Role of Delta-like 4 in solid tumours and response to radiation therapy



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Abstract

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Delta-like ligand 4 (DLL4) is a ligand for the Notch family of receptors. DLL4 is an important regulator of angiogenesis and DLL4 blockade promotes non-productive angiogenesis and delays tumour growth. The aim of this thesis was to investigate the effects of anti-DLL4 therapy in solid tumours in combination with a clinically relevant dose of ionising radiation (5 Gy; IR) and to analyse alterations in the Notch pathway induced by the treatments. Combining both treatments resulted in a greater than additive tumour growth delay in LS174T tumours, compared to either treatment alone. DLL4 blockade dysregulated vasculature and increased necrosis in LS174T and HCT-15 (DLL4-expressing and negative cell lines respectively) tumours within 3 days after treatment, but no changes were observed with IR alone. Additionally, combined IR and anti-DLL4 treatment of FaDu tumours (another DLL4-negative cell line) by our colleagues, also resulted in a supra-additive growth delay. These results show that combining IR with DLL4 blockade is an effective strategy for prolonging tumour growth delay and suggest that the stroma/vasculature provide the main therapeutic target for the anti-DLL4 therapy. Analysis of Notch pathway shows that IR upregulated Jag1 in tumour cells, and may inhibit Notch and downregulate DLL4 in the stroma. These changes may potentially affect tumour vessels and response to anti-DLL4 therapy. *In vitro,* anti-DLL4 therapy induced proliferation in quiescent contact-inhibited endothelial cells and also appeared to abrogate IR-induced inhibition of migration. These results suggest that DLL4 may be important in maintaining vessel quiescence and that IR may in part decrease migration through Notch signalling. Combining IR and DLL4 blockade to target tumour growth is an

effective and well tolerated strategy and warrants further validation and refinement to be translated into clinical practice.

~

To my parents,

and my uncle Mahmood Qasim

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List of abbreviations

ACL	Lung adenocarcinoma
ADAM	A disintegrin and metalloprotease
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
BSA	Bovine serum albumin
CADASIL	Cerebral Autosomal-Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy
CD11b	Cluster of differentiation molecule 11B
CD31	Cluster of differentiation 31
cDNA	Complementary DNA
CSL	CBF1, Suppressor of Hairless, Lag-1
DAB	3,3' Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DAPT	N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester
DBZ	Dibenzazepine
DEPC	Diethylpyrocarbonate
DLL	Delta-like
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNMAML1	dominant negative mastermind-like1
dNTP	Deoxyribonucleotide triphosphate
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
FBS	Foetal bovine serum
g	gravitational force
g	gram
GSI	γ-secretase inhibitor
Gy	Gray
HES	Hairy and Enhancer of Split
HEY	Hes-related protein families
HMVEC-d	Human dermal microvascular endothelial cell
HUVEC	Human umbilical vein endothelial cell
IKK	IκB kinase
IR	Ionising radiation
Jag	Jagged
Kg	Kilogram
LFng	Lunatic fringe
LLC	Lewis lung carcinoma
LNR	Lin-12/Notch repeat
M	Molar
mAb	Monoclonal antibody
MAML	Mastermind-like

MAPK	Mitogen-activated protein kinase
Mfng	Manic fringe
mg	Milligram
ml	Millilitre
mm	millimetre
MMTV	Mouse mammary tumour virus
mRNA	Messenger ribonucleic acid
N1CID	cleaved Notch1 intracellular domain
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ng	Nanogram
NICD	Notch intracellular domain
NNR	Negative regulatory region
NSCLC	Non-small cell lung cancer
p	P-value
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphoinositide 3-kinase
PTEN	Phosphatase and tensin homolog
qPCR	Quantitative polymerase chain reaction
RBPJk	Recombination signal binding protein for immunoglobulin κ J region
RNA	Ribonucleic acid
SCID	Severe combined immunodeficiency
SCLC	Small cell lung cancer
SDF1	Stromal cell-derived factor 1
SDS	Sodium dodecyl sulfate
shRNA	Short hairpin RNA
TACE	Tumor Necrosis Factor Alpha Converting Enzyme
TAD	Transactivation domain
TAM	Tumour-associated macrophage
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

Chapter 1

General Introduction

1. General Introduction

1.1. Notch signalling

1.1.1. A brief introduction to Notch.

The Notch pathway is highly evolutionarily conserved and is involved in the coordination of developmental processes such as cell differentiation, proliferation, morphogenesis and apoptosis. Components of the pathway have been recognised in a number of model organisms such as Drosophila melanogaster, Xenopus laevis, Caenorhabditis elegans and mice, as well as in humans (Artavanis-Tsakonas *et al.*, 1995, Robey, 1997, Artavanis-Tsakonas *et al.*, 1999, Ehebauer *et al.*, 2006). Notch was first identified by John S. Dexter during studies with fruit flies (*Drosophila melanogaster*) in 1914. The term "Notch" was derived from the characteristic notched wing phenotype of mutant flies with haploinsufficiency of the *Notch* gene (Dexter, 1914, Mohr, 1919). It was not until 1937 that the importance of Notch was highlighted. Analysis of homozygous *Notch* null mutants flies revealed an embryonic lethal phenotype characterised by neurogenic aberrations, where cells which would ordinarily give rise to the epidermis, instead acquired a neural fate (Poulson, 1937). The *Notch* locus was cloned by Artavanis-Tsakonas in 1983 (Artavanis-Tsakonas *et al.*, 1983) and since then widespread investigations into the mechanisms of Notch pathways as well as its role in developmental processes and disease states have followed. In humans, Notch signalling is important during T-cell development, vascular development and neurogenesis (Robey, 1997, Gridley, 2007), while aberrations in the pathway are linked to diseased states such as Cerebral Autosomal-Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL) (Arboleda-Velasquez *et al.*, 2005), Alagille syndrome (Li *et al.*, 1997, Oda *et al.*,

1997) and Spondylocostal dysostosis (Turnpenny *et al.*, 2003) as well as cancer (Bolos *et al.*, 2007, Wang *et al.*, 2010, Yin *et al.*, 2010).

1.1.2. The Notch receptor.

The Notch receptor family encodes single-pass transmembrane cell surface receptors. Four Notch receptors are recognised in mammals; Notch1, Notch2, Notch3 and Notch4 (figure1.1a). The receptor is translated as a single polypeptide but is constitutively cleaved in trans-Golgi by a furin-like convertase (S1 cleavage). It is then transported to the cell surface, where it exists as a heterodimer consisting of an extra cellular domain and a membrane-bound intracellular domain, non-covalently attached to each other (Blaumueller *et al.*, 1997, Logeat *et al.*, 1998).

The extracellular domain consists of tandem Epidermal growth factor-like (EGF-like) repeats, involved in interacting with the ligands, and cysteine-rich LIN/Notch repeats (LNR) (Wharton *et al.*, 1985, Rebay *et al.*, 1991). The intracellular domain contains a RAM domain adjacent to the transmembrane region, followed by CDC10/Ankyrin repeats, a transactivation domain (TAD) and a PEST sequence. The RAM domain appears to interact directly with the transcription factor CSL (CBF1, Suppressor of Hairless, Lag-1; also known as RBPJκ in vertebrates) (Tamura *et al.*, 1995). Together with RAM, the ankyrin repeats also participate in the interaction with RBPJκ and are necessary for the formation of the transcriptional activation complex (Roehl *et al.*, 1996, Tani *et al.*, 2001, Lubman *et al.*, 2004). Transcriptional activation domain together with the RAM domain and ankyrin repeats are important for binding with RBPJκ and transcriptional activity of Notch intracellular domain (Kurooka *et al.*, 1998). The c-terminal PEST sequence is important for

ubiquitination of the receptor and hence influences protein stability (Rogers *et al.*, 1986, Greenwald, 1994).

1.1.3. Notch ligands

The five canonical Notch ligands in mammals are grouped into two types; Delta-like (DLL1, DLL3 and DLL4) and Jagged (Jag1, Jag2), on the basis of homology to the *Drosophila* ligands Delta and Serrate respectively (D'Souza *et al.*, 2008). The ligands are single-pass transmembrane proteins consisting of a large extracellular domain and a small intracellular domain. The N-terminal extracellular domain consists of EGF-like repeats with the number of repeats differing between the ligands. Between the N-terminus and the EGF-like repeats is the DSL motif. The DSL motif along with the N-terminal region and first two EGF-like repeats are required for receptor binding (Shimizu *et al.*, 1999, Parks *et al.*, 2006). The Serrate/Jagged ligands have a cysteine-rich region, absent in Delta/Delta-like ligands, which has a homology to von Willebrand factor type C domain (Vitt *et al.*, 2001, D'Souza *et al.*, 2008). The intracellular domain are not well conserved between the ligands. The exception being that most contain multiple lysine residues and a C-terminal PDZ motif, needed for ligand signalling and cytoskeletal interactions respectively (Pintar *et al.*, 2007, D'Souza *et al.*, 2008).

1.1.4. Canonical Notch signalling pathway

The canonical Notch pathway (figure 1.1b) is activated upon interaction of the extracellular domain of the receptor with the ligand, allowing it to be cleaved by a disintegrin and metalloprotease/Tumour necrosis factor α converting enzyme (ADAM/TACE) within the juxtamembrane region (S2 cleavage) (Brou *et al.*, 2000, D'Souza

et al., 2008). The ligand and the bound extracellular domain of the receptor are then internalized by the signal sending cell. This endocytosis of the ligand, which leads to

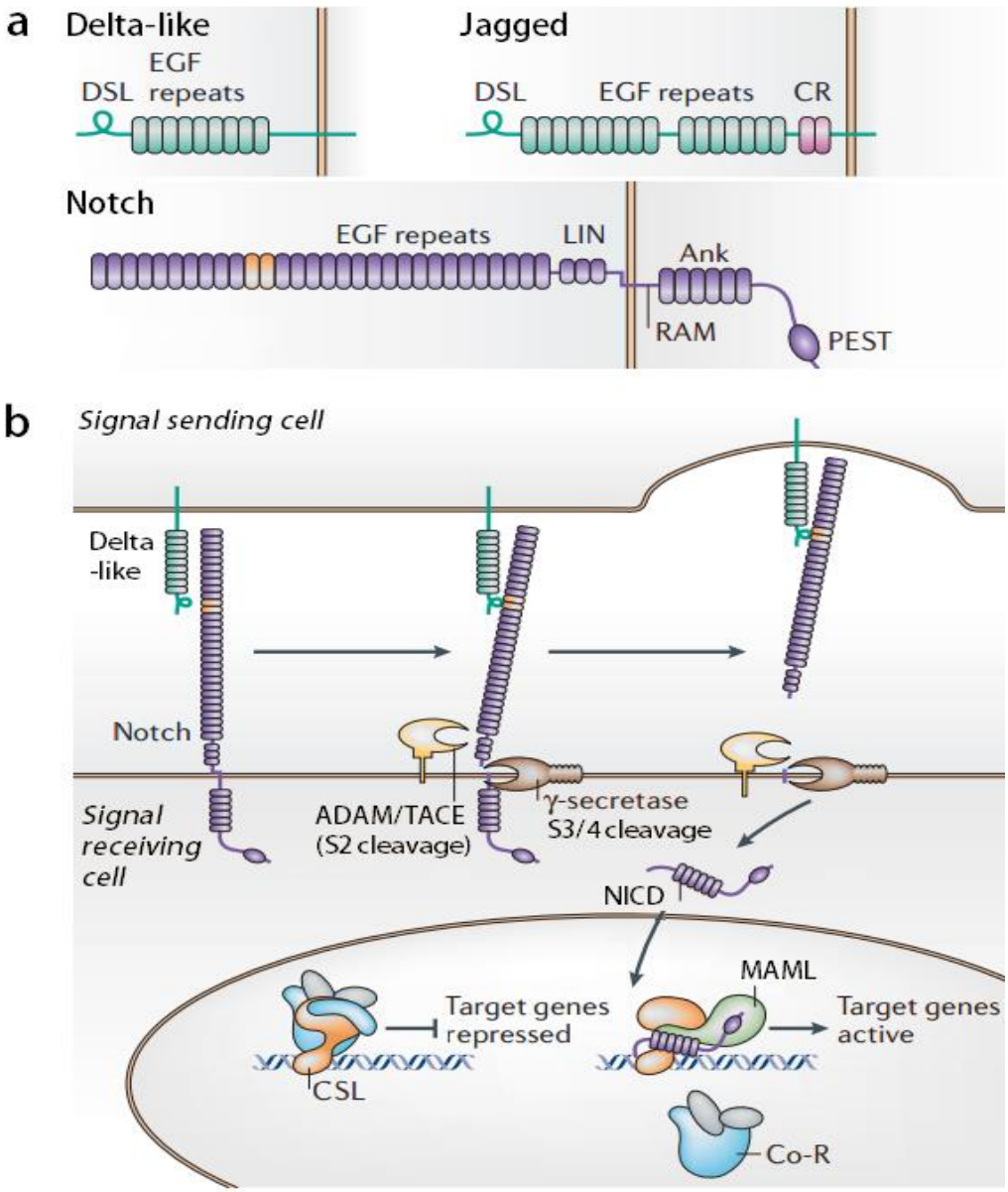


Figure 1.1. Notch signalling in mammals. **a,** Schematic representation of the Notch receptor and ligands illustrating positions of EGF-like repeats, Lin repeats (LIN), DSL domain (DSL), RAM-23 domain (RAM), ankyrin repeats (Ank), PEST domain and cysteine rich domain (CR). **b,** The canonical Notch signalling pathway. Notch receptor – ligand (Delta-like) interaction results in a conformational change of the receptor allowing two successive proteolytic cleavages S2 and S3/4 by ADAM or TACE metalloprotease and γ-secretase complex respectively. The cleaved Notch intracellular domain (NICD) translocates to the nucleus and interacts with the DNA binding CSL protein. Co-repressors (Co-R) are displaced while transcriptional activators such as Mastermind-like (MAML) are recruited allowing expression of target genes. Figure adapted from (Bray, 2006).

dissociation of the extracellular domain from the membrane spanning intracellular domain, has been suggested to be required for Notch activation (Klueg and Muskavitch, 1999, Parks *et al.*, 2000, Ahimou *et al.*, 2004, Nichols *et al.*, 2007). Following S2 cleavage, the receptor undergoes dual cleavage by γ-secretase within the transmembrane region (S3/S4 cleavage) releasing the Notch intracellular domain (NICD) (De Strooper *et al.*, 1999, Ray *et al.*, 1999, Okochi *et al.*, 2002, Chandu *et al.*, 2006). NICD then translocates to the nucleus where it interacts with transcriptional repressor CSL (RBPJκ in mammals) (Jarriault *et al.*, 1995, Tamura *et al.*, 1995, Schroeter *et al.*, 1998). CSL acts as a transcriptional repressor in the absence of NICD. Association with NICD leads to the displacement of the corepressor and recruitment of transcriptional coactivators such as Mastermind-like (MAML) resulting in the formation of a transcriptional activator complex (Dou *et al.*, 1994, Wu *et al.*, 2000, Zhou *et al.*, 2000, Wu *et al.*, 2002). The best characterised of the Notch targets are transcriptional repressors of the basic helix-loop-helix family; Hairy and Enhancer of Split (including Hes1, Hes5, Hes7) and Hes-related (including Hey1, Hey2, HeyL)(Sasai *et al.*, 1992, Jarriault *et al.*, 1995, Leimeister *et al.*, 1999, Iso *et al.*, 2003).

1.2. Notch signalling and cancer

1.2.1. Oncogenic roles of Notch

The Notch pathway has been implicated in a large variety of cancers, where it may act in an oncogenic or tumour suppressive fashion. One of the first reports associating Notch activation with cancer is from 1991, where 1% of T-cell acute lymphoblastic leukaemia (T-ALL) cases studied contained a chromosomal translocation resulting in a constitutive activation of Notch1 (Ellisen *et al.*, 1991). Subsequently a study revealed that over 50% of

all human T-ALLs contained activation mutations involving either the heterodimerisation domain or the PEST sequence of Notch1 (Weng *et al.*, 2004).

In breast cancer, it was discovered that insertion of mouse mammary tumour virus (MMTV) into the *Notch4* gene resulted in expression of a constitutively active truncated Notch4 product (Gallahan and Callahan, 1987). Notch1 has also been implicated in murine mammary tumours where truncated active Notch1 is produced due to MMTV integration into the *Notch1* locus (Dievart *et al.*, 1999). Notch has been shown to be tumourigenic in melanomas, where Notch1 signalling promotes progression of primary melanoma to an advanced stage (Balint *et al.*, 2005, Liu *et al.*, 2006). Increased levels of Notch1 have been observed in human melanoma samples and were necessary for transformation of melanocytes by Akt and hypoxia *in vitro* (Bedogni *et al.*, 2008). Aberrant Notch signalling has also been implicated in pancreatic cancer where ectopic activation promotes development of metaplastic ductal epithelium (Miyamoto *et al.*, 2003) and may be required for pancreatic cancer maintenance (Mullendore *et al.*, 2009). In colorectal cancers, increased transcription of Notch targets Hes1 and Hes5 due to increased IκB kinase (IKK) activity has been noted (Fernandez-Majada *et al.*, 2007). Hes1 was seen to be upregulated in human primary colorectal tumours as compared to normal colonic mucosa (Veenendaal *et al.*, 2008). Dysregulation of Notch signalling has also been reported in cervical (Maliekal *et al.*, 2008), renal (Aparicio *et al.*, 2011, Wu *et al.*, 2011), ovarian (Hopfer *et al.*, 2005), prostate (Wang *et al.*, 2010, Zhu *et al.*, 2013), esophageal (Liu *et al.*, 2013, Song *et al.*, 2013), hepatocellular (Ahn *et al.*, 2013, Hu *et al.*, 2013) and gastric (Brzozowa *et al.*, 2013) carcinomas and gliomas (Purow *et al.*, 2005).

Notch signalling may contribute to carcinogenesis by interacting with and promoting a number of oncogenic pathways. Constitutive activation of EGFR is a feature of many

tumours where it promotes cell growth, cell survival as well as resistance to cytotoxic drugs (McKenna *et al.*, 2003, Qiao and Wong, 2009). Notch signalling can induce tumour cell proliferation via activation of EGFR pathway as shown in glioma and breast cancer (Purow *et al.*, 2005, Dai *et al.*, 2009). Studies from breast cancer show that Notch may also promote tumourigenesis by inducing C-Myc (Efstratiadis *et al.*, 2007). C-Myc has also been reported to be targeted by Notch1 in Notch-dependent T-ALL cell lines (Weng *et al.*, 2006). Notch can induce the expression of cyclins including cyclin D1 (Cohen *et al.*, 2010) and cyclin A (Qi *et al.*, 2003). PI3K/Akt pathway may be upregulated by Notch, enhancing cancer cell survival (Nair *et al.*, 2003) possibly due to inactivation of PTEN (Parsons *et al.*, 2005). Nuclear factor κB (NFκB) can effect proliferation, migration and apoptosis in tumours (Dolcet *et al.*, 2005). Notch signalling has been shown to modulate NFκB in pancreatic cancer cells and effect tumour cell invasion (Wang *et al.*, 2006).

1.2.2. Notch ligands Jag1 and DLL4 in cancer.

Jag1 and DLL4 have been reported to be upregulated in a number of tumours. High levels of Notch1 as well as Jag1 have been associated with poor prognosis and significantly worse overall survival in human breast cancer (Reedijk *et al.*, 2005). Reedijk and colleagues examined a small cohort of human colon cancers and found that Jag1 and Notch1 were expressed in all tumours analysed (Reedijk *et al.*, 2008). Jag1 mRNA upregulation has been reported in pancreatic cancer (Miyamoto *et al.*, 2003). Overexpression of Jag1 at the protein level has been observed in brain (Cuevas *et al.*, 2005, Purow *et al.*, 2005), cervix (Gray *et al.*, 1999) and prostate (Santagata *et al.*, 2004) cancers.

DLL4 upregulation has been reported in pancreatic cancer (Mullendore *et al.*, 2009) and also in colon cancer where cancer cells expressed higher levels of DLL4 as compared to normal colon tissue (Jubb *et al.*, 2009, Qiao and Wong, 2009). DLL4 expression in tumour and stromal cells have been reported as prognostic markers in gastric cancer (Ishigami *et al.*, 2013). High DLL4 expression has been suggested as a predictor of poor prognosis in nasopharyngeal carcinoma patients (Zhang *et al.*, 2013).

1.2.3. Tumour-suppressive roles of Notch

One of the best examples of Notch functioning as a tumour suppressor comes from studies on skin cancers. In normal human epidermis Notch signalling functions to promote keratinocyte differentiation (Thelu *et al.*, 2002). However, lack of activation of Notch1 has been found in human basal cell carcinomas (Nickoloff *et al.*, 2003). Similarly specific inactivation of Notch1 in mouse skin has been shown to induce hyperproliferation of the epidermis followed by development of tumours (Nicolas *et al.*, 2003).

Down regulation of Notch1 has also been reported in non-small cell lung cancer (NSCLC) cell lines and constitutive activation of Notch1 been shown to induce cell death in lung adenocarcinoma (ACL). However, the study also observed that upregulation of Notch1 in hypoxic conditions was essential for cell survival in ACL (Chen *et al.*, 2007). In small cell lung cancers (SCLC) Notch1 and Notch2 have been observed to be expressed in low levels and overexpression of Notch resulted in growth inhibition in cancer cells (Sriuranpong *et al.*, 2001, Sriuranpong *et al.*, 2002). Tumour suppressive roles of Notch have also been suggested in liver (Croquelois *et al.*, 2005) and prostate (Shou *et al.*, 2001).

The tumour suppressive effect of Notch in the epidermis and in small cell lung cancer (SCLC) cell lines appears to be due to induction of p21, an important regulator of cell cycle

(Rangarajan *et al.*, 2001, Sriuranpong *et al.*, 2001). Activation of Notch1 in mouse keratinocytes led to an upregulation of p21 (Rangarajan *et al.*, 2001). Consequently, targeting Notch1 decreased p21 levels but increased β-catenin signalling and led to the formation of tumours (Nicolas *et al.*, 2003). Expression of Notch1 or Notch2 was also reported to upregulate p21 and cause cell cycle arrest (Sriuranpong *et al.*, 2001).

1.3. Notch signalling and tumour vasculature.

1.3.1. Development of vasculature.

Formation of vasculature is one of the earliest events during vertebrate organogenesis, occurring following the formation of early mesoderm via gastrulation, by a process termed vasculogenesis. Vasculogenesis involves *de novo* vessel formation from mesodermal progenitors 'angioblasts' to give rise to a primitive capillary plexus. This further expands by angiogenesis, the process of formation of new vasculature from pre-existing vessels (Risau and Flamme, 1995). Following expansion and remodelling by angiogenesis, pericytes and smooth muscle cells are recruited to stabilise the vascular network to gives rise to mature vessels.

Under normal physiological conditions in adults, the vasculature exists in a quiescent state in most instances, with a notable exception being the female reproductive system. Consequently, endothelial cells may survive for years, making them cells with one of the lowest turnover rates in the body apart from neurons. Pathological situations where angiogenesis is actively occurring include wound repair and tumour development.

1.3.2. Vasculature in solid tumours.

The existence of blood vessels within solid tumour has long been recognised, but it was not until the 1970s that seminal work by Judah Folkman highlighted the importance of tumour vasculature. Folkman proposed that diffusion of nutrients and waste would limit the growth of a tumour once it reached a diameter of 1-2mm, and angiogenesis would be required in order for the tumour to increase in size beyond this limit (Folkman, 1971). It was also proposed that targeting tumour vasculature would inhibit tumour growth and that regression of tumour vessels would result in regression of tumour mass, ultimately to an avascular tumour with a 1 to 2 mm diameter (Folkman, 1972).

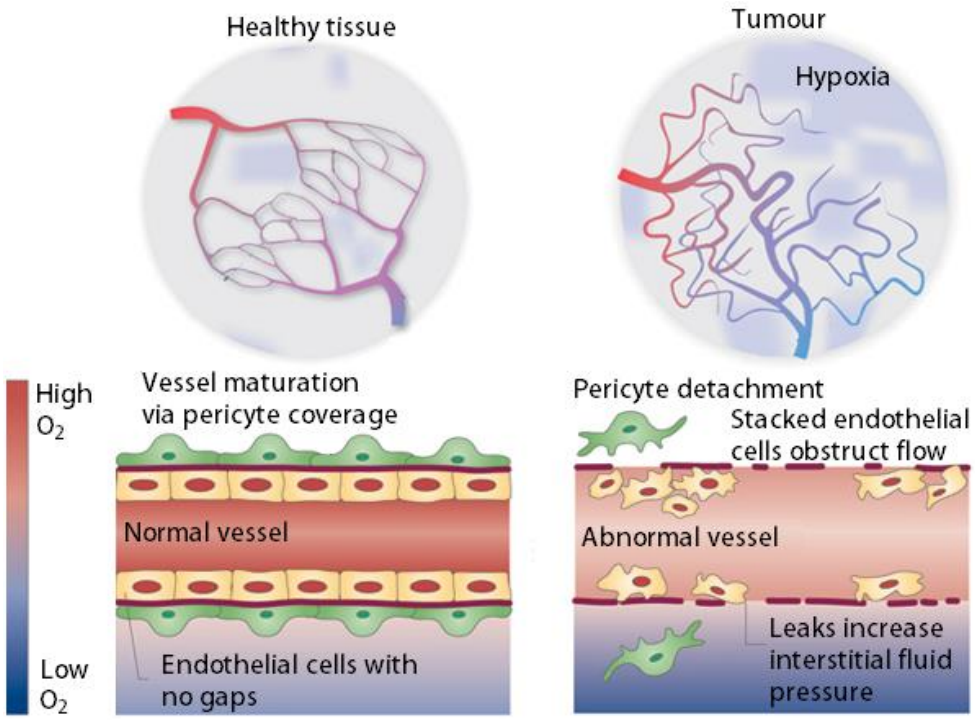


Figure 1.2. Physiological and tumour vasculature. In healthy tissues, blood vessels are non-tortuous and covered with pericytes, with endothelial cells arranged in a monolayer with tight junctions between them. In contrast, tumour vasculature is chaotic, tortuous, with poor pericyte coverage and gaps between endothelial cells, which may be stacked obstructing flow. This leads to increased interstitial fluid pressure, poor flow, and hypoxia. This causes a constant production of proangiogenic factors and promotes abnormal angiogenesis. Adapted from (Carmeliet and Jain, 2011).

In contrast to conditions such as wound healing where angiogenesis is switched on transiently (Carmeliet, 2005), permanent activation of angiogenesis is a feature of tumours. During growth, tumour mass increases beyond the ability of the existing vasculature to provide nutrients, oxygen and clear metabolic waste. Tumour cells respond to the resultant hypoxic condition by upregulating proangiogenic factors such as vascular endothelial growth factor (VEGF). Increasing proangiogenic signals stimulate nearby quiescent vessels and angiogenesis is induced in a process termed the 'angiogenic switch' (Bergers and Benjamin, 2003) allowing vessels to expand and support further growth.

Tumour vessels differ from normal physiological vasculature in many ways (illustrated in figure 1.2). Tumour vessels are often tortuous, chaotic and unevenly distributed, connecting to each other in a random fashion instead of being arranged into a well organised pattern of arteries, veins and capillaries. The vessels have uneven lumen and may be abnormally dilated or compressed by tumour or stromal cells (Bergers and Benjamin, 2003, Carmeliet and Jain, 2011). Endothelial cells are often irregularly shaped with loose intercellular contacts giving rise to highly permeable, leaky vessels resulting in increased interstitial fluid pressure. In addition to unperfused chronically hypoxic regions, continuous vasculature remodelling may transiently decrease blood flow to areas of the tumour, subjecting cells to acute hypoxia. (Chaplin *et al.*, 1986, Tozer *et al.*, 2005, Carmeliet and Jain, 2011).

Vessel development in normal physiology is tightly regulated and dependent on the balance of proangiogenic and antiangiogenic factors. Tumour vessels reflect a state where angiogenesis is constantly switched on resulting in a poorly functional vasculature, which fails to meet the demand of tumour for nutrients and oxygen, further promoting dysfunctional angiogenesis (Carmeliet and Jain, 2011).

1.3.3. Sprouting angiogenesis.

Two distinct modes of angiogenesis have been described: sprouting angiogenesis, which involves formation of new outgrowths from endothelial cells lining pre-existing vessels, and intussusceptive angiogenesis, which occurs when a vessel splits into two following development of transvascular pillars (Burri *et al.*, 2004).

Sprouting angiogenesis is initiated upon stimulation of endothelial cells lining the vessels wall by proangiogenic growth factors. This leads to increase in cell motility and secretion of proteases, including matrix metalloproteinases, enabling degradation of the basement membrane matrix. The endothelial cells proliferate forming outgrowths referred to as sprouts. These sprouts invade into the surrounding tissues, migrating via a concentration gradient of proangiogenic growth factors, towards the source of the stimuli. The endothelial sprouts are each led by a 'tip cell' which exhibit a non-proliferative, highly invasive, tubeless phenotype with long dynamic filopodia, studded with tyrosine kinase receptors such as VEGF receptors (VEGFR) to explore the environment for directional signals. The tip cells are followed by proliferating endothelial 'stalk cells', which extend the sprout and contribute to lumen formation (Adams and Alitalo, 2007). Adherens tight junctions are established between the stalk cells to prevent leakage (Dejana *et al.*, 2009). As the vessel matures, endothelial cell proliferation is reduced, extracellular matrix proteins are deposited into the subendothelial basement membrane and pericytes are recruited to stabilise the vessels (Adams and Alitalo, 2007).

1.3.4. Regulation of sprouting angiogenesis by Notch signalling.

The Notch receptors and ligands DLL4 and Jag1 are key regulators of sprouting angiogenesis and control the specification of tip and stalk cells (illustrated in figure 1.3a).

The process of sprouting angiogenesis is regulated by multiple growth factors, however VEGF plays a central role in the process. VEGF is secreted in response to local hypoxia and initiates sprouting as well as influences migration of sprouts along a VEGF concentration gradient (Stone *et al.*, 1995, Gerhardt *et al.*, 2003). Interestingly, upon VEGF stimulation only some cells lining the vessel wall are selected to become tip cells and initiate sprouting. VEGF is known to induce DLL4 expression in the endothelial cells (Hainaud *et al.*, 2006, Noguera-Troise *et al.*, 2006, Lobov *et al.*, 2007). These endothelial cells compete against each other for the tip cell position by a mechanism termed 'lateral inhibition'.

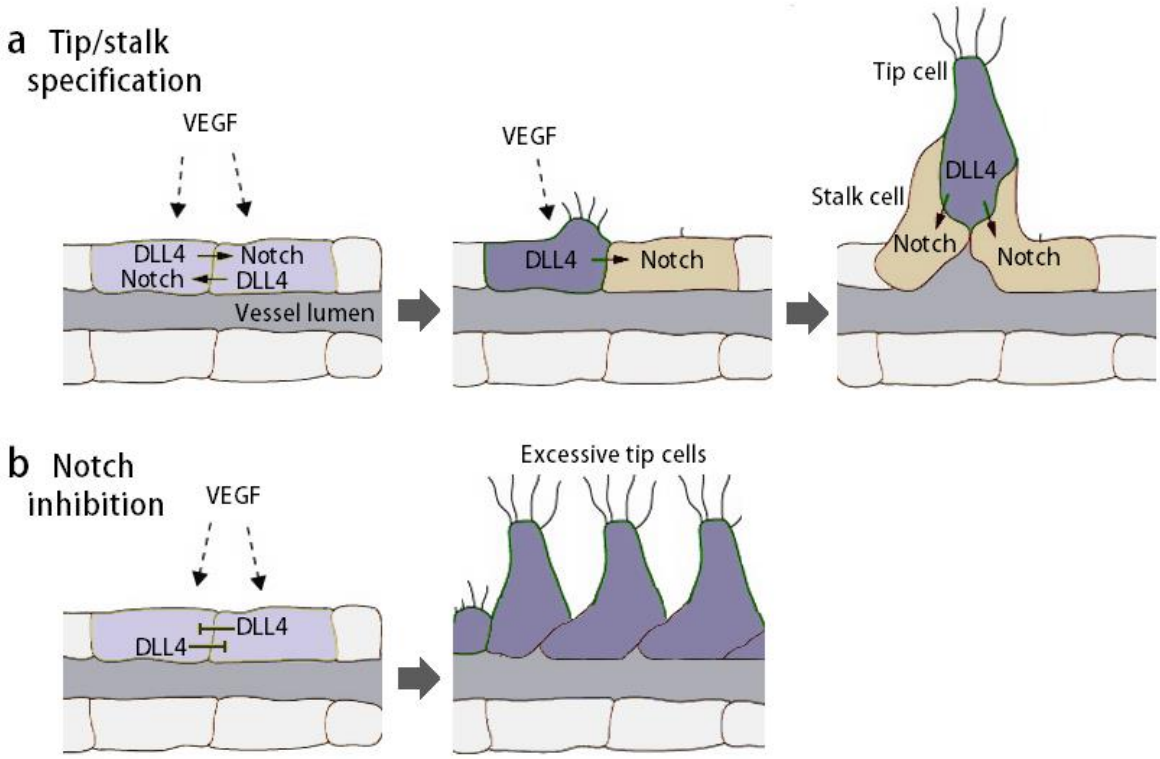


Figure 1.3. DLL4 – Notch signalling regulates tip and stalk cell specification during sprouting angiogenesis. **a**, VEGF secreted in response to hypoxia induces DLL4 expression (purple) in all endothelial cells. Cells with high DLL4 levels (dark purple) become tip cells. Surrounding cells (yellow) receive a strong Notch signalling, inhibiting DLL4 expression and specifying them as stalk cells. **b,** Following Notch inhibition, stalk cells are no longer specified, leading to excessive tip cell formation. Adapted from (Eilken and Adams, 2010).

Cells with the highest DLL4 expression acquire a tip cell fate, strongly activating Notch in neighbouring cells and specifying them as stalk cells (Phng and Gerhardt, 2009). It appears that the tip cell fate is the default outcome and is acquired in the absence of Notch signalling and thus Notch inhibition results in excessive tip cell formation and sprouting (figure1.3b). Hellstrom *et al.* have demonstrated in mouse retinas, that inhibition of Notch signalling by γ-secretase inhibitors, global deletion of one allele of DLL4 or inducible knockout of Notch1 specifically in the endothelium all results in excessive tip cell formation (Hellstrom *et al.*, 2007). DLL4 expression in mouse retinal vasculature has been shown to be predominantly at the leading front of growing vessels. Deletion of a single allele of DLL4, inhibition of DLL4 by a soluble DLL4-Fc (DLL4 fused to the human IgG1 Fc constant region) or DLL4 blocking antibody resulted in increased sprouting and endothelial cell proliferation (Lobov *et al.*, 2007). It has been observed that the absence of RBPJκ or DLL4 in zebrafish embryos results in excessive sprouting of intersegmental arteries while activation of Notch suppressed angiogenesis. Cells that lack RBPJκ are preferentially localised to the tip cell position in sprouts while cells with active Notch signalling excluded from the tip cell position (Siekmann and Lawson, 2007). While DLL4 expression is predominant in tip cells, strong Notch activation is observed in stalk cells (Hellstrom *et al.*, 2007).

Notch signalling also modulates angiogenesis by affecting the sensitivity of endothelium to VEGF ligands. VEGF ligands include secreted angiogenic and lymphangiogenic growth factors including VEGF-A (commonly referred to as simply VEGF) and VEGF-C. During angiogenesis, VEGF (VEGF-A) signals primarily through VEGFR2, while VEGF-C binds to VEGFR2 and VEGFR3 to activate tip cells. VEGFR1 has weak tyrosine kinase activity and may antagonise VEGFR2 by acting as a decoy for VEGF. In addition VEGFR1 may be

secreted as a soluble form trapping VEGF (Carmeliet and Jain, 2011). Studies suggest Notch activation may induce expression of VEGFR1, (Harrington *et al.*, 2008), while decreasing the expression of VEGFR2 (Taylor *et al.*, 2002, Li *et al.*, 2006, Williams *et al.*, 2006, Suchting *et al.*, 2007, Harrington *et al.*, 2008) and VEGFR3 (Siekmann and Lawson, 2007, Tammela *et al.*, 2008). As stalk cells receive a strong Notch signal from tip cells, this may repress transcription of the VEGF2 and VEGFR3 while inducing VEGFR1 and hence decrease the sensitivity of stalk cells to VEGF (Phng and Gerhardt, 2009). It has been observed that tip cells express VEGFR2 (Gerhardt *et al.*, 2003) and VEGFR3 (Tammela *et al.*, 2008) more strongly than stalk cells.

While the role of DLL4 during sprouting angiogenesis is well established, the function of Jag1 is less well understood. Benedito *et al.* have reported Jag1 to be predominantly expressed in stalk cells with weak expression in tip cells. Overexpression of Jag1 promoted sprouting angiogenesis in the neonatal mouse retina model with an increase in endothelial cell proliferation, vessel branching and tip cells, while deletion of Jag1 produced an opposite result. Jag1 appeared to antagonise DLL4 in this model, where deletion of Jag1 resulted in higher expression of Notch target gene Hey1 and this expression appeared to be predominantly in stalk cells. In Jag1 deficient retina, DLL4 expression was not confined to the vascular front as in a normal retina, but was observed throughout the endothelium (Benedito *et al.*, 2009). The Fringe family of glycosyltransferases (three homologues in mammals; Lunatic fringe, Radical fringe and Manic fringe) modulate Notch signalling by transfering N-acetylglucosamine to fucose on EGF-like repeats in the Notch receptor extracellular domain (Johnston *et al.*, 1997). Glycosylation of Notch by the Fringe enhances activation of the receptor by Delta/Delta-like ligands, but inhibit signalling by Serrate/Jagged ligands (Yang *et al.*, 2005). Complete

knockout of Lunatic Fringe (Lfng) resulted significantly increased sprouting in retinal endothelium. In cultured mouse endothelial cells, it was demonstrated that Jag1 and DLL4 coexpressing cells caused reduced Notch activation in adjacent Manic Fringe (Mfng) expressing cells, compared to when cultured with DLL4 only cells. These results indicate that Jag1 antagonises DLL4 induced Notch activation in the presence of Fringe expression.

1.3.5. Aberrant Notch signalling in tumour vasculature

A number of reports have focused on the differential expression of Notch signalling components in tumour vessels compared to normal vasculature. DLL4 has been observed to be higher in tumour vasculature compared to vessels in normal tissues (Mailhos *et al.*, 2001, Li *et al.*, 2010, Schadler *et al.*, 2010). In colon cancer, DLL4 expression was only observed in tumour endothelium and was absent in normal mucosa (Mailhos *et al.*, 2001, Jubb *et al.*, 2009). Expression of DLL4 in tumour vessels was not associated with poor patient outcome directly. However, DLL4 expression was found to be associated with high VEGF and hypoxia, both of which are correlated with poor outcome (Jubb *et al.*, 2009). In a Lewis lung carcinoma xenograft mouse model, DLL4 expression was seen predominantly in the tumour vasculature while only weak expression was observed in the surrounding skin vessels (Noguera-Troise *et al.*, 2006). Segarra *et al* have reported that overexpression of DLL4 in tumour cells resulted in smaller, less vascularised and more hypoxic tumours (Segarra *et al.*, 2008). In contrast to this, Li *et al* report that tumours derived from U87 glioblastoma cell line overexpressing DLL4, showed enhanced growth with fewer but larger and better perfused vessels, with bigger lumen (Li *et al.*, 2007). These effects suggest that the effects of ectopic DLL4 expression on tumour vessels may be tumour type specific. High levels of DLL4 in tumour vasculature has been reported to be

associated with reduced radio-resistance, likely due to a decrease in hypoxia (Koukourakis *et al.*, 2013).

The role of Jag1 is less well studied in this context. Urs *et al* observed that tumours derived from NIH3T3 fibroblasts expressing soluble Jag1 had a more extensive vascular network and more dilated vessels than the controls (Urs *et al.*, 2008). Zeng *et al* have observed an induction of Jagged1 by growth factors via MAPK pathway in head and neck squamous cell carcinoma cells. This induced Jag1 activated Notch in the endothelium leading to increased blood vessel development and faster tumour growth (Zeng *et al.*, 2005).

1.4. Targeting Notch signalling in cancer.

1.4.1. Targeting Notch in cancer with γ-secretase inhibitors

Increasing evidence of deregulation of the Notch pathway in cancer has placed Notch as a potential target for cancer therapy. One of the most actively investigated approaches targeting Notch signalling has been the use of γ-secretase inhibitors (GSI). GSIs have been employed in the past in targeting Aβ peptides associated with Alzheimer's disease, (Lichtenthaler *et al.*, 1997) but also target Notch signalling. Following Notch receptor activation, the transduction of Notch signal to the nucleus requires final proteolytic cleavages by γ-secretase to release the NICD. GSIs inhibit these cleavages and prevent NICD formation, suppressing downstream transcriptional processes.

A number of studies have reported the use of GSIs in targeting cancer cells. In a medulloblastoma xenograft model, treatment with the GSI N-[N-(3,5-difluorophenacetyl)-L-alanyl]-sphenylglycine-t-butyl ester (DAPT) resulted in decreased proliferation and increased apoptosis of tumour cells (Hallahan *et al.*, 2004). Another study revealed that

the GSI GS1 promoted apoptosis in breast cancer cell lines while showing a minimal effect on non-malignant breast cell lines (Rasul *et al.*, 2009). Use of GSI-XII on myeloma cells induced apoptosis as well as increased sensitisation to chemotherapeutic drugs doxorubicin and melphalan (Nefedova *et al.*, 2008). Induction of apoptosis by GSI was also observed in a xenograft model of Kaposi's sarcoma (Curry *et al.*, 2005) while inhibition of proliferation by induction of cell cycle arrest and apoptosis was seen in human nasopharyngeal carcinoma cells (Chen *et al.*, 2011). In T-ALL cell lines, treatment with GSI compound E inhibited growth and induced apoptosis (Weng *et al.*, 2003). Another compound GSI PF-03084014 has also shown to effect growth and promote apoptosis in T-ALL lines (Wei *et al.*, 2010).

GSI may also affect tumour development by targeting tumour initiating cells or cancer stem cells. A study with primary human melanoma cells show that GSI RO4929097 reduced proliferation and 3-dimentional colony formation *in vitro*, while decreased tumour volume and inhibition of subsequent tumour formation was noted in serial transplantation xenograft models (Huynh *et al.*, 2011). Similarly, in a transgenic mouse model of ERBB2 breast cancer, GSI MRK-003 inhibited survival of cells from tumours-spheres as well as their ability to form tumours on syngeneic mice. GSI treatment also resulted in inhibiting tumour cell proliferation, inducing apoptosis as well as tumour regression (Kondratyev *et al.*, 2012). Table 1.1 below (adapted from Espinoza *et al*., 2013) reviews the list of GSI currently under clinical development as well as their status.

1.4.2. Neutralizing antibodies against Notch

Blocking antibodies against Notch receptors so far, fall into two categories. The first type binds to the ligand binding EGF-like repeats of the extracellular domain of the receptor,

blocking ligand receptor interaction. The second type is directed towards the negative regulatory region (NRR), which comprises of the Lin/Notch repeats and the juxtamembrane heterodimerisation domain (Gordon *et al.*, 2009), preventing conformational change of the receptor required for ADAM/TACE cleavage (Espinoza and Miele, 2013). Antibodies to NNR of Notch1 have demonstrated anti-angiogenic effects and inhibited tumour growth (Wu *et al.*, 2010). Antibodies specific to NNR of Notch 2 and 3 have also been developed and are being studied (Li *et al.*, 2008, Wu *et al.*, 2010). Ligand blocking antibodies to the extracellular domain to Notch 1 have been generated and show successful downregulation of Notch target genes in human tumour cell lines (Aste-Amezaga *et al.*, 2010).

Agents against DLL4 reported include DLL4 blocking antibodies (Ridgway *et al.*, 2006) or DLL4-Fc fusion proteins (Noguera-Troise *et al.*, 2006, Scehnet *et al.*, 2007); that bind to but do not activate Notch receptors, thus competing with endogenous DLL4. Selective blockade of tumour DLL4 as well as stromal DLL4 in colon tumour xenografts has been shown to decrease proliferation of tumour cells and increase tumour growth delay. Targeting both tumour and stromal DLL4 together resulted in enhancing this effect (Hoey *et al.*, 2009). In patient-derived pancreatic cancer xenograft models, anti-human DLL4 antibody, OMP-21M18 has been demonstrated to reduce tumourigenicity, while treatment with anti-mouse DLL4 had minimal effect. However, both anti-human and anti-mouse DLL4 antibodies alone delayed tumour recurrence following termination of gemcitabine treatment, and combining anti-human and anti-mouse antibodies together resulted in an additive delay (Yen *et al.*, 2012). OMP-21M18 along with another anti-DLL4 antibody REGN421 are currently undergoing clinical development (Gurney and Hoey,

2011, Espinoza and Miele, 2013). Antibodies against the Notch pathway currently being studied in clinical settings, are listed in Table 1.1 below.

Table 1.1. γ-Secretase inhibitors and Notch pathway antibodies in clinical development. (adapted from Espinoza and Miele, 2013)

Agent	Compound	Condition	Trial
γ−Secretase Inhibitor (GSI)	RO4929097 (Roche)	Breast cancer Brain tumours Colorectal cancer Melanoma Solid tumours T−cell leukaemia	Phase I/II NCT01088763 NCT01198535 NCT01149356 NCT01141569 NCT01196416 NCT01218620 NCT01217411 NCT01270438 NCT01238133 NCT01208441 NCT01193868
	MRK−0752 (Merck)	Breast cancer Brain tumours Neoplasms Pancreatic cancer T−cell leukaemia	Phase I/II NCT00756717 NCT00803894 NCT01295632 NCT01098344 NCT00645333 NCT01243762 NCT00572182 NCT00106145 NCT00100152
	PF−03084014 (Pfizer)	Neoplasms Solid tumours Lymphoid leukaemia T−cell leukaemia	Phase I NCT00878189
Neutralizing antibodies	OMP−59R5 anti−Notch2/3 mAb (OncoMed Pharmaceuticals)	Solid tumours	Phase I NCT01277146
	OMP−21M18 anti−DLL4 mAb (OncoMed Pharmaceuticals)	Colorectal cancer Non-Small cell lung cancer Pancreatic cancer Solid tumours	Phase I/II NCT01189929 NCT01189942 NCT01189968 NCT00744562
	MEDI0639	Solid tumours	Phase I NCT01577745
	REGN-421	Solid tumors	Phase I NCT00871559

1.4.3. DLL4 blocking antibodies as antiangiogenic agents

Targeting endothelial cells in tumours presents a number of theoretical benefits. Endothelial cells may be more accessible to drugs than tumour cells, particularly in large poorly perfused tumours. Antiangiogenics and vascular targeting agents, specifically those which directly target endothelial cells, rather than growth factors produced by tumours, may avoid tumour drug resistance. Adult vasculature exists in a relatively quiescent state, except for particular instances such as during would healing. In contrast to this tumours represent sites of constantly active angiogenesis and vascular remodelling. Thus antiangiogenic therapies, specifically targeting signalling in proliferating endothelial cells should not present significant normal tissue toxicity. Finally, it has been suggested that a small number of endothelial cells may be responsible for supporting a large population of surrounding tumour cells (Denekamp, 1984). As continuous vessels are required in order to be functional, damage to a small vessel segment can potentially deprive a large tumour mass of blood flow and lead to growth arrest and cell death (Wachsberger *et al.*, 2003).

Understanding the importance of angiogenesis to tumour development has led to a focus on a number of antiangiogenic approaches, the best validated among which involves the blockade of VEGF signalling (Thurston *et al.*, 2007). Results from a clinical trial in 2004, showing a significant improvement in patient survival, led to the approval of the first antiangiogenic agent; Bevacizumab (anti-VEGF monoclonal antibody) in combination with chemotherapy against metastatic colorectal cancer (Hurwitz *et al.*, 2004). However not all tumours respond to anti-VEGF therapy and initially responsive tumours can develop resistance to the treatment (Kerbel *et al.*, 2001, Casanovas *et al.*, 2005, Jain *et al.*, 2006). Recently DLL4 has emerged as a potential target for antiangiogenic therapy. As DLL4 is a negative regulator of sprouting angiogenesis, blockade of DLL4 in tumour xenograft

models has been shown to increase tumour angiogenesis and lead to increased vessel density. However, the resultant vessels are poorly functional, decreasing tumour perfusion and increasing hypoxia. Therefore, DLL4 blockade results in delaying tumour growth (Noguera-Troise *et al.*, 2006, Ridgway *et al.*, 2006, Scehnet *et al.*, 2007). Promotion of excessive but non-functional vessel development represents a novel strategy in antiangiogenic therapy, where most therapies instead focus on blocking angiogenesis (illustrated in figure 1.4). DLL4 blockade has been shown to be even more efficient in delaying tumour growth, when combined with anti-VEGF treatment (Noguera-Troise *et al.*, 2006).

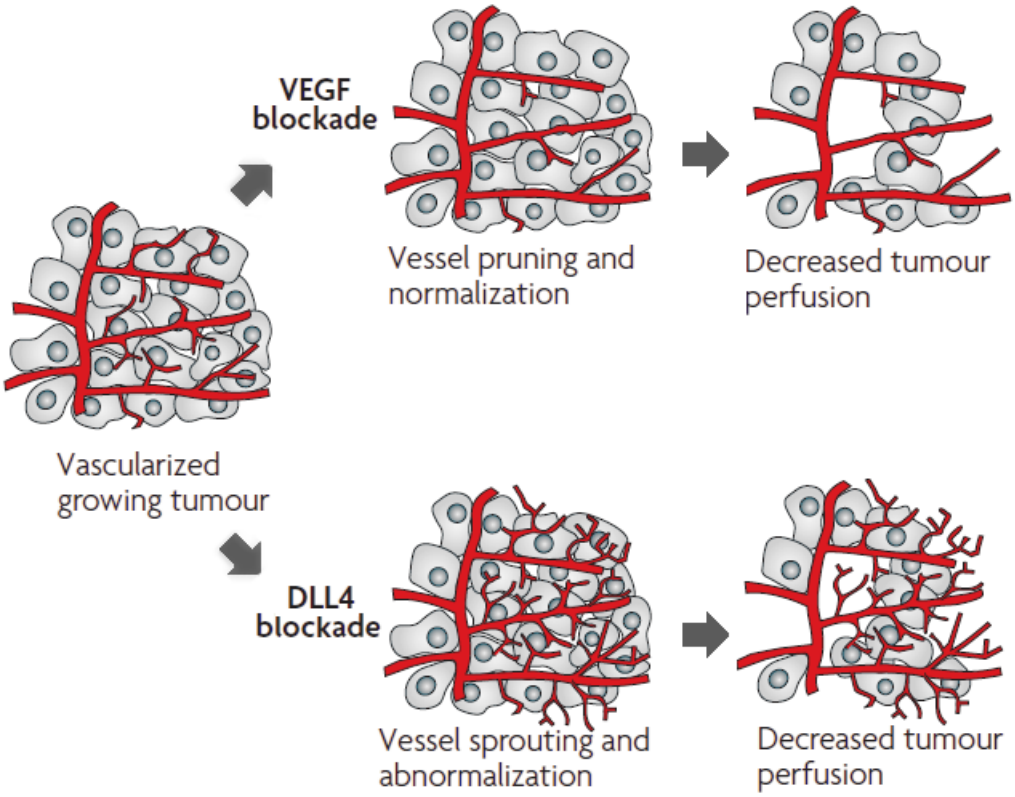


Figure 1.4. Targeting tumour vasculature by VEGF or DLL4 blockade. Angiogenesis is constantly active in growing tumours producing abnormal tumour vasculature. VEGF blockade results in decreasing angiogenesis, leading to vessel pruning and normalisation, consequently decreasing tumour perfusion and growth. By contrast, DLL4 blockade promotes sprouting and increases vessel density, but produces abnormal and poorly functional vessels and leads to a less well perfused tumour. Adapted from (Thurston *et al.*, 2007)

1.5. Targeting Notch with radiation therapy in cancer.

1.5.1. Combining antiangiogenics with radiation therapy for cancer treatment.

Radiotherapy remains an important modality in the treatment of solid tumours, with 50% or more of all cancer patients receiving either palliative or curative doses over the course of their treatment (Ringborg *et al.*, 2003). Therapeutic effects of ionising radiation are due to its potential to create DNA damage, principally double-strand breaks. Radiation may cause this by directly interacting with DNA, or indirectly by inducing radiolysis of water to produce free radicals, which may then react with and damage DNA. Oxygen acts as a radiosensitizer by promoting oxygen-centred free radical formation, as well as reacting with broken ends of DNA molecules and rendering them resistant to repair by the cell. A number of studies have shown that normoxic cells are more radiosensitive than hypoxic cells and that tumour hypoxia is strongly correlated to radioresistance (Moeller *et al.*, 2007, Mazeron *et al.*, 2011). Hence there is a concern that antiangiogenic treatments may decrease the effectiveness of radiation therapy by increasing hypoxia in tumours. Treatment of mice with mammary carcinoma with antiangiogenic agent TNP-470 during fractionated radiotherapy resulted in decreased radiocurability. However, administration of TNP-470 after the end of fractionated radiotherapy produced an additive growth delay in tumour growth assay (Murata *et al.*, 1997).

A number of studies have been published investigating the effects of combining antiangiogenic treatment with radiotherapy. The first such study, combining angiostatin; an endogenous inhibitor of angiogenesis, reported significant tumour regression in combination with fractionated radiotherapy (8-10 doses, 5Gy each) in multiple tumour xenograft models (Mauceri *et al.*, 1998). In another study, administration of anti-VEGF

antibodies before every 5Gy dose in a fractionated radiotherapy regimen, significantly delayed tumour growth in tumour xenograft models compared to radiotherapy alone (Gorski *et al.*, 1999). It has also been reported that treatment with VEGF receptor-2 (VEGFR2) blocking antibodies during fractionated radiotherapy led to a reduction in the radiation dose necessary for local tumour control (Kozin *et al.*, 2001). Lee *et al.* have observed that blockade of VEGF in LS174T colorectal carcinoma and U87 glioblastoma xenograft tumours prior to treatment with a single 20 Gy or higher radiation dose resulted in additive or supra-additive tumour growth delays respectively (Lee *et al.*, 2000). More recently, Hoang *et al.* have reported increased inhibition of tumour growth in tumour xenograft models when bevacizumab was concurrently given with radiation, compared to either of the modalities alone. (Hoang *et al.*, 2012).

Results above call attention to the potential of combining antiangiogenics with radiotherapy, but other studies show more modest results. Zips *et al.* did not observe any additional effects when PTK787/ZK222584 (inhibitor of VEGFR1, 2 and 3) was combined with fractionated radiation treatment (15 doses, 2Gy each), when the antiangiogenic drug was given before or during radiotherapy. However, retardation of tumour growth was seen when treatment with the drug was started after completion of radiotherapy (Zips *et al.*, 2003). Another study reported radiosensitisation with anti-VEGFR2 antibodies when given 1 hour prior to 13.5 Gy irradiation (IR), while no effects were seen when the antibodies were given 24 or 48 hours prior to or after IR (Truman *et al.*, 2010). Remarkably, none of the studies so far report toxicity to normal tissues (Kleibeuker *et al.*, 2012). Overall, the findings may be inconsistent and even contradictory as they are influenced by a number of factors including the dose of radiation, sequencing and duration of treatments and variability in tumour models.

Several clinical trials have been completed or are under way, investigating bevacizumab or receptor tyrosine kinase inhibitors such as sunitinib (which inhibit multiple proangiogenic pathways inclduding VEGFRs) in combination with chemo-radiation. Overall, results regarding patient survival and tumour response are positive. Encouraging response rates were seen in phase I trials when bevacizumab was combined with concurrent radiotherapy and capecitabine in cases with inoperable pancreatic adenocarcinoma (Crane *et al.*, 2006), as well as when bevacizumab, oxaliplatin and capecitabine were combined concurrently with radiation treatment in patients with rectal adenocarinoma (Czito *et al.*, 2007). A phase II study with bevacizumab as neoadjuvant to capecitabine and standard radiotherapy in locally advanced rectal cancer reported 38 out of 40 tumour resections with negative circumferential margins and primary tumour down staging in 15 cases (Gasparini *et al.*, 2012). Treatment specific toxicity has also been reported with common side effects being hypertension and mild bleeding. Wound-healing problems, proteinuria, thromboembolic disorder and gastrointestinal perforations have also been observed (Mazeron *et al.*, 2011). This list of clinical results is not exhaustive and is more comprehensively reviewed in Mazeron *et al.* and Kleibeuker *et al.* (Mazeron *et al.*, 2011, Kleibeuker *et al.*, 2012).

1.5.2. Effects of radiation therapy on the vasculature.

Tumour vasculature response to radiation treatment is an active area of investigation. Evidence so far suggest that low doses of radiation may promote angiogenesis while higher doses have an inhibitory effect. Vincenti *et al.* have observed that 1 Gy IR in human umbilical vein endothelial cells (HUVEC) increased *in vitro* tube formation (Vincenti *et al.*, 2011). Doses of radiation lower than or equal to 0.8 Gy enhanced endothelial cell

migration *in vitro*, while not affecting cell proliferation or survival. Treatment with 1 Gy doses however did not alter migration. In zebrafish models, it has been shown that low doses of radiation promoted sprouting during embryogenesis. Low dose IR increased angiogenesis and tumour growth and promoted metastasis (Sofia Vala *et al.*, 2010). Geng at al have observed that 2 Gy IR increased vasculature while 3 and 6 Gy IR produced a reduction in B16F0 melanoma tumour xenografts, while 3 Gy increased vasculature and 6 Gy decreased it in GL261 glioma tumours. However they show that 6 Gy IR induced minimal apoptosis in HUVECs and human microvascular endothelial cells (HMVECs) but anti-VEGF treatments increased radiation-induced apoptosis. It was also observed that quiescent endothelial cells *in vitro* were resistant to radiation induced apoptosis (Geng *et al.*, 2001). A similar observation was made *in vivo* with a higher dose of radiation, where 20 Gy IR did not induce apoptosis or alter quiescent mature dermal vessels, but pre-irradiation of the implantation site with this dose abrogated *de novo* angiogenesis in matrigel plug inserts (Imaizumi *et al.*, 2010). It has been described that treatment of mammary carcinoma tumour xenografts with 3 Gy IR for four days reduced tumour vascularity. Re-expansion of the vasculature was initially attributed to intussusceptive angiogenesis after which sprouting angiogenesis resumed (Hlushchuk *et al.*, 2008).

Radiation treatment may affect tumour vasculature indirectly, by inducing expression of proangiogenic growth factors in cancer and other stromal cells. Analysis of rectal carcinoma biopsies by Nozue *et al.* revealed that 12 out of 16 cases had higher VEGF expression after radiotherapy, while only 1 had lower levels (Nozue *et al.*, 2001). Upregulation of VEGF has been observed following two 20 Gy IR doses in Lewis lung carcinoma (LLC) tumours. In addition, radiation treatments between 5 – 20 Gy increased VEGF expression in LLC cells *in vitro* in a dose dependent manner (Gorski *et al.*, 1999). A

similar effect was seen in LS174T colorectal carcinoma xenografts, where treatment with either a single 20 Gy dose or 5 doses, 7 Gy each, increased VEGF expression (Solberg *et al.*, 2008). IR has also been shown to upregulate VEGF in tumour-associated macrophages (TAMs) and depletion of TAMs prior to IR increased the anti-tumour effects of radiation treatment (Meng *et al.*, 2010).

1.5.3. Radiation therapy and cellular infiltrates in tumours.

Cellular infiltrates such as monocytes/macrophages are an important part of the tumour stroma. TAMs are known to be strong promoters of angiogenesis via secretion of proangiogenic factors (Coffelt *et al.*, 2009). TAMs have been observed to accumulate in poorly vascularised, hypoxic areas of the tumour, and around tumour vessels (Leek *et al.*, 1999, Murdoch *et al.*, 2004, Ohno *et al.*, 2004) and usually correlate with poor prognosis (Bingle *et al.*, 2002, Lewis and Pollard, 2006, Aparicio *et al.*, 2011).

It has been shown that radiation treatment can cause recruitment of immune cell infiltrates into the tumour. It has been shown by Ahn *et al* that radiation treatment of an established tumour, or the tissue bed prior to tumour implantation, resulted in an increase in $CD11b^+$ cellular infiltrates. The study also suggests that these infiltrates promote vessel development and growth of tumours established on a tissue bed that has been irradiated with a 20 Gy dose (Ahn and Brown, 2008). Ahn *et al* demonstrate in a further study that radiation treatment initially decreases endothelial cells and pericytes but upon tumour recurrence, endothelial cell number are restored while pericytes coverage is partially restored. They also showed that $CD11b^+$cells numbers increase post irradiation and remain elevated upon tumour recurrence (Ahn *et al.*, 2010). More recent work by the same group has shown that radiation induced increase in $CD11b^+$ infiltrates in

an intracranial glioblastoma xenograft model, promotes vessel development and tumour regrowth following radiation (Kioi *et al.*, 2010). The role of notch signalling in macrophage associated angiogenesis remains to be dissected. It has been reported that DLL4 and Notch3 are expressed in macrophages and that DLL4 from co-cultured cells activated Notch on macrophages and further activated Akt, MAPK and NF-κB pathways, increased DLL4 expression and increased transcription of proinflammatory genes (Fung *et al.*, 2007). It is not known whether targeting Notch may influence $CD11b^{+}$ cell infiltrates including macrophages into tumours.

1.5.4. Notch signalling and radiation therapy.

Recently there have been a few reports suggesting that ionising radiation can influence the Notch pathway in tumour cells as well as endothelial cells. Treatment of human adult microvascular endothelial cells with 5Gy IR *in vitro* has been shown to transiently increase levels of Jag1 mRNA. Notch target gene Hey1 RNA was seen to increase at the same time and was shown to be abrogated by the addition of a GSI (Scharpfenecker *et al.*, 2009). Imaizumi *et al.* have also reported similar findings, where 15 Gy IR upregulated Hey1 in HUVEC cells (Imaizumi *et al.*, 2010). Treatment of breast cancer cell line MCF-7 monolayers with five fractionated doses of 3Gy of radiation resulted in an increase in Jag1 levels and increased Notch1 activation (Phillips *et al.*, 2006).

A few recent reports have suggested that Notch inhibition may increase radiosensitivity. Wang *et al.* have investigated the effects of GSI and radiation on stem cell enriched population of cells from glioma biopsy specimens. They report that GSI treatment increased radiation induced cell death and decreased clonogenic survival of glioma stem cells at clinically relevant doses (1-3 Gy). This effect was not observed with non-stem cell

populations. Knockdown of Notch1 and Notch2 also resulted in radiosensitisation of the stem cells. GSIs did not appear to alter DNA damage response in glioma stem cells but reduced radiation induced Akt activation and Mcl-1 levels. *In vitro* irradiation of stem cells expressing Notch1 or Notch2 shRNA, and subsequent injection in mice resulted in increased radiation-induced tumour growth delay in tumours with Notch1 and Notch2 knockdown (Wang *et al.*, 2010). In a glioblastoma explant model, it has been shown that GSI treatment decreased proliferation of tumour cells, decreased stem cell population and reduced endothelial cells. Combination of GSI treatment with radiation (10 Gy) results in a decrease in proliferation of tumour cells in explant cultures as compared to either of the treatment alone (Hovinga *et al.*, 2010). Inhibition of Notch signalling in CNE2 nasopharyngeal cells decreased cell viability and clonogenic survival and increased apoptosis following radiation treatment (Yu *et al.*, 2011). In a more recent study, radiation was observed to increase protein levels of Hes1 and Hey1 in Notch expressing lung cancer cell lines. Notch inhibition following IR decreased *in vitro* clonogenic survival and increased apoptosis in these cell lines. Treatment of lung carcinoma tumour xenografts with GSI and radiation showed that combining radiotherapy with GSI resulted in a greater tumour growth delay than either of the treatments alone (Mizugaki *et al.*, 2012). Using H460 NSCLC lines overexpressing Notch1 or expressing dominant negative mastermind-like1 (DNMAML1, mutant form of MAML which inhibits Notch signalling), Theys *et al.* have reported that increased or decreased Notch activation did not effect *in vitro* clonogenic survival following IR. However, when grown as tumour xenografts, control H460 tumours displayed a pronounced tumour growth delay following radiotherapy, while the growth delay in Notch1 overexpressing tumours was relatively modest (Theys *et al.*, 2013).

1.6. Aims of this thesis

Notch pathway has been reported to play an oncogenic role in a number of cancers. Additionally, DLL4-Notch signalling is a key regulator of angiogenesis and blocking DLL4 promotes non-productive angiogenesis and delays tumour growth. Given its dual functions in tumour and vascular cells, the Notch pathway presents an attractive target for cancer therapy. Radiotherapy is an important modality in the treatment of cancer. Radiation has been reported to alter tumour vasculature but its effects appear to be dependent on dose as well as tumour-type. Additionally, recent evidence indicates that radiation may alter Notch signalling in tumour cells and the endothelium. This thesis aims to investigate the effects of anti-DLL4 therapy in solid tumours in combination with a clinically relevant dose of ionising radiation (5 Gy; IR) and to analyse alterations in the Notch pathway induced by the treatments. Specifically,

- In chapter 4, Effects of combining IR and DLL4 blockade on LS174T cells *in vitro* and tumour growth delay *in vivo* are investigated.
- In chapter 5, Effects of combining the two treatments, on tumour cells and vasculature are examined in LS174T (DLL4 expressing) and HCT-15 (DLL4-negative) tumour models. Effects of the two treatments on Notch signalling in tumour and stromal cells are examined.
- In chapter 6, effects of the treatments on endothelial cell function is examined. Additionally, DLL4 and Jag1 expression in tumour vasculature and influx of proangiogenic immune cells into the tumour after treatments is studied.

Chapter 2

Materials and Methods

2. Materials and Methods

2.1. Cell lines and cell culture

Human colorectal adenocarcinoma cell lines LS174T and HCT-15 were obtained from American Type Culture Collection (ATCC). Cell lines were cultured in Dulbecco's modified Eagle medium (DMEM) containing 4.5 g/L glucose (Invitrogen, Paisley, UK) supplemented with 10% fetal bovine serum (Lonza, Slough, UK) and 1% penicillin–streptomycin (Invitrogen, Paisley, UK) (referred to as DMEM, 10% FBS). Human umbilical vein endothelial cells (HUVEC) were obtained from Lonza. Cells were maintained in EBM-2 media (Lonza, Slough, UK) supplemented with EGM-2 SingleQuots (referred to as EGM-2). All Cell lines were maintained at 37°C with 5% CO_2. Cell lines were passaged when they reached approximately 80% confluency. Cell lines were regularly tested with MycoAlert Assay kit (Lonza) according to manufacturer's instructions.

LS174T cells were infected with Cignal Lenti RBP-Jk Reporter luciferase (QIAGEN, Crawley, UK), according to manufacturer's instructions. To establish a stable pooled LS174T cell line harbouring this Notch-responsive luciferase reporter construct (LS174T RBPJκ-Luc), cells were cultured in medium containing 2 μg/mL puromycin (Sigma-Aldrich, Dorset, UK).

2.2. Cell based assays.

2.2.1. Immobilising recombinant DLL4 or BSA on plates

Plates were incubated with 0.2% gelatine (w/v) (Sigma-Aldrich) in Phosphate buffered saline (PBS) containing 1 μg/mL bovine serum albumin (BSA; Sigma-Aldrich) or 1 μg/mL rhDLL4 (R&D Systems), each was equivalent to 18nM protein solution. The plates were

incubated at 4°C for 24 hours followed by 37°C for 30 minutes. The solution was aspirated before seeding cells.

2.2.2. Bioluminescence imaging of luciferase activity

LS174T RBPJκ-Luc were seeded onto 24-well dishes (100 000 cells per well) onto which rDLL4 or BSA had been immobilised, in DMEM, 10% FBS containing vehicle (dimethyl sulfoxide; DMSO), 1 μg/mL mouse isotype immunoglobulin G1 (IgG1), (R&D Systems Europe Ltd), 20 nM dibenzazepine (DBZ) (Axon Medchem BV, Groningen, the Netherlands), or 1 μg/mL anti-DLL4 monoclonal antibody (DLL4 mAb; human IgG1, blocks human and mouse DLL4, kind gift from MedImmune LLC, Gaithersburg, MD) and incubated for 24 hours. To assess luciferase activity, 150 μg/mL D-luciferin (Gold BioTechnology, St Louis, MO) was added to the cultures incubated for 2 minutes. Luminescence was measured using Xenogen IVIS 200 imaging system (Caliper Life Sciences Ltd, Preston Brook, UK) and analyzed with Living Image 3.0 software (Caliper Life Sciences Ltd). The fold change in luciferase activity was calculated by dividing the measurement from the treatment group by that from the DMSO BSA control.

To analyse the effect of radiation on LS174T cells, LS174T RBPJκ-Luc cells were cultured for 3 days on BSA or rhDLL4 coated wells. Within this 3 day period, cells were irradiated using IBL 637 caesium irradiator (CIS Bio International) at a dose rate of 1.1Gy/min with a single 5 Gy IR dose, either 6 hours or 48 hour after plating (i.e. 3 days or 1 day before luciferase activity measurement).

For mock irradiation, the cells were treated the same as the irradiated cells, except no dose was given. The fold change in luciferase activity was calculated by dividing the luciferase activity in irradiated cells by that in the mock irradiated cells.

2.2.3. Network formation assay

Actively growing HUVEC cells were harvested and were plated onto wells in a 24-well plates containing 300µl polymerised BD Matrigel Basement Membrane Matrix (BD Biosciences) supplemented with 500µl EGM-2 media (50,000 cells/well). Appropriate plates immediately were exposed to 5 Gy radiation as previously described. Subsequently an additional 500µl of EGM-2 or EGM-2 containing 2µg/L DLL4 mAb was added to wells. Wells were photographed 8 hours later at low power (1 field per well) and number of branches and averaged branch lengths calculated using ImageJ. Three biological replicates were analysed for each experimental condition, experiments were repeated three times.

2.2.4. Migration assay

Actively growing HUVEC cells were harvested and were plated onto wells in 12-well plates containing 1 ml EGM-2 media. Cultures were allowed to reach confluence over 24 hours. The appropriate plates were exposed to 5 Gy IR and a physical scratch was created in the monolayers. EGM-2 media was replaced to remove cell debris and floating cells, with EGM-2 and EGM-2 containing 1µg/ml DLL4 antibody. The plates were incubated in and analysed with INCUCYTE Kinetic Imaging System (Essen BioScience, Ltd.). Scratched areas were detected and coverage of this area with HUVECs was measured over time. Three biological replicates were analysed for each experimental condition, experiments were repeated three times.

2.2.5. Proliferation assessment by immunocytochemistry

Actively growing HUVEC or LS174T cells were harvested and were plated onto wells in 8-well Nunc Lab-Tek II chamber slides (Thermo Scientific) containing 300µl EGM-2 media or EGM-2 media containing 1µg/ml DLL4 mAb. Appropriate slides were irradiated with 5 Gy immediately as described above. 15,000 or 60,000 HUVECs or 60,000 LS174T cells added per well and immunostained for Ki67, 24 hours later. Cells were washed once with PBS and incubated with 4% paraformaldehyde for 8 minutes. The remainder of the immunostaining and analysis was carried out as for Ki67 immunohistochemical staining below. Three biological replicates were analysed for each experimental condition, experiments were repeated three times.

2.2.6. Analysis of growth in cell cultures

Actively growing HUVEC or LS174T cells were harvested and were plated onto 6-well plates containing 2000µl EGM-2 media or EGM-2 media containing 1µg/ml DLL4 mAb. Appropriate plates were irradiated with 5 Gy immediately as described above. 30,000 HUVECs or 200,000 LS174T cells added per well. Cells numbers in each well were determined with NucleoCounter NC-100 (Chemometec). Growth of cultures was calculated by dividing final cell counts with the number of cells plated. Three biological replicates were analysed for each experimental condition, experiments were repeated three times.

2.3. Tumour xenograft studies

All animals purchased from Charles River, Tranent, UK or Harlan, Huntingdon, UK and housed in individually ventilated cages. 6-8 weeks old, female BALB/C nude mice were used in all experiments. All experiments were performed according to University of Oxford institutional guidelines and within the limits of the Project License issued by the Home Office, United Kingdom.

2.3.1. Tumour growth delay assay

LS174T RBPJκ-Luc cells (2×10^7 cells/ml), or HCT-15 cells (1×10^7/ml) were mixed 1:1 (v/v) in serum-free DMEM and Growth Factor Reduced Matrigel (Becton, Dickinson and Company, Oxford, UK) 100µl of the cell suspension was injected subcutaneously into the right flanks of mice. Tumour dimensions were measured by calipers and volumes estimated by the modified ellipse formula (volume = length × width^2 / 2).

To assess tumour growth delay in LS174T xenograft tumours, mice bearing tumours were treated with anti-DLL4 monoclonal antibody (DLL4 mAb; human IgG1, blocks human and mouse DLL4; kind gift from MedImmune LLC, Gaithersburg, MD) and/or 5 Gy radiation. Mice were randomly assigned to mock (n=7) , DLL4 mAb (n=7), IR (n=8) or DLL4 mAb + IR (n=8). Mice in the mock group received intra-peritoneal injection with 100µL PBS solution twice per week for the duration of the experiment. DLL4 mAb was administered twice per week at a dose of 5 mg/kg of body weight beginning on day 1 (DLL4 mAb group) or 30 minutes after treatment with ionizing radiation on day 1 (DLL4 mAb + IR) for the duration of the experiment. For irradiation, mice were restrained in a customized brass tube, exposing the tumour while shielding the remainder of the body. Tumours were irradiated

with 5 Gy using an Xstrahl RS320 x-ray system (Gulmay Medical Ltd, Chertsey, UK) at a dose rate of 2.77 Gy/min under anaesthesia. Luciferase activity was measured in 3 mice from each group by bioluminescence imaging, on the first day of the experiment, before treatments were given, and again 24 hours later (see below).

Tumour volumes were measured every 1-3 days and once tumours reached 4 times the starting volume, the mice injected intraperitoneally with the hypoxic cell marker EF5 (10μL/g of body weight of a 10mM solution in 0.9 % saline; kindly provided by Dr Cameron Koch, Hypoxia-Imaging Service Center, University of Pennsylvania). The mice were sacrificed by cervical dislocation 2.5 hours later, and their tumours were excised.

Three tumours were randomly chosen from each group and processed for immunohistochemistry. Tumours were incubated overnight at 4°C in 4% paraformaldehyde (Sigma-Aldrich) for fixation, then transferred to 25% glucose in PBS for 24 hours at 4°C. Tumours were frozen in isopentane (Sigma-Aldrich) cooled on dry ice and stored at -80°C.

To assess tumour growth delay in HCT-15 xenograft tumours, with 5 Gy radiation, the mice were randomly assigned to mock (n=5) or IR (n=6) groups. Mice were irradiated and tumour growth monitored as above. Once tumours reached 4 times the starting volume, mice were sacrificed by cervical dislocation and tumours were excised, immediately frozen in liquid nitrogen and transferred to -80°C for storage.

2.3.2. Early time-point studies

Xenograft tumours were established as above using LS174T RBPJκ-Luc or HCT-15 cells. To generate tumours for assessment of changes 3 days after DLL4 mAb or 5 Gy. Mice bearing LS174T or HCT-15 xenograft tumours were treated with DLL4 mAb and/or 5 Gy radiation

(IR). Mice were randomly assigned to mock, DLL4 mAb, IR or DLL4 mAb + IR; n=5 for all groups. Mice in the mock group received an intra-peritoneal injection with 100µL PBS solution. DLL4 mAb was administered once at the start of the experiment for DLL4 mAb group, and given 30 minutes after IR for mice in DLL4 mAb + IR group. Mice in the IR group were irradiated with 5 Gy as above. Three days after treatment, mice were injected intraperitoneally with the hypoxic cell marker EF5 (10µL/g of body weight of a 10mM solution in 0.9 % saline; kindly provided by Dr Cameron Koch, Hypoxia-Imaging Service Center, University of Pennsylvania). The mice were sacrificed by cervical dislocation 2.5 hours later, and their tumours were excised, divided into two halves and immediately frozen in liquid nitrogen and transferred to -80°C for storage.

2.3.3. Bioluminescence imaging of luciferase activity in tumours

Mice were anesthetised with 4% isofluorane (Baxter, Caxton Way, UK) and then placed inside Xenogen IVIS 200 imaging system (Caliper Life Sciences Ltd); anesthesia was maintained with 2% isofluorane throughout imaging. The mice were given intraperitoneal injection with D-luciferin (150 mg/kg body weight; Gold BioTechnology), 5 minutes before imaging. Luciferase signal was quantified using Living Image 3.0 software (Caliper Life Sciences Ltd). Luciferase signal measured 24 hours after treatment was expressed as a percentage of signal measured just before treatments (baseline).

2.4. Antibody-based techniques

2.4.1. Western Blotting

Cells were rinsed once with PBS, lysed in cold radioimmunoprecipitation assay lysis (RIPA) buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate; all from Sigma-Aldrich) containing Complete Protease Inhibitor Cocktail (Roche, Burgess Hill, UK) and phosphatase inhibitors (1 mM NaF, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate; all from Sigma-Aldrich), and sonicated for 1 minute.

The lysates were subjected to centrifugation at 10,000g at 4°C for 10 minutes and the supernatants collected. Protein concentrations of the lysates were determined by a Bradford protein assay (Bio-Rad, Hemel Hempstead, UK), denatured in sample buffer (40 mM Tris–HCl, pH 6.8, 2% SDS, 2 mM dithiothreitol, 4% glycerol, 0.01% bromophenol blue) at 95°C for 5 minutes. Proteins were separated by gel electrophoresis using Novex 4%–12% Tris–glycine gels (Invitrogen, Renfrew, UK) and transferred onto polyvinylidene fluoride membranes (Millipore, Watford, UK). Membranes were incubated for 1 hour at room temperature in 5% BSA in TBST (10 mM Tris–Base, 150 mM NaCl, 0.05% Tween-20; pH 7.4), then overnight at 4°C with primary antibody in 5% BSA in TBST. Membranes were washed three times for 10 minutes each in TBST, then incubated with a secondary antibody conjugated with horseradish peroxidase (Dako UK Ltd, Ely, UK) in 5% BSA in TBST, for 1 hour at room temperature. Membranes were washed a further three times for 10 minutes each with TBST. Bound antibodies were detected with ECL Plus (chemiluminescent) reagent (Amersham PLC, Little Chalfont, UK) and exposed to x-ray

film (FujiFilm, Bedfordshire, UK). Membranes were treated with Restore Western Blot Stripping Buffer (Thermo Scientific) before probing for β-actin.

Membranes were probed for DLL4 using Polyclonal Rabbit anti-human DLL4 (1:2000, Cell signaling Technology), Jag1 using Rabbit anti-Jagged1 (1:2000, clone 28H8, Cell signaling Technology), cleaved Notch1 intracellular domain using Rabbit anti-Cleaved Notch1 (Val1744) antibody (1:1000, clone D3B8, Cell Signaling Technology), Notch1 using Rabbit anti-Notch1 antibody (1:1000, clone D1E11, Cell Signaling Technology) and β-actin using Goat anti-actin (1:6000, clone I-19, Santa Cruz Biotechnology).

2.4.2. Immunohistochemical and immunofluorescent staining

All tumours were cryosectioned using cool-cut HM355s microtome (Mircrom International). 10μM thick sections were generated and dried overnight at 37^{o}C. For the LS174T tumour growth delay experiment antigen retrieval was necessary and was performed using 10mM sodium citrate, 0.05% Tween-20, pH 6.0 at 90^{o}C for 20 min. For tumours from all other experiments, tumours were not treated with a fixative before freezing and hence antigen retrieval was not performed. Instead tumours were treated with 4% paraformaldehyde (Sigma-Aldrich) for 8 minutes before starting immunostaining, unless stated otherwise. For all immunostaining, TNB blocking buffer (0.1 M Tris–HCl, pH 7.5, 150mM NaCl, 0.5% Tyramide Signal Amplification Blocking Reagent; PerkinElmer Inc, Seer Green, UK) was used.

Immunohistochemistry for Ki67 was performed using Mouse anti-human Ki67 Ig (1:100, clone 2531 ABD Serotec). Sections were pretreated with 0.3% hydrogen peroxide in PBS for 20 min. Endogenous biotin was blocked using Avidin/Biotin Blocking Kit (Vector) according to manufacturer's instructions followed by incubation with M.O.M blocking

reagent (Vector Laboratories) for 1 hr. Sections were incubated with TNB Blocking buffer for 5 min followed by the primary antibody in Blocking buffer for 30 min. Bound antibody was labelled with biotinylated Goat anti-mouse IgG antibody (1:500, Vector laboratories) followed by Vectastain ABC Elite reagent.

TUNEL staining was performed using Apoptag Peroxidase In Situ Apoptosis detection kit (Chemicon) according to manufacturer's instructions.

Immunohistochemistry for CD31 was performed using Rat anti-mouse CD31 Ig (1:50, clone SZ31, Dianova). Sections were pretreated with 0.3% hydrogen peroxide in PBS for 20 min, followed by TNB Blocking buffer for 30 min and primary antibody in blocking buffer for 1hr. Bound antibody was labelled with ImmPRESS™ Anti-Rat Ig (mouse adsorbed) Reagent (Vector) according to manufacturer's instructions.

Immunofluorescent staining for EF5 was performed using Mouse anti-EF5 Ig directly conjugated to Cy3 fluorophore (clone: Elk3-51, kindly provided by Dr. C. Koch, University of Pennsylvania). Stained sections were mounted in aqueous mounting media containing 4',6-diamidino-2-phenylindole (DAPI).

Immunohistochemistry for CD11b, F4/80, Jag1, DLL4 and cleaved Notch1 intracellular domain was performed using rat anti-CD11b (1:100, Abcam), rat anti-F4/80 (1:100, Abd Serotec), rabbit anti-Jag1 (1:200, Cell Signaling Technology), rabbit anti-DLL4 (1:500, Abcam) and rabbit anti-Cleaved Notch1 intracellular domain (1:100, Cell Signaling Technology) respectively. Sections were pretreated with 0.3% hydrogen peroxide in PBS for 20 min, followed by TNB Blocking buffer for 30 min and primary antibody in blocking buffer for 24hr at 4^{o}C. Bound antibody was labelled with ImmPRESS™ Anti-Rat Ig (mouse adsorbed) Reagent (Vector) or Anti-Rabbit Ig Reagent (Vector) according to manufacturer's instructions.

All immunohistochemical stainings were visualised with ImmPACT DAB Peroxidase Substrate (Vector Laboratories), counter-stained with hematoxylin Solution, Gill No. 1 (Sigma-Aldrich) and mounted using VectaMount Permanent Mounting Medium (Vector Laboratories).

Immunofluorescent co-staining for CD31 with Jag1 or DLL4 was performed using the antibodies above at concentrations above. Sections were incubated with TNB Blocking buffer for 30 min and primary antibody in blocking buffer for 24hr at 4^{o}C. Bound antibodies were labelled with Alexa Fluor 546 Goat Anti-Rabbit IgG and Alexa Fluor 633 Goat Anti-Rat IgG (Life Technologies). Stained sections were mounted in aqueous mounting media containing 4',6-diamidino-2-phenylindole (DAPI).

2.4.3. Analysis of immunostaining.

Sections immunostained for Ki67 and N1ICD were imaged at a magnification of 200x. 4-6 images were captured from random regions within each tumour section, avoiding areas of necrosis. Number of positively and negatively stained nuclei were determined for each image using ImageJ software (NIH) and Colour Deconvolution plugin (Ruifrok and Johnston, 2001) and positive cells were scored as a percentage of total cells for each image.

Sections immunostained for CD31 were viewed at a magnification of 100x to identify 3 areas of high vessel density. Vessel counts were determined at a magnification of 250x using a 25 point Chalkley eyepiece graticule as previously described (Fox *et al.*, 1994). Any CD31-positive staining that was separate from an adjacent microvessel was considered countable. The graticule was rotated in the eyepiece to a position where the maximum number of graticule points overlay immunohistochemically identified vessels or their

lumens. Chalkley vessel counts for individual tumors were generated by averaging the three graticule values.

Sections immunostained for EF5 were observed field by field at 150x magnification. Each field was assigned a class score between 0 to 4 based on the percentage of the tumour area positive for EF5 staining (0 = no staining observed, 1 = 1-5% positive 2 = 5 - 15% positive, 3 = 15 – 30% positive, 4 = 30 – 50 % positive, 5 = 50 - 75). Areas of necrosis were identified using DAPI staining and were not included when estimating hypoxia scores. Scores obtained for each field were converted to percentages by using the median values for the corresponding hypoxia scores (median values for each score were as follows: score 0: 0%, score 1: 2.5%, score 2: 10%, score 3: 22.5%, score 4: 40%, score 5: 62.5%). The mean percentage of hypoxic area for all scored fields was calculated for each tumor.

To quantify co-localisation of DLL4 or Jag1 with CD31 staining. Images were acquired by confocal microscopy (Leica microsystems). Channels with CD31 and DLL4 or Jag1 signal, were converted to 8-bit binary images, after manually determining thresholds to separate signal from background tissue auto-fluorescence. The same thresholds were used to generate binary images for all tumours. A Matlab (MathWorks) script written with our colleague Dr Danny Allen, was used to determine pixel overlap between the thresholded CD31 and DLL4 or jag1 channels. This value was expressed as a percentage of the total number of pixels in the CD31 channels (Pixels overlapping between CD31 and notch ligand images/ Total number of pixels in CD31 image x 100).

To quantity necrosis, H&E stained tissue sections were imaged at 10x. Images were taken spanning the entire section and realigned together using Adobe Photosphop CS5 (Adobe Systems Incorporated). Regions of necrosis were identified by microscopy, outlined in

images and area occupied by necrosis and total area determined using ImageJ software (NIH). Necrotic areas were expressed as a percentage of total area of the tumour section. Sections immunostained for CD11b and F4/80 were imaged at a magnification of 400x. 5 images were captured from random regions within each tumour section, avoiding areas of necrosis. Number of positive cells per field were counted for each image.

2.5. Quantitative Polymerase chain reaction (qPCR)

2.5.1. Isolation of tumour RNA

Total RNA was extracted from frozen tumours using RNeasy kit (Qiagen) according to manufacturer's instructions. Briefly, small pieces of the frozen tissue were cut and weighed to about 20mg. Tissues were immersed in 600µl of lysis buffer and homogenised. The lysates were centrifuged at 10,000 *g* at 4^{o}C for 3 minutes and the supernatant isolated. 600µl of 70% ethanol was added to the supernatant and the mixture was added onto the supplied columns. The columns were centrifuged at 8000 *g* for 15 seconds and the effluent was discarded. On column DNase digestion was performed using RNase-Free DNase Set (Qiagen) according to manufacturer's instructions. The columns were washed 3 times using the proprietary buffers RW1 and RPE provided at 8000 *g.* Finally 50µl of DEPC-treated water were added to the column and collected as effluent after centrifugation of the columns at 8000 *g*. The effluents were quantified using ND-1000 spectrophotometer (NanoDrop Technologies) using RNA-40 presets and stored at -80^{o}C.

2.5.2. Reverse transcription

Reverse transcription of the RNA was carried out using High Capacity cDNA Reverse transcription kit (Applied Biosystems, Warrington). I µg of RNA was diluted in 71µl of DEPC treated water in a 0.2 ml thermo-tube. 10µl of 10x RT buffer 10µl of Random Primers , 4µl of 25x dNTP, 5µl of Multiscribe Reverse Transcriptase was added to the RNA sample. The sample was incubated at 25^{o}C for 10 minutes, 37^{o}C for 2 hours and 4^{o}C for up to 12 hours before use. The cDNA concentration was assumed to be 10ng/µl.

2.5.3. Real time qPCR

Real time qPCR was performed using the Exiqon system (Roche Applied Science). A master mix containing 12.5µl SensiMix II Probe kit (Bioline), 1µl of 10µM forward primer and reverse primers, 0.25µl probe from Universal ProbeLibrary (Roche Applied Science) and 7.05µl DEPC treated water. Individual assays were prepared in triplicate using epMotion 5070 robot (Eppendorf). Each reaction contained 8µl of the above master mix and 2µl of 6.5ng cDNA/µl. Tubes were sealed and the qPCR assays performed using 7000 HT fast real-time PCR system (Applied Biosystems) using the following sequence: 2 min, 50^{o}C; 10 min, 95^{o}C; 40 cycles, 15 sec, 95^{o}C; 1 min, 60^{o}C. All reactions were performed using mouse and human specific assays previously validated by our group (table 2.1). Mouse and human β-actin were used as internal control genes, to normalise against variations in cDNA concentrations. All assays were expressed as fold changes relative to Mock.

Table 2.1. Primers and Exiqon ProbeLibrary™ probes used in real time qPCR assays.

Gene	Left primer	Right primer	Probe
hActB	cccagcacaatgaagatcaa	cgatccacacggagtacttg	63
hDLL4	ccctggcaatgtacttgtgat	tggtgggtgcagtagttgag	23
hHey1	cgagctggacgagaccat	ggaacctagagccgaactca	39
hHey2	gtaccatccagcagtgcatc	agagaattcagtcagggcattt	60
hHes1	agtgaagcacctccggaac	cgttcatgcactcgctga	60
hJag1	gaatggcaacaaaacttgcat	agccttgtcggcaaatagc	42
mActB	aaggccaaccgtgaaaagat	gtggtacgaccagaggcatac	56
mDLL4	aggtgccacttcggttacac	gggagagcaaatggctgata	106
mHey1	catgaagagagctcacccaga	cgccgaactcaagtttcc	17
mHey2	gaggaaacgacctccgaaa	gacctcatcactgagcttgtagc	85
mHes1	acaccggacaaaccaaagac	cgcctcttctccatgatagg	99
mJag1	tggccgaggtcctacactt	gccttttcaattatgctatcag	22
hActB	cccagcacaatgaagatcaa	cgatccacacggagtacttg	63
hDLL4	ccctggcaatgtacttgtgat	tggtgggtgcagtagttgag	23
hHey1	cgagctggacgagaccat	ggaacctagagccgaactca	39
hHey2	gtaccatccagcagtgcatc	agagaattcagtcagggcattt	60
hHes1	agtgaagcacctccggaac	cgttcatgcactcgctga	60
hJag1	gaatggcaacaaaacttgcat	agccttgtcggcaaatagc	42

2.6. Statistical analyses

Statistical analyses were performed using Graphpad Prism 6 (Graphpad Software, La Jolla, CA). Two means compared with two-tailed t-test. Multiple means compared with one-way ANOVA, with Bonferroni post test. Means displayed with 95% confidence intervals.

Chapter 3

Combining DLL4 blockade and ionising radiation to reduce tumour growth

3. Combining DLL4 blockade and ionising radiation to reduce tumour growth

3.1. Introduction

There is a rising interest in targeting tumour vasculature to inhibit blood flow to tumours and inhibit tumour growth. Within the last decade, the first antiangiogenic agent, a VEGF blocking monoclonal antibody, bevacizumab was approved for clinical use. Since then several antiangiogenic agents have been given approval, including four VEGF receptor tyrosine kinase inhibitors; sunitinib, sorafenib, pazopanib and vandetanib. A number of studies have focused on the combination of VEGF targeting agents with radiation therapy and have demonstrated that VEGF can be induced by radiation in tumour models, and combining VEGF blockade with radiotherapy increases tumour growth delay (Gorski *et al.*, 1999, Lee *et al.*, 2000, Abdollahi *et al.*, 2003). However, tumour resistance to VEGF blockade is common (Bergers and Hanahan, 2008) and has partly been attributed to the Notch signalling pathway (Noguera-Troise *et al.*, 2006, Li *et al.*, 2007).

The Notch signalling pathway consists of four receptors (Notch1, Notch2, Notch3 and Notch4) and five ligands (Jag1, Jag2, DLL1, DLL3 and DLL4) in mammals. Ligand binding triggers a conformational change in the receptor, allowing for two successive proteolytic cleavage by an ADAM family protease and γ-secretase respectively, which frees the Notch intracellular domain (NICD) allowing it to translocate to the nucleus, where it activates target genes (Kopan and Ilagan, 2009).

Notch pathway has been identified to act as an oncogene in a number of tumour types (Espinoza and Miele, 2013). Additionally, DLL4-Notch signalling is a key regulator of physiological and tumour angiogenesis (Phng and Gerhardt, 2009). Overexpression of

DLL4 in tumour cells activates Notch in the tumour stroma and results in larger and better functioning vessels, increasing tumour growth (Li *et al.*, 2007). Blocking DLL4 in tumour models has been shown to increase non-productive angiogenesis, increasing hypoxia and decreasing tumour growth (Noguera-Troise *et al.*, 2006, Ridgway *et al.*, 2006, Scehnet *et al.*, 2007, Hoey *et al.*, 2009). Anti-DLL4 therapy is a novel antiangiogenic strategy which focuses on promoting angiogenesis and vessel abnormalisation. Here we explore the combination of DLL4 blockade, with a clinically relevant dose of radiation, in a human colorectal carcinoma xenograft model and investigate the ability of the combination to delay tumour growth.

3.2. Results

3.2.1. Blocking DLL4 – Notch signalling in LS174T human colorectal carcinoma cells.

Notch signalling is important in the maintenance of normal intestinal and colonic physiology and has been mainly reported in an oncogenic context in colorectal cancers. Following a western blotting analysis of DLL4 expression in a number of colorectal cancer cell lines by Dr Stanley Liu, LS174T cells were selected as a model cell line to test the effects of DLL4 blockade alone and in combination with radiation treatment. LS174T cells were infected with a lentivirus containing a construct expressing firefly luciferase under the control of NICD binding RBPJκ sites. Together with Dr Stanley Liu, cells stably expressing this construct (LS174T RBPJκ-Luc) were generated following selection with puromycin containing media, and used to assess Notch activation by measuring luciferase activity. LS174T RBPJκ-Luc cells were cultured on plates with immobilised recombinant human DLL4 (rhDLL4) or bovine serum albumin (BSA), and treated with either DLL4 blocking monoclonal antibodies (DLL4 mAb) or γ-secretase inhibitor DBZ (figure 3.1a) for 24 hours. DMSO or isotype-control antibody (Isotype) treated cells were included as controls for DBZ and DLL4 mAb respectively. DMSO and Isotype treated cells cultured with rhDLL4 showed increased luciferase activity as compared to cells cultured with BSA. Both DLL4 mAb and DBZ treatments decreased Notch activity in cells cultured with BSA or rhDLL4, compared to DMSO controls. Notch activity inhibition observed with DLL4 mAb was comparable to that with DBZ, in both BSA and rhDLL4 exposed cells, indicating that Notch signalling may occur predominantly via DLL4 in LS174T cells *in vitro*.

Western blot analysis of LS174T cells treated with DBZ or DLL4 mAB (figure 3.1b, data contributed by Dr Stanley Liu) shows that both treatments cause an increase in DLL4 protein levels compared to DMSO control. However, the level of Notch ligand Jag1, also endogenously expressed by LS174T cells, was not altered by either treatments.

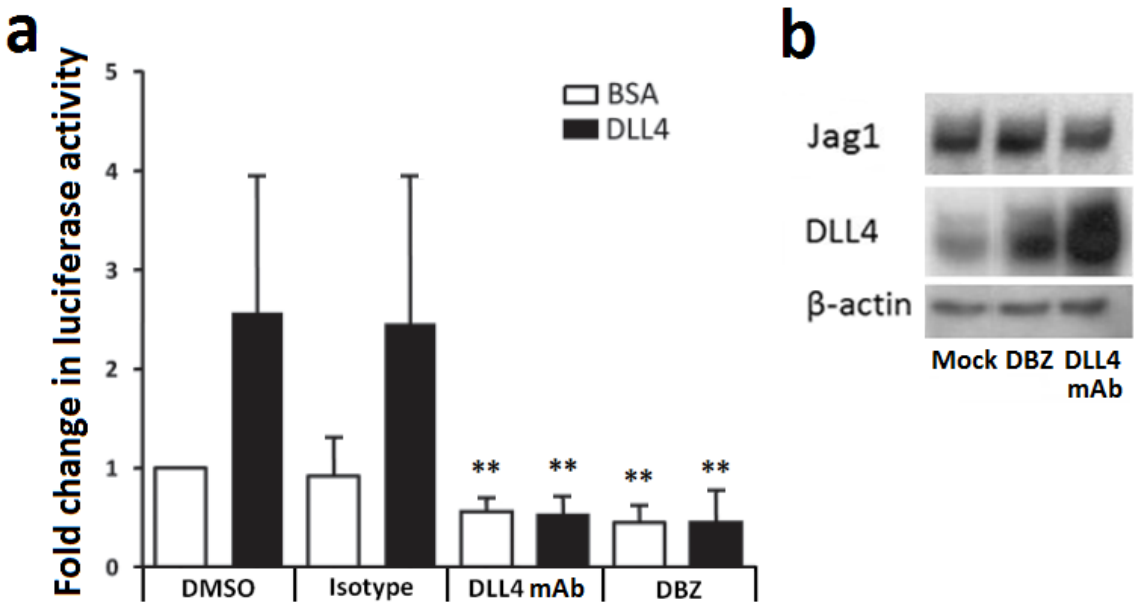


Figure 3.1. DLL4 blocking antibodies inhibit Notch signalling but upregulate DLL4 in LS174T cells. a, Changes in luciferase activity in LS174T cells transfected with Notch-responsive luciferase reporter. Cells were incubated on plates coated with recombinant DLL4 or BSA and incubated with DMSO, isotype-control antibody, DLL4 blocking antibody (DLL4 mAb) or DBZ for 24 hours before luciferase activity was measured. Measurements relative to BSA DMSO. *Columns*, mean; *error bars,* 95% CI, **p<0.01 vs. respective DMSO. **b**, Western blot analysis of Jag1 and DLL4 proteins in LS174T cells following treatment with DBZ or DLL4 overnight. B-actin as loading control.

3.2.2. Effects of ionising radiation on Notch signalling in LS174T cells.

As detailed above, ionising radiation has previously been reported to alter Notch pathway in cancer cell lines (Phillips *et al.*, 2006, Mizugaki *et al.*, 2012). To investigate the effects of radiation on Notch signalling in LS174T cells, we assessed Notch activation by measuring luciferase activity in LS174T RBPJκ-Luc cells (figure 3.2a). LS174T RBPJκ-Luc cells were

cultured for 3 days on BSA or rhDLL4 coated wells. During this period, cells were treated with a single 5 Gy IR dose, either 3 days or 1 day before luciferase activity measurement. After 3 days, luciferase activity was recorded and cells counts were determined to normalise for differences in cell numbers between IR treated samples and unirradiated controls. In cells cultured within BSA coated wells, treatment with radiation resulted in a reduction in Notch activity, 1 day as well as 3 days after IR. In cells exposed to exogenous rhDLL4, a significant decrease in Notch activity was seen only 3 days after irradiation. Radiation induced changes in Notch signalling was also explored via western blot analysis for cleaved Notch 1 intracellular domain (N1ICD), DLL4 and uncleaved Notch1 protein (figures 3.2b and c). Samples treated with 5 Gy IR exhibited a decrease in DLL4 protein

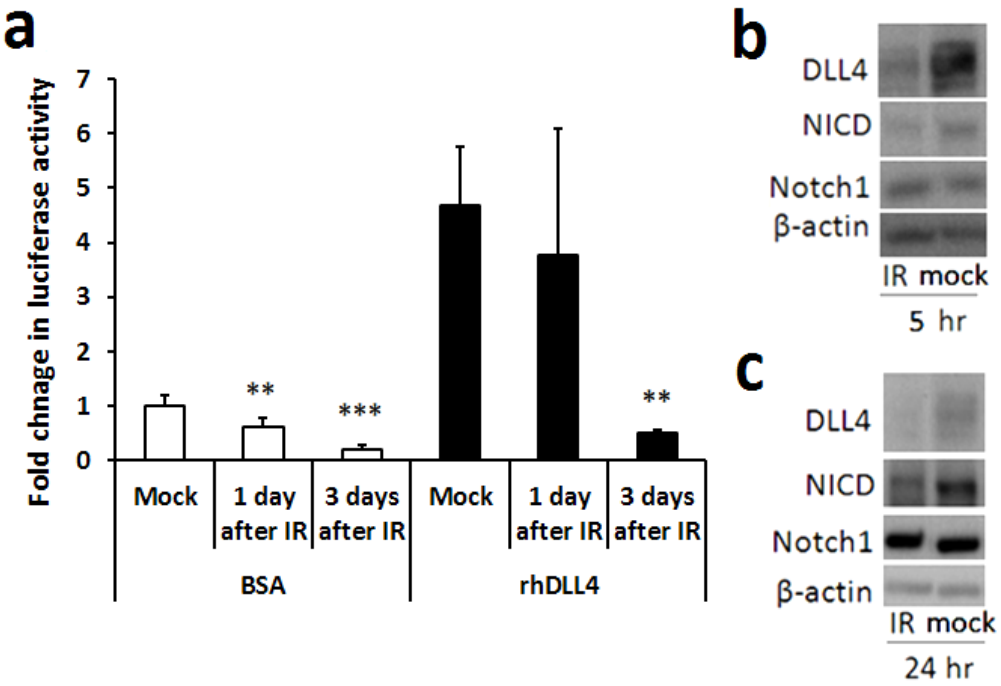


Figure 3.2. Ionising radiation suppresses Notch signalling in LS174T cells. a, Changes in luciferase activity in LS174T cells transfected with Notch-responsive luciferase reporter. Cells were incubated on plates coated with recombinant DLL4 or BSA, cultured for 3 days and treated with 5 Gy IR, 1 day or 3 days before luciferase measurements. Measurements relative to BSA mock. *Columns*, mean; *error bars,* 95% CI, **P<0.01 vs. BSA or rhDLL4 mock. **b**,**c,** Western blot analysis of DLL4, Notch1 and cleaved Notch1 intracellular domain (NICD) in LS174T cells harvested 5 hr (**b**) or 24hr (**c**) after treatment with 5 Gy IR . B-actin as loading control.

levels as well N1ICD levels within 5 hours, while Notch1 levels appeared to remain unchanged (figure 3.2b). A similar result was observed in samples analysed 24 hours after irradiation (figure 3.2c). The reduction in DLL4 levels observed may partly explain the reduced Notch activation observed following radiation treatment. These results collectively demonstrate that ionising radiation suppresses Notch signalling in LS174T cells *in vitro*.

3.2.3. Effects of radiation treatment and DLL4 blockade on proliferation and growth of LS174T cells.

Notch signalling has been observed to promote pro-proliferative pathways in cancer and Notch inhibition has been reported to decrease cancer cell proliferation and induce cell cycle arrest, as discussed in Chapter 1. Recent reports have also shown that targeting Notch may increase radiosensitivity, as detailed above. The effects of DLL4 blockade and IR on proliferation and growth of LS174T cells were examined next. LS174T cells were irradiated with 5Gy IR and/or treated with DLL4 mAb for 3 days before immunostaining for Ki67 (figure 3.3a). IR treatment caused a significant decrease in the number of cells with Ki67 positive nuclei compared to untreated mock. DLL4 blockade however did not appear to significantly alter proliferation when given alone, or lead to a further decrease in proliferation when combined with IR.

A similar result was observed when cell counts were measured after being subjected to 5Gy IR and allowed to grow for 3 days with or without DLL4 mAb (figure 3.3b). Cell counts measured show that cell numbers increased over 3 days in all treatment groups. However

cell numbers were lower in samples exposed to IR, compared to mock, while DLL4 did not significantly impact cell growth.

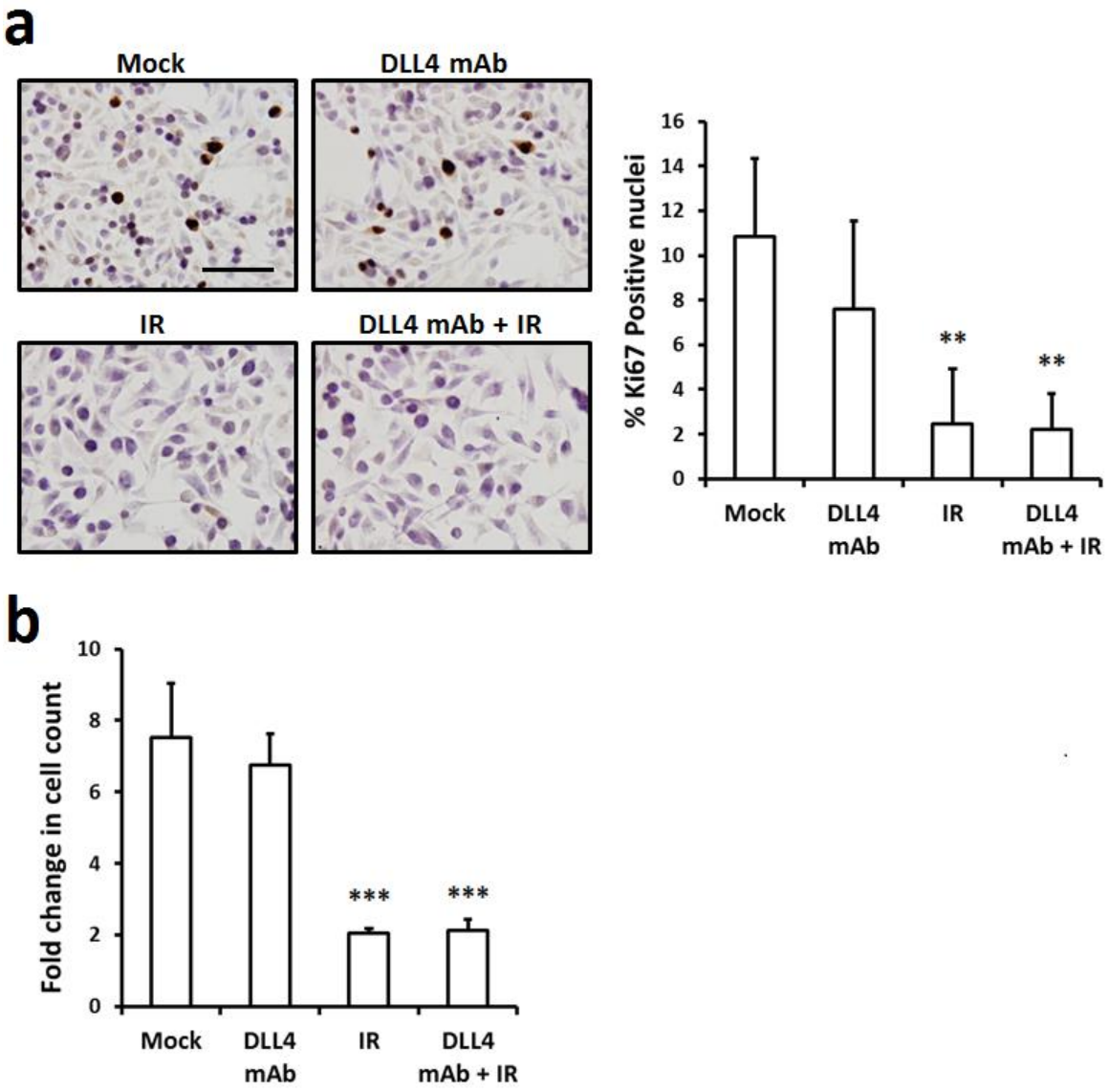
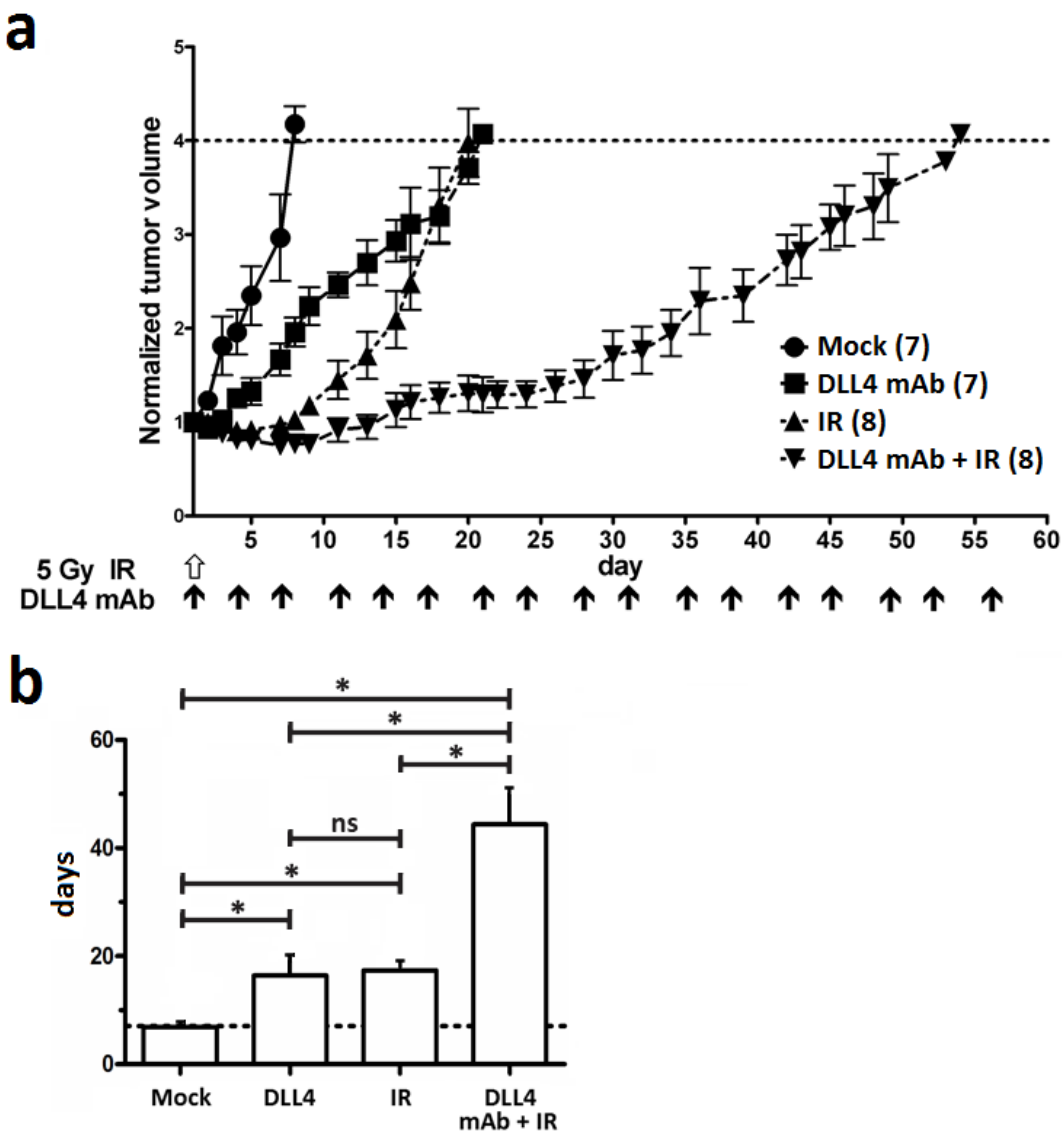


Figure 3.3. Effects of DLL4 blockade and radiation on proliferation and growth of LS174T cells *in vitro*. LS174T cells were treated with 5 Gy radiation or incubated with DLL4 blocking antibody (DLL4 mAb) or both. **a,** Proliferation analysed 3 days after treatments by Ki67 immunostaining. Representative images, left panels**, Scale bar***,* 50µm; quantified as percentage of positive nuclei, right panels. **b,** Total numbers of cells counted 3 days after treatment, measurements relative to mock. *Columns*, mean; *error bars,* 95% CI, **p<0.01, ***p<0.001 vs. mock

3.2.4. DLL4 blockade and IR inhibit Notch and delay LS174T tumour growth *in vivo*.

Solid tumours are complex structures which not only include cancer cells but also stromal elements such as inflammatory cells and vasculature (Mueller and Fusenig, 2004). Notch inhibition may affect tumour growth by directly acting on cancer cells or indirectly by targeting stromal components. To investigate the effects of combining DLL4 blockade and IR *in vivo*, we utilised a subcutaneous tumour xenograft model in athymic nude mice, established using LS174T RBPJk-luc cells. Once tumours reached 100mm^3, Dr Stanley Liu and I randomly assigned mice to Mock, DLL4 mAb, IR and DLL4 mAb + IR treatments, and



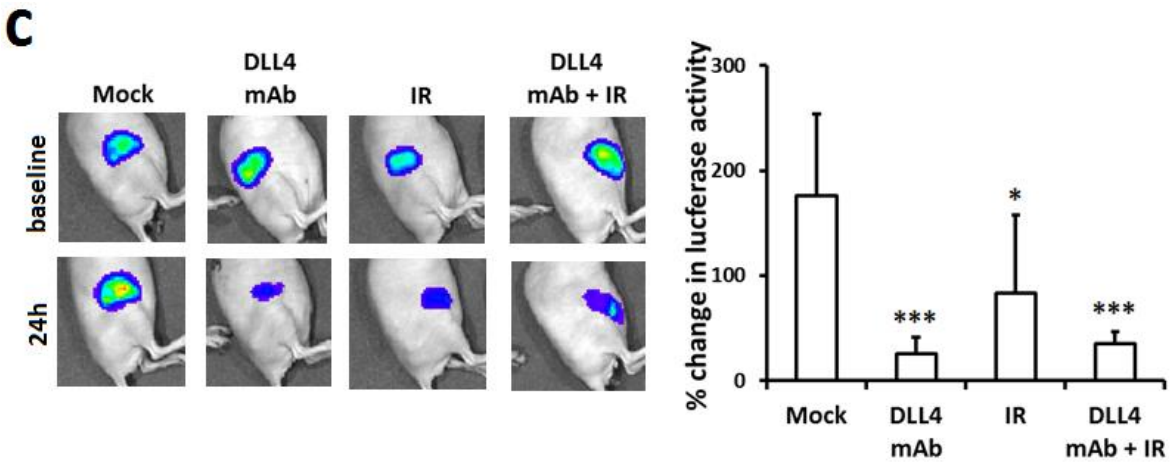


Figure 3.4. Combining DLL4 blockade and IR *in vivo*. Mice bearing subcutaneous LS174T tumours with a Notch responsive luciferase reporter were randomly assigned to treatment groups, Mock, anti-DLL4 antibody (DLL4 mAb), 5 Gy radiation (IR) or DLL4 mAb and IR, when tumour volumes were approximately 100 mm^3. **a,** Growth curves of tumours. Tumour volumes plotted relative to starting volumes, DLL4 mAb given twice a week (black arrowheads), IR given at the start of the experiment (white arrowhead). Mice sacrificed when tumours reached four times the starting volume (dotted line). Mice in each group shown in parentheses. **b,** Average times for tumours to reach four times the starting volume **c,** 3-5 mice from each treatment group injected intraperitoneally with luciferin, and tumour bioluminescence measured before and 24hr after treatment. Left panels, heat map overlying representative tumours displays relative luciferase activity. The mean fold change in luciferase activity is shown in graphical form in right. *Columns*, mean; *error bars,* 95% CI, *p<0.05, ***p<0.001 vs. mock.

treated relevant groups with a single dose of 5 Gy IR, followed by administration of DLL4 mAb (figure 3.4a). Subsequently, DLL4 mAb was administered twice per week for the remainder of the experiment. We scheduled the administration of DLL4 mAb after IR treatment as DLL4 blockade has been reported to increase tumour hypoxia and hence decrease radiosensitivity (as described in section 1.5.1). Tumours volumes were estimated from measurements using calipers throughout the experiment, and normalised to volumes at the start of treatment. Tumour volumes were monitored until they reached four times the starting volume (4RTV), when the mice were sacrificed and tumours resected for further analysis. Compared to mock tumours, the average time for tumours to reach 4RTV was longer for DLL4 mAb and IR treatments alone as well as when

combined together. Additionally, combining DLL4 mAb with IR led to a greater than additive growth delay than those observed with DLL4 mAb or IR.

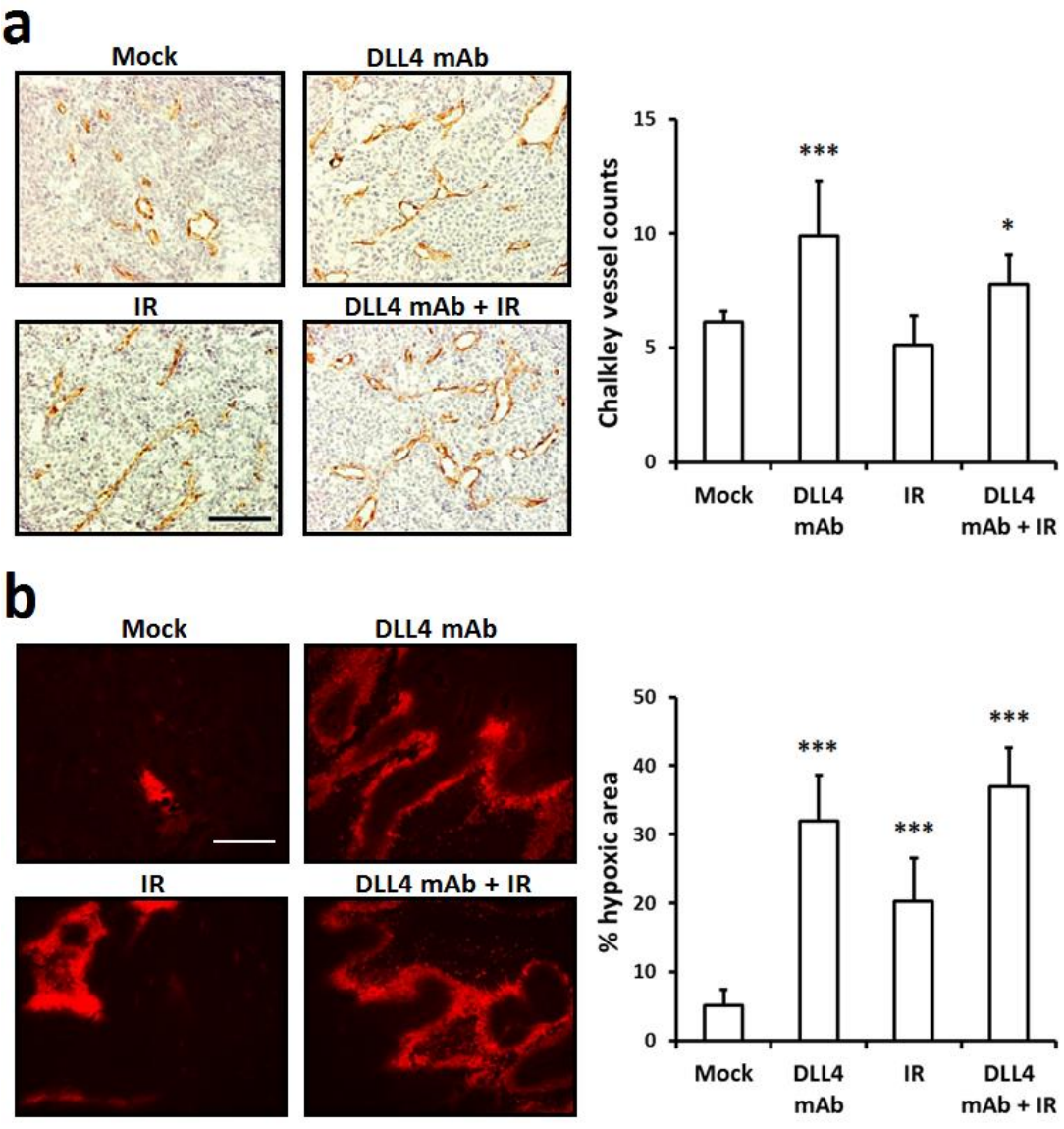
To examine the effects of IR on Notch signalling *in vivo* and to verify Notch inhibition by DLL4 mAb in LS174T tumours, Dr Stanley Liu measured luciferase activity before and 24 hours after IR and/or DLL4 mAb treatment (figure 3.4c). Treatment with DLL4 mAb decreased Notch activation alone and as well as when administered following IR. IR treatment alone also caused a decrease in Notch activity, which is in agreement with inhibition of Notch by IR in LS174T cells *in vitro* (section 3.2.2).

3.2.5. Effects of DLL4 blockade and IR on vessel density, hypoxia and necrosis in LS174T tumours.

DLL4-Notch signalling is a key regulator of angiogenesis and Notch inhibition has been shown to promote excessive but poorly-functional vasculature and may increase hypoxia, as discussed in Chapter 1. Treatment induced changes in vasculature, hypoxia and necrosis were examined by histological and immunohistochemical staining of tumours resected after 4RTV (section 3.2.4). Treated and untreated tumours were immunostained for CD31 and Chalkley vessel counts were conducted to determine changes in microvessel density (figure 3.5a). Tumours treated with DLL4 mAb with and without IR exhibited higher vessel counts compared to mock tumours, while no significant changes could be observed in tumours treated with IR alone.

Tumours were injected with EF5, a nitroimidazole which binds proteins in hypoxic cells (Lord *et al.*, 1993), before resection. Immunofluorescence staining of EF5-protein

conjugates was employed to detect and assess hypoxia (figure 3.5a). A higher percentage of EF5-positive area was observed in tumour treated with DLL4 mAb alone or following IR. To evaluate necrosis in tumours from the above experiment, sections were stained with haematoxylin and eosin and areas of necrosis were identified visually (figure 3.5a). Tumours in DLL4 mAb and combination treatment groups displayed a higher percentage of necrosis as compared to mock, while IR alone did not lead to significant increase. These results, together with increased vessel density and hypoxia observed in DLL4 mAb treated tumours show that DLL4 blockade increases angiogenesis but promotes poorly functional vasculature.



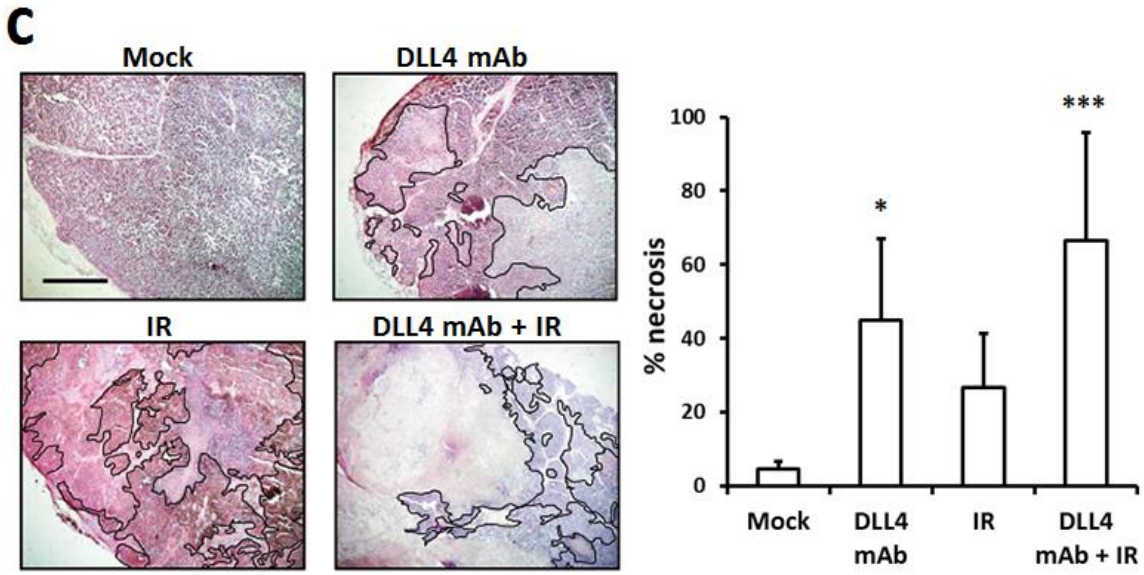


Figure 3.5. Assessment of vessel density, hypoxia and necrosis in LS174T tumours treated with radiation and DLL4 blocking antibodies. LS174T tumours treated with anti-DLL4 antibody (DLL4 mAb), 5 Gy radiation (IR) or DLL4 mAb and IR, and excised after assessment of growth delay for immunohistochemical analysis. **a,** Microvessel density assessed following CD31 immunostaining. Representative images, left panels, **scale bar** 100µm; quantified by Chalkley vessel counting**,** right panel. **b,** Hypoxia assessed by EF5 immunostaining. Representative images, left panels, **scale bar** 200µm; quantified by percentage of hypoxic area**,** right panel. **c,** Necrosis assessed following haematoxylin and eosin staining. Representative images, left panels, **scale bar** 1000µm; quantified as percentage of necrotic area**,** right panel. *Columns*, mean; *error bars,* 95% CI, *p<0.05, ***p<0.001 vs. mock.

3.2.6. Tumour cell proliferation and apoptosis in LS174T tumours treated with DLL4 blocking antibodies and radiation.

We also analysed proliferation and apoptosis in tumours collected after growth delay assessment with DLL4 mAb and/or IR. Proliferation was examined by staining for and counting cells with Ki67 positive nuclei (figure 3.6a). Compared with mock tumours, only tumours treated with DLL4 mAb alone showed a significant difference. The proportion of Ki67 positive cells did not appear to be different in combination treated tumours compared to mock. The growth delay in this group may be a result of the considerably decreased quantity of viable tumour cells due to necrosis. TUNEL staining revealed less

than 1% TUNEL positive cells observed for all treatment groups with no differences apparent between any groups (figure 3.6b).

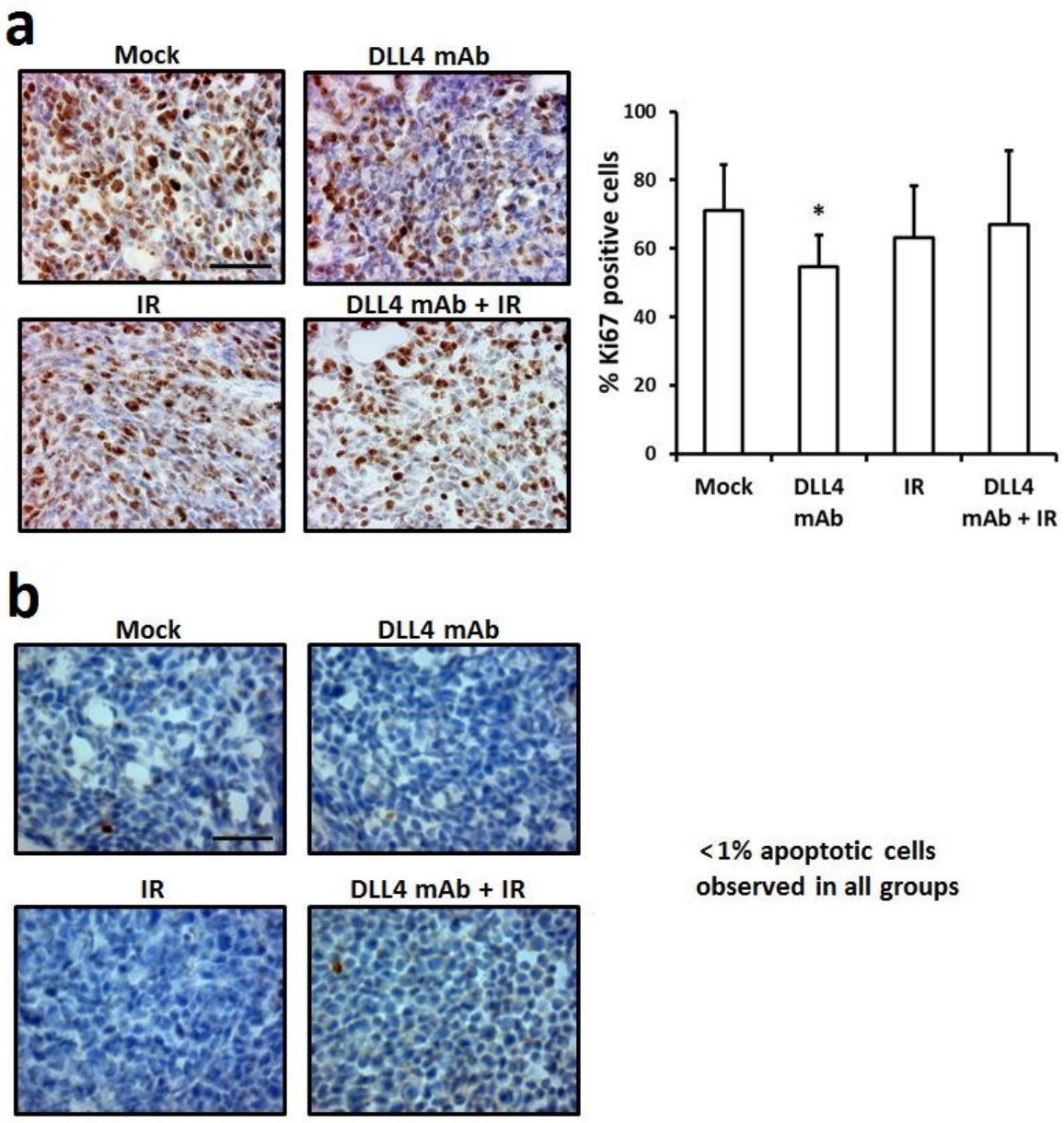


Figure 3.6. Proliferation and apoptosis in LS174T tumours treated with radiation and DLL4 blocking antibodies. LS174T tumours treated with anti-DLL4 antibody (DLL4 mAb), 5 Gy radiation (IR) or DLL4 mAb and IR, and excised after assessment of growth delay for immunohistochemical analysis. **a,** Proliferation assessed by Ki67 immunostaining. Representative images, left panels, **scale bar,** 50µm; quantified as percentage of positive nuclei**,** right panel. **b,** Apoptosis assessed by TUNEL staining. Representative images, left panels, **scale bar** 50µm. *Columns*, mean; *error bars,* 95% CI, *p<0.05.

3.3. Discussion

Reports prior to this study have identified the potential of combining Notch inhibition with radiation treatment in cancer (Hovinga *et al.*, 2010, Wang *et al.*, 2010, Yu *et al.*, 2011). However a majority of these results are based on *in vitro* models and do not account for the contribution of stromal and particularly vascular response to the treatments. Wang *et al.* noted increased tumour growth delay in xenograft tumours derived from glioma stem cells when Notch1 or Notch2 silencing was combined with radiation treatment. However, the cells were irradiated *in vitro* prior to implantation in mice, and Notch was silenced only in tumour cells. Therefore, this model did not directly account for stromal response to radiation or Notch inhibition. Here, we have explored the effects of DLL4 blockade with a clinically relevant dose of radiation in tumour xenografts. To our knowledge, the results presented here constituted the first report investigating the combination of Notch inhibition and radiation treatment in an *in vivo* model. Since then others have reported an increased tumour growth delay with a combination of γ-secretase inhibition and IR (Mizugaki *et al.*, 2012), and a reduction in radiation-induced growth delay upon overexpression of Notch1 (Theys *et al.*, 2013). Results presented here as well as experiments conducted by Dr Stanley Liu with pure populations of LS174T, show that blocking DLL4 does not affect proliferation, apoptosis or clonogenic survival *in vitro*. However, the treatments interacted favourably *in vivo*, resulting in a supra-additive tumour growth delay. In experiments conducted by Dr Stanley Liu, a supra-additive growth delay was also seen in the same tumour model when radiation treatment was combined with a γ-secretase inhibitor DBZ (Liu *et al.*, 2011) but no changes in apoptosis or clonogenic survival were seen *in vitro*. Theys *et al* have observed that Notch over-activation did not alter proliferation or radiosensitivity *in vitro* but induced

radioresistance *in vivo* (Theys *et al.*, 2013). These findings highlight the contribution of the tumour microenvironment to treatment response and provide a rationale for targeting Notch with radiation treatment in solid tumours.

Histological assessment of radiation treated tumours revealed no changes in vessel density. Dr Fokas of our group analysed tumours 2 and 5 days after IR and observed no changes in blood flow by ultrasound (Liu *et al.*, 2011). Yet, we did note an increase in hypoxia in IR treated tumours. Acute effects of radiation may not be apparent in histological analyses presented here, as the tumour had been excised a number weeks after radiation treatment. Assessment of tumours excised shortly after treatments would allow a better understanding of the effects of IR and its interaction with anti-DLL4 therapy *in vivo*.

Anti-DLL4 antibody promoted angiogenesis in tumours, as evidenced by increased microvessel density, but also increased in hypoxia and necrosis. Our colleague, Dr Emmanouil Fokas has also demonstrated that DLL4 blockade reduced blood flow in this tumour model by microbubble contrast ultrasound analysis. These results support prior reports that DLL4 blockade promotes non-productive angiogenesis (Noguera-Troise *et al.*, 2006, Ridgway *et al.*, 2006, Scehnet *et al.*, 2007). Interestingly tumours given anti-DLL4 therapy blockade, particularly in combination with radiation, exhibited necrotic centres with a viable rim of cancer cells toward the periphery of the tumour, an effect similar to that observed with vascular targeting agents (Horsman and Siemann, 2006). This may be due to proximity to normal vasculature outside the tumour mass, resulting in a better perfused rim. Hence, delivery of another dose of radiation at later point may target this better oxygenated mass, to achieve tumour control.

The precise mechanism of interaction of radiation treatment and DLL4 blockade in the tumour microenvironment remains unclear. In addition to directly targeting tumour cells, radiation treatment can affect tumour vasculature as well as influence influx of proangiogenic cellular infiltrates as described in detail in chapter 1. Additionally, radiation has been shown to alter Notch in endothelial cells (Scharpfenecker *et al.*, 2009, Imaizumi *et al.*, 2010) as well as tumour cells (Phillips *et al.*, 2006). In LS174T cells, IR suppressed Notch signalling and reduced DLL4 levels *in vitro*. Others have shown that DLL4 and Jag1 expressed on tumour cells can alter tumour vasculature (Zeng *et al.*, 2005, Li *et al.*, 2007). Similarly, Notch induced in tumour cells by stromal cells may promote tumourigenesis (Indraccolo *et al.*, 2009). Changes in tumour-stromal Notch signalling due to radiation and DLL4 blockade may alter growth of tumours, and merit further investigation.

In conclusion, these results demonstrate that anti-DLL4 therapy following ionising radiation in tumour, dysregulates vasculature and promotes tumour necrosis. This treatment has proven effective in retarding tumour growth in this pre-clinical study and merits further investigation as a therapy against solid tumours.

Chapter 4

Characterising tumour response to anti-DLL4 therapy and ionising radiation

4. Characterising tumour response to anti-DLL4 therapy and ionising radiation

4.1. Introduction

It is well established that ionising radiation leads to tumour cell death by inducing double strand breaks in the DNA. However, ionising radiation can also damage or inhibit endothelial cell function (Budach *et al.*, 1993, Imaizumi *et al.*, 2010). Ionising radiation (IR) has been reported to alter Notch signalling in endothelial cells. 5 Gy IR has been shown to increase *Jag1* and well as Notch downstream gene *Hey1* levels concomitantly in endothelial cells *in vitro*, a few hours after treatment (Scharpfenecker *et al.*, 2009). Another group has demonstrated similar results where a higher dose of radiation (15 Gy) increased *Hey1* expression in endothelial cells (Imaizumi *et al.*, 2010). It has been observed that expression of Notch ligands Jag1 and DLL4 on tumour cells can signal to, and alter tumour vasculature (Zeng *et al.*, 2005, Li *et al.*, 2007). Radiation may also alter Notch in tumour cells. In lung cancer cell lines that express Notch, radiation treatment increased protein levels of Hes1 and Hey1 (Mizugaki *et al.*, 2012). Treatment of a breast cancer cell line with fractionated radiotherapy was observed to increase Jag1 and promote Notch1 activation (Phillips *et al.*, 2006).

Studies examining the effects of ionising radiation on tumour vasculature have yielded conflicting results. It appears that high doses of radiation may inhibit angiogenesis while low doses may promote it (Imaizumi *et al.*, 2010, Sofia Vala *et al.*, 2010). Additionally, vasculature in two tumour models may respond differently to the same dose of radiation (Geng *et al.*, 2001). Radiation treatment may also induce expression of growth factors such as VEGF, in tumour cells and stroma and alter tumour vasculature in this fashion

(Gorski *et al.*, 1999, Nozue *et al.*, 2001, Solberg *et al.*, 2008). These findings indicate that radiation can affect tumour vasculature directly, or indirectly through tumour cells. The effects however are dependent of multiple factors including radiation dose and tumour type. In addition, radiation can alter Notch pathway in tumour as well as endothelial cells. As Notch pathway is a key regulator of angiogenesis and can also promote oncogenic pathways in tumour cells (as reviewed in chapter 1), these changes can have important implications for tumour development and treatment. Here we explored the effects of radiation treatment and anti-DLL4 therapy on tumour cells and vasculature and also examined alterations in Notch pathway in tumour cells and stroma by these treatments.

4.2. Results

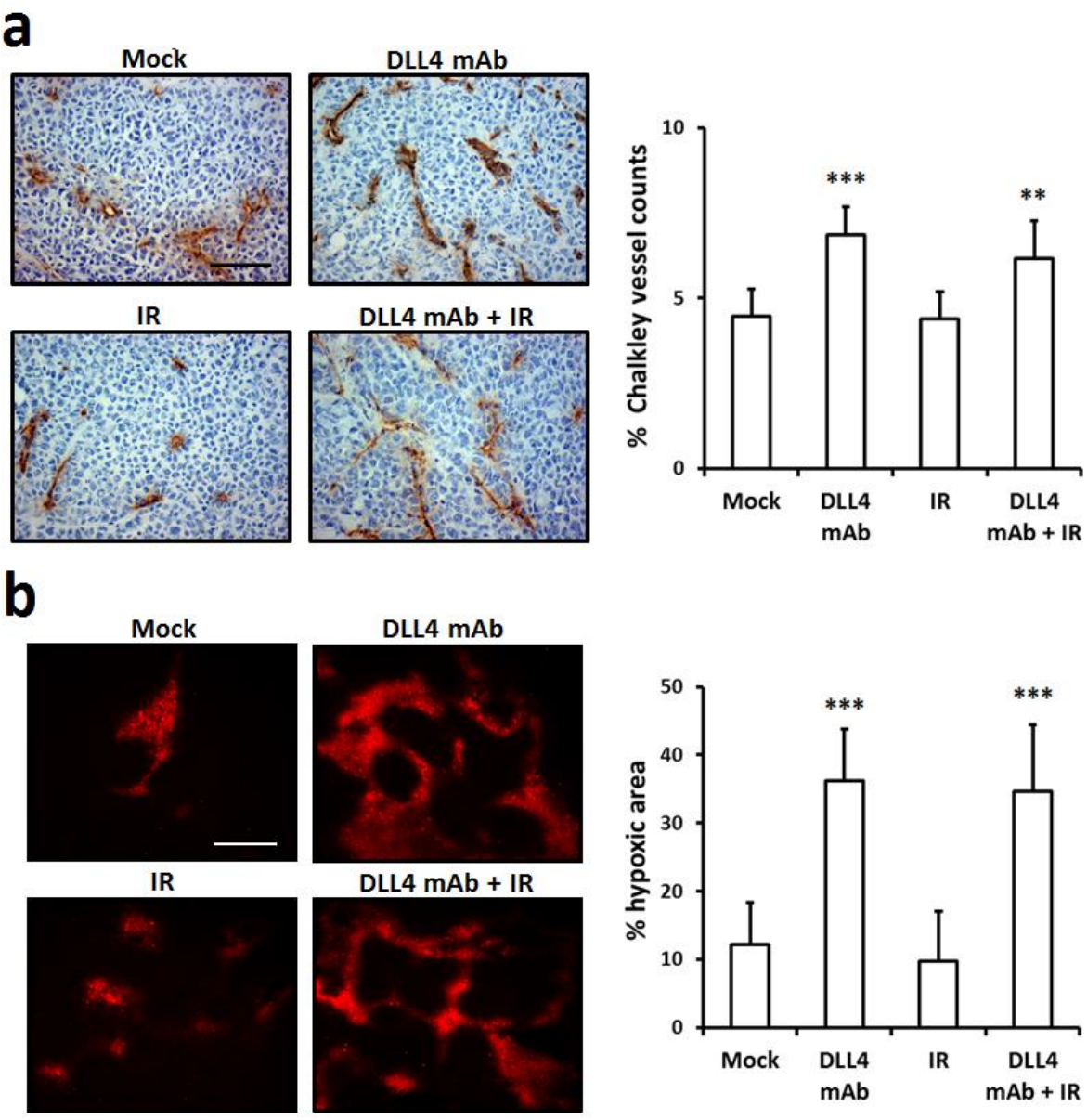
4.2.1. DLL4 blockade alters tumour vasculature within 3 days of treatment.

The results described in chapter 3 show maximal suppression of Notch signalling in LS174T cells *in vitro*, 3 days after IR treatment (figure 3.2a). Tumours analysed in the previous chapter, receiving IR alone or DLL4 mAb following IR, were on average excised 17 days and 44 days respectively after IR treatment respectively. We decided to explore changes caused by IR treatment *in vivo*, which may be apparent only at early time points, and to investigate interaction with DLL4 blockade in tumour cells and vasculature. LS174T RBPJκ-luc cells were grown as subcutaneous tumours in athymic nude mice. Once tumours reached 120mm^3, mice were randomly assigned to Mock, DLL4 mAb, IR or DLL4 mAb + IR treatment groups. Tumours were treated with a single dose of DLL4 mAb, 5 Gy IR or 5 Gy IR followed by DLL4 mAb. Mice were sacrificed 3 days later and tumours were excised. Tumours were immunostained for CD31 and Chalkley vessel count were performed to assess alterations in microvessel density (figure 4.1a). Tumours treated with DLL4 mAb alone and combination with IR displayed higher vessel counts compared to mock tumours. Microvessel density in tumours given IR only, appeared similar to mock.

As before, the hypoxic marker EF5 was administered to mice prior to sacrifice. Immunofluorescence detection and quantification of EF5 positive adducts showed an increased percentage of hypoxic area in tumours exposed to DLL4 mAb and combination therapy (figure 4.1b). Of note, hypoxic patches in these tumours were often observed bordering areas appearing to be necrotic. As with vessel density, distribution of hypoxia in IR treated tumours appeared similar that in mock.

Areas of necrosis in tumour sections were identified and quantified using haematoxylin and eosin staining (figure 4.1c). DLL4 mAb treatment resulted in an increase in necrosis compared to mock. Additionally, tumours treated with a combination of DLL4 mAb and IR displayed higher levels of necrosis compared to either of the treatments alone.

Together these results show that a single dose of DLL4 mAb can alter tumour vasculature within 3 days and lead to increased hypoxia and necrosis. IR does not appear to have a significant effect on vasculature when given alone, but may further enhance necrosis when combined with DLL4 mAb.



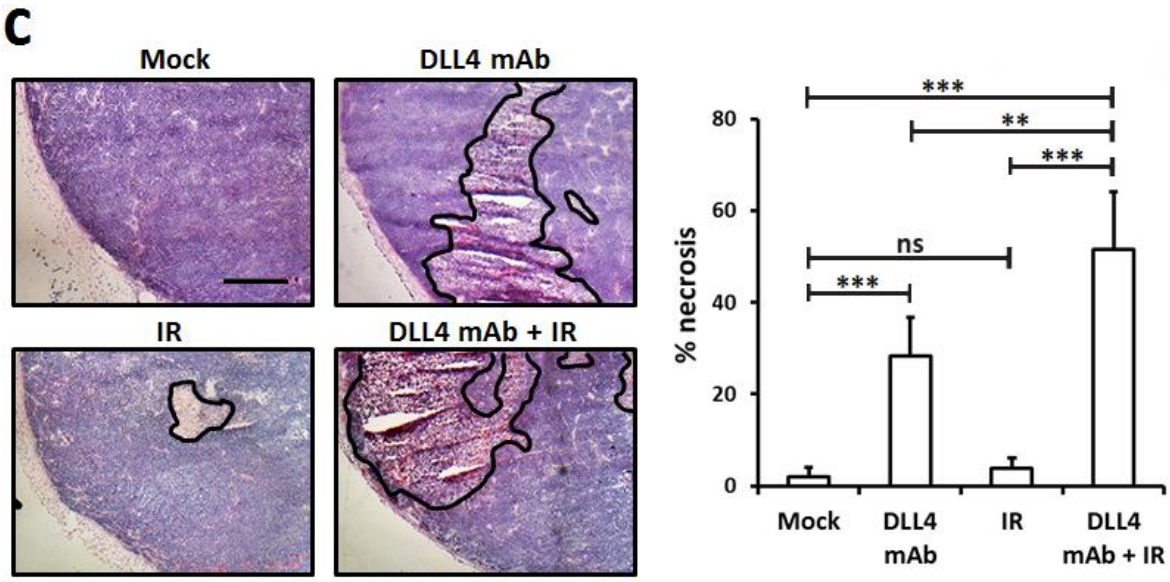


Figure 4.1. DLL4 blockade alters tumour vasculature and promotes hypoxia and necrosis within 3 days in LS174T tumours. Mice bearing subcutaneous LS174T tumours were treated with DLL4 mAb, 5 Gy or IR followed by DLL4 mAb 30 min later. Tumours resected 3 days later. **a,** Microvessel density assessed following CD31 immunostaining. Representative images, left panels, **scale bar** 100µm; quantified by Chalkley vessel counting**,** right panel. **b,** Hypoxia assessed by EF5 immunostaining. Representative images, left panels, **scale bar** 200µm; quantified by as percentage of hypoxic area**,** right panel. **c,** Necrosis assessed following haematoxylin and eosin staining. Representative images, left panels, **scale bar** 1000µm; quantified as percentage of necrotic area**,** right panel. *Columns*, mean; *error bars,* 95% CI, **p<0.01, ***p<0.001 vs. mock; n=5.

4.2.2. Ionising radiation decreases proliferation in LS174T cells *in vivo*.

LS174T cells exposed to IR *in vitro* displayed a decrease in proliferation within 3 days of treatment, as presented in the previous chapter (figure 3.3). To evaluate the effects of treatments after 3 days in LS174T tumour, we analysed proliferation by immunostaining for Ki67 and apoptosis by TUNEL staining (figure 4.2). IR treatment alone and in combination with DLL4 mAb resulted in a decrease in tumour cell proliferation compared to mock. DLL4 mAb on its own, did not cause a significant decrease in proliferation at this time point. However, the percentage of Ki67 positive cells was lower in tumours treated

with DLL4 mAb and IR compared to IR alone, suggesting that DLL4 mAb may increase effectiveness of IR at lowering tumour cell proliferation in this model.

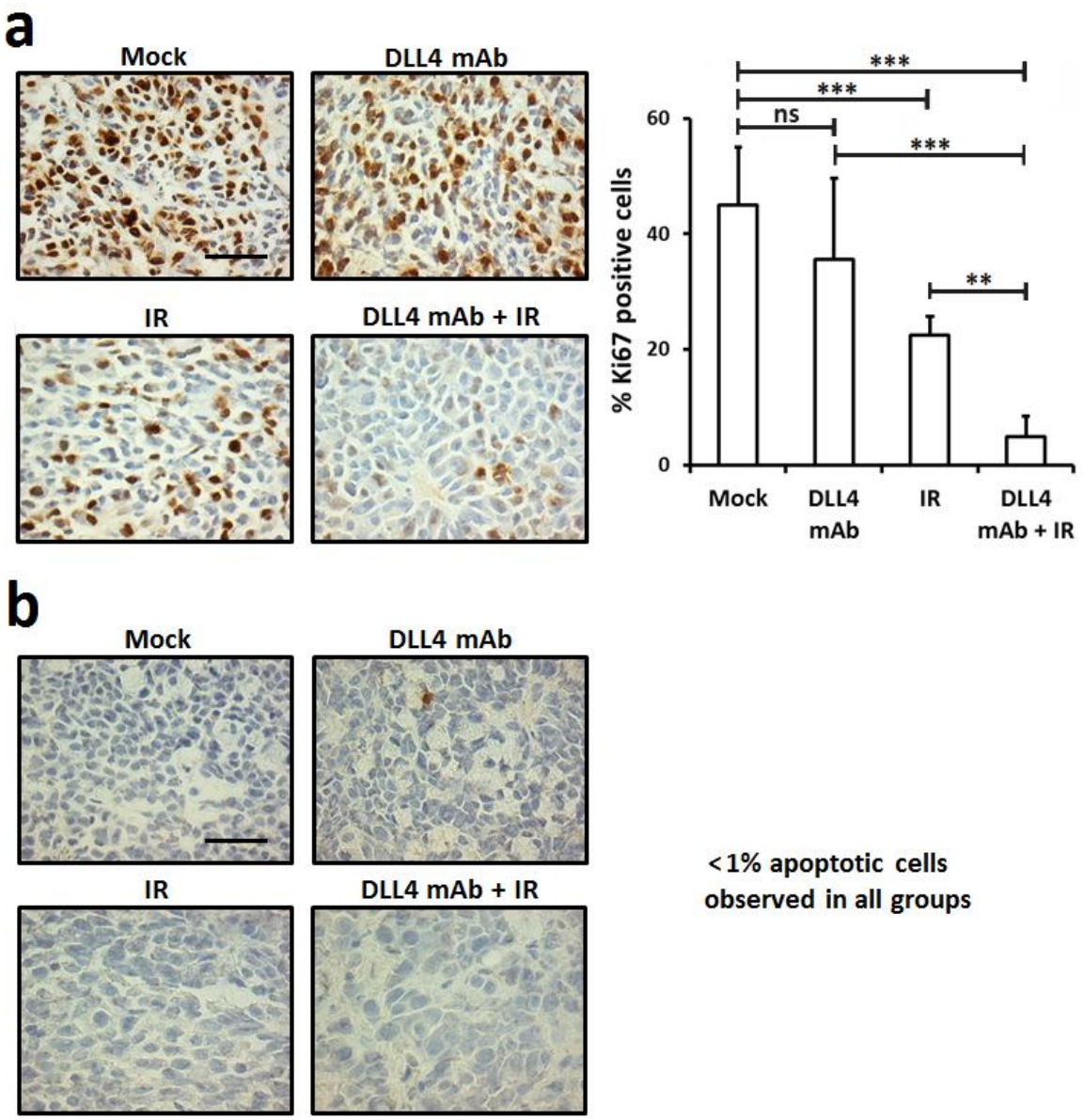


Figure 4.2. Combining radiation treatment with DLL4 blockade decreases proliferation but does not promote apoptosis in LS174T tumours. Mice bearing subcutaneous LS174T tumours were treated with DLL4 mAb, 5 Gy (IR) or IR followed by DLL4 mAb 30 min later. Tumours resected 3 days later. **a,** Proliferation assessed by Ki67 immunostaining. Representative images, left panels, **scale bar,** 50µm; quantified as percentage of positive nuclei**,** right panel. **b,** Apoptosis assessed by TUNEL staining. Representative images, left panels, **scale bar.** 50µm. *Columns*, mean; *error bars,* 95% CI, **p<0.01, ***p<0.001; n=5.

TUNEL staining of tumours revealed less than 1% TUNEL positive cells in all tumours studied regardless of treatment. This result is similar to the pattern of apoptosis observed in tumours analysed at the end of the tumour growth delay experiment presented in the previous chapter (figure 3.6). An *In vitro* caspase assay performed by Dr Liu with this cell line also show a lack of apoptosis with the treatments. These results suggest that apoptosis may not be a major contributor to cell death in this model, which may predominantly occur via necrosis or mitotic catastrophe.

4.2.3. HCT-15 cell line is DLL4 negative *in vitro* and *in vivo*.

Prior studies published by our group demonstrated that DLL4 expressed on tumour cells can activate Notch in tumour stroma and alter tumour vasculature *in vivo* (Li *et al.*, 2007). To investigate the effects of DLL4 blockade on tumour vasculature and radiation treatment independent of tumour DLL4 expression, we examined another colorectal adenocarcinoma cell line HCT-15. Lysates derived form HCT-15 cells were analysed for

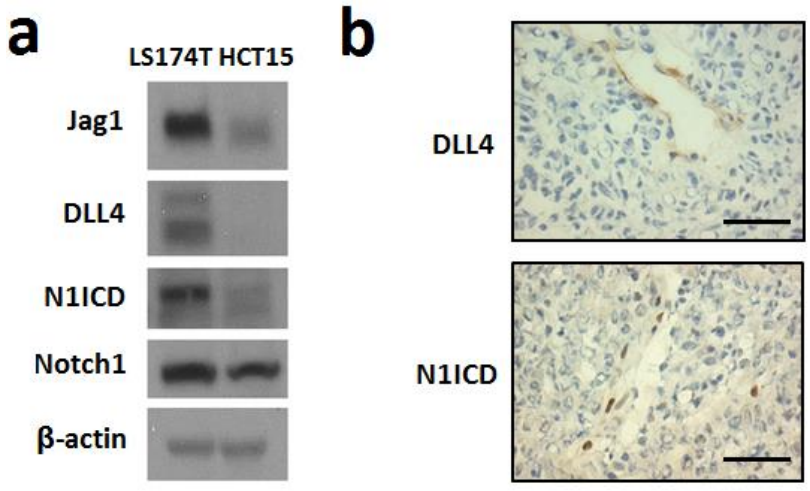


Figure 4.3. HCT-15 colorectal adenocarcinoma cells do not express DLL4. a, Western blot analysis of Jag1, DLL4, Notch1 and cleaved Notch1 intracellular domain (NICD) in LS174T and HCT-15 cells. **b,** DLL4 and cleaved Notch1 intracellular domain (N1ICD) immunostaining of tumours derived from HCT-15 cells. Representative images, **scale bar** 50μm, n=3.

Notch proteins by western blot (figure 4.3). No detectable levels of DLL4 protein were seen. However, the cells line did express intact Notch1 as well as low levels of Jag1 proteins. Low levels of cleaved Notch1 intracellular domain (N1ICD), were also observed indicating that this cell line has intact Notch signalling and can respond to Notch ligands.

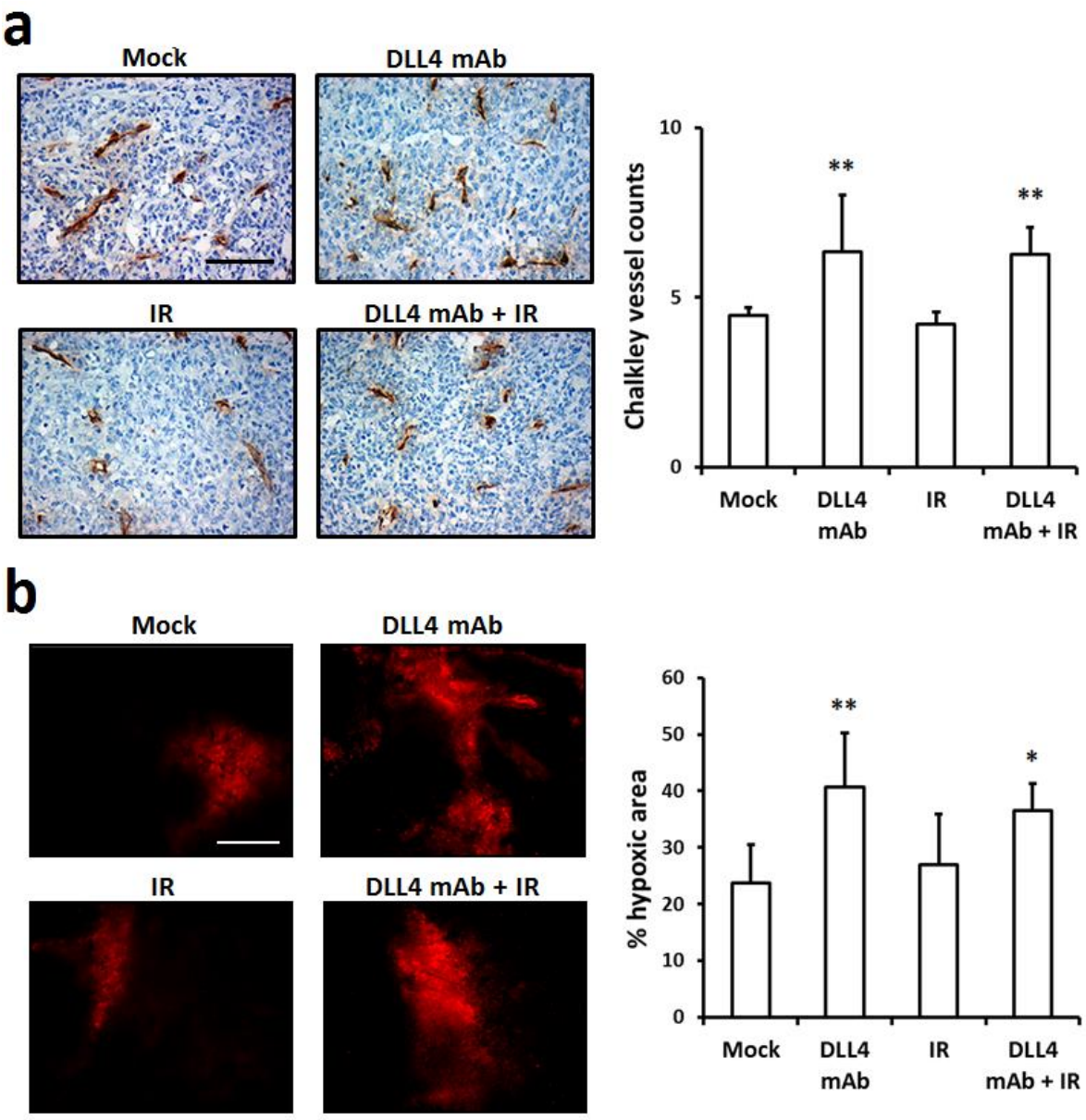
To confirm that HCT-15 cells do not express DLL4 *in vivo* and to determine growth characteristics of HCT-15 tumours for future experiments, we grew the cell line as subcutaneous tumours in athymic nude mice. Tumours immunostained for DLL4 showed no staining on HCT-15 cells (figure 4.3b). However, parts of vasculature within the tumour were observed to be DLL4 positive. Immunostaining tumours for N1ICD revealed no positive nuclei in HCT-15 cells, but once again positive cells were identified lining tumour vessels (figure 4.3b).

4.2.4. DLL4 blockade dysregulates tumour vasculature and increases hypoxia and necrosis in HCT-15 tumours.

To assess whether DLL4 on tumour cells is an essential target for anti-DLL4 therapy, we decided to evaluate the effectiveness of DLL4 blockade in tumours derived from a DLL4 negative cell line. We established subcutaneous HCT-15 tumours in athymic nude mice and treated tumours with a single dose of DLL4 mAb, 5 Gy IR or 5 Gy IR combined by DLL4 mAb. Mice were sacrificed 3 days later and tumours were excised and immunostained for CD31 or EF5 or with haematoxylin and eosin (figure 4.4a, b and c respectively). As in LS174T tumours before, HCT-15 tumours treated with DLL4 mAb alone or in combination with radiation, displayed increased microvessel density and hypoxia and higher levels of necrosis. However, combining IR with DLL4 mAb did not further alter vessel density,

hypoxia or necrosis compared to DLL4 mAb treatment alone. Additionally, in contrast to proliferation changes observed in LS174T tumour, IR did not alter proliferation in HCT-15 tumours, alone or with DLL4 mAb (figure4.4d).

Together these results demonstrate that DLL4 blockade is effective in dysregulating tumour vasculature and increasing necrosis regardless of DLL4 expression on tumour cells. Our colleague, Dr John Beech has used another DLL4 negative cell line to examine



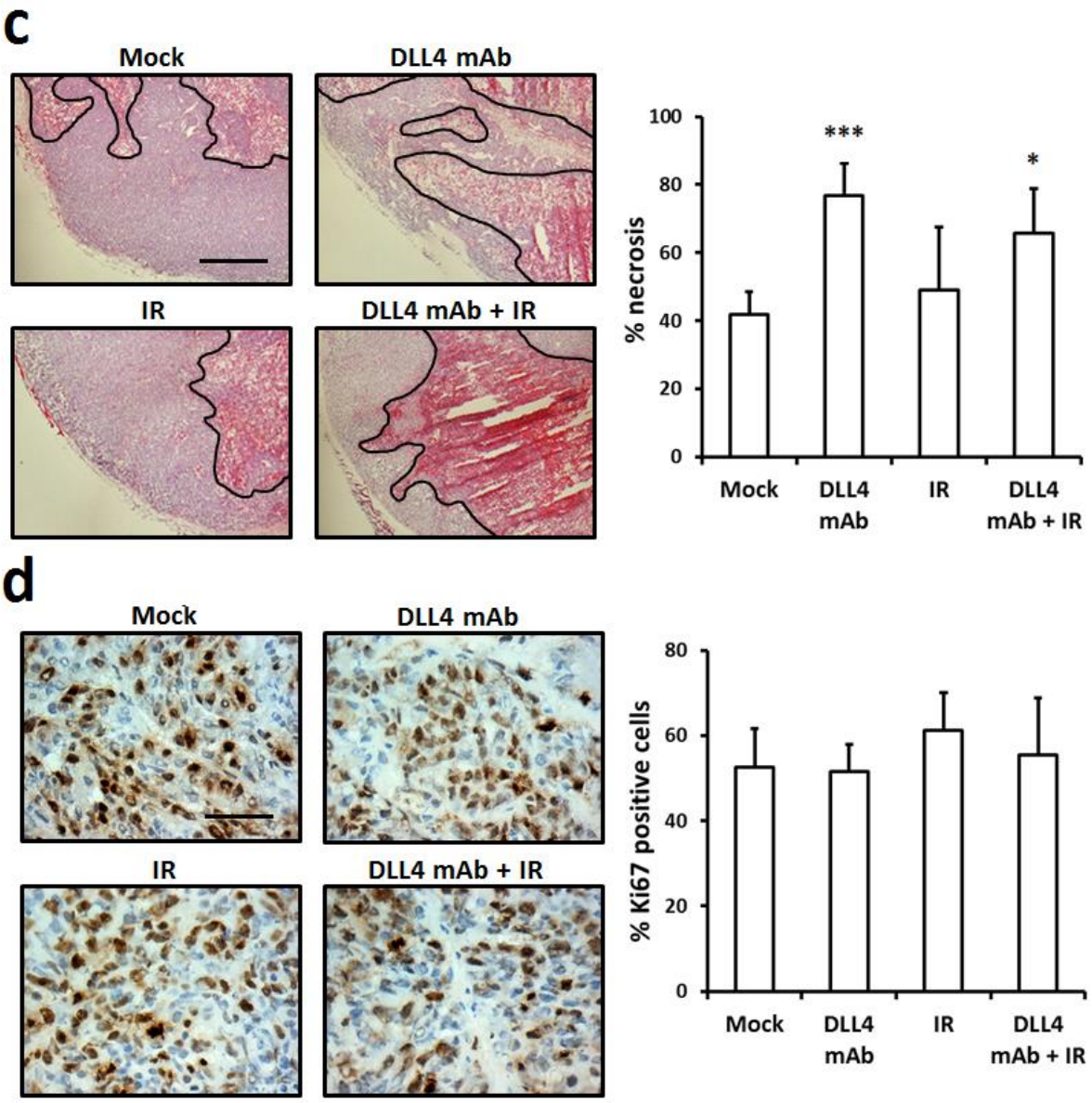


Figure 4.4. Vessel density, hypoxia, necrosis and proliferation in HCT-15 tumours following DLL4 blockade and radiation treatment. Mice bearing subcutaneous HCT-15 tumours were treated with DLL4 mAb, 5 Gy (IR) or IR followed by DLL4 mAb 30 min later. Tumours resected 3 days later. **a,** Microvessel density assessed following CD31 immunostaining. Representative images, left panels, **scale bar** 100µm; quantified by Chalkley vessel counting**,** right panel. **b,** Hypoxia assessed by EF5 immunostaining. Representative images, left panels, **scale bar** 200µm; quantified by as percentage of hypoxic area**,** right panel. **c,** Necrosis assessed following haematoxylin and eosin staining. Representative images, left panels, **scale bar** 1000µm; quantified as percentage of necrotic area**,** right panel. **d,** Proliferation assessed by Ki67 immunostaining. Representative images, left panels, **scale bar,** 50µm; quantified as percentage of positive nuclei**,** right panel. *Columns*, mean; *error bars,* 95% CI, *p<0.05, **p<0.01, ***p<0.001 vs. mock; n=5.

growth delay induced by DLL4 mAb and IR *in vivo.* In an experiment similar to the LS174T growth delay presented in the previous chapter (figure 3.4a,b), Dr Beech treated xenograft tumours derived from human hypopharyngeal squamous cell carcinoma FaDu, with 5Gy IR, DLL4 mAb or 5Gy IR followed by DLL4 mAb. Tumours in relevant treatment groups were given DLL4 mAb twice a week, and tumour sizes were monitored until tumours reached 4 times the starting volume. DLL4 mAb alone resulted in a significant delay in tumour growth, confirming that DLL4 mAb is effective regardless of apparent tumour DLL4 expression. IR alone did not significantly alter tumour growth but combining IR with DLL4 blockade resulted in a supra-additive growth delay (Liu *et al.*, 2011). Unfortunately, a similar experiment could not be repeated for HCT-15 tumours due to limited availability of the DLL4 mAb.

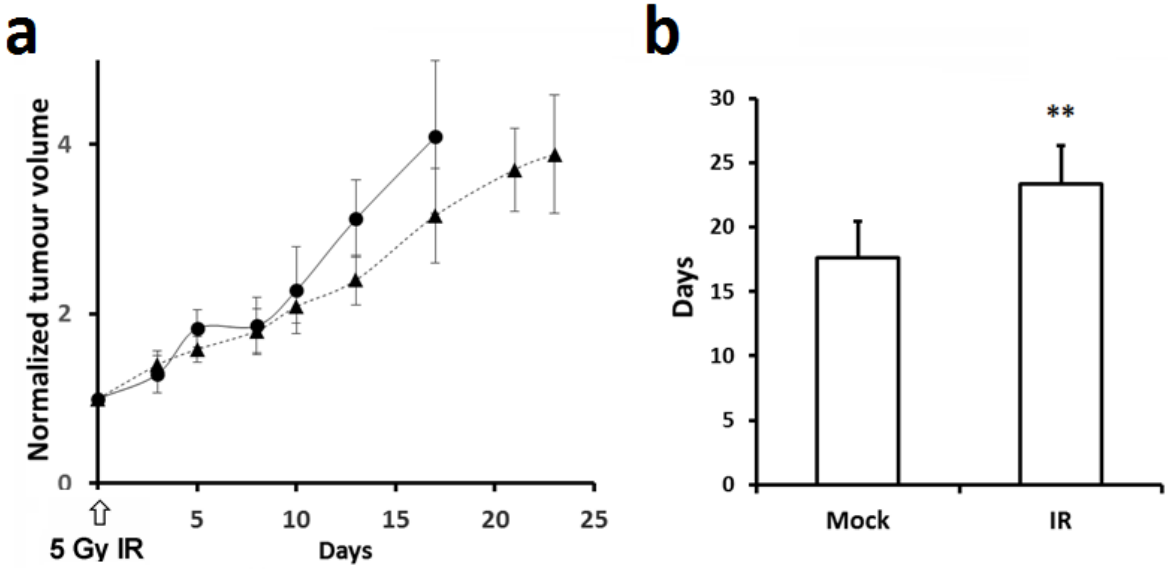


Figure 4.5. HCT-15 tumours are responsive to radiation treatment. Mice bearing subcutaneous HCT-15 tumours were randomly assigned to treatment Mock (n=5) or 5 Gy radiation (IR, n=6) treatment groups, when tumour volumes were approximately 130 mm^3. **a,** Growth curves of tumours. Tumour volumes plotted relative to starting volumes. Mice sacrificed when tumours reached four times the starting volume. **b,** Average times for tumours to reach four times the starting volume. *Columns*, mean; *error bars,* 95% CI, *p<0.05, **p<0.01 vs. mock.

4.2.5. Ionising radiation delays growth of HCT-15 xenograft tumours.

As we did not notice a difference in proliferation in HCT-15 tumours following IR and as untreated HCT-15 tumours were more hypoxic and necrotic than LS174T tumours, we decided to determine if HCT-15 tumours respond to IR. Tumours established in nude mice were treated with a single dose of 5 Gy IR and tumour sizes were monitored until they reached 4 times the starting volume (figure 4.5a). Tumours treated with IR showed a significant growth delay compared to untreated (figure 4.5b), demonstrating that 5Gy IR does affect tumour growth in this model.

4.2.6. Radiation and DLL4 blockade alter Notch signalling in LS147T tumours.

As detailed above, roles for Notch signalling have been described in tumour and stromal compartments in solid malignancies. Notch ligands DLL4 and Jag1 are important regulators of angiogenesis and changes in DLL4 or Jag1 expression in cancer cells have been reported to alter tumour vasculature. Recent reports show that radiation can alter Notch in cancer and endothelial cells and results in chapter 3 show IR induced suppression of Notch in LS174T cells and tumours (figure 3.2, 3.4). To investigate changes in Notch signalling in LS174T tumours by radiation and DLL4 blockade, we analysed tumours treated with DLL4 mAb and/or 5Gy IR and resected 3 days later. Tumours were immunostained for Jag1, DLL4 and cleaved Notch1 intracellular domain (N1ICD) (Figure 4.6). In agreement with *in vitro* and *in vivo* results described in chapter 3, a significant decrease in N1ICD was observed in tumours treated with IR as well as DLL4 mAb (figure 4.6a). None of the treatments appeared to significantly alter DLL4 levels (figure 4.6c), but

increased levels of Jag1 were observed in tumours treated with IR alone or in combination with DLL4 mAb (figure 4.6b).

RNA extracted from tumours was assayed for human and mouse *DLL4*, *Jag1*, *Hes1* and *Hey1* mRNA using species-specific primers, to detect changes in tumour (i.e. human) and stromal (i.e. mouse) compartments (figures 4.7 and 4.8). In tumour cells, DLL4 mAb

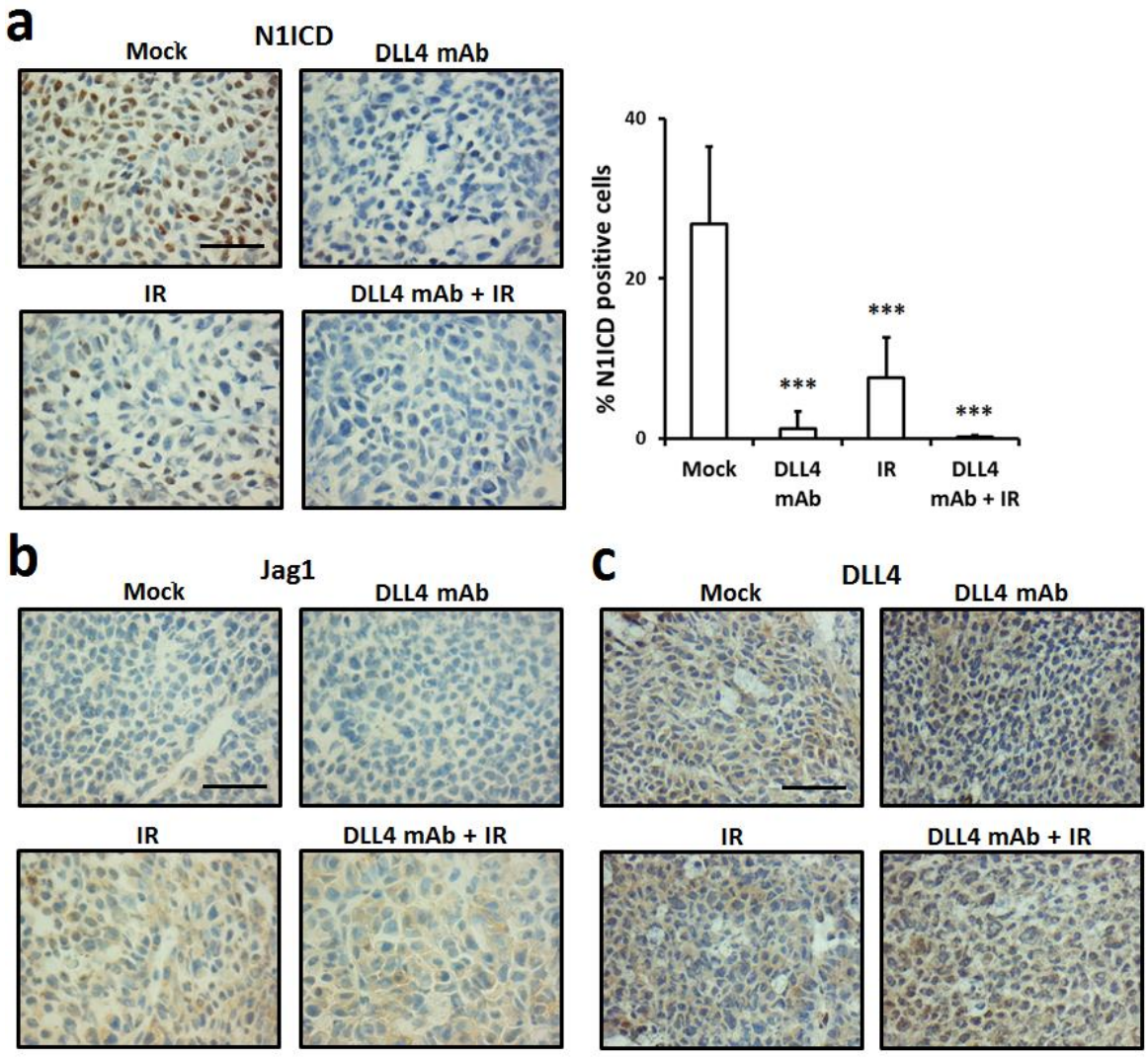


Figure 4.6. DLL4 blockade and radiation inhibit Notch but radiation upregulates Jag1 in LS174T tumours. Tumours treated with anti-DLL4 antibody (DLL4 mAb), 5 Gy radiation (IR) or DLL4 mAb and IR, and excised 3 days later for immunohistochemical analysis. **a,** Notch 1 activation assessed immunostaining for cleaved Notch1 intracellular domain (N1ICD). Representative images, left panels; quantified as percentage of positive nuclei**,** right panel. **b,** Jag1 immunostaining. **c,** DLL4 immunostaining**. Scale bars** 50µm. *Columns*, mean; *error bars,* 95% CI, ***p<0.001 vs. mock, n=5.

treatment decreased the expression of Notch target genes *hHes1* and *hHey1* as expected (figure 4.7a, b). Ionising radiation also appeared to decrease levels of *hHey1*, in agreement with previous results. Radiation treatment upregulated *hJag1* mRNA levels (figure 4.7c), which correlates with the immunostaining data above. Upregulation of *hDLL4* mRNA was observed in tumours treated with DLL4 mAb alone but combining DLL4 mAb with IR appears to inhibit this increase (figure 4.7d).

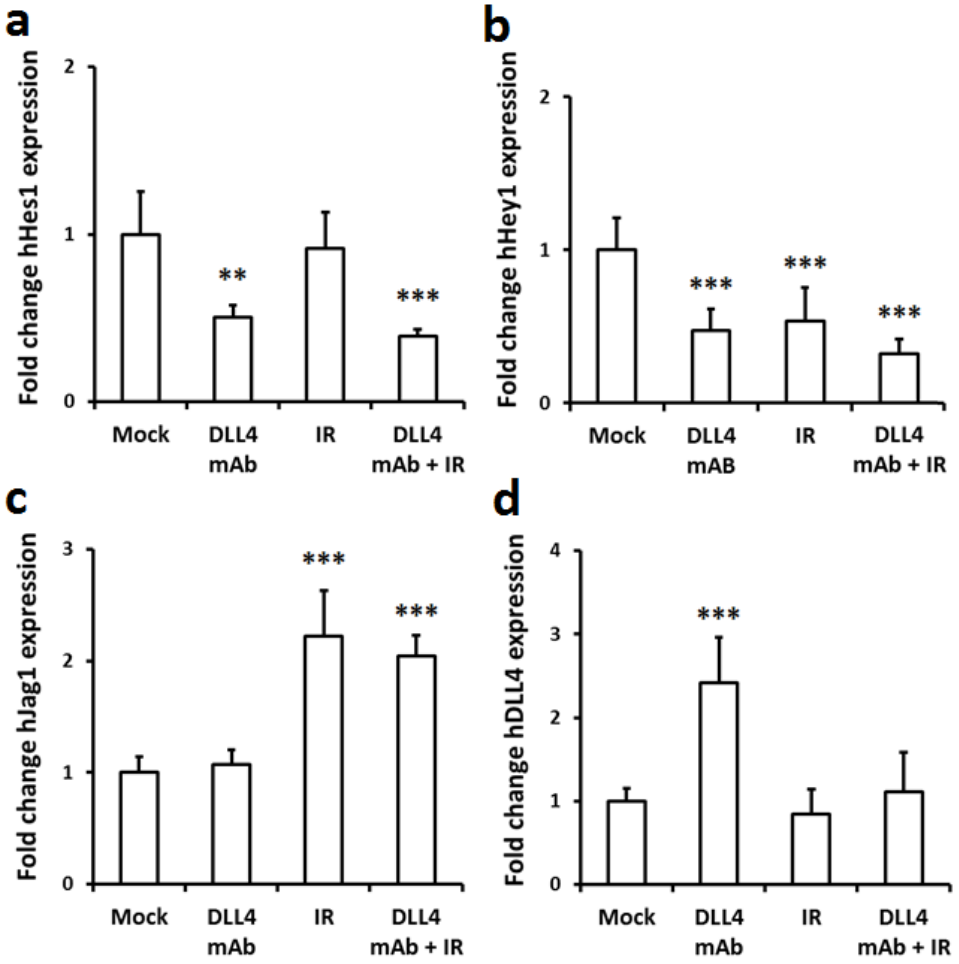


Figure 4.7. mRNA analysis of Notch genes in LS174T tumour cells *in vivo* following DLL4 blockade and radiation treatment. Tumours treated with anti-DLL4 antibody (DLL4 mAb), 5 Gy radiation (IR) or DLL4 mAb and IR, and excised 3 days later and RNA purified for gene expression analysis. Changes in **a,** Hes1; **b,** Hey1; **c,** Jag1 and **d,** DLL4 mRNA levels detected using human-specific qPCR assays. Values normalised to human β-actin expression, presented relative to Mock. *Columns*, mean; *error bars,* 95% CI, **p<0.01, ***p<0.001 vs. mock, n=5.

Assays with mouse specific primers to detect stromal mRNA (figure 4.8) indicate that DLL4 blockade alone or combined with IR, inhibited Notch signalling in the stroma, as evidenced by decreased *mHes1* and *mHey1* levels. No significant change was observed following IR treatment alone. *mJag1* levels did not change significantly with any treatment. However, IR appears to inhibit DLL4 levels in the stroma, as indicated by decreased expression in samples treated with IR alone or together with DLL4 mAb.

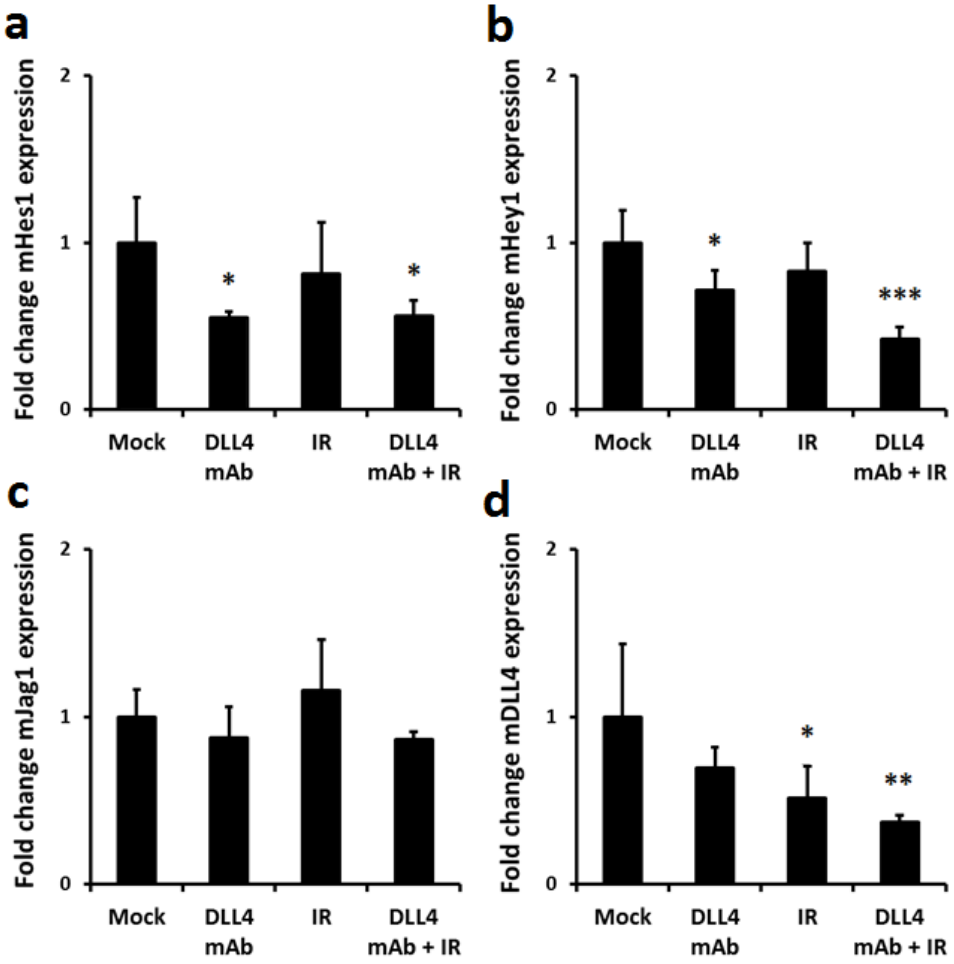


Figure 4.8. Changes in expression of Notch genes in LS174T tumour stroma following DLL4 blockade and radiation treatment. Tumours treated with anti-DLL4 antibody (DLL4 mAb), 5 Gy radiation (IR) or DLL4 mAb and IR, and excised 3 days later and RNA purified for gene expression analysis. Changes in **a,** Hes1; **b,** Hey1; **c,** Jag1 and **d,** DLL4 mRNA levels detected using mouse-specific qPCR assays. Values normalised to mouse β-actin expression, presented relative to Mock. *Columns*, mean; *error bars,* 95% CI, *p<0.05, **p<0.01, ***p<0.001 vs. mock, n=5.

Together these results show that DLL4 blockade inhibit Notch signalling in tumour and stromal cells in LS174T tumours. In tumour cells, DLL4 blockade alone also appears to upregulate DLL4 at the mRNA level but this could not be confirmed at the protein level by immunohistochemistry. IR increases Jag1 expression in LS174T tumour cells but this does appear not significantly alter Notch in the stroma.

4.2.7. Effect of radiation and DLL4 blockade on Notch signalling in HCT-15 tumours.

As tumour DLL4 can alter Notch in stromal cells (Li *et al.*, 2007), we decided to compare differences in expression pattern in a model that lacks a significant DLL4 signal from tumour cells to stroma. We analysed changes in Notch signalling in HCT-15 tumours treated with DLL4 mAb and/or 5Gy IR and excised 3 days later. As with LS174T tumours, N1ICD, Jag1, DLL4 proteins were detected in tumour cells by immunohistochemistry and RNA purified from tumours was assayed for DLL4, Jag1, Hes1 and Hey1 with human and mouse specific ssays. Immunostaining for N1ICD revealed no staining in tumour cells in any of the treatment groups (figure 4.9a). As before, structures that appeared to be vasculature, did contain some N1CD positive nuclei in all treatment groups. Positive staining for Jag1 was observed on all tumour cells but no differences between treatment groups could be observed visually. As before, tumour cells in untreated samples were negative for DLL4 (figure 4.9c) and the same was observed for all other treatments.

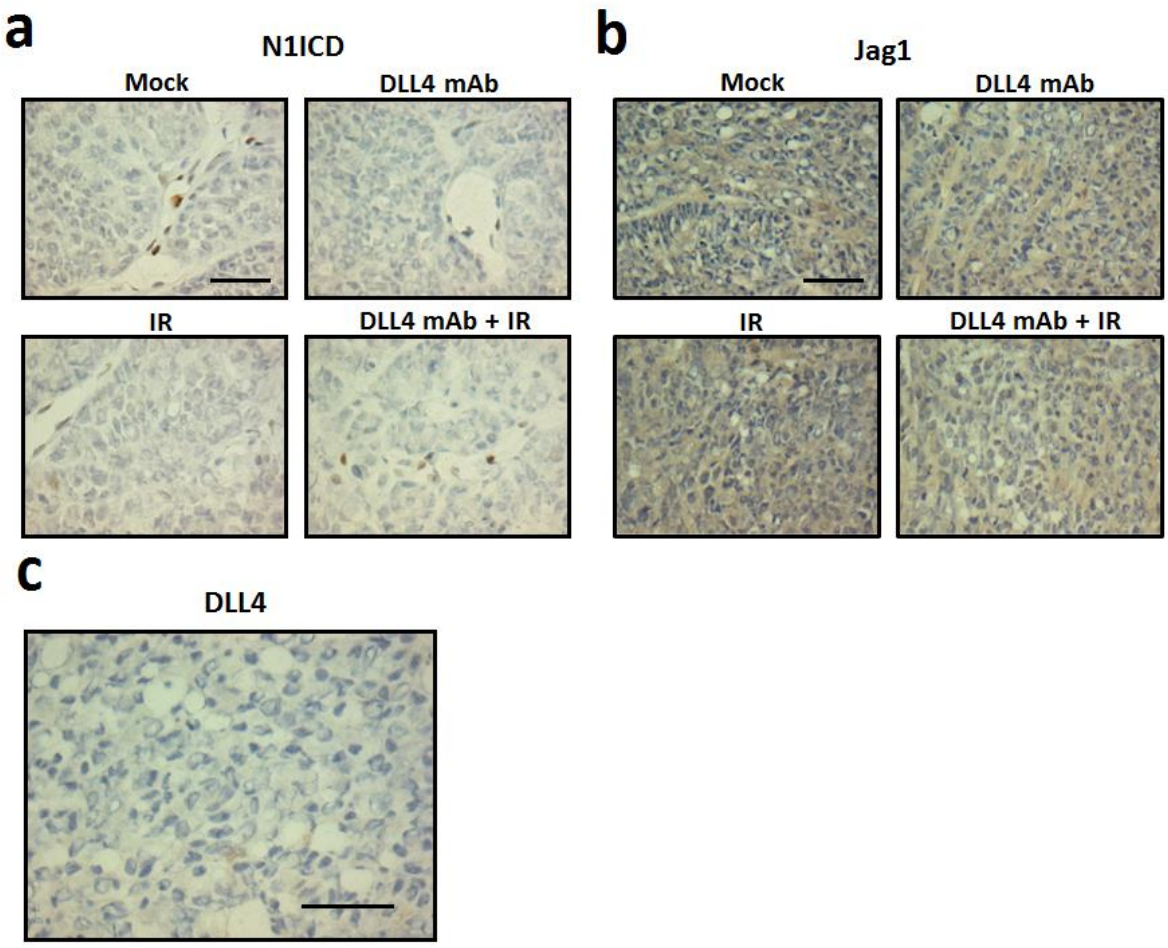


Figure 4.9. Immunostaining for Notch proteins in HCT-15 tumour after DLL4 blockade and radiation treatment. Tumours treated with anti-DLL4 antibody (DLL4 mAb), 5 Gy radiation (IR) or DLL4 mAb and IR, and excised 3 days later for immunohistochemical analysis. Immunostaining for **a,** Cleaved Notch1 intracellular domain (N1ICD); **b,** Jag1 and **c,** DLL4 (mock)**. Scale bars** 50µm.

Analysis of expression of *hHes1* and *hHey1* in tumour cells with human specific assays, showed that *hHes1* and *hHey1* could be detected in HCT-15 tumours. As N1ICD could not be detected by immunostaining, this may suggest that the cell-line may signal through other Notch receptors. It should be noted that low levels of N1ICD were detected in HCT-15 *in vitro* (figure 4.3). DLL4 blockade or IR do not significantly alter *hHes1* or *hHey1* levels. As this cell line is DLL4 negative (figure 4.3), the lack of change in Notch downstream genes indicate that stromal DLL4 may not contribute significantly to Notch activation in HCT-15 tumour cells. DLL4 mAb and IR increased hJag1 expression, but this

increase was less than that observed with IR in LS174T tumours. As mentioned above, this was not apparent in HCT-15 tumours by Jag1 immunostaining.

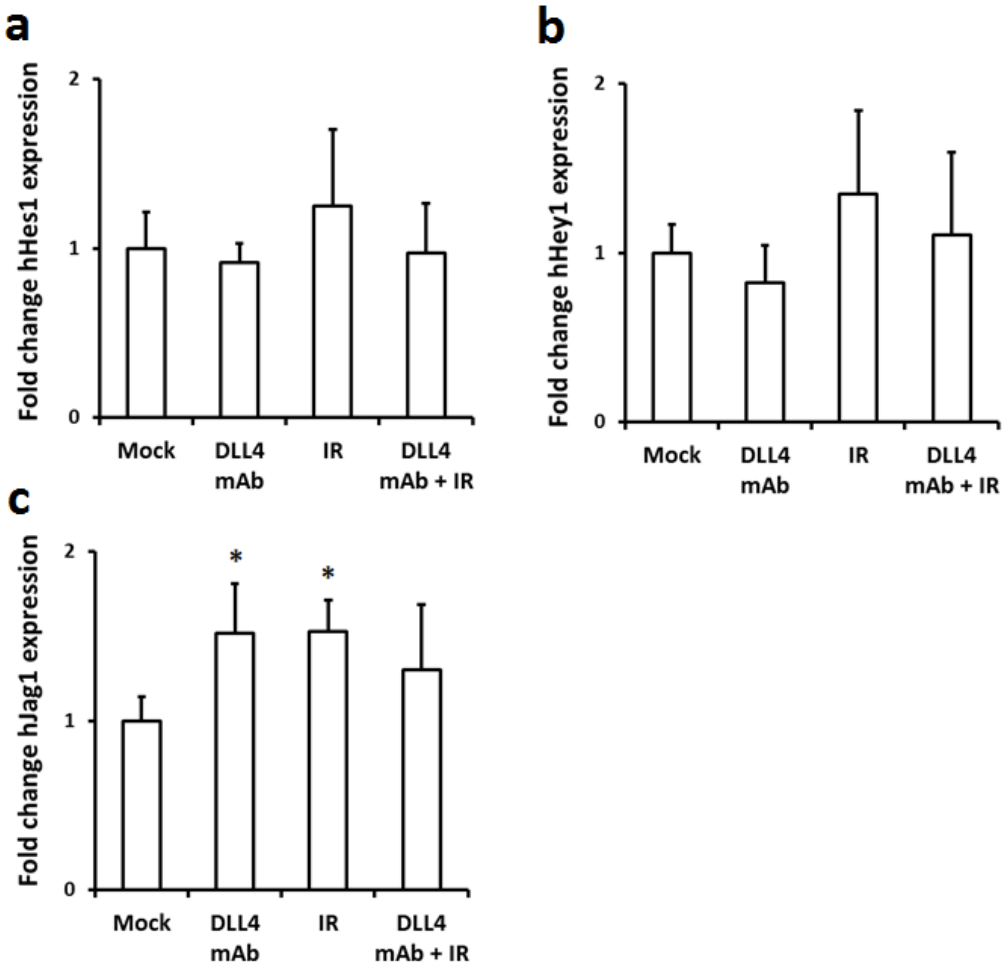


Figure 4.10. mRNA analysis of Notch genes in HCT-15 tumour cells *in vivo* following DLL4 blockade and radiation treatment. Tumours treated with anti-DLL4 antibody (DLL4 mAb), 5 Gy radiation (IR) or DLL4 mAb and IR, and excised 3 days later and RNA purified for gene expression analysis. Changes in **a,** Hes1; **b,** Hey1 and **c,** Jag1 mRNA levels detected using human-specific qPCR assays. Values normalised to human β-actin expression, presented relative to Mock. *Columns*, mean; *error bars,* 95% CI, *p<0.05 vs. mock, n=5.

In the stroma, radiation decreased Notch activation, as evidenced by decreased *mHes1* levels in IR and combination treated tumours and decreased *mHey1* in combination treated tumours. DLL4 blockade did not appear to reduce notch target levels, which may indicate that only a small subset of stromal cells express DLL4. In vasculature, DLL4 expression is largely restricted to tip cells and neighbouring stalk cells and almost entirely

absent in larger or more mature vessels. Reduction in *mDLL4* was seen in tumours given combination therapy. Of note IR decreased *mDLL4* levels in LS174T tumours, alone and with DLL4 mAb.

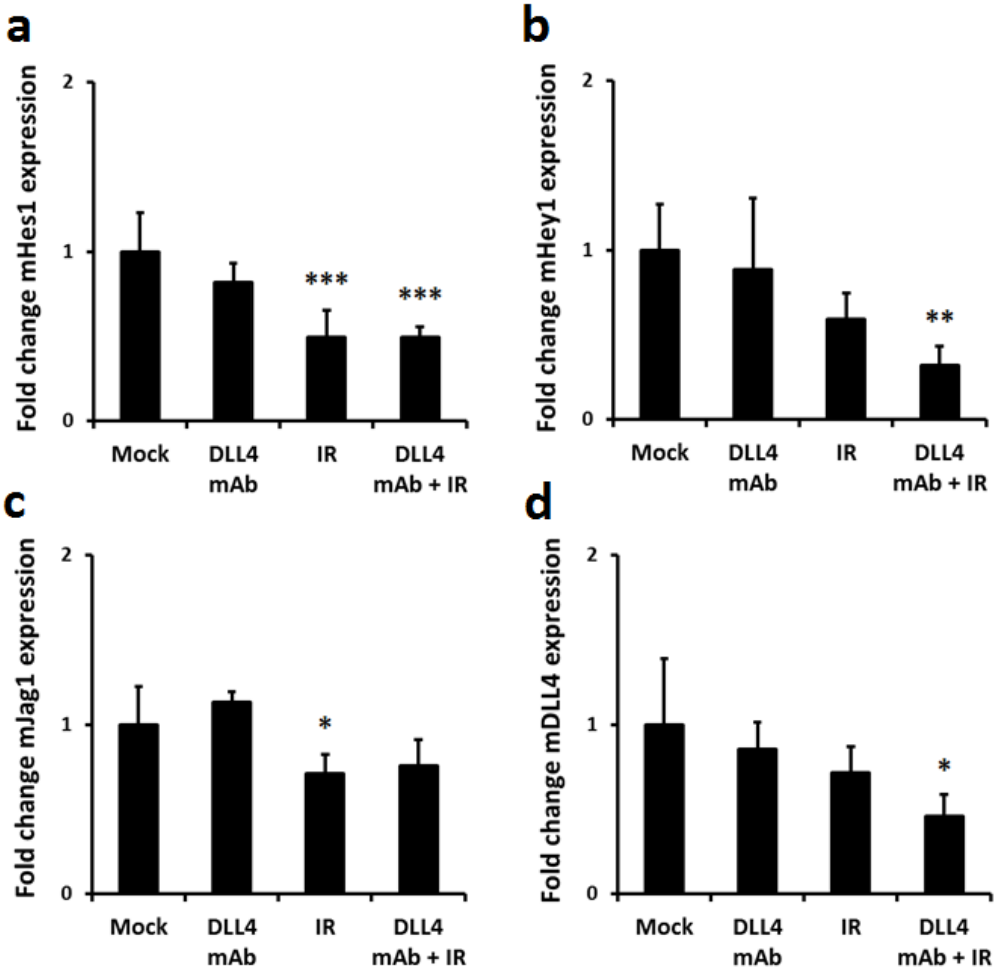


Figure 4.11. Changes in expression of Notch genes in HCT-15 tumour stroma following DLL4 blockade and radiation treatment. Tumours treated with anti-DLL4 antibody (DLL4 mAb), 5 Gy radiation (IR) or DLL4 mAb and IR, and excised 3 days later and RNA purified for gene expression analysis. Changes in **a,** Hes1; **b,** Hey1; **c,** Jag1 and **d,** DLL4 mRNA levels detected using mouse-specific qPCR assays. Values normalised to mouse β-actin expression, presented relative to Mock. *Columns*, mean; *error bars,* 95% CI, *p<0.05, **p<0.01, ***p<0.001 vs. mock, n=5.

4.3. Discussion

In the present study, we explored the interaction of radiation treatment and anti-DLL4 therapy in DLL4 positive and negative tumour xenografts. Here, we observe anti-DLL4 therapy was effective in dysregulating tumour vasculature within 3 days of treatment as evidenced by increased microvessel density and concurrent increases in hypoxia and necrosis in both cell lines. Surprisingly, the effect was independent of tumour DLL4 expression. In the LS174T tumour model, our colleague Dr Fokas has observed reduction in blood flow within 48 hours of anti-DLL4 therapy. Combined treatment of FaDu tumours, another DLL4-negative cell line, with DLL4 blocking antibodies and radiation also resulted in a supra-additive growth delay compared to either treatments alone (Liu *et al.*, 2011). Collectively, these results suggest that the tumour stroma/vasculature provides the main therapeutic target for anti-DLL4 therapy. In tumours, where angiogenesis is permanently switched on, blocking vascular DLL4 further stimulates angiogenesis, promoting vessel abnormalisation (an opposite effect to VEGF blockade) leading to hypoxia and tumour necrosis.

Ionising radiation has been reported to alter vasculature *in vivo*. However the effects appear to be dose dependent, with lower doses promoting and higher doses inhibiting angiogenesis (as reviewed in chapter 1 and Kleibeuker *et al*.). Here, we do not observe any significant changes in vessel density or hypoxia with a clinically relevant (5 Gy) dose of radiation in either tumour model. In line with these findings, Dr Fokas did not see changes in blood flow, 2 or 5 days after IR in LS174T tumours (Liu *et al.*, 2011). The addition of IR does not appear to affect vascular response to DLL4 blockade either. Geng *et al.* have reported that a 6 Gy IR dose altered tumour vasculature in a B16F0 melanoma tumour model but not in a GL261 glioma model. They also measured changes in blood flow via

ultrasound but the signal appeared to be coming from the vascularised tumour rim mainly. They note a decrease in blood flow 3 days after 6 Gy IR, but flow started to increase again after 7 days (Geng *et al.*, 2001). Earlier studies investigating the vascular response to radiation, show that a single 10 Gy dose increased tumour blood flow 3 to 4 days after radiation (Kallman *et al.*, 1972). These results indicate that radioresponse of tumour vasculature may not only be dose specific but may be influenced by tumour type and may vary between cell lines.

In the previous chapter, we did note a modest decrease in proliferation with prolonged anti-DLL4 treatment. Here, we observe a decrease in proliferation with radiation in LS174T tumours, and a further inhibition when anti-DLL4 therapy is combined with IR. Of note, Dr Stanley Liu observed an increase in DLL4 in LS174T cells incubated with anti-DLL4 antibody or DBZ *in vitro*. This may suggest a feedback loop where the cell line tries to compensate for loss in Notch activity by upregulating the ligand. Combining DLL4 blockade and IR may delay LS174T tumour growth by targeting proliferation as well as promoting non-productive angiogenesis. In contrast to this, radiation treatment or DLL4 blockade does not alter proliferation in HCT-15 tumours.

As expected, anti-DLL4 therapy reduced Notch1 activation and decreased Notch downstream gene expression (*hHes1, hHey1*) in LS1714T tumour cells *in vivo*. Anti-DLL4 therapy also decreased *mHes1* and *mHey1* levels in the stroma, suggesting that tumour DLL4 contributes to Notch signalling in the stromal compartment. In line with this, anti-DLL4 treatment did not alter tumour or stromal *Hes1* and *Hey1* levels in DLL4-negative HCT-15 tumours. Stromal DLL4 expression may be confined to a small subset of cells such as endothelial tip cells, and effects of blocking this may not be detectable by methods employed here.

In agreement with suppression of Notch by radiation detailed in the previous chapter, radiation decreased Notch1 activation as well as *Hey1* mRNA levels in LS1714T tumour cells. Radiation decreased expression of stromal Notch downstream genes in HCT-15 tumours but not in LS174T tumours, suggesting that the presence of tumour DLL4 may stimulate Notch in the stroma and mask this effect. We also detected upregulation of Jag1 protein as well as mRNA in LS174T tumours cells and Jag1 mRNA in HCT-15 cells, but these increases did not translate to increased Notch activation. Upregulation of Jag1 by radiation (5x 3 Gy) has also been reported in MCF-7 breast cancer cells (Phillips *et al.*, 2006). Increased Jag1 expression in tumours has been reported to promote angiogenesis increase tumour growth in tumours (Zeng *et al.*, 2005). Conversely, decreased stromal DLL4 expression was noted in LS174T tumours given IR alone and in combination with DLL4 mAb, as well as in HCT-15 tumours given combination therapy. Collectively these findings show that radiation alters Notch signalling in tumour and stromal cells, where it upregulated Jag1 in tumour cells, and may play a role in inhibiting Notch and downregulating DLL4 in the stroma. Blocking tumour derived DLL4 decreases Notch in the stroma, but in the absence of tumour DLL4 expression, no effects of DLL4 blockade could be detected in the stroma. However, the precise effects of treatments on the endothelium remain to be dissected.

Immune cell infiltrates are an important part of the tumour stroma, and tumour associated macrophages (TAMs) produce proangiogenic growth factors and promote angiogenesis in tumours (Coffelt *et al.*, 2009). It has been observed that high doses of radiation (such as 15, 20 Gy) promotes influx of bone marrow derived $CD11b^{+}$ cells into tumours where these may promote vessel development and tumour growth (Ahn and Brown, 2008, Ahn *et al.*, 2010, Kioi *et al.*, 2010). Blocking recruitment of these cellular

infiltrates has been shown to impair tumour growth following IR (Ahn *et al.*, 2010, Kioi *et al.*, 2010). DLL4 expression has been observed to promote inflammatory genes in macrophages (Fung *et al.*, 2007) and has been implicated in mononuclear cell recruitment in virus-induced demyelinating disease (Takeichi *et al.*, 2010). It is not known if a lower radiation dose used in this study (5 Gy) would cause recruitment of immune cell infiltrates into the tumour and whether DLL4 blockade may alter this.

In conclusion, these results demonstrate that anti-DLL4 therapy is effective irrespective of tumour DLL4 expression and thus may delay tumour growth by primarily targeting the stroma/vasculature. Radiation treatment can alter Notch signalling in the tumour cells and stroma, potentially affecting tumour vasculature and immune cell infiltrates.

Chapter 5

Effects of DLL4 blockade and IR on endothelial cells

5. Effects of DLL4 blockade and IR on the endothelial cells

5.1. Introduction

Angiogenesis is a tightly regulated process and is dependent on the ability of endothelial cells to migrate, proliferate and differentiate into tubules. Old and recent studies show that radiation can impair many of these functional aspects. It has been observed that radiation may alter migration in endothelial cells (Scharpfenecker *et al.*, 2009, Imaizumi *et al.*, 2010), and that this may in part, be due to alterations in Notch signalling (Scharpfenecker *et al.*, 2009). Other studies have reported decreased endothelial cell proliferation following radiation (Donker *et al.*, 2007). Radiation has also been reported to alter endothelial sprouting and induce senescence, and may affect proliferating and quiescent endothelial cells differently (Imaizumi *et al.*, 2010). As anti-DLL4 therapy affects tumour growth by altering angiogenesis in tumours, alterations in endothelial function may potentially affect the outcome of this therapy, particularly with multiple radiation doses such as in a fractionated radiotherapy schedule. Hence it is important to examine the response of irradiated endothelium to DLL4 therapy.

Recent reports have demonstrated that a high dose of radiation (20 Gy) inhibited local angiogenesis in tumours but promoted influx of bone marrow derived cells, composed mainly of $CD11b^+$ cells (Ahn and Brown, 2008, Ahn *et al.*, 2010). These cellular infiltrates increased vessel development and growth of tumours, established on a pre-irradiated site (Ahn and Brown, 2008). Blocking the recruitment of these cellular infiltrates with anti-CD11b antibodies resulted in enhanced tumour radiation response (Ahn *et al.*, 2010). DLL4-Notch signalling has been reported to induce inflammatory pathways in macrophages and DLL4 expression has been observed to co-localise with macrophages in

human atherosclerotic plaques (Fung *et al.*, 2007). Anti-DLL4 antibodies have also been observed to inhibit infiltration of mononuclear cells in spinal cords in virus-induced demyelinating disease (Takeichi *et al.*, 2010). It is not known whether lower doses of radiation elicits a similar influx of immune cells into the tumour and if anti-DLL4 therapy targets this potentially proangiogenic component of the tumour stroma.

We examined the effects of a moderate and clinically relevant dose (5 Gy) of radiation on endothelial cells and the response of irradiated cells to DLL4 therapy. We examined if this dose alters endothelial expression of Notch ligands, DLL4 and Jag1, which are important regulators of angiogenesis. We also explored whether this dose of radiation induces recruitment of proangiogenic immune cell infiltrates into the tumour and if anti-DLL4 therapy can affect this influx.

5.2. Results

5.2.1. DLL4 blockade and IR affect HUVEC migration.

Radiation has been reported to alter the ability of endothelial cells to migrate (Scharpfenecker *et al.*, 2009, Imaizumi *et al.*, 2010). To investigate the effects of DLL4 blockade on irradiated and unirradiated endothelium, cell migration was analysed with a

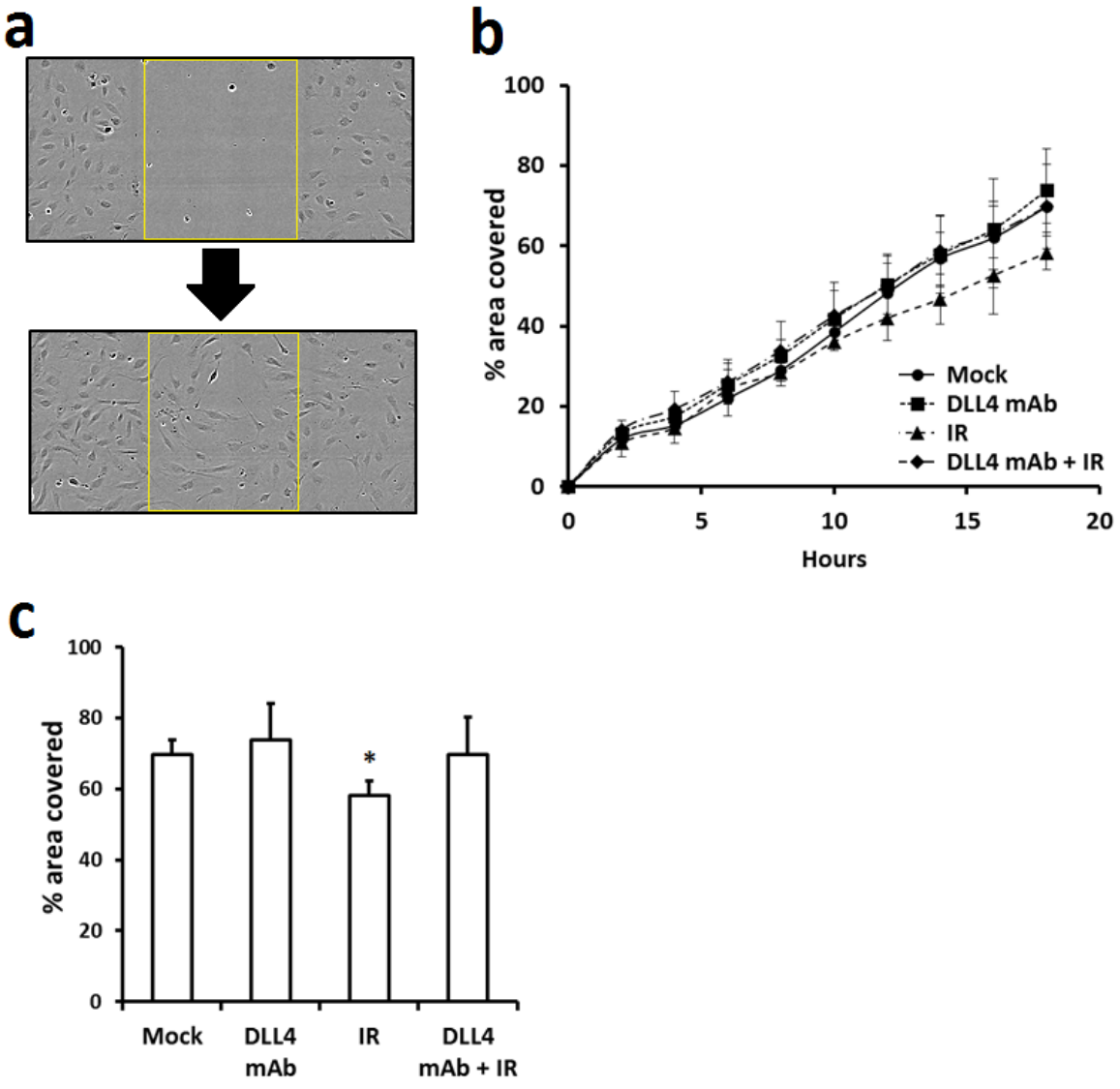


Figure 5.1. Endothelial cell migration following DLL4 blockade and radiation treatment. a, Confluent HUVEC monolayers were treated with DLL4 mAb, 5 Gy (IR) or DLL4 mAb and IR and scratched. Cell migration into the scratched area was quantified by measuring the scratched area covered by migrating cells over time. **b,** Scratched area covered over time. **c,** Percentage of cell coverage over scratched area at the end of the experiment. *Columns*, mean; *error bars,* 95% CI; *p<0.05.

scratch assay. Cells were treated with 5 Gy IR and/or DLL4 mAb and the migration of cells into a scratched area was quantified over time (figure 5.1a). Radiation treatment alone appeared to cause a decrease in cell migration but this effect was not seen when DLL4 mAb was combined with IR (figure 5.1b, c).

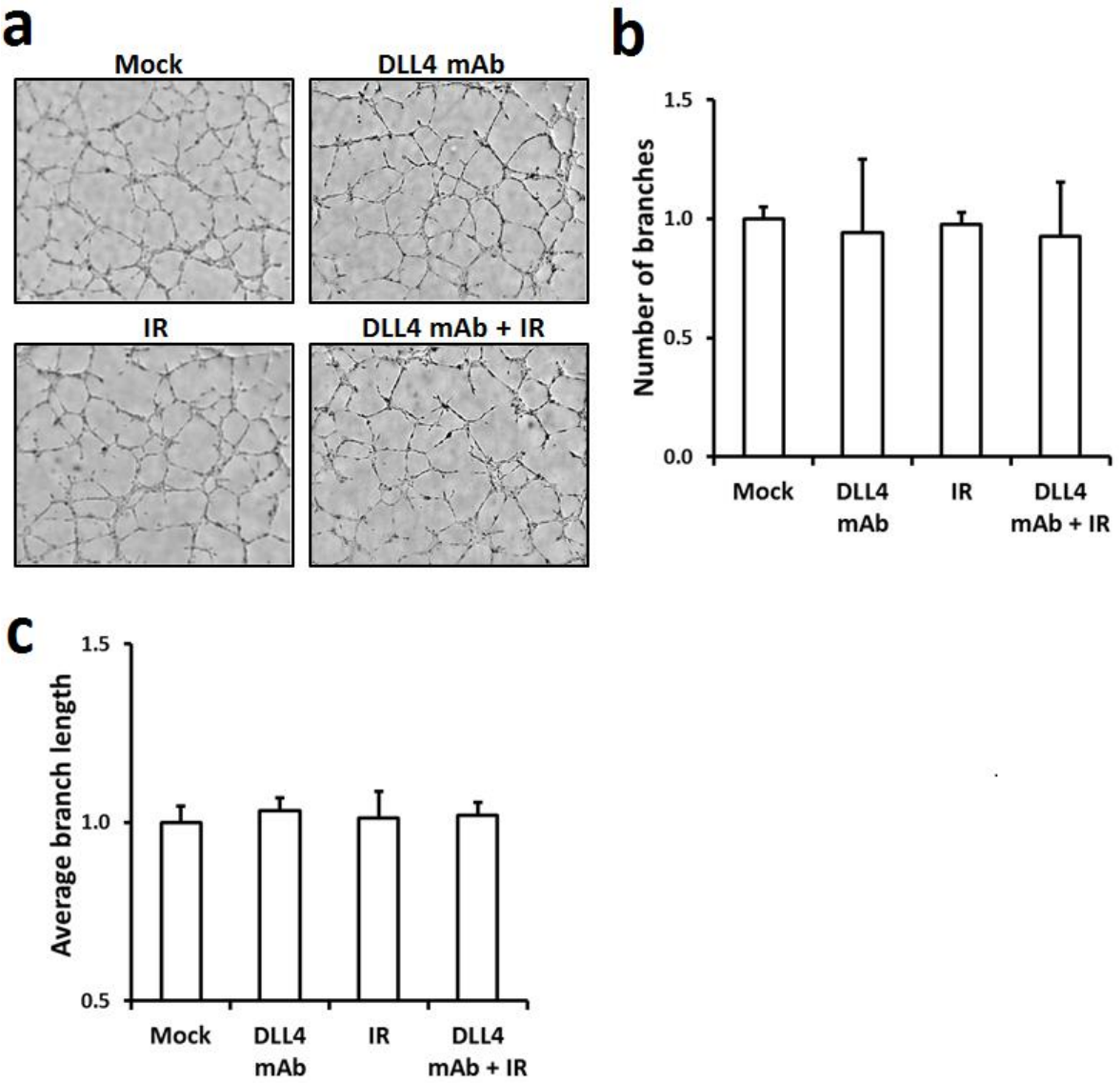


Figure 5.2. Endothelial network formation following DLL4 blockade and radiation treatment. HUVECs were trypsinised and introduced into wells contain Matrigel supplemented with EGM-2 media. Cells were treated with DLL4 mAb, 5 Gy (IR) or DLL4 mAb and IR and allowed to form networks. **a,** Representative images of endothelial networks, **b,** Number of branches and **c,** average branch length in endothelial networks quantified with ImageJ software. *Columns*, mean; *error bars,* 95% CI.

5.2.2. Effects of DLL4 blockade and IR on network formation in HUVECs.

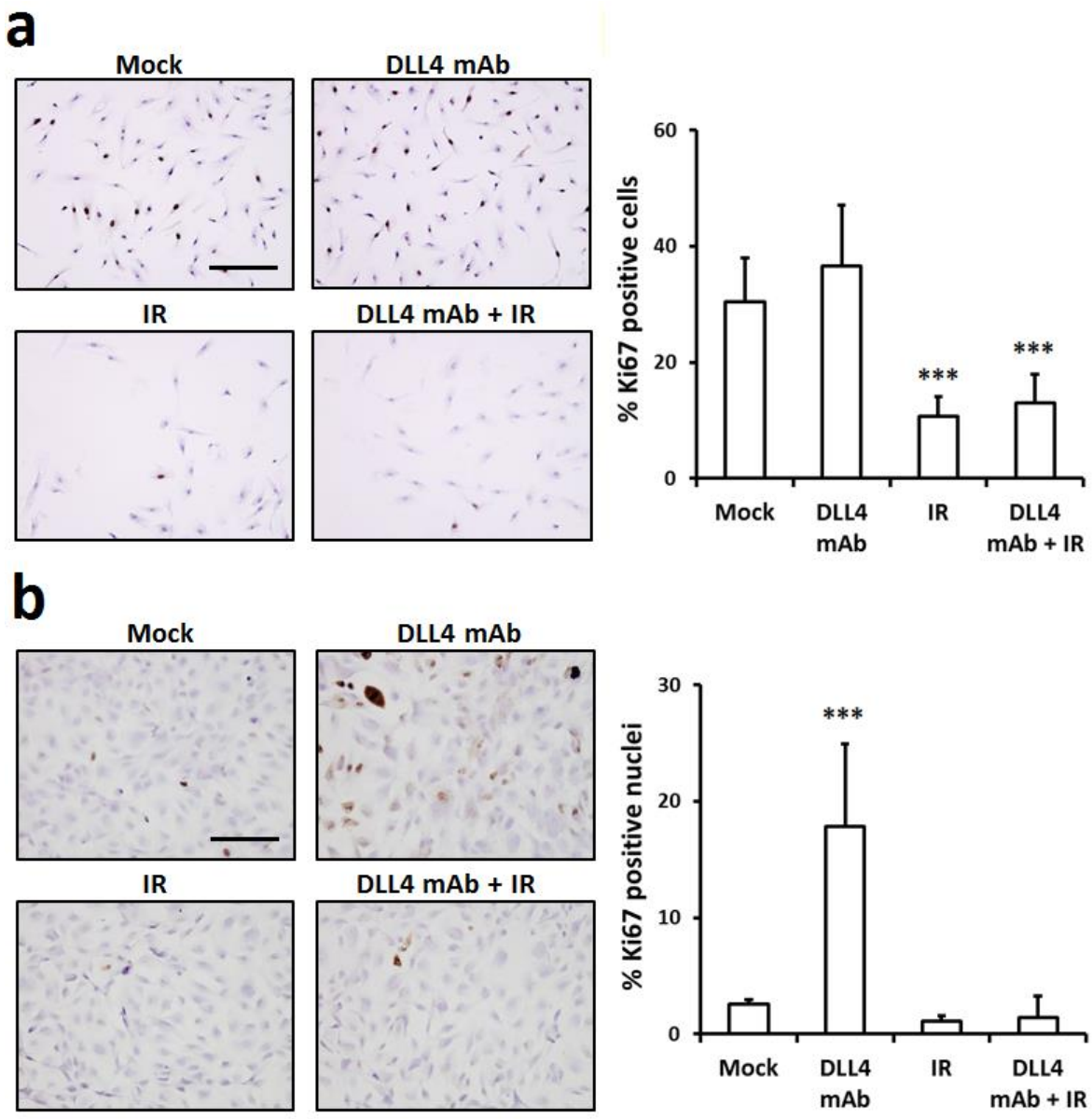
The capacity of endothelial cells to form networks is based on guidance pathways, migration, and differentiation into tubule-like structures. To determine if DLL4 blockade and IR altered the formation of vascular networks by endothelial cells, we assessed the formation of networks by HUVEC following 5Gy IR and/or DLL4 mAb (figure 5.2). Quantitative measurements of branch lengths and numbers show that none of the treatments affected network formation compared to mock (figure 5.2). Others have observed restoration of network formation following anti-DLL4 treatment but under growth factor deficient conditions (Scehnet *et al.*, 2007). We performed our assays in media supplemented with growth factor to more closely mimic conditions within the tumour, where angiogenesis is constantly activated due to growth factor secretion by tumour cells (Carmeliet and Jain, 2011).

5.2.3. DLL4 blockade and IR alter proliferation in HUVECs.

To investigate the effect of combining DLL4 blockade and IR on proliferation in endothelial cells, we treated confluent and non-confluent monolayers of HUVECs with DLL4 mAb and/or 5 Gy IR, and immunostained cells for Ki67 24 hours later (figures 5.3a, b). In actively growing, non-confluent cultures, IR reduced proliferation alone and with DLL4 mAb, However, cells treated with DLL4 mAb alone did not show any significant differences compared to mock. Untreated confluent cells displayed reduced levels of Ki67 positive cells compared to non-confluent cultures and this was not further reduced following IR treatment. Blocking DLL4 in unirradiated cells resulted in a significant

increase in Ki67 positive cells, but this increase was completely inhibited in cells that were irradiated.

We also assessed changes in cell numbers in sub-confluent *in vitro* HUVEC cultures, 3 days after treatment with DLL4 mAb and/or 5 Gy IR. In line with the above results, IR reduced cell numbers compared to mock, when given alone or in combination with DLL4 mAb, while DLL4 mAb treatment did not alter cell numbers. Together these results suggest that at the clinically relevant 5 Gy dose used in this study, IR inhibits proliferation in



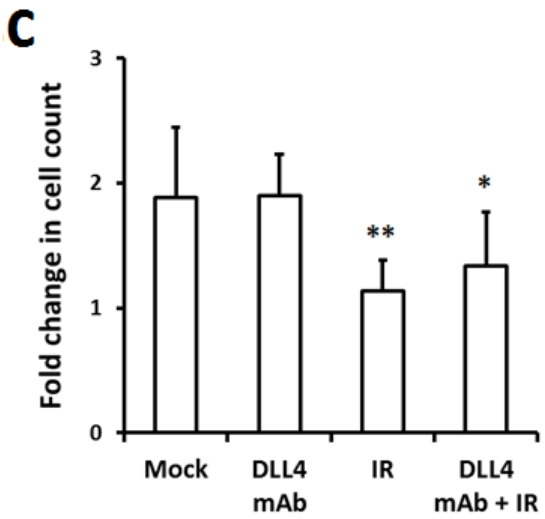


Figure 5.3. Effects of DLL4 blockade and radiation on proliferation and growth of endothelial cells. HUVECs were treated with DLL4 mAb, 5 Gy (IR) or DLL4 mAb and IR. Proliferation in **a,** sub-confluent and **b,** confluent HUVEC cultures, 24 hours after treatment analysed by Ki67 immunostaining. Representative images, left panels**, Scale bar**, 100µm; quantified as percentage of positive nuclei, right panels. **c,** HUVEC cell counts determined 3 days after treatment. Cells counts presented relative to cells plated. *Columns*, mean; *error bars,* 95% CI, *p<0.05, **p<0.01, ***p<0.01 vs. mock.

endothelial cells. Blocking DLL4 may induce proliferation in contact inhibited quiescent endothelial cells but does not rescue proliferation inhibition caused by IR.

5.2.4. Effects of DLL4 blockade and radiation on vascular Jag1 and DLL4.

As Notch ligands, DLL4 and Jag1 are important regulators of angiogenesis, we chose to investigate the impact on the treatments on these ligands in the tumour endothelium. LS174T tumours treated with DLL4 mAb and/or 5Gy IR and excised 3 days later, were immunofluorescently co-stained for CD31 and DLL4 or CD31 and Jag1 (figures 5.4a and b respectively). Analysis of co-localisation with CD31 revealed no significant differences in DLL4 or Jag1 expression in tumour vasculature between treatment groups. Of note, a high expression of DLL4 was observed in parts of vessels, as compared to DLL4 staining observed on LS174T. DLL4 is upregulated in endothelial tip cells but has also been

observed in tip and stalk cells in the vascular front in mouse retinas, and therefore lacks specificity as a tip cell marker (Hellstrom *et al.*, 2007, Lobov *et al.*, 2007).

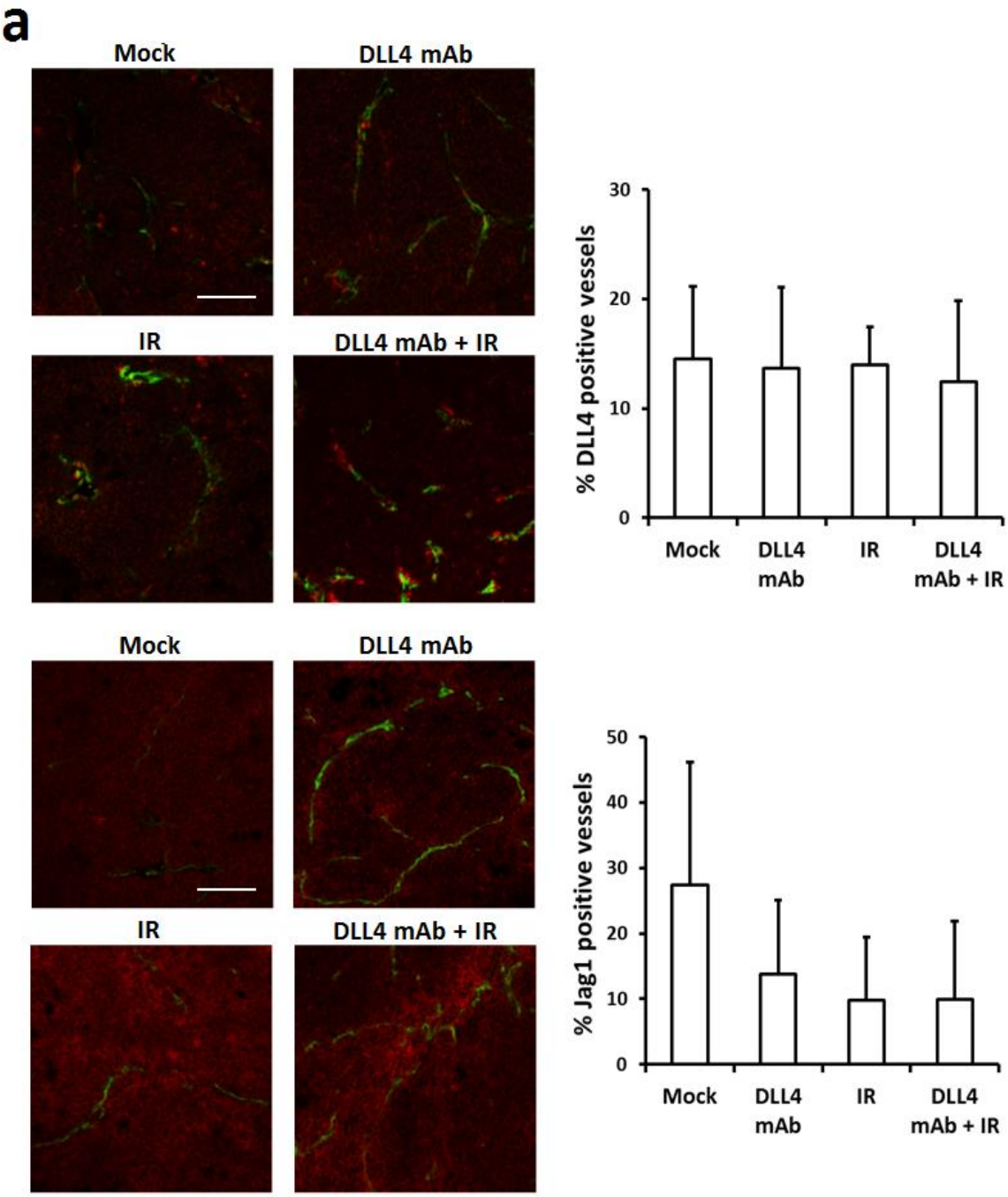


Figure 5.4. Vascular Jag1 and DLL4 expression in LS174T tumours following anti-DLL4 therapy and radiation treatment. Mice bearing subcutaneous LS174T tumours were treated with DLL4 mAb, 5 Gy (IR) or IR followed by DLL4 mAb 30 min later. Tumours resected 3 days later. **a,** DLL4 and **b,** Jag1 expression on tumour vessels assessed following co-immunostaining with CD31. Representative images, left panels; Jag1,DLL4 **red**, CD31 **green**, **scale bar** 100µm. Co-localisation quantified as a percentage of total CD31 positive area**,** right panels. *Columns*, mean; *error bars,* 95% CI; n=5.

5.2.5. DLL4 blockade does not affect radiation-induced influx of immune cells.

As mentioned in chapter 1, $CD11b^+$ immune cell infiltrates and specifically macrophages promote angiogenesis by secreting proangiogenic factors and may promote vasculature development in tumours. Additional, radiation treatment has been shown to stimulate the influx of these cells into the tumour. We explored whether DLL4 blockade would alter the numbers of $CD11b^+$ cells and macrophages in our tumour model alone or following radiation treatment. We immunostained LS174T tumours excised 3 days after treatment, for CD11b and the murine macrophage marker F4/80. Treatment with 5 Gy IR resulted in increased numbers of $CD11b^+$ cells and macrophages in the tumours compared to mock. Blocking DLL4, however, did not affect $CD11b^+$ cell or macrophage numbers compared to mock, or alter IR induced immune cell recruitment.

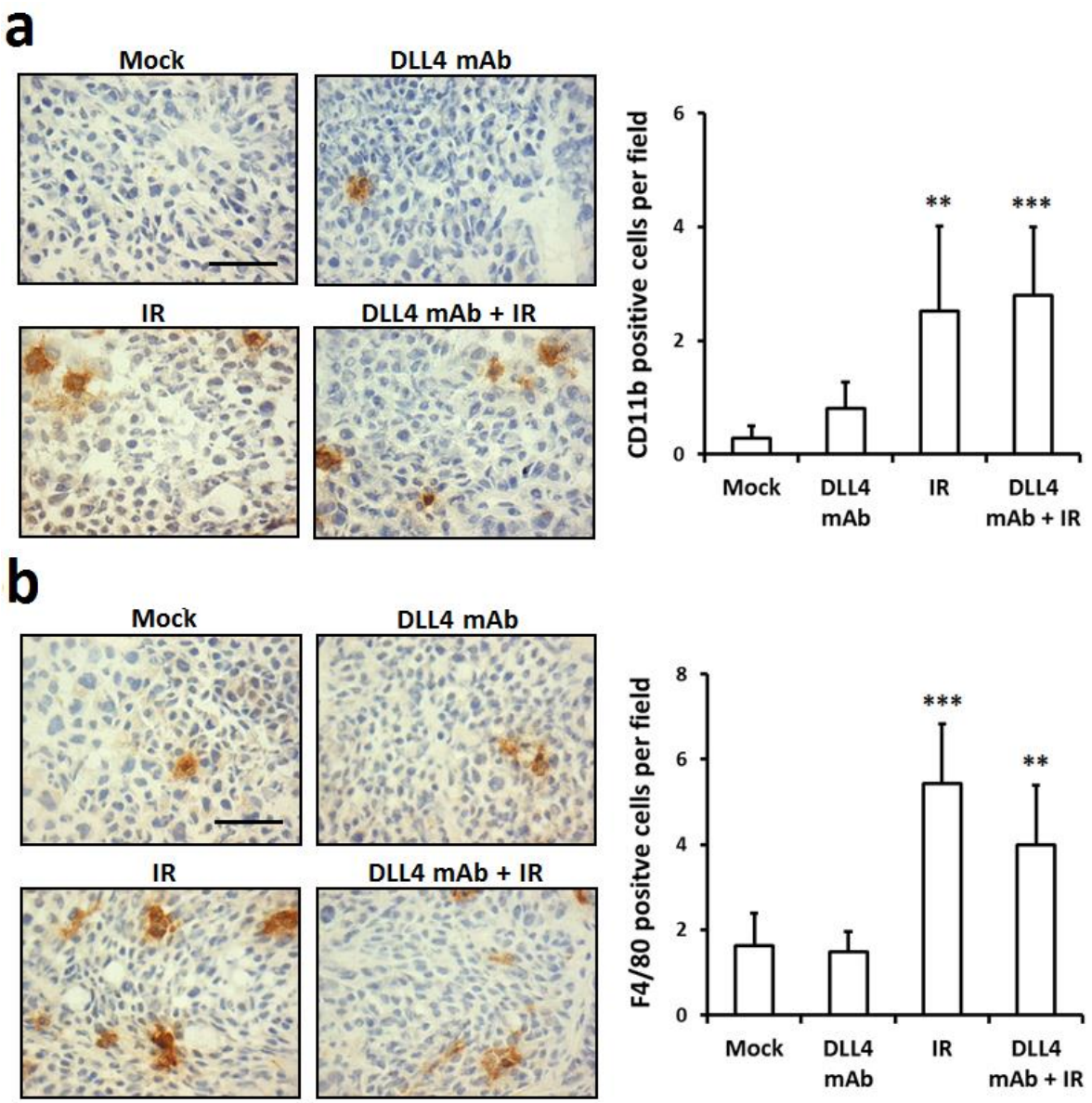


Figure 5.5. Immune cell infiltrates in LS174T tumours following DLL4 blockade and radiation treatment. Mice bearing subcutaneous LS174T tumours were treated with DLL4 mAb, 5 Gy (IR) or IR followed by DLL4 mAb 30 min later. Tumours resected 3 days later. **a,** $CD11b^+$ cells and **b,** macrophages visualised by immunostaining for CD11b and F4/80 antigens respectively. Representative images, left panels; **scale bar** 50μm. Positive cells per field of view counted**,** right panels. *Columns*, mean; *error bars,* 95% CI; **p<0.01, ***p<0.01 vs. mock; n=5.

5.3. Discussion

Tumour endothelium plays an important role in tumour development and response to radio and chemotherapy. There is accumulating evidence that endothelial cells are responsive to ionising radiation, which can directly affect endothelial function (as reviewed in chapter 1). Radiation can also alter gene expression profiles in endothelial cells, which may have implications for surrounding tumour cells. Here we investigate the effects of a moderate dose of radiation (5 Gy) on endothelial cells *in vitro*, in combination with anti-DLL4 therapy. Endothelial cell migration is a critical step during sprouting angiogenesis (Munoz-Chapuli *et al.*, 2004). Here we measured a decrease in HUVEC migration after radiation treatment, but this difference was no longer observed in irradiated cells incubated with anti-DLL4 antibody. Scharpfenecker *et al.* have also reported similar findings, where a 5 Gy dose of radiation decreased migration in human dermal microvascular endothelial cell (HMVEC-d) compared to unirradiated controls, but no significant difference was seen in irradiated cells treated with γ-secretase inhibitor. They also observed that Jag1 and Hey1 levels increased 3 hours after IR but subsided to the same level as control by 6 hours post-irradiation (Scharpfenecker *et al.*, 2009). We did not note increased Jag1 or DLL4 staining in tumour vasculature, 3 days after irradiation. Examination of tumours harvested a few hours after irradiation would help to resolve whether the *in vitro* upregulation of Jag1 occured *in vivo*. Collectively, these results show that ionising radiation impairs migration in endothelial cells which may in part be due to changes in Notch signalling.

Radiation has been shown to inhibit proliferation of endothelial cells at doses similar to this study (Abdollahi *et al.*, 2003, Donker *et al.*, 2007). In agreement with these findings,

we observe inhibition of proliferation and decreased cell numbers in cultures of irradiated non-confluent HUVECs. DLL4 has been shown to negatively regulate endothelial proliferation *in vitro* and *in vivo* (Liu *et al.*, 2006, Williams *et al.*, 2006, Benedito *et al.*, 2008). Additionally, it has been reported that the Notch pathway promotes cell cycle arrest in endothelial cells and may be involved in contact inhibition (Noseda *et al.*, 2004). Here we observe that blocking DLL4 did not significantly increase proliferation in actively growing HUVECs, but stimulated proliferation in contact inhibited quiescent endothelial monolayers. However, this increase was completely inhibited in irradiated cells. The Notch downstream gene HesR1 has been suggested to play a role in maintaining vascular homeostasis (Henderson *et al.*, 2001). The importance of DLL4 in tip cell specification during sprouting angiogenesis is well established. DLL4-Notch may also be involved in continued maintenance of a mature, quiescent vessel.

Higher doses of IR such as 15 Gy and 20 Gy have been demonstrated to promote the recruitment of $CD11b^+$ cells, primarily macrophages to the tumour (Ahn and Brown, 2008, Kozin *et al.*, 2010). Tumour associated macrophages are known to produce proangiogenic cytokines and affect vasculature (Coffelt *et al.*, 2009). In models involving IR mediated ablation of vasculature, it has been shown that redevelopment of vasculature requires an influx of $CD11b^+$ cells (Ahn and Brown, 2008, Ahn *et al.*, 2010) mediated via stromal derived factor 1 (SDF1) and that blocking SDF1 impairs tumour regrowth (Kioi *et al.*, 2010, Kozin *et al.*, 2010). Results here demonstrate that a single lower dose of 5 Gy significantly increases CD11b+ cells and macrophages infiltration into the tumour. This IR mediated influx of cells does not appear to be a response to necrosis as no more than 5% necrosis was present in tumours treated with radiation alone. In our model DLL4 blockade does not appear to affect radiation induced recruitment of $CD11b^+$ cell or macrophages.

In summary, the clinically relevant dose of radiation used in this study impairs migration and inhibits proliferation of endothelial cells *in vitro*. Blocking DLL4 may restore migration. DLL4 may be involved in maintaining quiescence in endothelial cells and anti-DLL4 therapy could possibly stimulate proliferation in contact inhibited cells. 5 Gy dose of radiation induced the influx of immune cells into the tumour and blocking DLL4 did not affect this recruitment.

Chapter 6

Conclusions and future perspectives

6. Conclusions and future perspectives

6.1. Introduction

Radiotherapy is an important treatment modality and is prescribed to approximately 50% of all cancer patients, either alone or in combination with chemotherapy or surgery (Begg *et al.*, 2011). Technological advances in the field over the past few decades have improved delivery of the dose to the tumour while reducing dose to the surrounding normal tissue, allowing higher doses of radiation to be used in treatments. Despites improvements, some patients respond poorly or develop recurrent tumours, highlighting the need to further improve efficacy of radiation treatment. The outcome of radiation treatment is dependent of several factors, including the intrinsic radioresistance of tumour cells as well as the extent of hypoxia.

Consequently, targeting tumour vasculature represents an important strategy in controlling tumour growth (Fens *et al.*, 2010). It has long been recognised that tumour hypoxia decreases the efficacy of radiotherapy (Moeller *et al.*, 2007). Hence, there is a concern that antiangiogenic therapy may decrease radiosensitivity. However, several preclinical and clinical studies reported favourable results with a combination of radiation treatment and antiangiogenic agents (reviewed in chapter 1).

As reviewed in chapter 1, the Notch pathway has been reported to play an oncogenic role in a number of cancers, and aberrant expression of DLL4 has been observed in a number of tumour types. Additionally, DLL4-Notch signalling is a key regulator of physiological and tumour angiogenesis. Given its dual functions in tumour and vascular cells, the Notch pathway presents an attractive target for cancer therapy. Consequently, a number of studies have explored the effects of Notch inhibition on tumour cells and vasculature as

well as its combination with conventional cancer therapies including radiation treatment. Specific inhibitors against different components of the pathway, including DLL4 are in development for treatment against a variety of malignancies (Table 1.1, chapter 1). For this thesis, we have investigated the effects of anti-DLL4 therapy on tumour cells and the endothelium and its combination with a clinically relevant dose of radiation.

Here we present the first report investigating the effects of combining anti-DLL4 treatment with radiation therapy. Combining the treatments resulted in a supra-additive growth delay compared to the treatments alone. We observed dysregulation of tumour vasculature within 3 days of DLL4 blockade. Our colleagues have shown the combination of the two treatments result in a supra-additive growth delay in xenograft tumours derived from FADU, a DLL4-negative cell line. Together these results suggest that DLL4 blockade following radiation therapy may be an effective strategy to target tumour growth, and indicate that that the tumour stroma may provide the main therapeutic target for anti-DLL4 therapy in solid tumours. Gene expression analysis of the Notch pathway in treated tumours shows that IR may upregulate Jag1 in tumour cells but may supress Notch signalling and downregulate DLL4 in the tumour stroma. As Jag1 and DLL4 are important regulators of angiogenesis, these changes may affect response to anti-DLL4 therapy. IR was also observed to decrease migration of cultured endothelial cells, but blocking DLL4 abrogated this effect. Dll4 blockade induced proliferation in quiescent confluent endothelial cultures. Collectively these results indicate a role for DLL4 in maintaining vessel quiescence.

6.2. DLL4 blockade as an antiangiogenic strategy.

Strategies targeting tumour vasculature can take a number of forms. They can be based around blocking tumour secreted growth factors, such as VEGF inhibitors (Thurston *et al.*, 2007). They can target distinct features of tumour vessels to disrupt vessel function. Examples include vascular targeting agents such as combretastatins (Iyer *et al.*, 1998). They can focus on endogenous anti-angiogenic molecules such as endostatin and angiostatin (Javaherian *et al.*, 2011). Conventional antiangiogenic therapies such as VEGF blockade aim to inhibit angiogenesis and prune existing vessels. Anti-DLL4 therapy represents a novel approach to targeting tumour vasculature selectively. Tumours represent sites of active, poorly balanced angiogenesis, resulting in a sub-optimal vascular network. Blocking DLL4 has been reported to further increase angiogenesis, resulting in even poorer perfusion and increased hypoxia, inhibiting tumour growth (Noguera-Troise *et al.*, 2006, Ridgway *et al.*, 2006, Scehnet *et al.*, 2007). Here, we also observed that anti-DLL4 therapy promoted non-productive angiogenesis and significantly inhibited tumour growth. We noted increases in vessel density and tumour hypoxia, impaired blood flow as well as significant induction of necrosis within 3 days of anti-DLL4 therapy.

In addition to functioning as a key regulator of tip and stalk cell specification during sprouting angiogenesis, Notch signalling may be important in maintaining vascular quiescence. Notch activation has been suggested to contribute contact inhibition in confluent endothelial cells (Noseda *et al.*, 2004). Overexpression of the Notch downstream target HESR1 has been reported to suppress endothelial cell proliferation and migration *in vitro* (Henderson *et al.*, 2001). We observed that blocking DLL4 in confluent quiescent HUVEC cultures initiated proliferation. We also noted that radiation induced inhibition of migration was no longer apparent when HUVECs were also treated

with anti-DLL4 antibodies. Recently it has also been reported that Angiopoetin-1 (Ang1), which has been reported to promote quiescence and vessel integrity (Brindle *et al.*, 2006), upregulated DLL4 in confluent endothelial cells, and induced deposition of extracellular collagen IV through DLL4-Notch signalling (Zhang *et al.*, 2011).

We and others noted that anti-DLL4 therapy is well tolerated with no apparent weight loss in animals treated (Ridgway *et al.*, 2006, Liu *et al.*, 2011). Notch signalling is important in the maintenance of intestinal crypt progenitor cells. Hence one serious side effect of treatment with γ-secretase inhibitors (GSI) is goblet cell metaplasia (Milano *et al.*, 2004, van Es *et al.*, 2005). Consequently, intermittent dosing is required to mitigate gastrointestinal toxicity (Tammam *et al.*, 2009). Additionally, γ-secretase is involved in a number of other signalling pathways, and cleaves other membrane proteins, increasing the potential for side effects (Fortini, 2002, Lleo, 2008). Targeting DLL4 specifically avoids these pitfalls, however prolonged DLL4 blockade has been reported to induce subcutaneous vascular neoplasms in rats, which appear to be benign. Liver pathology and upregulation of endothelial specific genes in the liver was also noted. The author suggest that DLL4 may be important in maintaining endothelial quiescence, which is consistent with our findings (discussed above) (Yan *et al.*, 2010). Frequency and dose of DLL4 blocking agents may have to be refined to minimise these side-effects in clinical settings.

6.3. Targeting DLL4 to improve the tumour radioresponse.

Other studies have explored the combination of antiangiogenic agents with radiation treatment to improve the radioresponse of tumours (as reviewed in chapter 1). However, the effects of combining anti-DLL4 therapy, an unconventional antiangiogenic therapy focussing on vascular abnormalisation, with radiation had not been explored *in vivo*.

Experiments by us and our colleague John Beech have demonstrated that the DLL4 blockade and ionising radiation (5 Gy) interact with each other and synergistically increase tumour growth delay (Liu *et al.*, 2011). We also observed that this effect occurred regardless of DLL4 expression in the tumour cell compartment, and suggest that the stromal/vascular DLL4 is the main therapeutic target for this treatment. This would indeed make the therapy broadly applicable in different tumour types. As listed in a table 1.1, a number of DLL4 blocking antibodies are being clinically developed for a number of solid tumours. Together with our colleagues, we have investigated the combination of anti-DLL4 therapy with radiation in two cancer (colorectal and squamous cell carcinoma). It remains to be determined whether the combination is effective in other malignancies as well. Additionally, the mechanism of interaction of anti-DLL4 therapy with ionising radiation remains unclear. We did not observe any changes in vasculature and our colleague Dr Fokas did not note any changes in perfusion between tumours treated with DLL4 antibody and those treated with a combination of antibody and radiation (Liu *et al.*, 2011).

Lee *et al.* examined the combination of VEGF blockade and high doses of radiation (20-40 Gy) in LS174T tumours, and found that the tumour growth delay was additive at best. Here, we show a supra-additive growth delay when DLL4 blockade was combined with a much lower dose (5 Gy). Our group also noted greater than additive tumour growth delays when the same dose of radiation was combined with DBZ, a γ-secretase inhibitor (Liu *et al.*, 2011). However, DBZ could only be administered for total of 5 doses (spanning 2 weeks) due to the risk of gastrointestinal toxicity. Hence, it is difficult to state whether γ-secretase inhibition combined with radiation treatment, would have resulted in further growth delay compared to anti-DLL4 therapy, if DBZ could be dosed throughout the

experiment. It was noted that tumours treated with DBZ alone, grew faster than tumours treated with anti-DLL4 antibody. Additionally, in contrast with anti-DLL4 therapy, DBZ treated tumours (which were excised before the cessations of DBZ treatment) did not display increased microvessel density or hypoxia compared to controls. Inhibition of Notch signalling was observed (via bioluminescence imaging) within 24 hours of DBZ or anti-DLL4 antibody treatment. However, as DBZ was administered once every 3 days, this inhibition may not have been sustained between doses as DBZ has been reported to have a plasma half-life of less than 12 hours (Milano *et al.*, 2004). A majority of biological drugs, including monoclonal antibodies, may have a half-life of several weeks (Javaherian *et al.*, 2011). The average half-life of a human IgG1 in SCID mice has been reported to be about 6 days (Bazin *et al.*, 1994). This may in part explain the differences in growth delay and vessel density between DBZ and anti-DLL4 antibody treated tumours.

Considering the preclinical nature of our findings, it would be important to investigate the combination of anti-DLL4 therapy in a fractionated radiotherapy schedule and determine effective scheduling of anti-DLL4 treatments to radiotherapy doses. Future experiments with xenograft tumours tumour models can be used to inform on the timescale of changes in tumour perfusion and hypoxia following cessation of anti-DLL4 treatment. Treatment of tumour bearing mice with DLL4 blocking antibodies, can be interrupted and tumour perfusion can be monitored via Power-Doppler ultrasound. Tumours can be resected at different time points and hypoxia and vessel density can be analysed via immunostaining. As poorly perfused, hypoxic tumours are less responsive to radiation treatment, these studies can inform on the optimal times to deliver subsequent doses of radiation during fractionated radiotherapy, particularly if anti-DLL4 therapy is to be maintained long term.

It has been observed with other antiangiogenic agents that the timing of antiangiogenic therapy in relation to radiotherapy influences the results of the treatment (Murata *et al.*, 1997, Zips *et al.*, 2003, Truman *et al.*, 2010). Here we observed increased hypoxia with DLL4 blockade within 3 days of treatment. The effects of cessation of anti-DLL4 therapy and the timescale of changes in tumour vasculature and hypoxia that may occur once anti-DLL4 therapy is paused, needs to be investigated to inform on incorporation into radiotherapy regimens. It is also important to determine the effects of different sequencing of treatments. Future experiments should focus on comparing tumour growth delay and necrosis in xenograft tumours upon delivery of radiation before, during or after anti-DLL4 treatment. These will help identify optimal time-windows when DLL4 blockade could be incorporated into fractionated radiotherapy schedules. As noted in chapter 3, despite extensive necrosis induced by anti-DLL4 treatment, an intact, viable rim of tumour tissue was observed near the tumour boundary, possibly due to its close proximity to normal vasculature outside the tumour. With careful scheduling of treatments, radiation and anti-DLL4 therapy may complement each other as anti-DLL4 therapy induces necrosis by targeting perfusion but spares the well oxygenated tumour rim, while radiation treatment preferentially affects areas with high oxygen tension.

In addition to vascular specific effects, anti-DLL4 therapy may directly target certain tumour types where DLL4-Notch may be acting in an oncogenic fashion. Notch activation can promote pro-survival, proliferation, invasion and drug resistance pathways in tumours, as reviewed in chapter 1. Upregulation of DLL4 has been noted in a number of tumour types including pancreatic (Mullendore *et al.*, 2009), colorectal (Jubb *et al.*, 2009), gastric (Ishigami *et al.*, 2013) and nasopharyngeal (Zhang *et al.*, 2013) carcinomas. Here we observe that DLL4 blockade potentiates the anti-proliferative effect of radiation in

(DLL4-positive) LS174T tumours but does not have any effect on (DLL4-negative) HCT-15 tumours. Therefore screening tumours for DLL4 expression may highlight cases where anti-DLL4 therapy would produce the maximal benefit by dually targeting tumour cell and vascular compartments.

Here we employed Ki67 immunostaining to assess proliferation in tumour and endothelial cells following IR and anti-DLL4 treatments. IR decreased proliferation in LS174T tumour cells *in vitro* and *in vivo*. Blocking DLL4 reduced proliferation alone and resulted in a further decrease when combined with radiation treatment. DLL4 blockade induced proliferation in contact inhibited endothelial cells *in vitro*, but IR treatment abrogated this effect. Further work investigating these changes is important to elucidate the mechanistic processes underlining the effects of DLL4 blockade and IR on proliferation. Cyclins are key factors that control the progression of cells through cell cycle and Notch signalling has been reported to directly alter expression of cyclins in tumours (as stated in section 1.2.1.). To determine the effects of the treatments on the expression of different cyclins, cyclin mRNA levels in treated and untreated cells may be analysed by qPCR. Immunostaining of treated cells for expression of cyclins D1, E, A and B1 may be used to identify cells in different compartments of cell cycle. Analysis by flow cytometry of treated cells labelled with bromodeoxyuridine (BrdU) together with propidium iodide staining, may also be used to study the distribution of cells in distinct phases of cell cycle and further define the growth arrest caused by IR treatment.

6.4. Effects of ionising radiation on tumour stroma and endothelium.

A few recent reports demonstrate that radiotherapy can alter Notch in tumour cells as well as endothelial cells (Phillips *et al.*, 2006, Scharpfenecker *et al.*, 2009, Imaizumi *et al.*,

2010). We observed upregulation that radiation upregulated Jag1 in tumour cells (increased mRNA levels and immunostaining in LS174T cells and increased mRNA in HCT-15 cells) that may potentially affect tumour vasculature. Zeng *et al.* reported that increased Jag1 in cancer cells can promote tumour angiogenesis (Zeng *et al.*, 2005). In DLL4-negative HCT-15 tumours, radiation appeared to decrease Notch signalling in the stroma, an effect which may be masked by DLL4 signalling from cancer cells in LS174T tumours. Radiation-induced changes in Notch signalling may be tumour specific but may affect tumour stroma/vasculature as well as response to anti-DLL4 therapy.

Current literature suggests that effects of radiation on vasculature may be dose-specific as well as dependent on tumour type. Lower doses of radiation have been suggested to promote angiogenesis while higher doses inhibit it (Kleibeuker *et al.*, 2012). Geng at al. show vascular changes with 6 Gy radiation in a B16F10 melanomas, while no changes were observed in a GL261 glioma model (Geng *et al.*, 2001). We did not note any vascular impairment in our *in vivo* studies following 5 Gy radiation (vessel density changes or tumour hypoxia). *In vitro* studies on pure populations of endothelial cells demonstrate that 5 Gy of radiation impaired migration and inhibited proliferation of endothelial cells. Others observed that a dose of 5 Gy (Scharpfenecker *et al.*, 2009) decreased endothelial cell migration. Imaizumi and colleagues have conducted a series of functional assays on endothelial cells *in vitro*, *ex vivo* and in matrigel plugs implanted subcutaneously into mice. They reported decreased angiogenesis and sprouting and migration as well as induction of senescence following radiation treatment (15-20 Gy). However, it is well established that a single large dose of radiation is not equivalent to a clinically relevant fractionated radiotherapy regimen (Wachsberger *et al.*, 2003). Further studies are

necessary to understand the effects of radiation doses commonly used in fractionated radiotherapy schedules, and their interaction with anti-DLL4 therapy.

In summary, anti-DLL4 therapy represents a novel strategy in targeting tumour vasculature. Additionally, DLL4-Notch signalling may play an oncogenic role in multiple tumour types. We explored the combination of anti-DLL4 therapy with radiation treatment and demonstrated that this combination is an effective and well-tolerated treatment that delayed tumour growth in a supra-additive manner. This approach may be clinically relevant in a number of tumour types and warrants further validation and refinement to be translated into clinical practice.

Chapter 7

References

7. References

Abdollahi, A., Lipson, K. E., Han, X., Krempien, R., Trinh, T., Weber, K. J., Hahnfeldt, P., Hlatky, L., Debus, J., Howlett, A. R. & Huber, P. E. (2003) SU5416 and SU6668 attenuate the angiogenic effects of radiation-induced tumor cell growth factor production and amplify the direct anti-endothelial action of radiation in vitro. *Cancer Res* **63**, 3755-3763.

Adams, R. H. & Alitalo, K. (2007) Molecular regulation of angiogenesis and lymphangiogenesis. *Nat Rev Mol Cell Biol* **8**, 464-478.

Ahimou, F., Mok, L. P., Bardot, B. & Wesley, C. (2004) The adhesion force of Notch with Delta and the rate of Notch signaling. *J Cell Biol* **167**, 1217-1229.

Ahn, G. O. & Brown, J. M. (2008) Matrix metalloproteinase-9 is required for tumor vasculogenesis but not for angiogenesis: role of bone marrow-derived myelomonocytic cells. *Cancer Cell* **13**, 193-205.

Ahn, G. O., Tseng, D., Liao, C. H., Dorie, M. J., Czechowicz, A. & Brown, J. M. (2010) Inhibition of Mac-1 (CD11b/CD18) enhances tumor response to radiation by reducing myeloid cell recruitment. *Proc Natl Acad Sci U S A* **107**, 8363-8368.

Ahn, S., Hyeon, J. & Park, C. K. (2013) Notch1 and Notch4 are markers for poor prognosis of hepatocellular carcinoma. *Hepatobiliary & pancreatic diseases international : HBPD INT* **12**, 286-294.

Aparicio, L. M., Villaamil, V. M., Gallego, G. A., Cainzos, I. S., Campelo, R. G., Rubira, L. V., Estevez, S. V., Mateos, L. L., Perez, J. L., Vazquez, M. R., Calvo, O. F. & Bolos, M. V. (2011) Expression of Notch1 to -4 and their ligands in renal cell carcinoma: a tissue microarray study. *Cancer genomics & proteomics* **8**, 93-101.

Arboleda-Velasquez, J. F., Rampal, R., Fung, E., Darland, D. C., Liu, M., Martinez, M. C., Donahue, C. P., Navarro-Gonzalez, M. F., Libby, P., D'Amore, P. A., Aikawa, M., Haltiwanger, R. S. & Kosik, K. S. (2005) CADASIL mutations impair Notch3 glycosylation by Fringe. *Hum Mol Genet* **14**, 1631-1639.

Artavanis-Tsakonas, S., Matsuno, K. & Fortini, M. E. (1995) Notch signaling. *Science* **268**, 225-232.

Artavanis-Tsakonas, S., Muskavitch, M. A. & Yedvobnick, B. (1983) Molecular cloning of Notch, a locus affecting neurogenesis in Drosophila melanogaster. *Proc Natl Acad Sci U S A* **80**, 1977-1981.

Artavanis-Tsakonas, S., Rand, M. D. & Lake, R. J. (1999) Notch signaling: cell fate control and signal integration in development. *Science* **284**, 770-776.

Aste-Amezaga, M., Zhang, N., Lineberger, J. E., Arnold, B. A., Toner, T. J., Gu, M., Huang, L., Vitelli, S., Vo, K. T., Haytko, P., Zhao, J. Z., Baleydier, F., L'Heureux, S., Wang, H., Gordon, W. R., Thoryk, E., Andrawes, M. B., Tiyanont, K., Stegmaier, K., Roti, G., Ross, K. N., Franlin, L. L., Wang, F., Chastain, M., Bett, A. J., Audoly, L. P., Aster, J. C., Blacklow, S. C. & Huber, H. E. (2010) Characterization of Notch1 antibodies that inhibit signaling of both normal and mutated Notch1 receptors. *PloS one* **5**, e9094.

Balint, K., Xiao, M., Pinnix, C. C., Soma, A., Veres, I., Juhasz, I., Brown, E. J., Capobianco, A. J., Herlyn, M. & Liu, Z. J. (2005) Activation of Notch1 signaling is required for beta-catenin-mediated human primary melanoma progression. *J Clin Invest* **115**, 3166-3176.

Bazin, R., Boucher, G., Monier, G., Chevrier, M. C., Verrette, S., Broly, H. & Lemieux, R. (1994) Use of hu-IgG-SCID mice to evaluate the in vivo stability of human monoclonal IgG antibodies. *Journal of immunological methods* **172**, 209-217.

Bedogni, B., Warneke, J. A., Nickoloff, B. J., Giaccia, A. J. & Powell, M. B. (2008) Notch1 is an effector of Akt and hypoxia in melanoma development. *J Clin Invest* **118**, 3660-3670.

Begg, A. C., Stewart, F. A. & Vens, C. (2011) Strategies to improve radiotherapy with targeted drugs. *Nat Rev Cancer* **11**, 239-253.

Benedito, R., Roca, C., Sorensen, I., Adams, S., Gossler, A., Fruttiger, M. & Adams, R. H. (2009) The notch ligands Dll4 and Jagged1 have opposing effects on angiogenesis. *Cell* **137**, 1124-1135.

Benedito, R., Trindade, A., Hirashima, M., Henrique, D., da Costa, L. L., Rossant, J., Gill, P. S. & Duarte, A. (2008) Loss of Notch signalling induced by Dll4 causes arterial calibre reduction by increasing endothelial cell response to angiogenic stimuli. *BMC developmental biology* **8**, 117.

Bergers, G. & Benjamin, L. E. (2003) Tumorigenesis and the angiogenic switch. *Nat Rev Cancer* **3**, 401-410.

Bergers, G. & Hanahan, D. (2008) Modes of resistance to anti-angiogenic therapy. *Nat Rev Cancer* **8**, 592-603.

Bingle, L., Brown, N. J. & Lewis, C. E. (2002) The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. *The Journal of pathology* **196**, 254-265.

Blaumueller, C. M., Qi, H., Zagouras, P. & Artavanis-Tsakonas, S. (1997) Intracellular cleavage of Notch leads to a heterodimeric receptor on the plasma membrane. *Cell* **90**, 281-291.

Bolos, V., Grego-Bessa, J. & de la Pompa, J. L. (2007) Notch signaling in development and cancer. *Endocr Rev* **28**, 339-363.

Bray, S. J. (2006) Notch signalling: a simple pathway becomes complex. *Nat Rev Mol Cell Biol* **7**, 678-689.

Brindle, N. P., Saharinen, P. & Alitalo, K. (2006) Signaling and functions of angiopoietin-1 in vascular protection. *Circulation research* **98**, 1014-1023.

Brou, C., Logeat, F., Gupta, N., Bessia, C., LeBail, O., Doedens, J. R., Cumano, A., Roux, P., Black, R. A. & Israel, A. (2000) A novel proteolytic cleavage involved in Notch signaling: the role of the disintegrin-metalloprotease TACE. *Molecular cell* **5**, 207-216.

Brzozowa, M., Mielanczyk, L., Michalski, M., Malinowski, L., Kowalczyk-Ziomek, G., Helewski, K., Harabin-Slowinska, M. & Wojnicz, R. (2013) Role of Notch signaling pathway in gastric cancer pathogenesis. *Contemporary oncology* **17**, 1-5.

Budach, W., Taghian, A., Freeman, J., Gioioso, D. & Suit, H. D. (1993) Impact of stromal sensitivity on radiation response of tumors. *J Natl Cancer Inst* **85**, 988-993.

Burri, P. H., Hlushchuk, R. & Djonov, V. (2004) Intussusceptive angiogenesis: its emergence, its characteristics, and its significance. *Developmental dynamics : an official publication of the American Association of Anatomists* **231**, 474-488.

Carmeliet, P. (2005) Angiogenesis in life, disease and medicine. *Nature* **438**, 932-936.

Carmeliet, P. & Jain, R. K. (2011) Molecular mechanisms and clinical applications of angiogenesis. *Nature* **473**, 298-307.

Carmeliet, P. & Jain, R. K. (2011) Principles and mechanisms of vessel normalization for cancer and other angiogenic diseases. *Nature reviews. Drug discovery* **10**, 417-427.

Casanovas, O., Hicklin, D. J., Bergers, G. & Hanahan, D. (2005) Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors. *Cancer Cell* **8**, 299-309.

Chandu, D., Huppert, S. S. & Kopan, R. (2006) Analysis of transmembrane domain mutants is consistent with sequential cleavage of Notch by gamma-secretase. *Journal of neurochemistry* **96**, 228-235.

Chaplin, D. J., Durand, R. E. & Olive, P. L. (1986) Acute hypoxia in tumors: implications for modifiers of radiation effects. *Int J Radiat Oncol Biol Phys* **12**, 1279-1282.

Chen, S. M., Liu, J. P., Zhou, J. X., Chen, C., Deng, Y. Q., Wang, Y. & Tao, Z. Z. (2011) Suppression of the notch signaling pathway by gamma-secretase inhibitor GSI inhibits human nasopharyngeal carcinoma cell proliferation. *Cancer Lett* **306**, 76-84.

Chen, Y., De Marco, M. A., Graziani, I., Gazdar, A. F., Strack, P. R., Miele, L. & Bocchetta, M. (2007) Oxygen concentration determines the biological effects of NOTCH-1 signaling in adenocarcinoma of the lung. *Cancer Res* **67**, 7954-7959.

Coffelt, S. B., Hughes, R. & Lewis, C. E. (2009) Tumor-associated macrophages: effectors of angiogenesis and tumor progression. *Biochim Biophys Acta* **1796**, 11-18.

Cohen, B., Shimizu, M., Izrailit, J., Ng, N. F., Buchman, Y., Pan, J. G., Dering, J. & Reedijk, M. (2010) Cyclin D1 is a direct target of JAG1-mediated Notch signaling in breast cancer. *Breast Cancer Res Treat* **123**, 113-124.

Crane, C. H., Ellis, L. M., Abbruzzese, J. L., Amos, C., Xiong, H. Q., Ho, L., Evans, D. B., Tamm, E. P., Ng, C., Pisters, P. W., Charnsangavej, C., Delclos, M. E., O'Reilly, M., Lee, J. E. & Wolff, R. A. (2006) Phase I trial evaluating the safety of bevacizumab with concurrent radiotherapy and capecitabine in locally advanced pancreatic cancer. *J Clin Oncol* **24**, 1145-1151.

Croquelois, A., Blindenbacher, A., Terracciano, L., Wang, X., Langer, I., Radtke, F. & Heim, M. H. (2005) Inducible inactivation of Notch1 causes nodular regenerative hyperplasia in mice. *Hepatology* **41**, 487-496.

Cuevas, I. C., Slocum, A. L., Jun, P., Costello, J. F., Bollen, A. W., Riggins, G. J., McDermott, M. W. & Lal, A. (2005) Meningioma transcript profiles reveal deregulated Notch signaling pathway. *Cancer Res* **65**, 5070-5075.

Curry, C. L., Reed, L. L., Golde, T. E., Miele, L., Nickoloff, B. J. & Foreman, K. E. (2005) Gamma secretase inhibitor blocks Notch activation and induces apoptosis in Kaposi's sarcoma tumor cells. *Oncogene* **24**, 6333-6344.

Czito, B. G., Bendell, J. C., Willett, C. G., Morse, M. A., Blobe, G. C., Tyler, D. S., Thomas, J., Ludwig, K. A., Mantyh, C. R., Ashton, J., Yu, D. & Hurwitz, H. I. (2007) Bevacizumab, oxaliplatin, and capecitabine with radiation therapy in rectal cancer: Phase I trial results. *Int J Radiat Oncol Biol Phys* **68**, 472-478.

D'Souza, B., Miyamoto, A. & Weinmaster, G. (2008) The many facets of Notch ligands. *Oncogene* **27**, 5148-5167.

Dai, J., Ma, D., Zang, S., Guo, D., Qu, X., Ye, J. & Ji, C. (2009) Cross-talk between Notch and EGFR signaling in human breast cancer cells. *Cancer Invest* **27**, 533-540.

De Strooper, B., Annaert, W., Cupers, P., Saftig, P., Craessaerts, K., Mumm, J. S., Schroeter, E. H., Schrijvers, V., Wolfe, M. S., Ray, W. J., Goate, A. & Kopan, R. (1999) A presenilin-1-dependent gamma-secretase-like protease mediates release of Notch intracellular domain. *Nature* **398**, 518-522.

Dejana, E., Tournier-Lasserve, E. & Weinstein, B. M. (2009) The control of vascular integrity by endothelial cell junctions: molecular basis and pathological implications. *Dev Cell* **16**, 209-221.

Denekamp, J. (1984) Vascular endothelium as the vulnerable element in tumours. *Acta radiologica. Oncology* **23**, 217-225.

Dexter, J. S. (1914) The Analysis of a Case of Continuous Variation in Drosophila by a Study of Its Linkage Relations. *The American Naturalist* **48**, 712-758.

Dievart, A., Beaulieu, N. & Jolicoeur, P. (1999) Involvement of Notch1 in the development of mouse mammary tumors. *Oncogene* **18**, 5973-5981.

Dolcet, X., Llobet, D., Pallares, J. & Matias-Guiu, X. (2005) NF-kB in development and progression of human cancer. *Virchows Archiv : an international journal of pathology* **446**, 475-482.

Donker, M., Van Furth, W. R., Mulder-Van Der Kracht, S., Hovinga, K. E., Verhoeff, J. J., Stalpers, L. J. & van Bree, C. (2007) Negligible radiation protection of endothelial cells by vascular endothelial growth factor. *Oncology reports* **18**, 709-714.

Dou, S., Zeng, X., Cortes, P., Erdjument-Bromage, H., Tempst, P., Honjo, T. & Vales, L. D. (1994) The recombination signal sequence-binding protein RBP-2N functions as a transcriptional repressor. *Molecular and cellular biology* **14**, 3310-3319.

Efstratiadis, A., Szabolcs, M. & Klinakis, A. (2007) Notch, Myc and breast cancer. *Cell Cycle* **6**, 418-429.

Ehebauer, M., Hayward, P. & Arias, A. M. (2006) Notch, a universal arbiter of cell fate decisions. *Science* **314**, 1414-1415.

Eilken, H. M. & Adams, R. H. (2010) Dynamics of endothelial cell behavior in sprouting angiogenesis. *Current opinion in cell biology* **22**, 617-625.

Ellisen, L. W., Bird, J., West, D. C., Soreng, A. L., Reynolds, T. C., Smith, S. D. & Sklar, J. (1991) TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. *Cell* **66**, 649-661.

Espinoza, I. & Miele, L. (2013) Notch inhibitors for cancer treatment. *Pharmacology & therapeutics* **139**, 95-110.

Fens, M. H., Storm, G. & Schiffelers, R. M. (2010) Tumor vasculature as target for therapeutic intervention. *Expert Opin Investig Drugs* **19**, 1321-1338.

Fernandez-Majada, V., Aguilera, C., Villanueva, A., Vilardell, F., Robert-Moreno, A., Aytes, A., Real, F. X., Capella, G., Mayo, M. W., Espinosa, L. & Bigas, A. (2007) Nuclear IKK activity leads to dysregulated notch-dependent gene expression in colorectal cancer. *Proc Natl Acad Sci U S A* **104**, 276-281.

Folkman, J. (1971) Tumor angiogenesis: therapeutic implications. *The New England journal of medicine* **285**, 1182-1186.

Folkman, J. (1972) Anti-angiogenesis: new concept for therapy of solid tumors. *Annals of surgery* **175**, 409-416.

Fortini, M. E. (2002) Gamma-secretase-mediated proteolysis in cell-surface-receptor signalling. *Nat Rev Mol Cell Biol* **3**, 673-684.

Fox, S. B., Leek, R. D., Smith, K., Hollyer, J., Greenall, M. & Harris, A. L. (1994) Tumor angiogenesis in node-negative breast carcinomas--relationship with epidermal growth factor receptor, estrogen receptor, and survival. *Breast Cancer Res Treat* **29**, 109-116.

Fung, E., Tang, S. M., Canner, J. P., Morishige, K., Arboleda-Velasquez, J. F., Cardoso, A. A., Carlesso, N., Aster, J. C. & Aikawa, M. (2007) Delta-like 4 induces notch signaling in macrophages: implications for inflammation. *Circulation* **115**, 2948-2956.

Gallahan, D. & Callahan, R. (1987) Mammary tumorigenesis in feral mice: identification of a new int locus in mouse mammary tumor virus (Czech II)-induced mammary tumors. *J Virol* **61**, 66-74.

Gasparini, G., Torino, F., Ueno, T., Cascinu, S., Troiani, T., Ballestrero, A., Berardi, R., Shishido, J., Yoshizawa, A., Mori, Y., Nagayama, S., Morosini, P. & Toi, M. (2012) A phase II study of neoadjuvant bevacizumab plus capecitabine and concomitant radiotherapy in patients with locally advanced rectal cancer. *Angiogenesis* **15**, 141-150.

Geng, L., Donnelly, E., McMahon, G., Lin, P. C., Sierra-Rivera, E., Oshinka, H. & Hallahan, D. E. (2001) Inhibition of vascular endothelial growth factor receptor signaling leads to reversal of tumor resistance to radiotherapy. *Cancer Res* **61**, 2413-2419.

Gerhardt, H., Golding, M., Fruttiger, M., Ruhrberg, C., Lundkvist, A., Abramsson, A., Jeltsch, M., Mitchell, C., Alitalo, K., Shima, D. & Betsholtz, C. (2003) VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *J Cell Biol* **161**, 1163-1177.

Gordon, W. R., Roy, M., Vardar-Ulu, D., Garfinkel, M., Mansour, M. R., Aster, J. C. & Blacklow, S. C. (2009) Structure of the Notch1-negative regulatory region: implications for normal activation and pathogenic signaling in T-ALL. *Blood* **113**, 4381-4390.

Gorski, D. H., Beckett, M. A., Jaskowiak, N. T., Calvin, D. P., Mauceri, H. J., Salloum, R. M., Seetharam, S., Koons, A., Hari, D. M., Kufe, D. W. & Weichselbaum, R. R. (1999) Blockage of the vascular endothelial growth factor stress response increases the antitumor effects of ionizing radiation. *Cancer Res* **59**, 3374-3378.

Gray, G. E., Mann, R. S., Mitsiadis, E., Henrique, D., Carcangiu, M. L., Banks, A., Leiman, J., Ward, D., Ish-Horowitz, D. & Artavanis-Tsakonas, S. (1999) Human ligands of the Notch receptor. *Am J Pathol* **154**, 785-794.

Greenwald, I. (1994) Structure/function studies of lin-12/Notch proteins. *Curr Opin Genet Dev* **4**, 556-562.

Gridley, T. (2007) Notch signaling in vascular development and physiology. *Development* **134**, 2709-2718.

Gurney, A. & Hoey, T. (2011) Anti-DLL4, a cancer therapeutic with multiple mechanisms of action. *Vascular cell* **3**, 18.

Hainaud, P., Contreres, J. O., Villemain, A., Liu, L. X., Plouet, J., Tobelem, G. & Dupuy, E. (2006) The role of the vascular endothelial growth factor-Delta-like 4 ligand/Notch4-ephrin B2 cascade in tumor vessel remodeling and endothelial cell functions. *Cancer Res* **66**, 8501-8510.

Hallahan, A. R., Pritchard, J. I., Hansen, S., Benson, M., Stoeck, J., Hatton, B. A., Russell, T. L., Ellenbogen, R. G., Bernstein, I. D., Beachy, P. A. & Olson, J. M. (2004) The SmoA1 mouse model reveals that notch signaling is critical for the growth and survival of sonic hedgehog-induced medulloblastomas. *Cancer Res* **64**, 7794-7800.

Harrington, L. S., Sainson, R. C., Williams, C. K., Taylor, J. M., Shi, W., Li, J. L. & Harris, A. L. (2008) Regulation of multiple angiogenic pathways by Dll4 and Notch in human umbilical vein endothelial cells. *Microvasc Res* **75**, 144-154.

Hellstrom, M., Phng, L. K., Hofmann, J. J., Wallgard, E., Coultas, L., Lindblom, P., Alva, J., Nilsson, A. K., Karlsson, L., Gaiano, N., Yoon, K., Rossant, J., Iruela-Arispe, M. L., Kalen,

M., Gerhardt, H. & Betsholtz, C. (2007) Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. *Nature* **445**, 776-780.

Henderson, A. M., Wang, S. J., Taylor, A. C., Aitkenhead, M. & Hughes, C. C. (2001) The basic helix-loop-helix transcription factor HESR1 regulates endothelial cell tube formation. *The Journal of biological chemistry* **276**, 6169-6176.

Hlushchuk, R., Riesterer, O., Baum, O., Wood, J., Gruber, G., Pruschy, M. & Djonov, V. (2008) Tumor recovery by angiogenic switch from sprouting to intussusceptive angiogenesis after treatment with PTK787/ZK222584 or ionizing radiation. *Am J Pathol* **173**, 1173-1185.

Hoang, T., Huang, S., Armstrong, E., Eickhoff, J. C. & Harari, P. M. (2012) Enhancement of radiation response with bevacizumab. *Journal of experimental & clinical cancer research : CR* **31**, 37.

Hoey, T., Yen, W. C., Axelrod, F., Basi, J., Donigian, L., Dylla, S., Fitch-Bruhns, M., Lazetic, S., Park, I. K., Sato, A., Satyal, S., Wang, X., Clarke, M. F., Lewicki, J. & Gurney, A. (2009) DLL4 blockade inhibits tumor growth and reduces tumor-initiating cell frequency. *Cell Stem Cell* **5**, 168-177.

Hopfer, O., Zwahlen, D., Fey, M. F. & Aebi, S. (2005) The Notch pathway in ovarian carcinomas and adenomas. *Br J Cancer* **93**, 709-718.

Horsman, M. R. & Siemann, D. W. (2006) Pathophysiologic effects of vascular-targeting agents and the implications for combination with conventional therapies. *Cancer Res* **66**, 11520-11539.

Hovinga, K. E., Shimizu, F., Wang, R., Panagiotakos, G., Van Der Heijden, M., Moayedpardazi, H., Correia, A. S., Soulet, D., Major, T., Menon, J. & Tabar, V. (2010)

Inhibition of notch signaling in glioblastoma targets cancer stem cells via an endothelial cell intermediate. *Stem Cells* **28**, 1019-1029.

Hu, L., Xue, F., Shao, M. H., Deng, A. M. & Wei, G. T. (2013) Aberrant expression of Notch3 predicts poor survival for hepatocellular carcinomas. *Bioscience trends* **7**, 152-156.

Hurwitz, H., Fehrenbacher, L., Novotny, W., Cartwright, T., Hainsworth, J., Heim, W., Berlin, J., Baron, A., Griffing, S., Holmgren, E., Ferrara, N., Fyfe, G., Rogers, B., Ross, R. & Kabbinavar, F. (2004) Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *The New England journal of medicine* **350**, 2335-2342.

Huynh, C., Poliseno, L., Segura, M. F., Medicherla, R., Haimovic, A., Menendez, S., Shang, S., Pavlick, A., Shao, Y., Darvishian, F., Boylan, J. F., Osman, I. & Hernando, E. (2011) The novel gamma secretase inhibitor RO4929097 reduces the tumor initiating potential of melanoma. *PloS one* **6**, e25264.

Imaizumi, N., Monnier, Y., Hegi, M., Mirimanoff, R. O. & Ruegg, C. (2010) Radiotherapy suppresses angiogenesis in mice through TGF-betaRI/ALK5-dependent inhibition of endothelial cell sprouting. *PloS one* **5**, e11084.

Indraccolo, S., Minuzzo, S., Masiero, M., Pusceddu, I., Persano, L., Moserle, L., Reboldi, A., Favaro, E., Mecarozzi, M., Di Mario, G., Screpanti, I., Ponzoni, M., Doglioni, C. & Amadori, A. (2009) Cross-talk between tumor and endothelial cells involving the Notch3-Dll4 interaction marks escape from tumor dormancy. *Cancer Res* **69**, 1314-1323.

Ishigami, S., Arigami, T., Uenosono, Y., Okumura, H., Kurahara, H., Uchikado, Y., Setoyama, T., Kita, Y., Kijima, Y., Nishizono, Y., Nakajo, A., Owaki, T., Ueno, S. &

Natsugoe, S. (2013) Clinical implications of DLL4 expression in gastric cancer. *Journal of experimental & clinical cancer research : CR* **32**, 46.

Iso, T., Kedes, L. & Hamamori, Y. (2003) HES and HERP families: multiple effectors of the Notch signaling pathway. *Journal of cellular physiology* **194**, 237-255.

Iyer, S., Chaplin, D. J., Rosenthal, D. S., Boulares, A. H., Li, L. Y. & Smulson, M. E. (1998) Induction of apoptosis in proliferating human endothelial cells by the tumor-specific antiangiogenesis agent combretastatin A-4. *Cancer Res* **58**, 4510-4514.

Jain, R. K., Duda, D. G., Clark, J. W. & Loeffler, J. S. (2006) Lessons from phase III clinical trials on anti-VEGF therapy for cancer. *Nat Clin Pract Oncol* **3**, 24-40.

Jarriault, S., Brou, C., Logeat, F., Schroeter, E. H., Kopan, R. & Israel, A. (1995) Signalling downstream of activated mammalian Notch. *Nature* **377**, 355-358.

Javaherian, K., Lee, T. Y., Tjin Tham Sjin, R. M., Parris, G. E. & Hlatky, L. (2011) Two Endogenous Antiangiogenic Inhibitors, Endostatin and Angiostatin, Demonstrate Biphasic Curves in their Antitumor Profiles. *Dose-response : a publication of International Hormesis Society* **9**, 369-376.

Johnston, S. H., Rauskolb, C., Wilson, R., Prabhakaran, B., Irvine, K. D. & Vogt, T. F. (1997) A family of mammalian Fringe genes implicated in boundary determination and the Notch pathway. *Development* **124**, 2245-2254.

Jubb, A. M., Turley, H., Moeller, H. C., Steers, G., Han, C., Li, J. L., Leek, R., Tan, E. Y., Singh, B., Mortensen, N. J., Noguera-Troise, I., Pezzella, F., Gatter, K. C., Thurston, G., Fox, S. B. & Harris, A. L. (2009) Expression of delta-like ligand 4 (Dll4) and markers of hypoxia in colon cancer. *Br J Cancer* **101**, 1749-1757.

Kallman, R. F., DeNardo, G. L. & Stasch, M. J. (1972) Blood flow in irradiated mouse sarcoma as determined by the clearance of xenon-133. *Cancer Res* **32**, 483-490.

Kerbel, R. S., Yu, J., Tran, J., Man, S., Viloria-Petit, A., Klement, G., Coomber, B. L. & Rak, J. (2001) Possible mechanisms of acquired resistance to anti-angiogenic drugs: implications for the use of combination therapy approaches. *Cancer Metastasis Rev* **20**, 79-86.

Kioi, M., Vogel, H., Schultz, G., Hoffman, R. M., Harsh, G. R. & Brown, J. M. (2010) Inhibition of vasculogenesis, but not angiogenesis, prevents the recurrence of glioblastoma after irradiation in mice. *J Clin Invest* **120**, 694-705.

Kleibeuker, E. A., Griffioen, A. W., Verheul, H. M., Slotman, B. J. & Thijssen, V. L. (2012) Combining angiogenesis inhibition and radiotherapy: a double-edged sword. *Drug resistance updates : reviews and commentaries in antimicrobial and anticancer chemotherapy* **15**, 173-182.

Klueg, K. M. & Muskavitch, M. A. (1999) Ligand-receptor interactions and trans-endocytosis of Delta, Serrate and Notch: members of the Notch signalling pathway in Drosophila. *Journal of cell science* **112 (Pt 19)**, 3289-3297.

Kondratyev, M., Kreso, A., Hallett, R. M., Girgis-Gabardo, A., Barcelon, M. E., Ilieva, D., Ware, C., Majumder, P. K. & Hassell, J. A. (2012) Gamma-secretase inhibitors target tumor-initiating cells in a mouse model of ERBB2 breast cancer. *Oncogene* **31**, 93-103.

Kopan, R. & Ilagan, M. X. (2009) The canonical Notch signaling pathway: unfolding the activation mechanism. *Cell* **137**, 216-233.

Koukourakis, M. I., Giatromanolaki, A., Sivridis, E., Gatter, K. C. & Harris, A. L. (2013) High DLL4 expression in tumour-associated vessels predicts for favorable radiotherapy

outcome in locally advanced squamous cell head-neck cancer (HNSCC). *Angiogenesis* **16**, 343-351.

Kozin, S. V., Boucher, Y., Hicklin, D. J., Bohlen, P., Jain, R. K. & Suit, H. D. (2001) Vascular endothelial growth factor receptor-2-blocking antibody potentiates radiation-induced long-term control of human tumor xenografts. *Cancer Res* **61**, 39-44.

Kozin, S. V., Kamoun, W. S., Huang, Y., Dawson, M. R., Jain, R. K. & Duda, D. G. (2010) Recruitment of myeloid but not endothelial precursor cells facilitates tumor regrowth after local irradiation. *Cancer Res* **70**, 5679-5685.

Kurooka, H., Kuroda, K. & Honjo, T. (1998) Roles of the ankyrin repeats and C-terminal region of the mouse notch1 intracellular region. *Nucleic acids research* **26**, 5448-5455.

Lee, C. G., Heijn, M., di Tomaso, E., Griffon-Etienne, G., Ancukiewicz, M., Koike, C., Park, K. R., Ferrara, N., Jain, R. K., Suit, H. D. & Boucher, Y. (2000) Anti-Vascular endothelial growth factor treatment augments tumor radiation response under normoxic or hypoxic conditions. *Cancer Res* **60**, 5565-5570.

Leek, R. D., Landers, R. J., Harris, A. L. & Lewis, C. E. (1999) Necrosis correlates with high vascular density and focal macrophage infiltration in invasive carcinoma of the breast. *Br J Cancer* **79**, 991-995.

Leimeister, C., Externbrink, A., Klamt, B. & Gessler, M. (1999) Hey genes: a novel subfamily of hairy- and Enhancer of split related genes specifically expressed during mouse embryogenesis. *Mechanisms of development* **85**, 173-177.

Lewis, C. E. & Pollard, J. W. (2006) Distinct role of macrophages in different tumor microenvironments. *Cancer Res* **66**, 605-612.

Li, B., Tang, S.-b., Hu, J., Gao, Y., Zhang, G., Lin, S.-f., Chen, J.-h. & Li, B.-j. (2006) Protective effects of transcription factor HESR1 on retinal vasculature. *Microvascular Research* **72**, 146-152.

Li, J. L., Jubb, A. M. & Harris, A. L. (2010) Targeting DLL4 in tumors shows preclinical activity but potentially significant toxicity. *Future oncology* **6**, 1099-1103.

Li, J. L., Sainson, R. C., Shi, W., Leek, R., Harrington, L. S., Preusser, M., Biswas, S., Turley, H., Heikamp, E., Hainfellner, J. A. & Harris, A. L. (2007) Delta-like 4 Notch ligand regulates tumor angiogenesis, improves tumor vascular function, and promotes tumor growth in vivo. *Cancer Res* **67**, 11244-11253.

Li, K., Li, Y., Wu, W., Gordon, W. R., Chang, D. W., Lu, M., Scoggin, S., Fu, T., Vien, L., Histen, G., Zheng, J., Martin-Hollister, R., Duensing, T., Singh, S., Blacklow, S. C., Yao, Z., Aster, J. C. & Zhou, B. B. (2008) Modulation of Notch signaling by antibodies specific for the extracellular negative regulatory region of NOTCH3. *The Journal of biological chemistry* **283**, 8046-8054.

Li, L., Krantz, I. D., Deng, Y., Genin, A., Banta, A. B., Collins, C. C., Qi, M., Trask, B. J., Kuo, W. L., Cochran, J., Costa, T., Pierpont, M. E., Rand, E. B., Piccoli, D. A., Hood, L. & Spinner, N. B. (1997) Alagille syndrome is caused by mutations in human Jagged1, which encodes a ligand for Notch1. *Nat Genet* **16**, 243-251.

Lichtenthaler, S. F., Ida, N., Multhaup, G., Masters, C. L. & Beyreuther, K. (1997) Mutations in the transmembrane domain of APP altering gamma-secretase specificity. *Biochemistry* **36**, 15396-15403.

Liu, J., Fan, H., Ma, Y., Liang, D., Huang, R., Wang, J., Zhou, F., Kan, Q., Ming, L., Li, H., Giercksky, K. E., Nesland, J. M. & Suo, Z. (2013) Notch1 is a 5-fluorouracil resistant and

poor survival marker in human esophagus squamous cell carcinomas. *PloS one* **8**, e56141.

Liu, S. K., Bham, S. A., Fokas, E., Beech, J., Im, J., Cho, S., Harris, A. L. & Muschel, R. J. (2011) Delta-like ligand 4-notch blockade and tumor radiation response. *J Natl Cancer Inst* **103**, 1778-1798.

Liu, Z. J., Xiao, M., Balint, K., Smalley, K. S., Brafford, P., Qiu, R., Pinnix, C. C., Li, X. & Herlyn, M. (2006) Notch1 signaling promotes primary melanoma progression by activating mitogen-activated protein kinase/phosphatidylinositol 3-kinase-Akt pathways and up-regulating N-cadherin expression. *Cancer Res* **66**, 4182-4190.

Liu, Z. J., Xiao, M., Balint, K., Soma, A., Pinnix, C. C., Capobianco, A. J., Velazquez, O. C. & Herlyn, M. (2006) Inhibition of endothelial cell proliferation by Notch1 signaling is mediated by repressing MAPK and PI3K/Akt pathways and requires MAML1. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **20**, 1009-1011.

Lleo, A. (2008) Activity of gamma-secretase on substrates other than APP. *Curr Top Med Chem* **8**, 9-16.

Lobov, I. B., Renard, R. A., Papadopoulos, N., Gale, N. W., Thurston, G., Yancopoulos, G. D. & Wiegand, S. J. (2007) Delta-like ligand 4 (Dll4) is induced by VEGF as a negative regulator of angiogenic sprouting. *Proc Natl Acad Sci U S A* **104**, 3219-3224.

Logeat, F., Bessia, C., Brou, C., LeBail, O., Jarriault, S., Seidah, N. G. & Israel, A. (1998) The Notch1 receptor is cleaved constitutively by a furin-like convertase. *Proc Natl Acad Sci U S A* **95**, 8108-8112.

Lord, E. M., Harwell, L. & Koch, C. J. (1993) Detection of hypoxic cells by monoclonal antibody recognizing 2-nitroimidazole adducts. *Cancer Res* **53**, 5721-5726.

Lubman, O. Y., Korolev, S. V. & Kopan, R. (2004) Anchoring notch genetics and biochemistry; structural analysis of the ankyrin domain sheds light on existing data. *Molecular cell* **13**, 619-626.

Mailhos, C., Modlich, U., Lewis, J., Harris, A., Bicknell, R. & Ish-Horowicz, D. (2001) Delta4, an endothelial specific notch ligand expressed at sites of physiological and tumor angiogenesis. *Differentiation* **69**, 135-144.

Maliekal, T. T., Bajaj, J., Giri, V., Subramanyam, D. & Krishna, S. (2008) The role of Notch signaling in human cervical cancer: implications for solid tumors. *Oncogene* **27**, 5110-5114.

Mauceri, H. J., Hanna, N. N., Beckett, M. A., Gorski, D. H., Staba, M. J., Stellato, K. A., Bigelow, K., Heimann, R., Gately, S., Dhanabal, M., Soff, G. A., Sukhatme, V. P., Kufe, D. W. & Weichselbaum, R. R. (1998) Combined effects of angiostatin and ionizing radiation in antitumour therapy. *Nature* **394**, 287-291.

Mazeron, R., Anderson, B., Supiot, S., Paris, F. & Deutsch, E. (2011) Current state of knowledge regarding the use of antiangiogenic agents with radiation therapy. *Cancer treatment reviews* **37**, 476-486.

McKenna, W. G., Muschel, R. J., Gupta, A., Hahn, S. & Bernhard, E. J. (2003) Signaling inhibition with radiation in colorectal cancer: clinical trials. *Semin Oncol* **30**, 56-67.

Meng, Y., Beckett, M. A., Liang, H., Mauceri, H. J., van Rooijen, N., Cohen, K. S. & Weichselbaum, R. R. (2010) Blockade of tumor necrosis factor alpha signaling in tumor-associated macrophages as a radiosensitizing strategy. *Cancer Res* **70**, 1534-1543.

Milano, J., McKay, J., Dagenais, C., Foster-Brown, L., Pognan, F., Gadient, R., Jacobs, R. T., Zacco, A., Greenberg, B. & Ciaccio, P. J. (2004) Modulation of notch processing by gamma-secretase inhibitors causes intestinal goblet cell metaplasia and induction of genes known to specify gut secretory lineage differentiation. *Toxicol Sci* **82**, 341-358.

Miyamoto, Y., Maitra, A., Ghosh, B., Zechner, U., Argani, P., Iacobuzio-Donahue, C. A., Sriuranpong, V., Iso, T., Meszoely, I. M., Wolfe, M. S., Hruban, R. H., Ball, D. W., Schmid, R. M. & Leach, S. D. (2003) Notch mediates TGF alpha-induced changes in epithelial differentiation during pancreatic tumorigenesis. *Cancer Cell* **3**, 565-576.

Mizugaki, H., Sakakibara-Konishi, J., Ikezawa, Y., Kikuchi, J., Kikuchi, E., Oizumi, S., Dang, T. P. & Nishimura, M. (2012) gamma-Secretase inhibitor enhances antitumour effect of radiation in Notch-expressing lung cancer. *Br J Cancer* **106**, 1953-1959.

Moeller, B. J., Richardson, R. A. & Dewhirst, M. W. (2007) Hypoxia and radiotherapy: opportunities for improved outcomes in cancer treatment. *Cancer Metastasis Rev* **26**, 241-248.

Mohr, O. L. (1919) Character Changes Caused by Mutation of an Entire Region of a Chromosome in Drosophila. *Genetics* **4**, 275-282.

Mueller, M. M. & Fusenig, N. E. (2004) Friends or foes - bipolar effects of the tumour stroma in cancer. *Nat Rev Cancer* **4**, 839-849.

Mullendore, M. E., Koorstra, J. B., Li, Y. M., Offerhaus, G. J., Fan, X., Henderson, C. M., Matsui, W., Eberhart, C. G., Maitra, A. & Feldmann, G. (2009) Ligand-dependent Notch signaling is involved in tumor initiation and tumor maintenance in pancreatic cancer. *Clin Cancer Res* **15**, 2291-2301.

Munoz-Chapuli, R., Quesada, A. R. & Angel Medina, M. (2004) Angiogenesis and signal transduction in endothelial cells. *Cellular and molecular life sciences : CMLS* **61**, 2224-2243.

Murata, R., Nishimura, Y. & Hiraoka, M. (1997) An antiangiogenic agent (TNP-470) inhibited reoxygenation during fractionated radiotherapy of murine mammary carcinoma. *Int J Radiat Oncol Biol Phys* **37**, 1107-1113.

Murdoch, C., Giannoudis, A. & Lewis, C. E. (2004) Mechanisms regulating the recruitment of macrophages into hypoxic areas of tumors and other ischemic tissues. *Blood* **104**, 2224-2234.

Nair, P., Somasundaram, K. & Krishna, S. (2003) Activated Notch1 inhibits p53-induced apoptosis and sustains transformation by human papillomavirus type 16 E6 and E7 oncogenes through a PI3K-PKB/Akt-dependent pathway. *J Virol* **77**, 7106-7112.

Nefedova, Y., Sullivan, D. M., Bolick, S. C., Dalton, W. S. & Gabrilovich, D. I. (2008) Inhibition of Notch signaling induces apoptosis of myeloma cells and enhances sensitivity to chemotherapy. *Blood* **111**, 2220-2229.

Nichols, J. T., Miyamoto, A., Olsen, S. L., D'Souza, B., Yao, C. & Weinmaster, G. (2007) DSL ligand endocytosis physically dissociates Notch1 heterodimers before activating proteolysis can occur. *J Cell Biol* **176**, 445-458.

Nickoloff, B. J., Osborne, B. A. & Miele, L. (2003) Notch signaling as a therapeutic target in cancer: a new approach to the development of cell fate modifying agents. *Oncogene* **22**, 6598-6608.

Nicolas, M., Wolfer, A., Raj, K., Kummer, J. A., Mill, P., van Noort, M., Hui, C. C., Clevers, H., Dotto, G. P. & Radtke, F. (2003) Notch1 functions as a tumor suppressor in mouse skin. *Nat Genet* **33**, 416-421.

Noguera-Troise, I., Daly, C., Papadopoulos, N. J., Coetzee, S., Boland, P., Gale, N. W., Lin, H. C., Yancopoulos, G. D. & Thurston, G. (2006) Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis. *Nature* **444**, 1032-1037.

Noseda, M., Chang, L., McLean, G., Grim, J. E., Clurman, B. E., Smith, L. L. & Karsan, A. (2004) Notch activation induces endothelial cell cycle arrest and participates in contact inhibition: role of p21Cip1 repression. *Molecular and cellular biology* **24**, 8813-8822.

Nozue, M., Isaka, N. & Fukao, K. (2001) Over-expression of vascular endothelial growth factor after preoperative radiation therapy for rectal cancer. *Oncology reports* **8**, 1247-1249.

Oda, T., Elkahloun, A. G., Pike, B. L., Okajima, K., Krantz, I. D., Genin, A., Piccoli, D. A., Meltzer, P. S., Spinner, N. B., Collins, F. S. & Chandrasekharappa, S. C. (1997) Mutations in the human Jagged1 gene are responsible for Alagille syndrome. *Nat Genet* **16**, 235-242.

Ohno, S., Ohno, Y., Suzuki, N., Kamei, T., Koike, K., Inagawa, H., Kohchi, C., Soma, G. & Inoue, M. (2004) Correlation of histological localization of tumor-associated macrophages with clinicopathological features in endometrial cancer. *Anticancer research* **24**, 3335-3342.

Okochi, M., Steiner, H., Fukumori, A., Tanii, H., Tomita, T., Tanaka, T., Iwatsubo, T., Kudo, T., Takeda, M. & Haass, C. (2002) Presenilins mediate a dual intramembranous gamma-secretase cleavage of Notch-1. *The EMBO journal* **21**, 5408-5416.

Parks, A. L., Klueg, K. M., Stout, J. R. & Muskavitch, M. A. (2000) Ligand endocytosis drives receptor dissociation and activation in the Notch pathway. *Development* **127**, 1373-1385.

Parks, A. L., Stout, J. R., Shepard, S. B., Klueg, K. M., Dos Santos, A. A., Parody, T. R., Vaskova, M. & Muskavitch, M. A. (2006) Structure-function analysis of delta trafficking, receptor binding and signaling in Drosophila. *Genetics* **174**, 1947-1961.

Parsons, D. W., Wang, T. L., Samuels, Y., Bardelli, A., Cummins, J. M., DeLong, L., Silliman, N., Ptak, J., Szabo, S., Willson, J. K., Markowitz, S., Kinzler, K. W., Vogelstein, B., Lengauer, C. & Velculescu, V. E. (2005) Colorectal cancer: mutations in a signalling pathway. *Nature* **436**, 792.

Phillips, T. M., McBride, W. H. & Pajonk, F. (2006) The response of CD24(-/low)/CD44+ breast cancer-initiating cells to radiation. *J Natl Cancer Inst* **98**, 1777-1785.

Phng, L. K. & Gerhardt, H. (2009) Angiogenesis: a team effort coordinated by notch. *Dev Cell* **16**, 196-208.

Pintar, A., De Biasio, A., Popovic, M., Ivanova, N. & Pongor, S. (2007) The intracellular region of Notch ligands: does the tail make the difference? *Biology direct* **2**, 19.

Poulson, D. F. (1937) Chromosomal Deficiencies and the Embryonic Development of Drosophila Melanogaster. *Proc Natl Acad Sci U S A* **23**, 133-137.

Purow, B. W., Haque, R. M., Noel, M. W., Su, Q., Burdick, M. J., Lee, J., Sundaresan, T., Pastorino, S., Park, J. K., Mikolaenko, I., Maric, D., Eberhart, C. G. & Fine, H. A. (2005) Expression of Notch-1 and its ligands, Delta-like-1 and Jagged-1, is critical for glioma cell survival and proliferation. *Cancer Res* **65**, 2353-2363.

Qi, R., An, H., Yu, Y., Zhang, M., Liu, S., Xu, H., Guo, Z., Cheng, T. & Cao, X. (2003) Notch1 signaling inhibits growth of human hepatocellular carcinoma through induction of cell cycle arrest and apoptosis. *Cancer Res* **63**, 8323-8329.

Qiao, L. & Wong, B. C. (2009) Role of Notch signaling in colorectal cancer. *Carcinogenesis* **30**, 1979-1986.

Rangarajan, A., Talora, C., Okuyama, R., Nicolas, M., Mammucari, C., Oh, H., Aster, J. C., Krishna, S., Metzger, D., Chambon, P., Miele, L., Aguet, M., Radtke, F. & Dotto, G. P. (2001) Notch signaling is a direct determinant of keratinocyte growth arrest and entry into differentiation. *The EMBO journal* **20**, 3427-3436.

Rasul, S., Balasubramanian, R., Filipovic, A., Slade, M. J., Yague, E. & Coombes, R. C. (2009) Inhibition of gamma-secretase induces G2/M arrest and triggers apoptosis in breast cancer cells. *Br J Cancer* **100**, 1879-1888.

Ray, W. J., Yao, M., Mumm, J., Schroeter, E. H., Saftig, P., Wolfe, M., Selkoe, D. J., Kopan, R. & Goate, A. M. (1999) Cell surface presenilin-1 participates in the gamma-secretase-like proteolysis of Notch. *The Journal of biological chemistry* **274**, 36801-36807.

Rebay, I., Fleming, R. J., Fehon, R. G., Cherbas, L., Cherbas, P. & Artavanis-Tsakonas, S. (1991) Specific EGF repeats of Notch mediate interactions with Delta and Serrate: implications for Notch as a multifunctional receptor. *Cell* **67**, 687-699.

Reedijk, M., Odorcic, S., Chang, L., Zhang, H., Miller, N., McCready, D. R., Lockwood, G. & Egan, S. E. (2005) High-level coexpression of JAG1 and NOTCH1 is observed in human breast cancer and is associated with poor overall survival. *Cancer Res* **65**, 8530-8537.

Reedijk, M., Odorcic, S., Zhang, H., Chetty, R., Tennert, C., Dickson, B. C., Lockwood, G., Gallinger, S. & Egan, S. E. (2008) Activation of Notch signaling in human colon adenocarcinoma. *Int J Oncol* **33**, 1223-1229.

Ridgway, J., Zhang, G., Wu, Y., Stawicki, S., Liang, W. C., Chanthery, Y., Kowalski, J., Watts, R. J., Callahan, C., Kasman, I., Singh, M., Chien, M., Tan, C., Hongo, J. A., de Sauvage, F., Plowman, G. & Yan, M. (2006) Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis. *Nature* **444**, 1083-1087.

Ringborg, U., Bergqvist, D., Brorsson, B., Cavallin-Stahl, E., Ceberg, J., Einhorn, N., Frodin, J. E., Jarhult, J., Lamnevik, G., Lindholm, C., Littbrand, B., Norlund, A., Nylen, U., Rosen, M., Svensson, H. & Moller, T. R. (2003) The Swedish Council on Technology Assessment in Health Care (SBU) systematic overview of radiotherapy for cancer including a prospective survey of radiotherapy practice in Sweden 2001--summary and conclusions. *Acta Oncol* **42**, 357-365.

Risau, W. & Flamme, I. (1995) Vasculogenesis. *Annual review of cell and developmental biology* **11**, 73-91.

Robey, E. (1997) Notch in vertebrates. *Curr Opin Genet Dev* **7**, 551-557.

Roehl, H., Bosenberg, M., Blelloch, R. & Kimble, J. (1996) Roles of the RAM and ANK domains in signaling by the C. elegans GLP-1 receptor. *The EMBO journal* **15**, 7002-7012.

Rogers, S., Wells, R. & Rechsteiner, M. (1986) Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. *Science* **234**, 364-368.

Ruifrok, A. C. & Johnston, D. A. (2001) Quantification of histochemical staining by color deconvolution. *Anal Quant Cytol Histol* **23**, 291-299.

Santagata, S., Demichelis, F., Riva, A., Varambally, S., Hofer, M. D., Kutok, J. L., Kim, R., Tang, J., Montie, J. E., Chinnaiyan, A. M., Rubin, M. A. & Aster, J. C. (2004) JAGGED1 expression is associated with prostate cancer metastasis and recurrence. *Cancer Res* **64**, 6854-6857.

Sasai, Y., Kageyama, R., Tagawa, Y., Shigemoto, R. & Nakanishi, S. (1992) Two mammalian helix-loop-helix factors structurally related to Drosophila hairy and Enhancer of split. *Genes Dev* **6**, 2620-2634.

Scehnet, J. S., Jiang, W., Kumar, S. R., Krasnoperov, V., Trindade, A., Benedito, R., Djokovic, D., Borges, C., Ley, E. J., Duarte, A. & Gill, P. S. (2007) Inhibition of Dll4-mediated signaling induces proliferation of immature vessels and results in poor tissue perfusion. *Blood* **109**, 4753-4760.

Schadler, K. L., Zweidler-McKay, P. A., Guan, H. & Kleinerman, E. S. (2010) Delta-like ligand 4 plays a critical role in pericyte/vascular smooth muscle cell formation during vasculogenesis and tumor vessel expansion in Ewing's sarcoma. *Clin Cancer Res* **16**, 848-856.

Scharpfenecker, M., Kruse, J. J., Sprong, D., Russell, N. S., Ten Dijke, P. & Stewart, F. A. (2009) Ionizing radiation shifts the PAI-1/ID-1 balance and activates notch signaling in endothelial cells. *Int J Radiat Oncol Biol Phys* **73**, 506-513.

Schroeter, E. H., Kisslinger, J. A. & Kopan, R. (1998) Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. *Nature* **393**, 382-386.

Segarra, M., Williams, C. K., Sierra Mde, L., Bernardo, M., McCormick, P. J., Maric, D., Regino, C., Choyke, P. & Tosato, G. (2008) Dll4 activation of Notch signaling reduces tumor vascularity and inhibits tumor growth. *Blood* **112**, 1904-1911.

Shimizu, K., Chiba, S., Kumano, K., Hosoya, N., Takahashi, T., Kanda, Y., Hamada, Y., Yazaki, Y. & Hirai, H. (1999) Mouse jagged1 physically interacts with notch2 and other notch receptors. Assessment by quantitative methods. *The Journal of biological chemistry* **274**, 32961-32969.

Shou, J., Ross, S., Koeppen, H., de Sauvage, F. J. & Gao, W. Q. (2001) Dynamics of notch expression during murine prostate development and tumorigenesis. *Cancer Res* **61**, 7291-7297.

Siekmann, A. F. & Lawson, N. D. (2007) Notch signalling limits angiogenic cell behaviour in developing zebrafish arteries. *Nature* **445**, 781-784.

Sofia Vala, I., Martins, L. R., Imaizumi, N., Nunes, R. J., Rino, J., Kuonen, F., Carvalho, L. M., Ruegg, C., Grillo, I. M., Barata, J. T., Mareel, M. & Santos, S. C. (2010) Low doses of ionizing radiation promote tumor growth and metastasis by enhancing angiogenesis. *PloS one* **5**, e11222.

Solberg, T. D., Nearman, J., Mullins, J., Li, S. & Baranowska-Kortylewicz, J. (2008) Correlation between tumor growth delay and expression of cancer and host VEGF, VEGFR2, and osteopontin in response to radiotherapy. *Int J Radiat Oncol Biol Phys* **72**, 918-926.

Song, S., Maru, D. M., Ajani, J. A., Chan, C. H., Honjo, S., Lin, H. K., Correa, A., Hofstetter, W. L., Davila, M., Stroehlein, J. & Mishra, L. (2013) Loss of TGF-beta adaptor beta2SP activates notch signaling and SOX9 expression in esophageal adenocarcinoma. *Cancer Res* **73**, 2159-2169.

Sriuranpong, V., Borges, M. W., Ravi, R. K., Arnold, D. R., Nelkin, B. D., Baylin, S. B. & Ball, D. W. (2001) Notch signaling induces cell cycle arrest in small cell lung cancer cells. *Cancer Res* **61**, 3200-3205.

Sriuranpong, V., Borges, M. W., Strock, C. L., Nakakura, E. K., Watkins, D. N., Blaumueller, C. M., Nelkin, B. D. & Ball, D. W. (2002) Notch signaling induces rapid degradation of achaete-scute homolog 1. *Molecular and cellular biology* **22**, 3129-3139.

Stone, J., Itin, A., Alon, T., Pe'er, J., Gnessin, H., Chan-Ling, T. & Keshet, E. (1995) Development of retinal vasculature is mediated by hypoxia-induced vascular endothelial growth factor (VEGF) expression by neuroglia. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **15**, 4738-4747.

Suchting, S., Freitas, C., le Noble, F., Benedito, R., Breant, C., Duarte, A. & Eichmann, A. (2007) The Notch ligand Delta-like 4 negatively regulates endothelial tip cell formation and vessel branching. *Proc Natl Acad Sci U S A* **104**, 3225-3230.

Takeichi, N., Yanagisawa, S., Kaneyama, T., Yagita, H., Jin, Y. H., Kim, B. S. & Koh, C. S. (2010) Ameliorating effects of anti-Dll4 mAb on Theiler's murine encephalomyelitis virus-induced demyelinating disease. *International immunology* **22**, 729-738.

Tammam, J., Ware, C., Efferson, C., O'Neil, J., Rao, S., Qu, X., Gorenstein, J., Angagaw, M., Kim, H., Kenific, C., Kunii, K., Leach, K. J., Nikov, G., Zhao, J., Dai, X., Hardwick, J., Scott, M., Winter, C., Bristow, L., Elbi, C., Reilly, J. F., Look, T., Draetta, G., Van der Ploeg, L., Kohl, N. E., Strack, P. R. & Majumder, P. K. (2009) Down-regulation of the Notch pathway mediated by a gamma-secretase inhibitor induces anti-tumour effects in mouse models of T-cell leukaemia. *Br J Pharmacol* **158**, 1183-1195.

Tammela, T., Zarkada, G., Wallgard, E., Murtomaki, A., Suchting, S., Wirzenius, M., Waltari, M., Hellstrom, M., Schomber, T., Peltonen, R., Freitas, C., Duarte, A., Isoniemi, H., Laakkonen, P., Christofori, G., Yla-Herttuala, S., Shibuya, M., Pytowski, B., Eichmann, A., Betsholtz, C. & Alitalo, K. (2008) Blocking VEGFR-3 suppresses angiogenic sprouting and vascular network formation. *Nature* **454**, 656-660.

Tamura, K., Taniguchi, Y., Minoguchi, S., Sakai, T., Tun, T., Furukawa, T. & Honjo, T. (1995) Physical interaction between a novel domain of the receptor Notch and the transcription factor RBP-J kappa/Su(H). *Current biology : CB* **5**, 1416-1423.

Tani, S., Kurooka, H., Aoki, T., Hashimoto, N. & Honjo, T. (2001) The N- and C-terminal regions of RBP-J interact with the ankyrin repeats of Notch1 RAMIC to activate transcription. *Nucleic acids research* **29**, 1373-1380.

Taylor, K. L., Henderson, A. M. & Hughes, C. C. (2002) Notch activation during endothelial cell network formation in vitro targets the basic HLH transcription factor HESR-1 and downregulates VEGFR-2/KDR expression. *Microvasc Res* **64**, 372-383.

Thelu, J., Rossio, P. & Favier, B. (2002) Notch signalling is linked to epidermal cell differentiation level in basal cell carcinoma, psoriasis and wound healing. *BMC dermatology* **2**, 7.

Theys, J., Yahyanejad, S., Habets, R., Span, P., Dubois, L., Paesmans, K., Kattenbeld, B., Cleutjens, J., Groot, A. J., Schuurbiers, O. C., Lambin, P., Bussink, J. & Vooijs, M. (2013) High NOTCH activity induces radiation resistance in non small cell lung cancer. *Radiother Oncol*.

Thurston, G., Noguera-Troise, I. & Yancopoulos, G. D. (2007) The Delta paradox: DLL4 blockade leads to more tumour vessels but less tumour growth. *Nat Rev Cancer* **7**, 327-331.

Tozer, G. M., Kanthou, C. & Baguley, B. C. (2005) Disrupting tumour blood vessels. *Nat Rev Cancer* **5**, 423-435.

Truman, J. P., Garcia-Barros, M., Kaag, M., Hambardzumyan, D., Stancevic, B., Chan, M., Fuks, Z., Kolesnick, R. & Haimovitz-Friedman, A. (2010) Endothelial membrane remodeling is obligate for anti-angiogenic radiosensitization during tumor radiosurgery. *PloS one* **5**, e12310.

Turnpenny, P. D., Whittock, N., Duncan, J., Dunwoodie, S., Kusumi, K. & Ellard, S. (2003) Novel mutations in DLL3, a somitogenesis gene encoding a ligand for the Notch signalling pathway, cause a consistent pattern of abnormal vertebral segmentation in spondylocostal dysostosis. *J Med Genet* **40**, 333-339.

Urs, S., Roudabush, A., O'Neill, C. F., Pinz, I., Prudovsky, I., Kacer, D., Tang, Y., Liaw, L. & Small, D. (2008) Soluble forms of the Notch ligands Delta1 and Jagged1 promote in vivo tumorigenicity in NIH3T3 fibroblasts with distinct phenotypes. *Am J Pathol* **173**, 865-878.

van Es, J. H., van Gijn, M. E., Riccio, O., van den Born, M., Vooijs, M., Begthel, H., Cozijnsen, M., Robine, S., Winton, D. J., Radtke, F. & Clevers, H. (2005) Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. *Nature* **435**, 959-963.

Veenendaal, L. M., Kranenburg, O., Smakman, N., Klomp, A., Borel Rinkes, I. H. & van Diest, P. J. (2008) Differential Notch and TGFbeta signaling in primary colorectal tumors

and their corresponding metastases. *Cellular oncology : the official journal of the International Society for Cellular Oncology* **30**, 1-11.

Vincenti, S., Brillante, N., Lanza, V., Bozzoni, I., Presutti, C., Chiani, F., Etna, M. P. & Negri, R. (2011) HUVEC respond to radiation by inducing the expression of pro-angiogenic microRNAs. *Radiation research* **175**, 535-546.

Vitt, U. A., Hsu, S. Y. & Hsueh, A. J. (2001) Evolution and classification of cystine knot-containing hormones and related extracellular signaling molecules. *Mol Endocrinol* **15**, 681-694.

Wachsberger, P., Burd, R. & Dicker, A. P. (2003) Tumor response to ionizing radiation combined with antiangiogenesis or vascular targeting agents: exploring mechanisms of interaction. *Clin Cancer Res* **9**, 1957-1971.

Wang, J., Wakeman, T. P., Lathia, J. D., Hjelmeland, A. B., Wang, X. F., White, R. R., Rich, J. N. & Sullenger, B. A. (2010) Notch promotes radioresistance of glioma stem cells. *Stem Cells* **28**, 17-28.

Wang, Z., Banerjee, S., Li, Y., Rahman, K. M., Zhang, Y. & Sarkar, F. H. (2006) Down-regulation of notch-1 inhibits invasion by inactivation of nuclear factor-kappaB, vascular endothelial growth factor, and matrix metalloproteinase-9 in pancreatic cancer cells. *Cancer Res* **66**, 2778-2784.

Wang, Z., Li, Y., Banerjee, S., Kong, D., Ahmad, A., Nogueira, V., Hay, N. & Sarkar, F. H. (2010) Down-regulation of Notch-1 and Jagged-1 inhibits prostate cancer cell growth, migration and invasion, and induces apoptosis via inactivation of Akt, mTOR, and NF-kappaB signaling pathways. *Journal of cellular biochemistry* **109**, 726-736.

Wang, Z., Li, Y. & Sarkar, F. H. (2010) Notch Signaling Proteins: Legitimate Targets for Cancer Therapy. *Curr Protein Pept Sci*.

Wei, P., Walls, M., Qiu, M., Ding, R., Denlinger, R. H., Wong, A., Tsaparikos, K., Jani, J. P., Hosea, N., Sands, M., Randolph, S. & Smeal, T. (2010) Evaluation of selective gamma-secretase inhibitor PF-03084014 for its antitumor efficacy and gastrointestinal safety to guide optimal clinical trial design. *Molecular cancer therapeutics* **9**, 1618-1628.

Weng, A. P., Ferrando, A. A., Lee, W., Morris, J. P. t., Silverman, L. B., Sanchez-Irizarry, C., Blacklow, S. C., Look, A. T. & Aster, J. C. (2004) Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. *Science* **306**, 269-271.

Weng, A. P., Millholland, J. M., Yashiro-Ohtani, Y., Arcangeli, M. L., Lau, A., Wai, C., Del Bianco, C., Rodriguez, C. G., Sai, H., Tobias, J., Li, Y., Wolfe, M. S., Shachaf, C., Felsher, D., Blacklow, S. C., Pear, W. S. & Aster, J. C. (2006) c-Myc is an important direct target of Notch1 in T-cell acute lymphoblastic leukemia/lymphoma. *Genes Dev* **20**, 2096-2109.

Weng, A. P., Nam, Y., Wolfe, M. S., Pear, W. S., Griffin, J. D., Blacklow, S. C. & Aster, J. C. (2003) Growth suppression of pre-T acute lymphoblastic leukemia cells by inhibition of notch signaling. *Molecular and cellular biology* **23**, 655-664.

Wharton, K. A., Johansen, K. M., Xu, T. & Artavanis-Tsakonas, S. (1985) Nucleotide sequence from the neurogenic locus notch implies a gene product that shares homology with proteins containing EGF-like repeats. *Cell* **43**, 567-581.

Williams, C. K., Li, J. L., Murga, M., Harris, A. L. & Tosato, G. (2006) Up-regulation of the Notch ligand Delta-like 4 inhibits VEGF-induced endothelial cell function. *Blood* **107**, 931-939.

Wu, K., Xu, L., Zhang, L., Lin, Z. & Hou, J. (2011) High Jagged1 expression predicts poor outcome in clear cell renal cell carcinoma. *Japanese journal of clinical oncology* **41**, 411-416.

Wu, L., Aster, J. C., Blacklow, S. C., Lake, R., Artavanis-Tsakonas, S. & Griffin, J. D. (2000) MAML1, a human homologue of Drosophila mastermind, is a transcriptional co-activator for NOTCH receptors. *Nat Genet* **26**, 484-489.

Wu, L., Sun, T., Kobayashi, K., Gao, P. & Griffin, J. D. (2002) Identification of a family of mastermind-like transcriptional coactivators for mammalian notch receptors. *Molecular and cellular biology* **22**, 7688-7700.

Wu, Y., Cain-Hom, C., Choy, L., Hagenbeek, T. J., de Leon, G. P., Chen, Y., Finkle, D., Venook, R., Wu, X., Ridgway, J., Schahin-Reed, D., Dow, G. J., Shelton, A., Stawicki, S., Watts, R. J., Zhang, J., Choy, R., Howard, P., Kadyk, L., Yan, M., Zha, J., Callahan, C. A., Hymowitz, S. G. & Siebel, C. W. (2010) Therapeutic antibody targeting of individual Notch receptors. *Nature* **464**, 1052-1057.

Yan, M., Callahan, C. A., Beyer, J. C., Allamneni, K. P., Zhang, G., Ridgway, J. B., Niessen, K. & Plowman, G. D. (2010) Chronic DLL4 blockade induces vascular neoplasms. *Nature* **463**, E6-7.

Yang, L. T., Nichols, J. T., Yao, C., Manilay, J. O., Robey, E. A. & Weinmaster, G. (2005) Fringe glycosyltransferases differentially modulate Notch1 proteolysis induced by Delta1 and Jagged1. *Molecular biology of the cell* **16**, 927-942.

Yen, W. C., Fischer, M. M., Hynes, M., Wu, J., Kim, E., Beviglia, L., Yeung, V. P., Song, X., Kapoun, A. M., Lewicki, J., Gurney, A., Simeone, D. M. & Hoey, T. (2012) Anti-DLL4 has

broad spectrum activity in pancreatic cancer dependent on targeting DLL4-Notch signaling in both tumor and vasculature cells. *Clin Cancer Res* **18**, 5374-5386.

Yin, L., Velazquez, O. C. & Liu, Z. J. (2010) Notch signaling: emerging molecular targets for cancer therapy. *Biochem Pharmacol* **80**, 690-701.

Yu, S., Zhang, R., Liu, F., Hu, H. & Wang, H. (2011) Down-regulation of Notch signaling by a gamma-secretase inhibitor enhances the radiosensitivity of nasopharyngeal carcinoma cells. *Oncology reports* **26**, 1323-1328.

Zeng, Q., Li, S., Chepeha, D. B., Giordano, T. J., Li, J., Zhang, H., Polverini, P. J., Nor, J., Kitajewski, J. & Wang, C. Y. (2005) Crosstalk between tumor and endothelial cells promotes tumor angiogenesis by MAPK activation of Notch signaling. *Cancer Cell* **8**, 13-23.

Zhang, J., Fukuhara, S., Sako, K., Takenouchi, T., Kitani, H., Kume, T., Koh, G. Y. & Mochizuki, N. (2011) Angiopoietin-1/Tie2 signal augments basal Notch signal controlling vascular quiescence by inducing delta-like 4 expression through AKT-mediated activation of beta-catenin. *The Journal of biological chemistry* **286**, 8055-8066.

Zhang, J. X., Cai, M. B., Wang, X. P., Duan, L. P., Shao, Q., Tong, Z. T., Liao, D. Z., Li, Y. Y., Huang, M. Y., Zeng, Y. X. & Shao, J. Y. (2013) Elevated DLL4 expression is correlated with VEGF and predicts poor prognosis of nasopharyngeal carcinoma. *Medical oncology* **30**, 390.

Zhou, S., Fujimuro, M., Hsieh, J. J., Chen, L., Miyamoto, A., Weinmaster, G. & Hayward, S. D. (2000) SKIP, a CBF1-associated protein, interacts with the ankyrin repeat domain of NotchIC To facilitate NotchIC function. *Molecular and cellular biology* **20**, 2400-2410.

Zhu, H., Zhou, X., Redfield, S., Lewin, J. & Miele, L. (2013) Elevated Jagged-1 and Notch-1 expression in high grade and metastatic prostate cancers. *American journal of translational research* **5**, 368-378.

Zips, D., Krause, M., Hessel, F., Westphal, J., Bruchner, K., Eicheler, W., Dorfler, A., Grenman, R., Petersen, C., Haberey, M. & Baumann, M. (2003) Experimental study on different combination schedules of VEGF-receptor inhibitor PTK787/ZK222584 and fractionated irradiation. *Anticancer research* **23**, 3869-3876.