stoeltzing et al. 2003). ANGPT-1 was induced by both hypoxia and DMOG in Caco-2

cells, suggesting HIF-independence. It was only detected in 1 of the 6 donors (in which it was strongly upregulated), supporting reports that it is rarely expressed in primary CRC tissue (Ahmad et al. 2001a). Hypoxic induction of pro-angiogenic ANGPT-2 has been reported in CRC cell-lines (Gu et al. 2006), but in my data this was only seen in one of 6 donors. In Caco-2 cells it was upregulated by hypoxia but not DMOG, suggesting HIF-independence. ANGPT-1 and -2 both signal via the Tie-2 (TEK) receptor. Hypoxia regulation of the Tie-2/TEK receptor has not been previously investigated in CRC cells, although induction has been reported in monocytes (Murdoch et al. 2007). In the present study, Tie-2 expression in TDCs was reduced by hypoxia, the significance of which warrants further investigation.

The structural complexity of ANGPTL-4 underlies its remarkably diverse roles (Zhu et al. 2012), and it can have both pro- and anti-tumourigenic roles (Tan et al. 2012). Elevated expression levels have been detected in cancers including CRC, and correlate with venous invasion and metastasis (Nakayama et al. 2011). ANGPTL-4 is upregulated by hypoxia via HIF-1 α (Kim et al. 2011), supporting my data, and by TGF- β 1 (Padua et al. 2008). It was upregulated in both TDCs (hypoxia) and Caco-2 cells (hypoxia and DMOG). In the latter, data suggested a reciprocal relationship between HIF-1 α and HIF-2 α regulation of ANGPTL-4. ANGPTL-3, which differs from ANGPTL-4 in expression patterns, regulation and function (Li 2006), has been reported to have similar angiogenic potency to VEGF in stimulating endothelial cell adhesion and migration via integrin-mediated signalling (Camenisch et al. 2002). To my knowledge, hypoxic induction of ANGPTL-3 as seen in TDCs has not been reported previously.

Adhesion Factors/Molecules And Matrix Remodelling Factors

As discussed in Chapter 3, upregulation of MMP-2 and MMP-9 and downregulation of TIMP3 are associated with angiogenesis, advanced tumour stage, increased metastasis and poorer survival in CRC (Grigioni et al. 1994; Zeng and Guillem 1996; Zeng et al. 1996; Kim and Kim 1999; Kikuchi et al. 2000; Bai et al. 2007). Hypoxia induction of both MMP-2 and -9 has been previously reported in the CRC cell-line LoVo (Wu et al. 2008), supporting my Caco-2 (MMP-9) and TDC (MMP-2) data. The hypoxic downregulation of TIMP shown here in TDCs has been reported in breast cancer cell-lines (Fu et al. 2009), although the isoforms differed (TIMP-3 in my study *versus* TIMP-4 in breast). Interestingly, IL-8, which was also hypoxia-induced in TDCs, has been shown to upregulate both MMP-2 and -9 in endothelial cells (Li et al. 2003a).

ANPEP which has similar enzymatic activity to MMPs, promotes human umbilical vein endothelial cell (HUVEC) proliferation, migration and capillary tube formation *in vitro* (Fukasawa et al. 2006). ANPEP expression has been reported in Caco-2 cells (Howell et al. 1992), and tissue expression correlates with increased tumour size and poor prognosis in several

cancers including CRC (Hashida et al. 2002; Wickström et al. 2011). In endothelial cells it is induced by hypoxia, VEGF and TNF- α (Bhagwat et al. 2001). Hypoxia induction, as seen in TDCs, has not been previously reported.

Urokinase-type plasminogen activator (uPA/PLAU) is a component of the urokinase plasminogen activating system (uPAS). As well as being implicated in angiogenesis, uPAS function can influence tumour cell proliferation, adhesion and migration, intravasation and metastasis (Blasi and Carmeliet 2002; Choong and Nadesapillai 2003). Increased expression of uPAS components has been reported to correlate to poor outcomes in numerous tumours including CRC. In CRC, uPA and its receptor (uPAR) accumulate at the invading tumour edge, and expression levels correlate with advanced Dukes' stage and poor prognosis (Buo et al. 1995; Tatsuta et al. 1997). A link between hypoxia and the uPAS system is suggested by the fact that uPAR and HIF-1 α co-localise at the tumour edge in CRC (Pyke et al. 1991), and supported by the finding that hypoxia-induced invasion in the HCT116 CRC cell-line is attenuated by anti-uPAR antibodies (Krishnamachary et al. 2003). The hypoxia-induction of uPA in TDCs in my study is in line with these findings.

A necessary step in the initiation of invasion of epithelial tumours is disruption of the basement membrane (BM), the major structural component of which is Collagen type IV alpha 3 (α -3(IV), encoded by the COL4A3 gene) (Tanjore and Kalluri 2006). Downregulation of α -3(IV) has been reported in CRC (Polette et al. 1997) and lung cancer (Nakano et al. 2001). Recombinant α -3(IV) potently inhibits angiogenesis and tumour growth in an *in vivo* melanoma model (Petitclerc et al. 2000), via mechanisms involving cleavage of α -3(IV) by MMP-9, or suppression by α -3(IV) of MMP-2 activation (Martinella-Catusse et al. 2001; Hamano et al. 2003). The hypoxic downregulation of α -3(IV) seen in TDCs has not been previously described. The α -3(IV) detected in the TDCs may derive from tumour or stromal cells. Nevertheless, the suppressive effect of hypoxia on α -3(IV) might be anticipated to be tumourigenic, either through a reduction of its potent angiostatic effects, or through disruption of its structural role in the BM.

Summary

Analysis of the array data revealed a putative TDC Signature, comprising of a set of 18 angiogenic genes that are regulated by hypoxia in TDCs. As discussed above, these genes are implicated in numerous signalling pathways and cellular processes, often interacting with each other in complex networks, which enhance the ability of tumour cells to proliferate, invade local tissue, and metastasise.

The methodology employed in this chapter represents a novel model for the investigation of hypoxia-induced angiogenesis in CRC. The use of cells derived directly from donor tumours avoids some of the problems associated with cell-lines that limit the strength of conclusions that can be drawn from their use. The TDCs established displayed similar characteristics to the Caco-2 cell-line such as expression of epithelial and CRC markers. Furthermore, the angiogenic responses to hypoxia of both cell-types showed some similarity, which provides a degree of validation of the Caco-2 data in Chapters 3 and 4. There was significant variation in the responses of different donors. This may be partly due to the mixed cell-types in the TDCs, but may also reveal true biological differences between the tumours of each donor, and further work is necessary to elucidate this (see Further Work). In addition, there were significantly more hypoxia-responsive genes in the TDCs than in the Caco-2 cells. This discrepancy further underlines the inability of the cell-lines to truly represent the behaviour of tumour cells *in vivo*.

Chapter 6

6 CONCLUDING REMARKS

The aims of this study were firstly to investigate the relative contribution of HIF-1 α and HIF-2 α in the hypoxia regulation of CRC (objectives 1 and 2), and secondly to compare the hypoxia-induced angiogenesis response of an established CRC cell-line (Caco-2) with primary cultures (TDCs) (objective 3).

Caco-2 hypoxia-responses were characterised (Chapter 3), and 3 novel hypoxia-induced angiogenesis genes (ANGPTL-4, EFNA-3 and TGF- β 1) were identified using a PCR angiogenesis array. The effect of selective silencing of the two HIF isoforms was investigated (Chapter 4), and the roles of HIF-1 α and HIF-2 α in the regulation of Caco-2 hypoxia-responses were found to be different, thus disproving the first null hypothesis.

In the second part of the study, similarities in the hypoxia response of Caco-2 cells and TDCs in terms of angiogenesis genes were identified (Chapter 5), thus disproving the second null hypothesis. Of particular note was the heterogeneity of hypoxia-induced angiogenesis responses from patient to patient.

6.1 General Discussion

As discussed in Section 1.3, evidence for the divergent roles of HIF-1 α and HIF-2 α in mediating gene expression in response to changes in oxygen tension has accumulated in recent years (Hu et al. 2003). It has been shown in several cell-lines and tissue types that they have different target genes (Wang et al. 2005; Carroll and Ashcroft 2006), albeit with some overlap. In addition, isoform-specific regulatory mechanisms have been described (Thrash-Bingham and Tartof 1999; Koh et al. 2011). Evidence from experiments in renal cancer suggests that HIF regulation can switch from HIF-1 α to HIF-2 α dominance, with upregulation of the latter suppressing expression of the former, resulting in a more aggressive phenotype (Koh et al. 2011). In CRC, there is controversy as to which isoform, if any, is dominant (Yoshimura et al. 2004; Franovic et al. 2009; Imamura et al. 2009; Rasheed et al. 2009), and as to whether this is functionally significant.

My data described in this thesis has demonstrated that both HIF- α isoforms are involved in hypoxia-regulated angiogenesis gene expression in Caco-2 CRC cells. Each of four genes (ANGPTL-4, EFNA-3, TGF- β 1 and VEGF) was induced by HIF-1 α . TGF- β 1 was also induced by HIF-2 α , and there was a trend (not statistically significant) towards HIF-2 α induction for VEGF and EFNA-3. Interestingly, expression of ANGPTL-4 was suppressed by HIF-2 α , and there was a trend towards the same for VEGF and TGF- β 1. This supports evidence from breast and renal cancer cell-lines of a reciprocal relationship between the two isoforms (Raval et al. 2005; Carroll and Ashcroft 2006), which has not been previously described in CRC. The functional significance of these HIF-isoform specific changes in gene expression requires further investigation. Preliminary experiments conducted in normoxia suggested an effect of HIF-2 α Caco-2 proliferation, but technical difficulties were encountered with a functional assay of angiogenesis.

Hypoxia-induction of BNIP-3 was exclusively HIF-1 α -regulated in Caco-2 cells, as has been shown in numerous cell-types. Of 4 other apoptosis genes investigated, 2 were downregulated by hypoxia (survivin, BAX), while 2 were unchanged (XIAP, Bcl-2). The functional relevance of these conflicting changes in apoptosis gene expression requires further examination (see section 0). Three cell-adhesion molecules were investigated, of which two (CD-151 and VE-Cadherin) were unaffected by hypoxia or DMOG. However, hypoxia-regulation of STAB-1 was seen. STAB-1 has a variety of functions in several cell-types, and its role in cancer is poorly understood. Hypoxia has been reported to both up- and downregulate expression in different cell-types (Movahedi et al. 2010; Ong et al. 2010). Interestingly, data indicated hypoxic suppression when confluence was below 50%, and induction above 50%. Modulation of hypoxic responses by cell-cell contacts is supported in data from bladder cancer cell-lines, in which

hypoxic VEGF induction in sub-confluent cells was abolished upon reaching confluence (Jones et al. 2001). However this reversal of regulation has not been previously described. Repeat experiments are necessary to confirm this finding, which would be interesting to explore further.

The differences in HIF isoform specificity between my data and the literature may reflect cell-line specificity. Confirmation of this could be achieved by repeating the Caco-2 experiments in different cell-lines, selected according to factors known to influence hypoxia responses, using criteria such as mutation status of common oncogenes/tumour suppressors (e.g. p53, K-ras and BRAF). This may help to tease apart the mechanisms that drive this variability in cell-line responses, and build up a more accurate picture of the behaviour of cancers *in vivo*.

The question still remains: how accurately can cell-lines model “real” CRC cells? The considerable heterogeneity of CRC makes translation of this kind of data to individual patients problematic. Drawing from considerable previous experience in our laboratory of using short-term primary cultures, I sought to examine angiogenic responses to hypoxia of cells derived directly from patients’ tumours. I was able to establish cultures of mixed cells that responded to hypoxia by altering their expression of angiogenesis genes. Angiogenesis array data showed some similarity between the responses of these TDCs and Caco-2 cells, with notable differences. For example, the TDCs expressed more genes overall (70 were detected on average, compared to 53 in Caco-2). In addition, whereas only upregulation was seen following hypoxia-stimulation in Caco-2 cells, downregulation of some genes was also seen in TDCs. This may be a reflection of the mixed cell-types in the TDCs, and further work to characterise the composition of TDCs is required.

An interesting finding was the significant inter-donor variation in the pattern of angiogenic responses in TDCs. This is best appreciated from the scatter plots of the array data (Figure 5.7 to Figure 5.12). For example, Donors 1 and 2 showed both strong induction and suppression of several genes, Donor 3 showed mainly upregulation, and Donor 4 showed mainly downregulation. This inter-donor variation persisted when analysis was limited to four genes, namely EFNA-3, ANGPTL-4, TGF- β 1 and VEGF. While no clear correlation with clinicopathological parameters was seen, the small sample-size was a significant limitation, and this is being addressed in an on-going project in our laboratory. The continued use of PCR array analysis is precluded by prohibitive costs. Therefore, the study is increasing patient numbers and analysing hypoxia-induced expression of a group of 14 genes including EFNA-3, ANGPTL-4, TGF- β 1 and VEGF by Q-PCR. It is hypothesised that correlations will emerge between patterns of hypoxia-induction and clinicopathological parameters.

Should this be confirmed, a number of possibilities follow. Firstly, it would provide proof-of-concept of this “live sampling” technique. This could be developed in a number of ways. Firstly, modification of the protocol may improve success rates for establishing TDCs and increase RNA (and protein) yields. This could potentially be applied to any solid cancer tissue. Refinement of the angiogenesis hypoxia-response signature may be achieved by increasing the sample size (n number) and expanding the PCR array data, allowing data analysis utilising robust statistical/bioinformatic techniques. Examination of protein expression (e.g. cytokine arrays) may improve this further. Numerous commercially available PCR and protein arrays could be used to examine tumourigenic processes other than angiogenesis, such as apoptosis, cell-motility and EMT. The use of stimuli other than hypoxia, such as cytokines and growth factors, would expand this further, allowing the investigation of specific signalling pathways.

6.1.1 Limitations

My Caco-2 work focussed primarily on gene expression, and due to time constraints, protein expression and functional studies were limited. Novel hypoxia-regulated genes were identified, but whether these findings are reflected at the protein level, and whether there are any functional consequences, is unclear. Similarly, although differences in HIF-isoform regulation of several genes involved in CRC pathogenesis was seen, the effect at the protein and functional levels was not investigated (see section 6.2.1). Furthermore, translation of results from a single immortalised cell-line is problematic.

The aim of the TDC work was to reliably establish viable short-term cultures whose hypoxia responses could be investigated in a manner comparable to Caco-2 cells. The small size of tissue samples resulted in low cell yield, which in turn meant that limited protein/RNA material would be available for analysis. Therefore the initial focus was on using the available material to establish that hypoxia responses could be consistently analysed, before turning attention to accurate characterisation of culture composition. As a result, while the presence of CRC cells in the TDCs is supported by the expression of CEA, Ep-CAM and VE-Cadherin, the precise composition of these cultures is unclear. It may therefore be that the differences in hypoxia-response between donors are due to different TDC composition, rather than the characteristics of the originating lesion. A larger data set may reveal correlations between TDC response patterns and tumour biology, but the numbers in this study are insufficient (see section 6.2.2).

As discussed in section 5.1, the inability of immortalised cell-lines to accurately represent the behaviour of cancers *in situ* is a major weakness of their use. My study sought to address this by comparing a cell-line with freshly-derived CRC cultures. However, HIF-isoform roles were only investigated in Caco-2 (see section 6.2.1). There were similarities between Caco-2 and TDC hypoxia-responses, which provides a degree of mutual validation of the data. However, the

strength of this validation depends absolutely on two conditions. Firstly, the TDC data may provide support for Caco-2 cells as a model of CRC *providing the TDCs are (predominantly) composed of CRC cells*. This has not been shown definitively (see section 6.2.2). Secondly, as it is a well-established CRC cell-line, the Caco-2 data may provide support for the TDC model *providing the Caco-2 cells are representative of the original CRC tumour from which it was derived*. This has also not been shown (see section 6.2.1).

6.2 Future Work

6.2.1 *Caco-2*

RNA and Protein

Further characterisation of Caco-2 hypoxia responses, and the effects of HIF- α knockdown, should examine protein expression, particularly of the three novel genes EFNA-3 (ELISA Kit, Antibodies-online Inc.; Atlanta, USA), ANGPTL-4 (Duoset © ELISA Kit, R&D Systems) and TGF- β 1 (ELISA Kit, Abcam). Antibodies are also available for Western Blotting of whole cellular extracts (Invitrogen, Paisley, UK; Santa Cruz Biotechnology; Abcam).

Confirmation of the HIF specificity data should include further knockdown experiments using different siHIF-1 α and siHIF-2 α sequences, and by overexpression studies using a plasmid or viral vector. To further investigate the possible reciprocal effects of the HIF- α isoforms, and to examine possible HIF-independent effects, double-knockdown experiments, or knockdown of the common HIF- β -subunit, should be performed. Cell-viability at the end of transfection should be confirmed using trypan blue stain.

It would be interesting to investigate the roles of the three novel genes, particularly for EFNA-3, since induction of this gene by hypoxia has not previously been described in CRC, and very little is known about its function. As well as examining protein expression, siRNA knockdown followed by functional studies (see below) should be performed.

The downregulation of HIF-1 α and HIF-2 α mRNA by hypoxia highlighted the complexity of HIF regulation. The role of the PHDs and FIH could be further investigated by selective knockdown (and overexpression) of the respective genes followed by examination of the effects on HIF mRNA and protein, and on cell function (see below). HIF activity should also be assessed using the TransAM™ HIF- α binding assay (Active Motif, Rixensart, Belgium), which measures binding to HRE.

Functional Studies

Functional studies are required to investigate the relevance of the Caco-2 HIF- α knockdown data. Preliminary experiments were performed using a BrdU assay (section 7.1), which require optimisation and replication. Apoptosis assays should include a cell-death assay (e.g. MTT Assay, Roche, Burgess Hill, UK) and fluorescence-activated cell sorting (FACS) with Annexin V-labelling.

Angiogenesis requires induction of endothelial cell function. Human Umbilical Vein Endothelial Cells (HUVEC) are primary endothelial cells regularly used in our laboratory to

investigate angiogenesis. A co-culture technique has previously been applied in our laboratory to assess the angiogenic potential of CRC cell-lines (T. Khong, unpublished data). HUVEC and Caco-2 cells are co-cultured in a double chamber which allows angiogenic cytokines released by Caco-2 cells in the bottom chamber to cross a semi-permeable membrane into the HUVEC chamber (Figure 6.1). The HUVEC migrate towards the Caco-2 along a chemotactic gradient. The number of HUVEC that migrate in a given time period is proportional to the potency of the angiogenic stimulus from the Caco-2 cells. Initial attempts used conditioned medium from hypoxia-stimulated transfected Caco-2 cells (avoiding direct exposure of HUVEC to hypoxia). This was unfortunately unsuccessful, in part due to inconsistent HUVEC responses to positive controls. Substitution of HUVECs with human microvascular endothelial cells (HMEC) showed some promise. This requires further optimisation.

Further techniques for assaying angiogenic function include the Matrigel (BD Biosciences) and Angiokit (TCS Cell Works, UK) Assays which allow quantitative measurement of tubule development in HUVECs. Finally, Caco-2 have been shown to be tumorigenic in mouse xenografts. Should these assays indicate functionally diverse roles of the HIF isoforms, transplantation of HIF-isoform deficient or overexpressing Caco-2 cells into immunodeficient mice would serve to confirm this. HIF silencing would require more stable transfection than siRNA achieves, for example with shRNA.

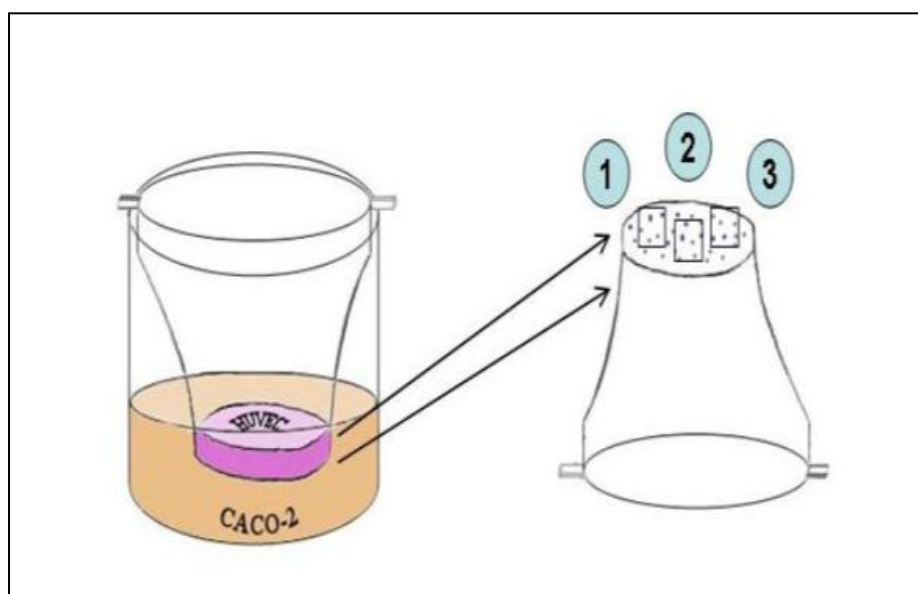


Figure 6.1 HUVEC Migration Assay

Caco-2 cells, adherent to the lower chamber, are exposed to various experimental conditions (e.g. transfection, hypoxia) prior to the introduction of HUVECs, which are cultured independently within cell inserts. HUVECs migrate in response to a chemical gradient of angiogenic mediators released by the CRC cells. Following removal of unmigrated cells from the inner surface of cell inserts, the number of migrated cells on the undersurface of the cell insert membrane will be counted, and a mean calculated. (Courtesy T. L. Khong).

Cell-lines

The Caco-2 cell-line is derived from a primary tumour with relatively low tumourigenicity. It would be interesting to compare hypoxia-expression profiles and HIF-isoform roles with cell-lines of metastatic origin (e.g. SW620), or with a more aggressive phenotype such as HT-29 (intermediate) and HCT116 (high) (Yeung et al. 2010). Caco-2 cells were obtained directly from ATCC. A study investigating cell-line contamination and mis-identification did not identify this cell-line as being prone to these issues (O'Brien 2001), but for completeness, testing could be performed using short tandem repeat profiling (Masters et al. 2001). Passage number should be carefully recorded for all experiments to address any possible confounding effects.

6.2.2 TDCs

Enlarge Data Set

Data from the 13 patients for whom TDCs analysed by PCR (array and/or Q-PCR) suggests variation in hypoxia-induced angiogenesis gene expression, which is hypothesised to be related to the biological behaviour of the originating tumours. Correlation of expression patterns with clinico-pathological parameters would support this, but the numbers in this study are insufficient to confirm this. Increasing the sample size forms a significant part of an on-going follow-up project. Expression patterns of genes including VEGF, EFNA-3, ANGPTL-4 and TGF- β 1 are being evaluated by Q-PCR. Demographic and pathological data is being collected from medical notes and routine histopathology reports, including tumour site, stage, differentiation grade, vascular invasion, use of neoadjuvant chemo-radiotherapy, morbidity and mortality. As the numbers increase, it is hoped that factors will emerge that influence the success of the TDC establishment method, so that data yields may be increased.

Characterisation

In order for strengthen the validity of comparisons of hypoxia responses between donors, it is important to confirm whether the composition of TDCs is consistent. Fuller characterisation of the TDCs should be carried out using FACS to identify the constituent cell-types. This may also assist with interpretation of gene expression patterns. Inconsistency in TDC composition would not automatically invalidate inter-donor comparisons, since the composition may itself be influenced by clinically relevant biological characteristics of the tumour. In addition, lack of any correlation between clinical variables and expression of the selected genes may simply indicate a weakness in the “signature” identified, rather than invalidating the hypothesis entirely. Further work to refine the signature may include investigation of protein expression using ELISA and Western Blotting (and/or protein/cytokine arrays to identify novel proteins). Functional assays to

investigate the ability of TDCs to induce endothelial cell function (section 6.2.1) should also be used.

Controls

Since the aim of this study was to investigate differences in hypoxia-induced angiogenic responses between TDCs from different donors, the controls used were unstimulated (normoxic) cells from the same TDC. In order to fully characterise the TDCs, a further control would be to use cells derived from normal colon tissue from the same patient. In order that sufficient tissue was obtained, this would be taken from the resection margins of the same specimen, since obtaining a sample of adequate size from the remaining colon would increase the risk of post-operative leak. Further controls could include cells derived from non-cancer operations, such as bowel resections for diverticular disease or IBD. Such samples would control for any inflammatory component to the behaviour of TDCs. In addition, tissue could be obtained from benign colonic lesions (e.g. adenomas) and/or biopsies of normal mucosa, obtained via colonoscopy. However, these would likely yield small amounts of tissue, and in the case of normal mucosal biopsies would increase the risk to the patient (bleeding, perforation). Finally, it would be interesting to establish TDCs from metastatic deposits, particularly where comparison to the primary lesion were possible.

Inhibitors

It would be interesting to investigate the role of HIF in the TDC hypoxia responses, by treating the cultures with HIF inhibitors prior to hypoxia experiments. These are commercially available (e.g. Biomol, Exeter, UK). Further possibilities include the use of angiogenesis inhibitors, or other commonly-used therapeutic agents, which may give some insight into their therapeutic utility in specific patients. In addition, pathways other than hypoxia-induced angiogenesis could be investigated using different stimuli and the appropriate readouts (e.g. doxorubicin or 5-FU to induce apoptosis followed by evaluation of apoptosis gene and protein expression (Q-PCR or PCR Array), MTT assay, FACS).

6.3 Clinical Implications

The potential for HIF inhibition as a therapeutic strategy in CRC has been recognised for some time (Semenza 2002b). Evidence suggests that HIF-induction enhances resistance to chemotherapy (Rohwer et al. 2010) and radiotherapy (Moeller et al. 2007), and it may be involved in the development of resistance to novel biological therapies such as imatinib (Zhao et al. 2010). HIF induction may be a feature of response to antiangiogenic therapy, since effective disruption of tumour blood supply would result in hypoxia. It has been suggested that HIF inhibition may improve the efficacy of antiangiogenic drugs, particularly in patients with documented HIF overexpression in biopsy or resection specimens (Semenza 2012). Many anti-cancer drugs designed to target non-HIF mechanisms have been shown to inhibit HIF-1 α mRNA and/or protein (e.g. mTOR inhibitors such as temsirolimus; topoisomerase inhibitors such as topotecan) (Semenza 2007). Some of these drugs are known to be HIF-1 α specific (Semenza 2012), while for others (including some used in CRC) the effect on HIF-2 α is unknown. The involvement of both HIF- α isoforms in the regulation of Caco-2 angiogenesis responses seen in my experiments supports data indicating that simultaneous inhibition of both isoforms may be desirable (Burkitt et al. 2009). In view of the possibility of compensatory mechanisms, as in the case of ANGPTL-4 mRNA regulation in Caco-2 seen in my data for example, the inhibition of HIF-1 α alone may in fact be undesirable (Carroll and Ashcroft 2006). Although some publications discussing HIF inhibition continue to consider anti-HIF-1 α activity alone (Wang et al. 2011a; Xia et al. 2012), drugs that inhibit both isoforms are under development (Bohonowych et al. 2011; Chen and Sang 2011). Thus, mounting evidence underlines the importance of considering both HIF isoforms.

Conventional biomedical research techniques typically begin with assessment of tumour specimens for surrogate markers of tumourigenicity, such as distorted anatomy/morphology (e.g. differentiation, invasion, MVD), and altered patterns of gene and protein expression. The rapid and continued development of new molecular biology techniques (e.g. DNA sequencing, gene and protein (micro-)arrays), has greatly enhanced the degree and sophistication with which this type of sampling can be performed. In combination with powerful statistical techniques (and an exponential increase in the computational power of the micro-chip), correlations can be identified between such biomarkers and clinical outcomes that have prognostic and predictive value. However robust such statistical associations may be, the relation of these markers to tumour cell function can generally only be inferred. An understanding of function is crucial for the development of interventions that will improve outcomes. *In vitro* experimental techniques, for example using cell-line models, provide powerful tools for the investigation of the function of specific genes, proteins and signalling pathways. This experimental data can then be used to validate the observed clinico-pathological correlations (as well as identifying new hypothetical markers).

Significant advances in our understanding of cancer biology, and the attendant improvements in clinical outcomes, are testament to the success of this paradigm. However, a weakness of this approach is the remove between *in vitro* models and the *in vivo* milieu. Deep insights into the huge complexity and heterogeneity of cancer cell biology have accrued from *in vitro* research. When one considers that *in vitro* model systems are simplified by necessity, in order that a limited number of variables can be investigated, it can be assumed that this complexity is even greater *in vivo*. A further layer of heterogeneity is introduced by the stromal cells with which cancer cells interact *in vivo* (Hanahan and Weinberg 2011; De Palma and Hanahan 2012), increasing the complexity by orders of magnitude. This can limit the validity applying experimental results to the clinical setting, and may contribute to issues such as side-effects and variable responses to novel therapies (Kerbel and Ebos 2010; Nikolinakos et al. 2010), and to the long delays that intervene between basic research findings and clinical applications. The short-term TDC model may address some of these problems by virtue of retaining some of the inter-cellular interactions that are lost with cell-lines.

This gap between the bench and the clinic has been the driving force behind the development over the past 20 years of the field of Translational Research, which has seen a shift in emphasis from “basic” research (which may be characterised purely theoretical) to research that can be more directly related the patient. A critical component of translational research is the concept of Personalised Medicine, an important goal of which is the identification of biomarkers which when applied to an individual patient will aid accurate prognostication, and selection of appropriate therapeutic strategies. This is an extension of traditional staging of tumours (e.g. TNM), whereby tumours can be stratified into categories which, although having useful prognostic/predictive power, are often too broad to accurately reflect the heterogeneity of tumour behaviour. Personalised medicine aims to achieve classification with much finer resolution, increasing predictive accuracy, and avoiding the unnecessary use of often expensive, potentially toxic drugs.

Perhaps the most exciting possibility arising from the short-term TDC model is its potential for use in personalised medicine. It may be possible to treat TDCs from an individual patient with antiangiogenic agents, and measure the attenuation, if any, of the gene response to hypoxia. Such a “chemosensitivity and resistance assay” (CSRA) technique, analogous to antibiotic sensitivity testing for bacterial infections, has been utilised with some success in ovarian and bladder cancer (Havaleshko et al. 2007; Ferriss and Rice 2010). CSRAs measure cell-death/apoptosis of tumour-derived cells treated with specific chemotherapy regimes. Tests of this nature, combining therapeutic and diagnostic elements, have been referred to as “Theranostics”, and form an important part of the personalised medicine approach. In a retrospective study, Gallion *et al.* reported that in patients receiving a treatment regime to which a CSRA had

classified their tumour as “resistant”, a 3-fold increased risk of progression was predicted (Gallion et al. 2006). A small RCT reported a trend towards better outcomes in patients receiving a CSRA-selected chemotherapy regime, compared to those for whom treatment was selected according to “physician’s choice” (Cree et al. 2007). The TDC short-term model investigated in my study would aim to use hypoxia-induced alterations in angiogenic function as a readout, rather than cell-death, and as such may prove to be a more sensitive method of predicting angiogenic drug response.

The short-term TDC model offers the prospect of direct measurement of the angiogenic capacity of a tumour. It has the potential to be applied to other tumourigenic processes and different cancers, and may have both diagnostic and therapeutic applications.

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Chapter 7

7 APPENDIX

7.1 Supplementary Caco-2 Data

Angiogenesis Array

Table 7.1 Genes not detected by PCR Array in Caco-2 Cells

A total of 31 genes were not detected by PCR Array (Ct values > 30).

BAI1	CXCL9	IFNB1	PGF
CCL11	TYMP	IFNG	PLG
CDH5	FGF1	IL6	PTGS1
CXCL1	FGF2	LECT1	TEK
CXCL10	FIGF	LEP	TIMP3
CXCL3	HAND2	MDK	TNF
CXCL5	HGF	NOTCH4	TNFAIP2
CXCL6	IFNA1	PF4	

Preliminary Functional Assays

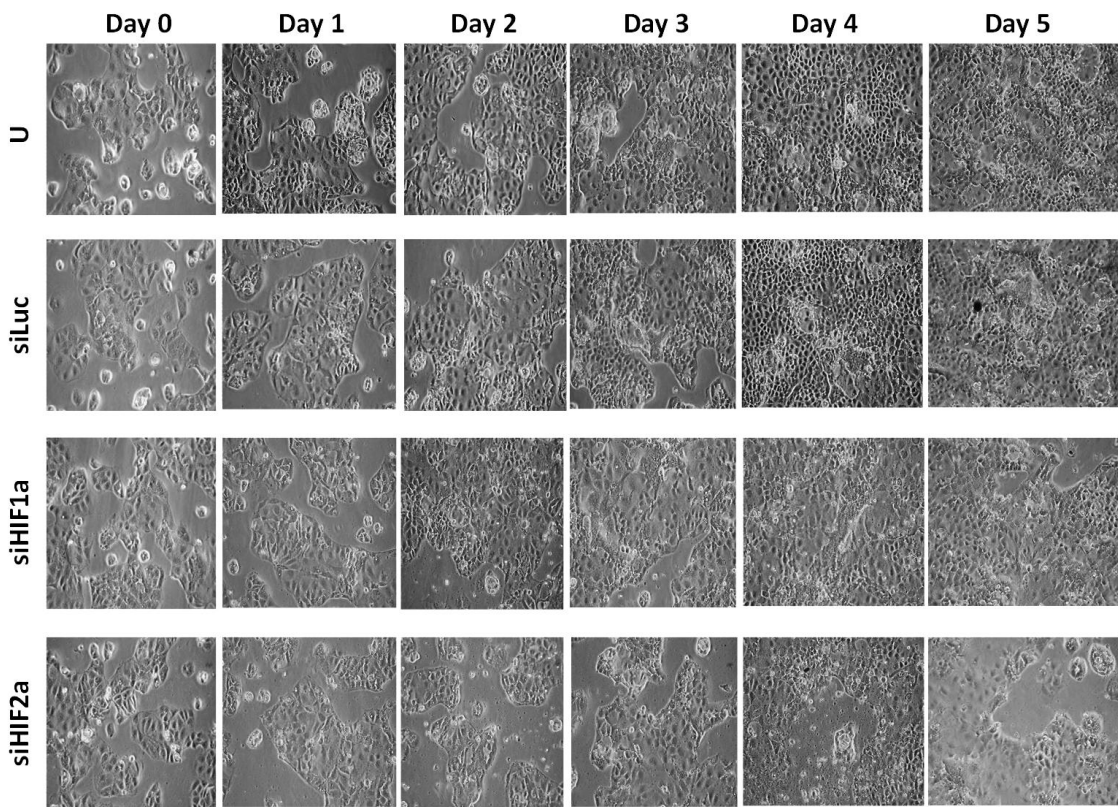


Figure 7.1 Caco-2 cell growth in normoxia following HIF knockdown

Caco-2 cells transfected with siHIF-1 α or siHIF-2 α and incubated in normoxia for 5 days, and photographed daily. At the end of the experiment, cells were trypsinised and counted (Figure 7.2).

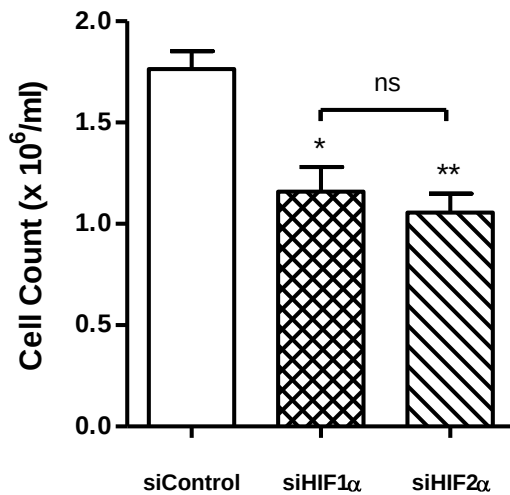


Figure 7.2 **Caco-2 cell counts after 5 days of normoxia following HIF knockdown**

Caco-2 cells transfected with siHIF-1 α or siHIF-2 α and incubated in normoxia for 5 days (see Figure 7.1). At the end of the experiment, cells were trypsinised and counted by the trypan blue exclusion method. Graph shows cell counts (x10⁶/ml). Data are mean $\pm$ SEM from a single experiment. (* $p < 0.05$, ** $p < 0.01$, ns = not significant, 1-way ANOVA *versus* siControl unless otherwise stated).

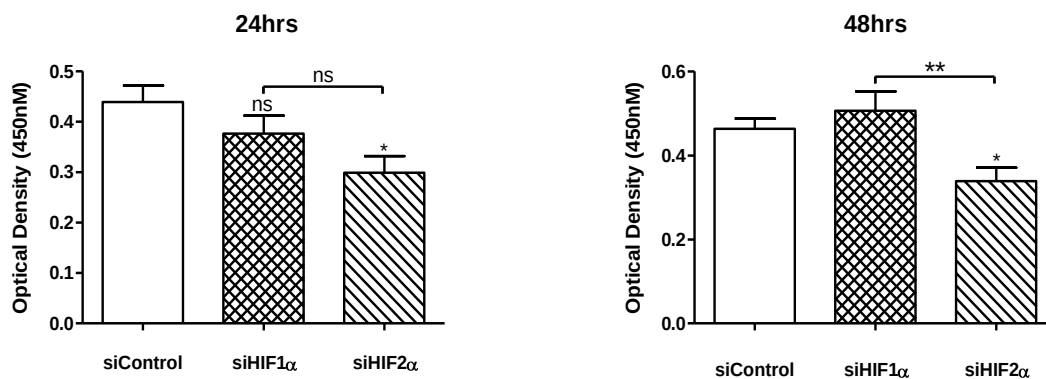


Figure 7.3 **Caco-2 proliferation following HIF knockdown**

Caco-2 cells transfected in 6-well plates with siControl siHIF-1 α or siHIF-2 α . 100 μ l BrdU (Roche, Burgess Hill, UK) was added to each well at the end of transfection and the cells incubated in normoxia for 24 or 48 hours. At each time-point, cells were trypsinised and transferred to a 96-well ELISA plate. The plate was centrifuged at 1500rpm for 20 mins, supernatants were discarded, and the plate allowed to air dry. Peroxidase-labelled anti-BrdU antibody was added, followed by TMB substrate, resulting in a colourimetric reaction which was measured using a microplate reader at 450nm wavelength (Thermo Labsystems). Graphs show optical density at 24 hours (left) and 48 hours (right). Data are mean $\pm$ SEM from a single experiment. (* $p < 0.05$, ** $p < 0.01$, ns = not significant, 1-way ANOVA *versus* siControl unless otherwise stated.)

7.2 Supplementary Information CRC Patients

Table 7.2 Demographic and pathology characteristics of donors analysed by Q-PCR

Table shows age, gender, site (proximal or distal colon, or rectum) and differentiation grade for the 13 donors analysed by Q-PCR.

Donor	Age (yrs)	Gender	Site	Dukes' Stage	Differentiation
1	66	F	Rectum	A	moderate
2	73	M	Proximal	B	moderate
3	68	M	Distal	A	moderate
4	64	M	Rectum	C	moderate
5	59	M	Distal	C	moderate
6	55	F	Distal	C	moderate
7	70	F	Distal	A	moderate
8	85	M	Distal	B	moderate
9	81	F	Distal	B	poor
10	76	M	Rectum	D	poor
11	51	F	Distal	C	poor
12	80	M	Proximal	C	moderate
13	77	M	Proximal	D	poor

Table 7.3 Q-PCR patients pathology statistics

Table shows pathology statistics for donors analysed by array.

Parameter	n	%	Full Cohort (n=34) %
Site			
Proximal colon	3	23.1	50.0
Distal colon	7	53.8	23.5
Rectum	3	23.1	26.5
Dukes' Stage			
A	3	23.1	12.1
B	3	23.1	36.4
C	5	38.5	39.4
D	2	15.4	12.1
Differentiation			
moderate	9	69.2	63.6
poor	4	30.8	33.3
undifferentiated	0	0	3.0

7.3 Publications and Presentations

The work presented in this thesis has been presented at the following scientific meetings (poster presentations unless otherwise stated):

HIF-Isoforms Have Divergent Roles in the Angiogenesis of Colorectal Cancer

N. Thairu, S. Kiriakidis, P. Dawson, E. Paleolog.

- Society of Academic and Research Surgery, Nottingham, January 2012 (Oral)
- 5th London Surgical Symposium, Imperial College, September 2011 (Oral)
- Association of Coloproctologists of Great Britain & Ireland, Birmingham, June 2011
- Association of Surgeons of Great Britain & Ireland, Bournemouth, May 2011
- 4th International Meeting on Angiogenesis, Amsterdam, March 2011

Abstracts:

- **Br J Surg**, 2012; 99(S4):6-40.
- **Colorectal Disease**, 2011; 13(S4):15-47
- **Br J Surg**, 2011; 95(S3):80-217
- **Angiogenesis**, 2011; 14(1):111-112

Short-Term Cultures Of Tumour-Derived Colorectal Cancer Cells – A Novel In Vitro Model for the Evaluation of Angiogenesis in Colorectal Cancer

N. Thairu, S. Kiriakidis, E. Paleolog, P. Dawson.

- Association of Coloproctologists of Great Britain & Ireland, Dublin, July 2012
- Association of Surgeons of Great Britain & Ireland, Liverpool, May 2012 (Oral)

Abstracts:

- **Br J Surg**, 2012; 99(S6):1-82
- **Colorectal Disease**, 2012; 14: 12-40

The section on bevacizumab therapy in the Introduction (section 1.2.4.1) is based on the following review paper:

Angiogenesis as a Therapeutic Target in Rheumatoid Arthritis: Learning the Lessons of the Colorectal Cancer Experience.

Thairu N., Kiriakidis S., Dawson P., Paleolog, E.

Angiogenesis, 2011 Sep; 14(3):223-34