The Functional and Biological Implications of EphB4 Receptor Overexpression and Knockout in Colorectal Cancer

ELIF KADIFE

THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Principal Supervisor: Associate Professor Kulmira Nurgali

Associate Supervisor: Dr. Paul V. Senior

Associate Supervisor: Dr. Rodney B. Luwor

Victoria University, Melbourne, Australia

2018

Abstract

Colorectal cancer (CRC) is one of the most frequently diagnosed cancers in Australia and globally. Early detection and intervention is vital for the longevity of patients with any cancer, however, this appears to be most challenging with CRC, as it is largely asymptomatic. For this reason, most cases are not diagnosed until the cancer has metastasised, primarily to the liver. At this late stage of diagnosis, 5-year patient's survival is predicted to be less than 10%. However, even when CRC is diagnosed and treated in the initial stages of neoplastic growth, high recurrence rates in patients still present as a serious issue. The problems associated with treatment and recurrence raise the need to identify molecular targets, so that specific and aggressive therapeutic interventions may be designed and developed. One such potential target is the **e**rythropoietin-**p**roducing **h**epatocellular B4 (EphB4) receptor.

The Ephs constitute the largest family of tyrosine kinase receptors. The activation of Eph receptors is achieved through association with their corresponding cell-bound 'Eph receptor interacting' (Ephrin) ligands. The signalling by the Eph receptors and their membrane-bound ligands, the Ephrins, is unique among the tyrosine kinases as both the receptor and ligand are found on the cell surface. Bidirectional interaction results in the phenomena of 'forward' signalling via the Eph receptor carrying cells and 'reverse' signalling in those cells expressing the Ephrin ligands. Several members of the Eph receptor receptor family, including EphB4, have been implicated with progression of many different types of cancer. However, EphB4 receptor's contribution towards CRC yields the most

contradictory findings. Some studies suggest that EphB4 is upregulated in late and metastatic stages of CRC, while others argue that EphB4 expression is often silenced in the progressive state of the disease. Due to the promising results achieved in other types of cancers, it is important to elucidate the role of EphB4 receptors in CRC in order to develop more specific and aggressive cancer therapies.

The overall aim of this study is to elucidate the influence of EphB4 receptor expression on the development and progression of CRC. To achieve this, we used modified derivatives of multiple human and a mouse CRC cell line in *in vitro* and *in vivo* experiments. *In vitro* experiments were utilised to study effects of EphB4 overexpression and knockout on proliferative aptitude, migratory and invasive abilities of human and mouse CRC cells. *In vivo* subcutaneous models of CRC were used to evaluate the ability of high, low and knockdown of EphB4 receptor expression to influence morphological changes, rate of growth, vascularization and tumour-stromal interactions. The time course and rate of metastasis of CRC cells to the liver were studied in *in vivo* orthotopic and intra-splenic metastasis models. The level of *EPHB4* and *EPHRINB2* expression was investigated using databases to determine their correlation with survival and disease-free outcomes of CRC patients.

The results of this study provide evidence that high EphB4 receptor expression significantly increases the rate of proliferation, migration and invasion of CRC cells *in vitro*, and enhances tumour growth *in vivo* due to enhanced vascularisation. Knockout of EphB4 expression reduces these effects. EphrinB2 appears to inhibit proliferation in cells overexpressing EphB4 and its expression correlates with poor patient outcome.

Candidate Declaration

I, Elif Kadife, declare that the PhD thesis entitled 'The Functional and Biological Implications of EphB4 Receptor Overexpression and Knockout in Colorectal Cancer' is no more than 100,000 words in length including quotes, exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

Signature 

Date: 01/04/2018

Declarations of Contributed Work

CHAPTER 2: Dr. Paul Senior has contributed to designing and engineering of plasmids. The fluorescent EphB4 and EphrinB2 knockout cells were generated by Dr. Paul Senior.

Acknowledgements

I wish to thank all those who helped me throughout my PhD, without you I could not have completed it. Being a hugely sentimental person, I could write pages about each of you, so I will do my best to keep it short.

I would like to express my gratitude to **A/Prof Kulmira Nurgali**- you have spent countless hours reading my work to ensure I achieve my best. You have contributed enormously to developing my scientific writing and a huge support in getting my research published. Thank you for ongoing engagement and support throughout these years, knowing I could rely on you always made a world of difference to me. I admire your diligence and your commitment to research is truly inspiring. You make personal sacrifices to support the work of your students and want the absolute best for us, which is something I really truly appreciate.

I would like to thank, **Dr. Paul V Senior**- you have provided me with extensive training, guidance and assistance throughout these years. I have learnt so much from you, during the time that I had the privilege of being your student. Being inspired by you, those many years ago, was one of the main reasons I decided to pursue a career in research. As an undergraduate student, you introduced me to the wonderful and complex world of Eph receptors, it used to take me a week just to read and understand one paper. Now I think about how far I have come, and I feel thankful to you, because you were always there for me, ready to share your knowledge and experience to develop my research skills.

I would also like to thank my associate supervisor, **Dr. Rodney Luwor-** you were always encouraging and supportive. Thank you for taking me on so late into my

PhD, without your intellectual and reagent contributions, it would not have been possible to complete some sections of this PhD. Becoming your student, led me to new opportunities and advanced my skill set, for that I will always be grateful. I admire your problem-solving skills and focus, which has helped me when I needed it most.

I would like to specifically acknowledge the following individuals, for going out of their way to try and help me, without expecting anything in return. Firstly, I would like to say a big thank you to renowned Eph receptor expert, **Dr. Peter Janes** (Monash University) for taking time out to correspond and meet with me, giving me feedback about my results and for generously gifting me antibodies. I will always tell everyone about your kindness. Your help came at a very crucial time and it contributed to shaping my PhD. To **Prof. Steven Tuck Foo Chan** (Western Health)- you are one of the nicest people I have met, thank you for helping me with applications, finding examiners and statistic related questions. To **Dr. Stanley S Stylli** (Melbourne University)- I met you in the final months of my PhD, yet you have given me your time and reagents, your heartening chats always helped to turn my day around. To **Mrs. Varsha Lal** and **A/Prof Alan Hayes**- thank you for ensuring I have space to work in the lab, also for always being fair and accommodating.

I would like to acknowledge the KN lab members, **Valentina Jovanoska** (lab mum), **Dr. Suh Youn Ko, Dr. Vanesa Stojanovska, Dr. Rachel McQuade, Elizabeth Donald, Rhian Stavely, Ainsley Robinson, Sarah Miller**- PhD is long and difficult, thank you for making it a more pleasant journey with the countless laughs, supportive chats and for sharing your reagents with me. Also, all students

and staff at WCHRE for the ongoing encouragement, advice and willingness to help.

Last, but not the least, I would like to thank my family and loved ones for being there for me throughout it all. To my mother **Emine Kadife**, and my father **Mutlu Kadife**- I could never repay you and all the sacrifices you have made for me. Thank you for all that you have done to ensure I achieve my goals, for emotionally supporting me when I felt I could not possibly endure. I dedicate this thesis to you, without your support none of this would be possible. I am truly lucky to have such loving, caring and selfless parents, who want nothing more than seeing me happy. To my fiancé and best friend, **Troy Gosetti**- you are one of the best things this PhD has brought to me. Thank you for always encouraging me to persevere, being my shoulder to cry on, making me see possibility, giving me hope and for putting a smile on my face. Thank you for driving me to the lab late at night and on weekends and waiting for me for hours while I did experiments, thank you for buying snacks to put in my car and preparing lunches to ensure I eat when I was too busy to, while driving between labs. Thank you for understanding my stress, long working days. I do not know how I would have survived this journey without you.

Publications

Publications from this Thesis

Elif Kadife, Thomas Michael Benjamin Ware, Rodney Brian Luwor, Steven Tuck Foo Chan, Kulmira Nurgali & Paul Vincent Senior (2018) Effects of EphB4 receptor expression on colorectal cancer cells, tumour growth, vascularization and composition. *Acta Oncologica*, 1-14. DOI: 10.1080/0284186X.2018.1429650.

Oral Presentations

1. **Kadife E**, Senior P, Nurgali K 2014. The role of EphB4 receptors in the progression and metastasis of colorectal cancers. *The Annual Victoria University HDR Student Conference*, Melbourne, Australia.
2. **Kadife E**, Senior P, Nurgali K 2014. The role of EphB4 receptors in the progression and metastasis of colorectal cancers. *Western Health Kendall Francis Presentation*, Melbourne, Australia.
3. **Kadife E**, Senior P, Nurgali K 2015. The role of EphB4 receptors in the progression and metastasis of colorectal cancers. *ASMR Victorian Student Research Symposium*, Melbourne, Australia.
4. **Kadife E**, Senior P, Nurgali K 2015. The role of EphB4 receptors in the progression and metastasis of colorectal cancers. *Victoria University College of Health and Biomedicine Post-Graduate Research Conference*, Melbourne, Australia.

Poster Presentations

1. **Kadife E**, Senior P, Nurgali K, 2013. The role of EphB4 receptors in the progression and metastasis of colorectal cancers. *Melbourne Protein Group Student Symposium*, Melbourne, Australia.
2. **Kadife E**, Ware TMB, Luwor RL, Chan STF, Nurgali K, Senior PV 2016. The role of EphB4 receptors in colorectal cancer. *Lorne Cancer Conference*, Melbourne, Australia.

Research Activity

Melbourne Protein Group Student Symposium 2013 - Student volunteer

Australian Society for Medical Research - Victorian Student Research Symposium 2016 - Organising subcommittee member

Table of Contents

List of Figures

List of Tables

List of Abbreviations

ADAM	A disintegrin and metalloproteinase
Akt	Protein kinase B
APC	Adenomatous polyposis coli
ATP	Adenosine triphosphate
BRAF	B-raf proto-oncogene, serine/threonine kinase
CBP	Cyclic adenosine monophosphate response element-binding protein
Cdc42	Cell division cycle 42
cDNA	Complementary DNA
CDK4	Cyclin-dependent kinase
CI	Cellular impedance
CIN	Chromosomal instability
CK1α	Serine/threonine kinases casein kinase 1 alpha
CRC	Colorectal cancer
CRD	Cysteine rich domain
Creb	cAMP response element-binding protein
CRISPR	Clustered regularly interspaced short palindromic repeats
DAB	3-3´-diaminobenzidine chromogen
DAPI	4′,6-diamidino-2-phenylindole
DII4	Delta-like ligand 4
DNA	Deoxyribonucleic acid
DSH	Dishevelled protein
EEA-1	Early endosome antigen-1
EC	Endothelial cell
EDTA	Ethylenediaminetetraacetic acid
EGFR	Endothelial growth factor receptor
EEM-1	Early endosome marker-1
Eph	Erythropoietin-producing hepatocellular receptor
Ephexin	Eph-interacting exchange factor
Ephrin	Eph interacting ligand

Erk	Extracellular signal regulated kinases
EV	Empty vector
Fc	The fragment crystallizable region of antibody
FN3	Fibronectin-3 domain
GDP	Guanine diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GPI	Glycosylphosphotidylinositol
GSK3	Glycogen synthase kinase 3
GTP	Guanosine-5'-triphosphate
HCL	Hydrochloric acid
HDR	Homology directed repair
HP	Hydrophobic
HUVEC	Human umbilical vein cells
IgG	Immunoglobulin G
ISC	Intestinal stem cells
JAK	Janus kinase
JM	Juxtamembrane domain
KO	Knockout
KRAS	K-ras proto-oncogene, GTPase
KSR1	Kinase suppressor of Ras-1
LEF	Lymphoid enhancing factor
LBD	Ligand binding domain
LOH	Loss of heterozygosity
LRP	Low-density lipoprotein-related protein
MAPK	Mitogen-activated protein kinase
MLH	mutL homologues
MMP	Matrix metalloproteinase
MMR	Mismatch repair
MO	Morpholino oligonucleotides
mRNA	Messenger RNA
MSH	MutS heterodimer proteins
MSI	Microsatellite instability

mRNA	Messenger ribonucleic acid
MTT	2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide
NADH	Nicotinamide adenine dinucleotide
NHEJ	Non-homologous end joining
PAM	Protospacer motif
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene-glycol
PDZ	Post-synaptic density protein/disc large/zona occludens domain
PI3K	Phosphoinositide 3-kinases
precrRNA	Precursor crRNA
Rac	Ras-related C3 botulinum toxin substrate
Ras-MAP	Ras- mitogen activated protein kinase
RBD	Receptor binding domain
RFP	Red fluorescent protein
RhoA	Ras homolog gene family, member A
RNA	Ribonucleic acid
RNAi	RNA interference
SAM	Sterile α-motif domain
SCID	Severe combined immunodeficiency
SDF-1	Stromal cell-derived factor-1
SFM	Serum-free medium
shRNA	Small hairpin RNA
SH2	Src homology 2
siRNA	Small interfering RNA
Src	Sarcoma proto-oncogene, non-receptor tyrosine kinase
STAT3	Signal transducer and activator of transcription 3
TCF	T-cell factor
TMD	Transmembrane domain
tracrRNA	Trans-activating RNA
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
VEGF	Vascular endothelial growth factor

Wnt	Wingless-type
WST-1	Water soluble tetrazolium (sodium 5-(2,4-disulfophenyl)-2-(4-iodophenyl)-3-(4-nitrophenyl)-2H-tetrazolium inner salt)
WT	Wild-type

CHAPTER 1

Literature Review

1.1 Introduction

Colorectal cancer (CRC) is one of the most frequently diagnosed cancers in Australia and globally. CRC makes up approximately 10% of all the cancers diagnosed worldwide, with over 600,000 new cases being identified each year (Ferlay et al. 2013; Gandomani et al. 2017). Highest rates of incidence are observed in Australia and New Zealand. It is estimated that of the 17,004 new cases diagnosed in 2017, approximately 9,000 of the affected were male and 8,000 were female (AIHW 2016; AIHW 2017). These statistics make CRC the second most commonly diagnosed type of cancer. Risk of developing CRC increases with age and is usually observed in individuals over the age of 50, with death rates tripling between the ages of 50-85 (ABS 2015). For this reason, national bowel cancer screening programs have been introduced for individuals who are over the age of 50 in Australia (AGDHA 2005). With the introduction of these and other improved screening programs, the number of CRC diagnosed cases have increased. In the period between 2002 and 2012, CRC reported cases in men have increased from 6,902 to 8,239 and in women from 5,641 to 6,718 cases (AIHW 2016; AIHW 2017). Despite the increasing number of CRC cases, mortality rates have since fallen slightly due to early diagnosis and screening (Arnold et al. 2017). Nonetheless, it is the second most common cause of cancer related in Australia (AIHW 2017). Early detection and intervention is vital for the longevity of patients with any cancer, however, this appears to be most challenging with CRC as it is largely asymptomatic. As a result, most cases are still not diagnosed until the cancer has metastasised, primarily to the liver. At this late stage of diagnosis, 5-year patient's survival is predicted to be less than 10% (ABS 2015). The very few symptoms that

do present are often vague and overlooked by individuals; they can range from abdominal pain/discomfort, change in bowel habits, tiredness and at times bloody stools (Cancer Council 2015). A problem with these types of symptoms is that, they are often experienced by elderly individuals and are perceived as age related events. However, even when CRC is diagnosed and treated in the initial stages of neoplastic growth, high recurrence rates in patients still present as a serious issue.

Although an exact cause for CRC development has not been clearly identified, many factors are thought to contribute in its initiation and consequent progression. It is often difficult to directly link poor lifestyle choices with cancer occurrence, as many of these cannot be assessed as a stand-alone factor. However, a combination of these choices often leads to poor health outcomes and increase the likelihood of developing cancer (Cancer Council 2015). There has been a collection of evidence for many years suggesting that smoking and alcohol consumption increases this risk (Bagnardi et al. 2015; Nishihara et al. 2013). In CRC, it is predicted that these factors can even contribute to specific and highly mutated epigenotypes of this cancer and absence or cessation of use can be a preventative measure (Nishihara et al. 2013). Furthermore, dietary components like high meat consumption and low fruit and vegetable intake are predicted to contribute to CRC (Aune et al. 2011; Chan et al. 2011; Norat et al. 2013). Consequently, increased dietary fibre intake through whole grain foods, fruit and vegetables is found to have strong links with being defensive against CRC. In addition, lack of physical activity, high body mass index and abdominal fat-induced inflammation are also thought to be clinically relevant in the onset, progression and even recurrence of CRC (Riondino et al. 2014). As such, it is assumed that some CRC cases can be prevented through

lifestyle modifications. Emerging evidence suggests that CRC patients can have altered gut microbiota than healthy individuals indicating that it may contribute to the initiation of cancer (Gao et al. 2015; Raskov et al. 2017; Van Raay et al. 2017; Wang et al. 2012).

Factors more strongly associated with the onset of CRC, arise from hereditary links and diseases of the bowel. Although most cases of CRC are sporadic, the extent and duration of inflammatory bowel diseases like Crohn's disease and ulcerative colitis are thought to increase the risk of developing CRC (Castaño-Milla et al. 2014; Grivennikov 2013). The risk is even more evident in inherited genetic mutations which are collectively termed hereditary CRC syndromes which represent approximately 30% of cases (Jasperson et al. 2010). A prominent hallmark for these hereditary cancers is that the age of cancer onset can be as low as 20 years old which is considerably lower than that of sporadic CRC cases. Some conditions that comprise hereditary CRC syndromes include Lynch syndrome, familial adenomatous polyposis (classic and attenuated), MutY DNA glycosylase-associated polyposis and hamartomatous polyposis conditions. Lynch syndrome and familial polyposis patients carry approximately 70% risk of developing CRC; hamartomatous polyposis conditions above 40%, while MutY DNA glycosylase-associate polyposis cases approximately 4% (Burt et al. 2004; Jasperson et al. 2010; Stoffel et al. 2009).

1.2 Molecular determinants of CRC initiation & progression

1.2.1 Microsatellite instability mutations in CRC

Many genetic and epigenetic mutations are thought to initiate the transition of healthy gut epithelia to neoplastic adenomas and consequently determine CRC progression and severity. CRC arises through two main pathways (Colussi et al. 2013). Firstly, the less frequent pathway is that of aberrant DNA Mismatch Repair (MMR) systems, which lead to microsatellite instability (MSI). MSI-associated CRC mostly occurs with Lynch syndrome and a small proportion of sporadic CRCs. The MMR system in cells is a post-replication control which detects inaccurately placed nucleotides with mutS heterodimer proteins MSH2/MSH6 or MSH2/MSH3 (Kloor et al. 2013). After this, mutL homologues MLH1, MLH3, PMS1, and PMS2 are recruited to remove the wrong nucleotides and replace them with correct amino acids. Abnormalities arising in this system cause changes in number of nucleotides in DNA, hence termed MSI, and interfere with the translation of functional proteins (Kloor et al. 2013; Munteanu et al. 2014). Inactivation germline mutations in MSH2, MLH1, MSH6, and PMS2 are key characteristics of CRC that arise in people with Lynch syndrome (Pawlik et al. 2004; Boland et al. 2010). On the other hand, sporadic tumours with MSI exhibit a loss of expression through hypermethylation in the promoters of MLH1 and PMS2 protein. Another main difference between Lynch syndrome and sporadic MSI initiated tumours is that Lynch tumours have activating mutation in the *KRAS* gene but never in *BRAF*, while sporadic tumours most often exhibit activating *BRAF* mutations associated with CpG island methylator phenotypes. Nonetheless, both genes are implicated in cellular growth and division in the colon leading to the initiation of adenomas (Le

Rolle et al. 2016; Morkel et al. 2015; Tsai et al. 2016). Alongside these mutations in MSI tumours, tumour suppressor genes which function to inhibit epithelial proliferation are also silenced in the progression of cancer.

1.2.2 Loss of heterozygosity in CRC

The second and most commonly observed pathway in CRC onset is described as the loss of heterozygosity (LOH). This is characterised by inactivation of tumour suppressor genes and other chromosomal abnormalities like insertion of multiple copies of oncogenes (Colussi et al. 2013). Collectively, these are termed as chromosomal instabilities (CIN). Oncogenic *Ras* and tumour suppressor genes like *p53* and *p27* are amongst the regularly affected in the progression of CRC. Ras family of proteins are activated upon stimulation of receptor tyrosine kinases (Zenonos et al 2013). Ras proteins ultimately bind transcriptional factors that upregulate the expression of genes implicated in cellular growth and survival. Members of the Ras family of proteins like *KRAS* and *BRAF* are frequently mutated in CRC, which often render the Ras related pathways constitutively activated (Zenonos et al 2013). In association with these type of activation mutations, silencing of tumour suppressors like *p53* and *p27* cell cycle regulators, further deregulates the synchronised cycle of cell death and survival, leading to continuous proliferation of cancer cells while avoiding apoptotic cues (Harris et al. 1993; Wu et al. 2013). Aside from these genetic mutations, one of the most important examples of CIN, found in almost all cases of CRC, is the changes that occur in the Wnt signalling pathway.

1.3 Wnt signalling in CRC

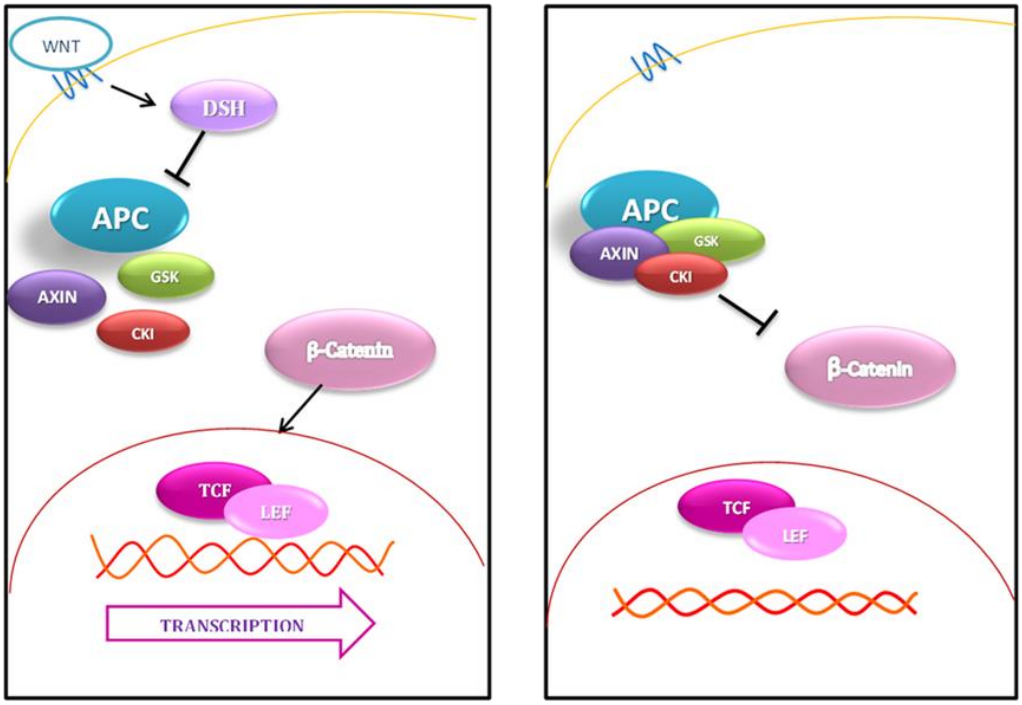
Allelic inactivation mutations to the *adenomatous polyposis coli* (*APC*) tumour suppressor gene of the Wnt signalling pathway, can be found in both sporadic CRC and inherited as germline mutation in 'familial adenomatous polyposis' cases (Colussi et al. 2013). It has been shown that animals with transitional *APC* silencing develop tumours within the intestine and colon, while restoring the *APC* expression in these animals, reversed tumourigenic potential in animals that still had *p53* and *KRAS* mutations (Dow et al. 2015). This also appears to be the case in most human CRCs, where the *APC* gene mutation has been widely accepted as the key initiator in driving the onset of CRC.

Wnt signalling pathway can be subdivided as canonical, which is propagated with β-catenin, and non-canonical which is independent of β-catenin activity. Wnt ligands are secreted glycoproteins that are assembled within the endoplasmic reticulum and transported to the Golgi complex, where the transmembrane protein Wntless assists their translocation to the cell membrane and secretion (de Sousa et al. 2011; Novellasdemunt et al. 2015). They are secreted in short range signalling and act upon the Frizzled and low-density lipoprotein-related protein (LRP) receptors on the cell membranes. In canonical Wnt pathway, the binding of Wnt ligands to their target receptors results in the accumulation of Wnt integral component and signal transducer, β-catenin, in the cytoplasm (**Figure 1.1**) (Song et al. 2015). β-catenin is then translocated to the nucleus where it binds to several transcriptional factors, lymphoid enhancer-binding factor/T-cell factor (LEF/TCF) family, leading to the expression of Wnt target genes. Aside from interacting with

transcriptional factors, another role of β-catenin is to regulate cellular adhesion, as it links E-cadherin adhesion receptor to the actin-cytoskeleton.

In the absence of a Wnt signal, destruction complexes negatively regulate free β-catenin molecules that are not participating in cellular adhesion (Novellasdemunt et al. 2015). These destruction complexes are formed with serine/threonine kinases casein kinase 1 alpha (CK1α), glycogen synthase kinase 3 (GSK3), axin protein and adenomatous polyposis coli (APC) protein. The APC and axin protein act as scaffolding proteins enabling the assembly of the destruction complex, while CK1α and GSK3 phosphorylate β-catenin permitting the recruitment of ligase β-Transducin. This leads to the ubiquitination of β-catenin, resulting in its degradation within the proteasomes. Upon Wnt ligand stimulation, dishevelled (DSH) protein binds to and guides axin away from the destruction complex disrupting its configuration and consequent degradation of β-catenin, allowing β-catenin to fulfil its partial role in transcription activation (**Figure 1.1**).

Figure 1.1. Schematic representation of the Wnt signalling pathway. *Left:* Wnt signal binding to a frizzled receptor activates dishevelled (DSH) protein. This protein inhibits the destructive complex formation with APC and associated proteins, allowing β-catenin to bind to and activate transcriptional factors, T-cell factor (TCF) and lymphoid enhancing factor (LEF). *Right:* In the absence of a Wnt signal the destruction complex involving APC protein degrades β-catenin, preventing transcriptional activation (adapted from Lau et al. 2007).


WNT
DSH
APC
GSK
AXIN
CKI
β-Catenin
TCF
LEF
TRANSCRIPTION
APC
AXIN
GSK
CKI
β-Catenin
TCF
LEF

Wnt signalling has been well characterised in the colonic crypts as a key driving force in maintaining the stem cell populations. In the colonic crypts, cellular maturation and migration occurs in an upward gradient (Krausova et al. 2013; Melo et al. 2010). The intestinal stem cells occupy the bottom most compartments of the crypts and they move up as they differentiate into transient amplifying cells. Ultimately at the apex of the crypts, the mature epithelial cells are shed off into the lumen as they fulfil their roles and are replaced. Wnt signalling gradient also follows this path, where its activity is predominant in the stem cell niches and declines at the top of the crypts. In cancer, when gene mutations cause an inactivation or dysfunction of the APC protein, β-catenin accumulates in the cell and travels to the nucleus to mediate transcription (Anastas et al. 2013; Burgess et al. 2011). This leads to the overexpression of Wnt target genes, causing dysregulation of normal cell proliferation and function, contributing to tumourigenesis. The non-canonical Wnt pathways, that function independent of β-catenin mediated transcription, regulate cellular polarity and migration. For this reason, Wnt over-activation through acquired mutations in non-stem cell populations, like the mature epithelium, is thought to aid epithelial-mesenchymal transition, where cancer cells gain stem cell-like properties and can proliferate infinitely and have enhanced migratory and invasive abilities (Basu et al. 2016). Among the target genes regulated by the Wnt pathway are members of the Eph/Ephrin family. β-catenin has been shown to control cell positioning in the crypts by regulating EphB receptor expression, and these receptors also appear to have an important role in CRC progression (Battle et al. 2002).

1.4 The Eph receptors and Ephrin ligands

1.4.1 Overview

Originally discovered and characterised in **e**rythropoietin-**p**roducing **h**epatocellular carcinoma cell lines, the Ephs constitute the largest family of tyrosine kinase receptors (Hirai et al. 1987; Eph Nomenclature Committee 1997). Based on their amino acid sequence composition and binding affinities Eph receptors are subdivided into A and B types. The subclasses consist of ten EphA and six EphB receptors, however, EphA9 and EphB5 (previously known as Cek9 and Hek9) are largely exclusive to avian species (Eph Nomenclature Committee 1997; Gale et al. 1996; Himanen 2012; Sasakia et al. 2003). The activation of Eph receptors is achieved through association with their corresponding cell-bound 'Eph receptor interacting' (Ephrin) ligands (Davis et al. 1994). Ephrins have nine members, six of which are type A and three are type B and they are categorized based on structural differences and sequence similarities (Himanen 2012).

The signalling by the Eph receptors and their membrane-bound ligands, the Ephrins, is unique among the tyrosine kinases as both the receptor and ligand are found on the cell surface. Receptor-ligand interaction is initiated by cell-cell contact and phosphorylated tyrosines activate various downstream effectors resulting in bidirectional signalling in both cells (Herath et al. 2010; Merlos-Suárez et al. 2008). Bidirectional signalling results in the phenomena of 'forward' signalling via the Eph receptor carrying cells and 'reverse' signalling in those cells expressing the Ephrin ligands. The distinctive signalling that occurs between the Eph receptors and Ephrins is made possible by their structural composition, complex assembly upon interaction and activation of signalling dynamics.

1.4.2 The structure of Eph receptors and Ephrin ligands

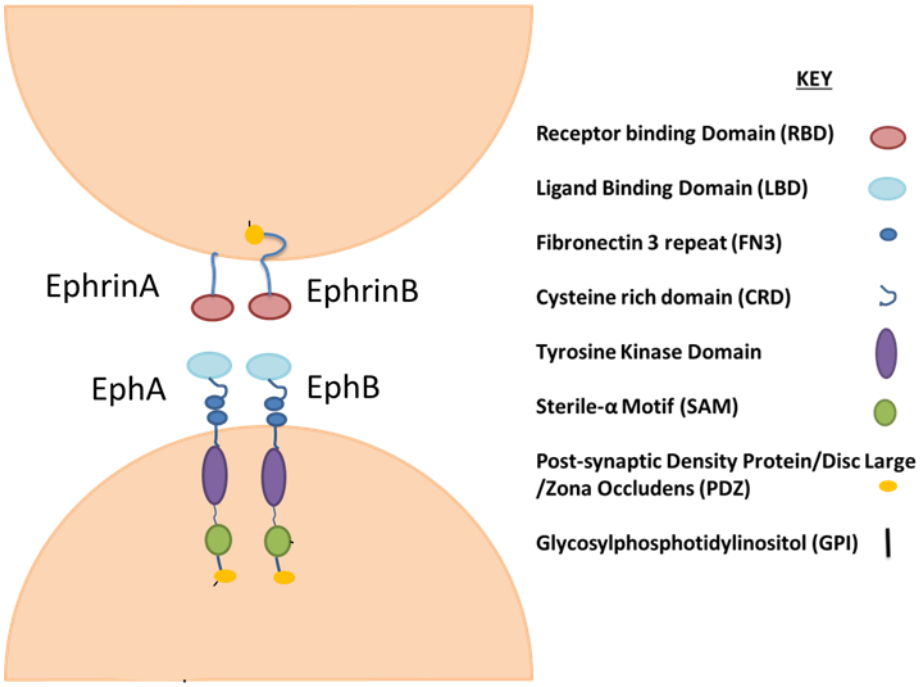
All Eph receptors are composed of extracellular, transmembrane and cytoplasmic regions (**Figure 1.2**). The extracellular segment of the Eph receptors contains three major sections which are N-terminal (amine) ligand binding domain (LBD), a cysteine-rich domain (CRD) and two fibronectin III (FN3) repeats (Arvanitis et al. 2008; Cheng et al. 2002; Himanen et al 1998; Himanen et al. 2010). The cytoplasmic region is composed of four functionally distinctive domains which are Src Homology 2 (SH2) docking sites, a dual lobe tyrosine kinase domain, sterile α-motif (SAM), post-synaptic density protein/disc large/zona occludens (PDZ) domains. Amongst receptor tyrosine kinases, the Eph receptor family are unique in having a cytoplasmic module, other than the protein kinase domain.

In comparison to the Eph receptors, the structural composition of Ephrins is much simpler. All Ephrins have an extracellular receptor binding domain (RBD). Type A Ephrins are attached to the membrane by glycosylphosphotidylinositol (GPI) anchors and type B Ephrins extend into the cell with a short cytoplasmic domain and a PDZ-binding domain (Egea et al. 2007; Herath et al. 2012; North et al. 2012; Pasquale 2010; Vearing et al. 2005).

In general, the two classes of Eph receptors, Type A and B, interact with and are activated by the same class divisions of Ephrins. However, these interactions are not exclusive and cross class associations are noted to occur between EphA3, EphA4 and EphB2 receptors which can interact with EphrinB2, EphrinB3 and EphrinA (Arvanitis et al. 2008; Gale et al. 1996; Hinamen 2004; North et al. 2012; Pasquale 2004). Furthermore, co-clustering and communications between

the different subclasses of Ephs have also been demonstrated to regulate the activation and function of these receptors (Janes et al. 2011).

Figure 1.2. Schematic representation of Eph receptor and Ephrin ligand molecular structure. Eph receptors consist of ligand binding domain, cysteine rich domain, two fibronectin 3 repeats and span into the membrane with tyrosine kinase, sterile α-motif domains. EphrinA are attached to the cellular membrane via GPI anchors and EphrinB have a short cytoplasmic tail with a PDZ domain (adapted from Vearing et al. 2005).


EphrinA
EphrinB
EphA
EphB
KEY
Receptor binding Domain (RBD)
Ligand Binding Domain (LBD)
Fibronectin 3 repeat (FN3)
Cysteine rich domain (CRD)
Tyrosine Kinase Domain
Sterile-α Motif (SAM)
Post-synaptic Density Protein/Disc Large /Zona Occludens (PDZ)
Glycosylphosphotidylinositol (GPI)

1.4.2.1 The ligand binding domain

Eph receptor class divisions and specificity are determined by their amino acid residue sequences. Some of the conserved residues in Eph receptors accommodate their rather undiscriminating interactions with several Ephrin ligands. One receptor that stands out amongst the other Ephs, for its level of specificity for the EphrinB2 ligand, is the EphB4 receptor. Even though only few papers exist on the biochemical structure of EphB4 receptor, these provide useful insight on the receptor's interaction with the EprinB2 ligand. Unlike other Ephs, the EphB4 receptor has several compositional and structural differences that allow it to bind strongly with EphrinB2, while having only weak interactions with EphrinB1 and B3 ligands (Chrencik et al. 2006*a*). EphrinA4 ligand is found to be the most promiscuous Ephrin ligand being able to bind most EphA and EphB receptors, yet it was not found to interact with EphB4 receptor (Noberini et al. 2011). In experiments done with EphrinB1, B2 and B3 Fc in prostate and lung cancer cell lines, only the EphrinB2-Fc was able to pull down the EphB4 receptor. This was further demonstrated at low concentration of EphrinB2-Fc, where it continued to pull down the EphB4 receptor, more successfully than other types of Ephrins (Noberini et al. 2011). However, EphrinB2 ligand can bind several other Eph receptors. EphB4, like other type-I tyrosine kinase receptors, has an N-terminal ligand binding domain and its intracellular domain contains a C-terminal domain and a tyrosine kinase domain. Ligand interaction stabilises the ligand binding interface of EphB4 (Chrencik et al. 2006*a*). The ligand binding domain of EphB4 is hydrophobic and consists of 13 antiparallel β sheets arranged in a β sandwich. In this sandwich, there is a concave and a convex sheet also amino acid links that connect them and form loops. The ligand binding domain consists of α helices, β

sheets and amino acid links. The D-E and J-K loops form the peptide binding channel of the EphB4 receptor (Chrencik et al. 2006*b*). Also, the D-E, E-F- G-H and J-K sheets are associated with dimerization and the H-I loops are involved in the tetramerization of the receptor. EphB4 binding channel has several distinct features which are not conserved through other Eph receptors, which make them specific to the EphrinB2 ligand.

Through 'conformational selection' Eph receptors recognise their ligands and interaction begins while the Ephs are still in a closed state (Dai et al. 2014). Certain residues are thought to enable initial recognition and 'anchoring' of receptor and ligand together. As the interaction advances, more residues are recruited at the binding interfaces. Some unique anchor residues have also been observed in EphB4 and EphrinB2 interactions and are thought to be part of the receptors selectivity (Dai et al. 2014). Upon initial encounter two pairs of salt bridges form between the Asp-39, Arg-65 in EphB4 receptor and Lys-57, Glu-116 residues (respectively) in the EphrinB2 ligand, guiding the ligand into the binding domain of the receptor, then breaking away. Interaction between EphB4 and EphrinB2 heterodimer complexes occur with the insertion of the EphrinB2 G-H loop into the hydrophobic upper convex surface of the EphB4 receptor (Chrencik et al 2006*a*). Despite certain differences, EphB4 is often compared to EphB2 for their high level of sequence similarity. Additionally, since EphrinB2 also binds to EphB2, these comparisons provide useful insight into the reasons behind high selectivity of EphB4 toward EphrinB2. It was discovered that the Arginine in position 95 of the EphB2 result in steric clash with the EphrinB2 ligand's Phe-120 residue (Chrencik et al 2006*a*). In EphB4, position 95 is occupied by a Leucine instead of Arginine, allowing for van

der Waals interactions with Phe-120 of the ligand. This interaction allows for the ligand to be buried in the binding cleft of the receptor rather than be directed toward the surface, as it does with EphB2. The Leu-95 residue is thought to be a key driver behind the EphB4 receptors specificity to EphrinB2. Following the Phe-120 residue that the EphrinB2 ligand uses to interact with EphB4, Leucine and Tryptophan residues occupy positions 121 and 122 (Guo et al. 2014). Interestingly, these two residues participate in cross class interaction between EphrinB2 and the EphA4 receptor. The EphA4 receptor's flexible J-K loop is reported to physically adapt to EphrinB2 upon interaction (Bowden et al. 2009; Singla et al. 2010). Flexible loops lining the LBD of EphB2, are also reported to assist this receptor in binding with A and B type Ephrins (Goldgur et al. 2009). The J-K loop sequences of type B Eph receptors are markedly different, with EphB4 having the least amount of similarity between residues 151-167 (Bowden et al. 2009). The Pro-151 and Gly-152 residues that form the tip of the J-K loop, interact with ligand residues and are also suggested to contribute to ligand specificity of EphB4 (Chrencik et al. 2006*a*).

Certain EphrinB2 ligand sequences are also thought to contribute to EphB4 receptors specificity. The EphrinB2 ligand is largely identical in sequence to the other EphrinB ligands except for a Leucine at position 124 (Chrencik et al 2006*a*; Ran et al. 2008). Upon binding with EphB2 receptor, EphrinB2 is proposed to induce conformational changes to the receptor's ligand binding domain (Ran et al. 2008). When ectodomains of EphrinB2 bound EphB2 and EphB4 are compared, structurally no vast differences can be observed between the two receptor-ligand complexes. However, the Leu-124 of EphrinB2 is smaller compared to the bulky Tyrosine found at this position in the EphrinB1 receptor. As such, this residue

allows for the EphrinB2 ligand to embed into the ligand binding cavity of the EphB4 receptor without the need for the EphB4 receptor to undergo a large conformational change (Chrencik et al 2006*a)*. Furthermore, a phenylalanine residue at position 120 within the EphrinB2 ligand adopts a unique position when interacting with EphB4 in comparison to the EphB receptors. It is buried within the hydrophobic binding cleft interacting with the residue Leu-95 and the Cysteine-61-Cysteine- 184 disulphide bridges of the EphB4 receptor. On the other hand, the EphrinB3 receptor has a Tyrosine residue at the Phe-120 position which result in a steric clash with EphB4, explaining why EphB4 has a weak affinity for EphrinB3 and high affinity for EphrinB2. Furthermore, EphB4 has other non-conserved amino acid residues within the dimerization and tetramer interfaces (Chrencik et al 2006*b*). These unique residues assist the ligand selectivity of EphB4, as steric incompatibility leads unfavourable interaction with other EphrinB ligands.

1.4.2.1.1 Inhibiting EphB4 by targeting its highly specific ligand binding domain

Many inhibitors have been designed against various domains of Eph receptors and EphB4 (Herington et al. 2014; Lamminmaki et al. 2015). However, a potent inhibitor which binds to the ectodomain of EphB4 receptor, interfering with its highly specific LBD, is accepted to be one of the most potent. Using this inhibitor has provided more evidence into the specificity and interaction of EphB4 with EphrinB2. In phage display experiments, a 15-amino acid peptide (amino acid sequence Tyr-Asn-Tyr-Leu-Phe-Ser-Pro-Asn-Gly-Pro-Ile-Ala-Arg-Ala-Trp TNY LFSPNGPIARAW, designated as **TNYL-RAW**) was found to fix to the ligand binding domain of EphB4 with very high affinity (Koolpe et al. 2005). Amongst

the three EphB receptors that were tested (EphB1, EphB1 and EphB4), certain peptides with a high affinity for EphB1 and EphB2 were found to not bind to EphB4. These EphB1 and EphB2 binding peptides revealed that they had very similar sequences, whereas the EphB4 binding peptides were different. This was interesting as EphB1 and EphB2 receptors also bind EphrinB2, the preferred ligand for EphB4. It was noted that the binding peptides sequences of EphB1 and EphB4 matched different regions of the EphrinB2 G-H loops (Koolpe et al. 2005). The peptides that were tested had a proline which matched the tryptophan found at the end of Ephrin G-H loops. This amino acid produces a bend, which is like that of the ligand G-H loop, being structurally favourable. In EphB4 binding peptides, a glycine comes before the proline residue, which further adds to the peptide's structural advantage in producing high affinity interactions. The TNYL-RAW peptide is structurally stable and avoids large conformational change in binding EphB4 which maintains the high affinity relationship.

A disadvantage of this peptide, is a short half-life in both culture and animal experiments. In culture, TNYL-RAW was found to be cleaved by proteases, as such, addition of the peptide in culture along with protease inhibitors, might improve this issue (Noberini et al. 2011). In animal plasma, the peptide was cleared within a very short time frame of 30 minutes, hence it is suggested that introducing small modification to the peptide can increase its half-life without taking away from its high affinity binding to EphB4. The modification tested were fusing TNYL-RAW with Fc portion of human IgG1, streptavidin and covalent coupling with a PEG polymer. These modifications were shown to not affect the function of the peptide and while inhibiting EphB4/EprinB2 binding and phosphorylation as tested

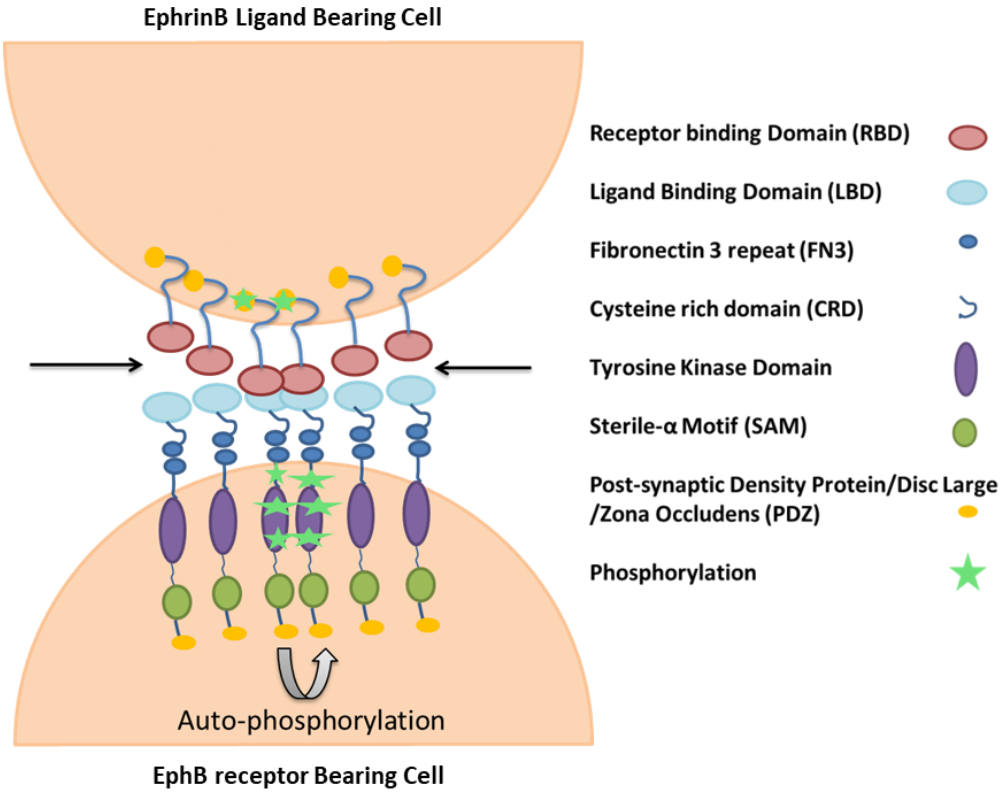
in B16 melanoma cells, epicardial mesothelial cells and human umbilical vein endothelial cells. Most modifications improved the half-life of TNYL-RAW, but PEG integrated peptide showed the best results in increased half-life in cell culture and in mouse serum. The PEGylated peptide was also shown to prevent EphrinB2 phosphorylation whereas the PEG control used in the experiments showed no such effect. This could be useful in therapeutic application since EphrinB2 can interact with and be activated by Eph receptors other than EphB4 and still have an effect. The inhibitory peptide has also been used in studies of non-invasive imaging of EphB4 in grafted tumours with positron emission tomography/computed tomography (Xiong et al. 2011). A radiometal chelator sequence DOTA was introduced to the N-terminal of TNYL-RAW. This addition reduced the interaction affinity of the peptide, which was rescued with the addition of a Cu^{2+} ion to the DOTA (64Cu-DOTA-TNYL-RAW). Contrary to previous reports, this study found that some additions to the N-terminal of TNYL-RAW can affect binding of the peptide. This peptide was then introduced into animals bearing tumours of EphB4 positive or negative cell lines. In the positive CT26 colon cancer and PC-3M prostate cancer cell lines, the peptide was shown to be taken up rapidly while in the EphB4 negative cell line there was minimal uptake. The persistence of the peptide in the tumours lasted between 4-24 hours, which confirms findings of the previous study where TNYL-RAW was reported to have a short half-life. In this study, the peptide-EphB4 off-rate was found to be slow, therefore, it is suggested that level of receptor expression in the tumours may be the preliminary factor behind peptide uptake and retention. Aside from this large amount of accumulation in the liver, spleen and kidney of the animals, this would need to be addressed in future studies. Nonetheless, this peptide proves useful for transient imaging of tumours. Other

modification to the TNYL-RAW peptide have been introduced to allow for near-infrared imaging which is said to be better than radioactive alternative also being sensitive and inexpensive (Zhang et al. 2011). A dual-labelled TNYL-RAW-CCPM nanoparticle was developed where each nanoparticle contained 60 TNYL-RAW peptides attached to it. Addition of the peptide to the nanoparticle is said to enhance its stability and half-life by 2 times better than the TNYL-RAW peptide alone in the blood of the animals, which in turn improves tumour uptake. The multimeric interaction produced by the nanoparticles with EphB4 overexpressing tumour cells was said to enhance imaging quality with both single-photon emission computed tomography and infrared imaging. Other peptides binding competitively with TNYL-RAW to the LBD of EphB4 have also been identified (Duggineni et al. 2013). These studies provide some useful insight into using binding peptides as potential experimental and therapeutic purposes; however, the mode of application and consequences still need be explored in more depth.

1.4.2.2 Eph/Ephrin interaction and clustering

After the initial recognition and interaction of Eph and Ephrins, their activation and signal propagation are rather complex series of events. This family of tyrosine kinase receptors are different to others as dimerization is not sufficient for their activation. In an unbound state Ephs and Ephrins are found in specialized domains or cholesterol-rich lipid rafts on the cell surfaces (Bruckner et al. 1999; Chavent et al. 2016; Marquardt et al. 2005). Upon initial interaction EphBs and EphrinBs lead to the formation of heterodimers (Himanen et al. 2001; Janes et al. 2012). Then with increasing concentration, aggregate to form tetramers, where each ligand interacts with two receptors and vice versa hence producing oligomer clusters (**Figure 1.3**).

Figure 1.3. Schematic representation of the interaction of EphB receptors and EphrinB ligands. Upon engagement between two receptors and two ligands a heterodimer forms leading to recruitment of other nearby Eph receptors and Ephrins. EphB receptors are auto- and trans- phosphorylated through their tyrosine kinase domains, while EphrinB ligands are phosphorylated through the Src family of kinases (Adapted from Himanen et al. 2007).


EphrinB Ligand Bearing Cell
Receptor binding Domain (RBD)
Ligand Binding Domain (LBD)
Fibronectin 3 repeat (FN3)
Cysteine rich domain (CRD)
Tyrosine Kinase Domain
Sterile-α Motif (SAM)
Post-synaptic Density Protein/Disc Large /Zona Occludens (PDZ)
Phosphorylation
Auto-phosphorylation
EphB receptor Bearing Cell

Tetramer clustering is supported through interfaces found in ligand-receptor interaction sites and other domains. Upon encounter, receptor and ligand become energetically favourable, leading to the attraction and recruitment of more receptors and ligands to the heterodimers. The ways in which the receptors and ligands are inserted into the membrane are also no coincidence (Himanen et al. 2001). The positioning of carboxyl terminal of the receptor and ligand on juxtaposed cells are in opposite orientation to facilitate binding, and when bound, the tetramer clusters become stable and fix the kinase domains in place to allow auto-phosphorylation of receptors termed 'forward signalling'. In these complexes the ligands adopt an open configuration and their kinase domains are activated, hence, resulting in 'reverse signalling'. Since the receptors and their cell bound ligands can transmit downstream signalling, this type of interaction and activation between the Ephs and Ephrins is referred to as 'bidirectional signalling'. In simple terms, Eph receptor activation, hence forward signalling, is thought to cause cellular repulsion/migration and reverse signalling through Ephrin ligands leads to cellular adhesion (Aharon et al. 2014; Zimmer et al. 2003). However, many studies have shown that the type of signalling can depend on various factors, such as size of the clusters produced through interaction, the composition of these clusters and the ratio of receptor ligand expression in cellular populations.

As an example, in experiments using synthetic dimerizers, modified EphB2 receptors were induced to form dimers, small clusters or large clusters (Schaupp et al. 2014). It was observed that oligomers made of pentamers and hexamers did not enhance the overall signalling of receptors, when compared to smaller oligomers consisting of trimers and tetramers. This observation suggests that smaller clusters

are capable of maximising receptor activation and signalling. Also, clusters that have a higher ratio of inactive dimers do not influence strength of signalling, on the other hand, high-order oligomer clusters with higher ratio of engaged multimers enhance signal strength. As such, some very interesting work conducted by Egea and associates (2005) has shown that a constitutively active EphA4 receptor, with a kinase that is functioning at high capacity, can still form high order clusters upon Ephrin ligand stimulation. Therefore, it is suggested that upon dimerization with the proximity of the two kinases, of Eph receptors are auto-phosphorylation and oligomerisation does not increase kinase activity, rather facilitates the recruitment of other cytoplasmic effectors, increasing signal strength. This was further supported with the finding that Ephexin1, a type of cytoplasmic effector that interacts with the cytoplasmic domain of EphA4, is only recruited to this site upon high order clustering of the receptor with an Ephrin ligand. Another interesting concept in Eph receptor clustering suggests that ectodomain structures and clustering mode of Eph receptors can alter cellular response (Seiradake et al. 2013). In cells transfected with EphA2 and EphA4 receptors, EphA4 receptor expressing cells demonstrated enhanced cellular rounding under ligand stimulation and adhered more strongly to EphrinA5-Fc coated plates, even though both receptors bind to EphrinA5 ligand with the same affinity. When ectodomain properties of these two receptors were investigated, it was found that EphA2 clustering was mediated through two clustering interfaces, one in the H-I loop of the LBD and the other in the CRD. In contrast, only the CRD segment and not the H-I loop of the EphA4 receptor was found to be involved in Eph-Eph clustering in these experiments. To test the functional relevance of this information, cells were transfected with constructs, where the ectodomain of EphA2 and EphA4 were

switched on the full length transmembrane portions of these receptors. While the type of response did not change, possibly due to the function of transmembrane domain of the Eph receptors, the EphA4 ectodomain expressing cells enhanced cellular rounding, indicating that the ectodomains of these receptors regulated the intensity of cellular response (Seiradake et al. 2013). Also, worth noting was that the size of EphA2 receptors were found to produce larger clusters than EphA4 receptor. This is particularly interesting because as previously discussed the composition within the clusters was important in determining cellular response, nonetheless it seems the mode of clustering can also affect this process (Schaupp et al. 2014; Seiradake et al. 2013).

Mutant Eph receptors lacking an intracellular domain were capable of clustering on cell surfaces, suggesting high order clustering involves the extracellular domains of these receptors (Wimmer-Kleikamp 2004). It had previously been reported that. the Ephrin ligand G-H loops facilitate dimerization of the ligand and C-D loops participate in the tetramerization of the receptor (Himanen et al. 2001). However, ligand independent clustering has also been shown with Eph receptors (Wimmer-Kleikamp et al. 2004). These clusters appear not to be limited in size and all nearby Ephs and Ephrins not already occupied can be recruited. The level of recruitment and size of the clusters are thought to determine the nature of the signal that is propagated and the cellular response that is produced (Nievergal et al. 2012; Nikolov et al. 2013; Wimmer-Kleikamp et al. 2004). As such, these interactions occurring on the same cell surface have been termed '*cis*', as opposed to conventional receptor-ligand binding on opposing cells which has been termed '*trans*' interaction. *Cis* interactions can impact the level of Eph and Ephrin available

for binding in *trans*, for this reason *cis* engagement between ligand and receptor is classified as being inhibitory (Carvalho et al. 2006; Dudanova et al. 2011). These associations are tuned and synchronized to control cellular response. The type of response appears to depend on level of Ephrin expression in cells. In low level of Ephrin expression *trans* interactions with Ephs are noted to be greater. When Ephrin expression is high the ligands bind to Eph receptors in *cis* occupying them and as a result limiting the receptors that engage in *trans* interaction. EphB receptor knockout mice exhibit minimal side effects to their neuronal organisation despite the EphB receptors being expressed on all lateral motor column neurons (Wimmer-Kleikamp et al. 2004). This is thought to be because *cis* inhibition imposed on EphB receptors by high level of EphrinB expression that also exist within these neurons.

1.4.2.3 The cysteine-rich domain

The cysteine-rich domain (CRD) immediately follows the LBD and links onto the fibronectin repeats. As with most of the Eph structure, it appears that this domain too has a specialised function contributing to the oligomerisation of the receptor (Nikolov et al. 2014). In crystallisation experiments using the ectodomain of unliganded or liganded EphA2 it was revealed that both have comparable structures with only minute movement of the liganded receptor (Himanen et al. 2010). The LBD, as previously discussed, was found to be the only compartment that encounters the ligand, however, upon tetramerisation two types of clusters were observed. In one of the clusters, LBD exclusively governed the clustering process between ligand and receptor but also between the heterotetramic receptors. This formation was likened to that of the clusters formed by EphB2. This type of clustering was not affected by the removal of CRD in EphA2 or EphA4 receptors

(Himanen et al. 2010; Xu et al. 2013). A second type of cluster formation involved only Eph-Eph interactions and it did not rely on ligand interaction. These interactions involve all CRD containing proteins. In this formation clustering is supported by the LBD and CRD. Two neighbouring receptors form salt bridges and hydrogen bonds between the residues in the LBD. The CRD interface is characterised by leucine-zipper-like motifs and receptors link when vast amount of the CRD buries into each neighbouring Eph (Xu et al. 2013). This region is said to be conserved across Eph receptors, hence, suggesting this type of clustering applies to all (Himanen et al. 2010). When Eph receptor expression is high this type of clustering is deemed possible. In functional experiments, the CRD interaction residues were mutated, which revealed that the mutants greatly impacted ligand independent phosphorylation of Ephs. When the LBD of the EphA2 receptor was mutated, using confocal microscopy it was observed that the recruitment of these receptors into clusters were not different to Wild-type (WT) receptors, indicating strong interactions being supported through the CRD. An antibody raised against the CRD of EphB4, was shown to inhibit receptor phosphorylation and is predicted to interfere with ligand binding (Stephenson et al. 2015). Nonetheless, the structural and biological function of the EphB4 receptor's CRD remains to be investigated.

1.4.2.4 Fibronectin repeat domain

The thickness and the composition of the cell membrane can also influence the type and structure of the interaction formed by the TMD of Ephs (Bocharov et al. 2010). Eph receptors and Ephrin ligands are thought to exist in preclustered forms in specialised lipid rafts in the cell membrane. These rafts are said to be thicker than rest of lipid bilayer of the membrane which is thought to be able to support to TMD

helix interaction and stabilize the dimeric formation of the receptors. Furthermore, there are suggestions that the fibronectin repeats (FN) found in the Eph receptor's ectodomain can directly interact with the lipid rafts influencing the structural stance of at least the EphA2 receptor (Chavent et al. 2016). A degree of flexibility exists between the two FN repeats of Eph receptors allowing for rotation and positioning of the receptor in the membrane. In stimulation experiments of EphA2 monomer and dimerised ectodomains and the TMDs, it was discovered that the receptors uptake two likely arrangements; one where it lies almost flat on the cell membrane when it is 'unliganded' and the other where it is standing up right when it is bound to a ligand (Chavent et al. 2016). It is suggested that at low density the receptor can be flat on the surface while high density and preclustering promotes the upright position. It is noted that the flat position does not interfere with ligand binding as the LBD still faces up, nonetheless, this is proposed to have limitations on receptor activity since additional binding sites outside of the LBD may be masked and the kinase domains of dimers may be too far apart for auto-phosphorylation (Chavent et al. 2016). These constraints imposed by the flat configuration of the receptor can be an inhibitory mechanism ensuring receptor activation and signalling in the absence of a ligand is limited. The findings of this study should be further verified with other Eph receptors.

1.4.2.5 Transmembrane domain

In the transmembrane domain (TMD) spanning the cellular membrane, connecting extracellular and cytoplasmic regions, two different sequences may exist; one a heptad repeat, the other a glycine zipper motif (Sharonov et al. 2014). These sequences have structural importance in receptor dimerization and activation in

opposing ways. Since the Eph receptors contain a single helix TMD, determining which sequence is expressed is of importance. The EphA2 receptor was found to express variants of both sequences and experiments were conducted to determine how mutations influence its function. In experiments where the heptad repeat is mutated, the EphA2 receptor was more readily activated, suggesting that this repeat is involved in conserving an inactive configuration of the receptor (Sharonov et al. 2014). On the other hand, glycine zipper mutations in the same receptor resulted in decreased phosphorylation, signifying that it helps the receptor obtain a configuration which favours activation. As previously mentioned, Eph receptors largely exist as dimers on the cell surface and this is their inactive state (Wimmer-Kleikamp et al. 2004). Through molecular modelling, it is revealed that the heptad repeats in dimerized TMDs EphA2 receptors, results in a small angle in between two rod-like helixes. With the glycine zipper motif, the angle between the two 'rods' is vastly increased, and the helixes take up a scissor looking shape, being split at the cytoplasmic end. It is presumed that in an inactive form, the kinase domains of the Eph receptors are in a closed dimer configuration. In order for activation to occur, the angle between the two TMDs need to increase, helixes need to rotate, and the cytoplasmic ends need to separate considerably, opening the up the kinase domain for phosphorylation (Sharonov et al. 2014). As such, the glycine motif result in a 'pro-active' configuration, making it easier for phosphorylation of the kinase domain. Hence, when the receptors are in a 'contra-active' configuration through heptad repeat engagement, receptor activation will need to involve an approximate 160° rotation and large degree of separation at the cytoplasmic end of the TMD. Since Eph receptors can act ligand independently in *cis*, it was noted that overexpressing EphA2 does not spontaneously result in more pro-active

configuration of the receptor. Even though overexpression leads to an increase in dimer formation upon the receptors, only a portion of the receptors adopt a proactive configuration while others are still in in inactive state (Sharonov et al. 2014). As expected when the heptad repeat is mutated in these cells, receptors once again engage in *cis* and become more readily activated. Ligand interaction on the other hand turns all configurations to active form.

1.4.2.6 Kinase domain and activation through phosphorylation

The kinase domains of Ephs are bilobed and flexible enough to allow for the domain to assume an open or closed configuration (Wybenga-Groot et al. 2001). However, the configuration of this catalytic domain is usually closed, where the two bound lobes create an interfacial nucleotide binding site and catalytic cleft. In an inhibited state, the catalytic domain still resembles the structure of an active one. The juxtamembrane (JM) interacts directly with the N-terminal of the kinase domain and loosely with the C-terminal, which is superimposed. These JM interactions alter helix structure, causing it to take up an inactive conformation. Upon phosphorylation of the JM residues, steric and electrostatic forces release the bonds with the N-terminal kinase, allowing for the domain to rearrange into an active conformation (Wybenga-Groot et al. 2001). Mutating residues in EphB2 and EphA4 JM results in a more active kinase domain with the inhibition removed (Wiesner et al. 2006; Wybenga-Groot et al. 2001). The specific JM residues may be regulatory sites as they are highly conserved, and the inhibitory regulation imposed on the kinase domain is likely to apply to all Ephs. In more recent times experiments done with EphA2 reveal that tyrosine kinase domain of this receptor can stay in an inactive configuration even when the inhibition from the JM is

removed (Wei et al. 2014). EphA2 receptor JM, just like the inactive EphB2, interacts with the kinase domain and masks the activation sites. However, the JM of EphA2 is positioned differently, as it faces away from the cleft it typically occupies (Wybenga-Groot et al. 2001). As a result, the authors propose that the EphA2 receptor JM segment is unique amongst other Ephs (Wei et al. 2014). The JM segment is still found to undergo configuration change with activation however, in this instance it is thought to be a result of the activation, rather than the cause. As demonstrated by this study, mode of inhibition and activation maybe different in Eph receptors. EphB4 receptor's juxtamembrane domain have not been studied fully and it is still unknown if this receptor, which is most comparable to EphB2 in structure, is also governed by the same auto-inhibitory mechanisms that is observed with EphB2. In one study, the conserved EphB receptor phosphorylation sites were inactivated in EphB4. The mutants were then stably transfected into mouse bone marrow cells and showed that the effects of EphB4 overexpression were inhibited. By losing these key sites, EphB4 is said to lose the ability to auto-phosphorylate. It is also suggested that, through these mutations EphB4 loses the ability bind and interact with SH2 domain proteins, which can also bind the phosphorylation sites of the JM. However, these predictions were not confirmed through experimentations. For this reason, mode of inhibition in auto-phosphorylation in EphB4 remains unclear.

Part of the issue with studying the structural composition and topology of the intracellular domains of EphB4 is its instability in *E. coli* cells. Although Eph receptors have a high degree of sequence conservation, few differences that do exist are thought to influence their affinity for different substrates. As such, EphB2

kinase phosphorylation is likely toxic to *E. coli* cells, however, when expressed in catalytically inhibited form this problem can be overcome (Overman et al. 2013a). These issues are not solved as easily for the EphB4 receptor, which is suspected to have an inherently low intrinsic stability, when compared to other EphB receptor. A possible explanation for this stability issue could be that *in vivo* kinase folding or stability may be supported by chaperon complexes, as EphB4 kinase expression was enhanced in the presence of these complexes in the *E. coli* cells (Overman et al. 2013a). In denaturing experiments to investigate protein unfolding, EphB4 and EphB3 were observed to be the least tolerant of the EphB receptors being investigated, by beginning to unfold at low concentrations. Furthermore, despite a relatively small amount of difference in sequence identity between the EphB receptors, in thermal stability experiments, it was found that there was rather a large difference of approximately 17 degrees between EphB4 and EphB1 (Overman et al. 2013a). EphB4 kinase domain melting temperature was found to be the lowest of the four EphB receptors investigated. In looking for differences in the binding regions of the EphB receptors kinases, EphB4 receptor's active site was found to have a glycine in position 699 which was conserved in all other EphBs except EphB3 in which it is a cysteine.

To overcome the low stability and solubility issue of EphB4 in *E. coli*, Overman and associates (2013b), tried rational library engineering of EphB4. This was done using previously available data on the structure and sequence of EphB4 kinase domain, where the designed variant was tested for construct length, functional mutations and stability grafting. The mutant variations were compared to EphB1 and EphB2 in structure as these two receptors show high level of thermal stability

and the aim was to design EphB4 so that it had their stable properties. Hydrophobic mutations (EphB4 HP) in the core were determined to be characteristic amongst the clones which offered the most stability and solubility of EphB4 kinase domain. The staurosporine bound EphB4 HP crystals revealed that there is minimal change to the secondary structure of the C terminal lobe. One of the introduced mutations protrudes out of the kinase core, increasing hydrophobic interactions between the pair of kinase helixes. This is thought to be a key factor in increasing EphB4 stability (Overman et al. 2013b). Some structural differences are noted to exist in the N terminal lobe of HP. One of the introduced mutations being in a phosphorylation site, HP might presume different crystallisation behaviour. In enzymatic assays performed to assess phosphorylation profiles of EphB4 HP and Wild-type (WT) EphB4, the catalytic efficiency for HP was found to be higher (Overman et al 2013b; Marangoni 2003). Despite this, the excitable HP mutant was not found to have a different molecule binding profile, as it had similar half maximal inhibitor concentration (IC_{50}) values as the WT with two different inhibitors that were used. Furthermore, EphB4 constructs of various lengths, containing JM region and SAM domain were found to be soluble in the HP mutants, suggesting they can be added for analysis in future studies.

Despite the low stability of EphB4 kinase domain, in crystallisation screening experiments, unexpectedly the WT kinase domain was the most readily crystallised amongst other EphB receptors (Overman et al. 2014). It had a 6% hit rate in proteinaceous crystalline conditions that were tested in comparison to 2% in EphB1 and EphB2. This is deemed possible as previous reports indicate that stability is not the key factor determining protein crystallisability. In these experiments, EphB4

was co-complexed with an alkaloid, staurosporine, which was used to reveal the apoprotein structure through molecular replacement with previous data that was available to the authors. Through these means it was revealed that EphB4 kinase domain does obtain the typical bi-lobeled fold, however, it adopts the most closed configuration around the staurosporine that it had bound (Overman et al. 2014). In this configuration the glycine rich loop folded over the molecule while in EphB1 and EphB2 this loop remained disordered. Furthermore, EphB4 activation loop become ordered upon stimulation. Also, the active sites of the EphB4 and EphB1 kinases were found to be similar and designing inhibitors that only target one of these receptors' kinase domains was difficult. Nonetheless, some inhibitors have been designed and optimised toward the EphB4 receptor's kinase domain. In a three-part series, Bardelle and associates (2008, 2008, 2010) describe in detail different types of inhibitors.

1.4.2.7 The SAM domain

Intracellular domains of the Eph receptors are also thought to participate in the oligomerisation process. The SAM domain was identified in yeast as the 'sterile alpha motif' (SAM) factor (Stapleton et al. 1999). The SAM domains of Eph receptors are highly conserved and are followed by the PDZ domain binding site (Torres et al. 1998; Wang et al. 2016). SAM domains mediate dimerization of transcriptional factor, transcriptional repressors and scaffolding proteins. The SAM termini of Eph receptors are thought to aid oligomerization and bringing catalytic elements of the receptor near and in correct orientation for auto-phosphorylation (Wei et al. 2014). Despite this, hindrance to receptor clustering due to cytoplasmic protein recruitment to the SAM and PDZ domains during ligand stimulation is

thought to limit and therefore regulate signalling (Schaupp et al. 2014). As such, improved receptor clustering and signalling were observed in experiments where SAM and PDZ deletion mutations were introduced to EphB2 receptors *in vitro*. In EphrinB2, removing the tyrosine residues in the cytoplasmic tail along with the PDZ domain had deleterious effects on embryo lymphatic development (Mäkinen et al. 2005). Hence, under biological conditions the SAM and PDZ domains could possibly act as inhibitory regulators of Eph-Ephrin signalling but also can mediate with downstream effectors upon activation.

1.5 Ending Eph/Ephrin interaction and cellular consequences post-interaction

Eph and Ephrin relation is further complicated and unique in the way in which interaction is terminated. It is facilitated through *cis* or *trans* proteolytic cleavage of one of the proteins, followed by transcytosis of the complexes into either the receptor or ligand bearing cell (Lisabeth et al. 2013). Metalloproteinase interaction with EphAs and EphrinAs has been reported. A type of distintegrin and metalloprotease (ADAM10), was found to form a stable ternary complex when incubated together with EphA-EphrinA complexes (Janes et al. 2009). When Eph receptors become phosphorylated, the JM domain is said to assume a relaxed position extending the kinase domain. The metalloproteinase then binds to a recognition motif. This formation was found to be primarily supported through the Eph receptor LBD and ADAM10 CRD. When EphA3/ADAM10 expressing cells were exposed to preclustered ligands, *trans* cleavage of EphrinA5 is observed (Janes et al. 2005). It is interesting to note that, the co-expression of this proteinase with EphrinA did not promote cleavage of the ligand even in the presence of

preclustered EphA antibodies. Furthermore, interfering with cleaving action of ADAM10 with the use of inhibitors, short interfering RNA and expressing truncated ADAM10, showed a reduction in EphrinA5 cleavage and inhibition in internalisation (Atapattu et al. 2012; Janes et al. 2005). Meltrinβ/ADAM19, is another reported metalloproteinase that can EphrinA5 (Yumoto et al. 2008). Meltrinβ was found to be expressed at the same cell surface with EphA4 at the neuromuscular junction. Since this ADAM is not usually expressed at the cell surface, its insertion into the membrane is suggested to be regulated by EphA4 expression. Meltrinβ is said to cleave EphrinA5 in *trans* only. When it was expressed in EphrinA5 bearing cell, *cis* cleaving of EphrinA5 by Meltrinβ did not occur even with EphA4 exposure.

EphrinB ligand interaction and cleavage by matrix metalloproteinase (MMPs) have also been studied. EphrinB1 activation was shown to enhance the trafficking of MMP-8 from the cytoplasm to the membrane, from where it was secreted (Tanaka et al. 2007). Once secreted MMP-8 appears to cleave EphrinB1 in the ectodomain. Hence this is representative of *cis* cleaving process taking place on the same cell surface as the MMP-8 was released. This type of cleavage also appears to downregulate the EphrinB1 and EphB2 communication. Experiments done in human embryonic kidney cells revealed that EphrinB1 and EphrinB2 can be cleaved by ADAM13 but not ADAM10, ADAM12 or ADAM19 (Wei et al. 2010). Furthermore, ADAM13 was not found to cleave EphrinA and EphrinB3 ligands. This cleavage was found to remove the entire ectodomain of EphrinB2. This is proposed to reduce signalling, since the intracellular domain of EphrinB2 cannot interact with any other Eph receptors and the shed ectodomain is said to be not

functional. On the other hand, cleaving of EphrinB2, the preferred ligand for EphB4, is reported by another paper to be regulated by ADAM10 (Ji et al. 2014). When ADAM10 was knocked down in embryonic tissue, loss of EphrinB2 expression was prevented, whilst a knockdown of ADAM13 or ADAM17 did not result in changes to the ligand expression (Ji et al. 2014). The specific site of cleavage by ADAM10 was determined to be in the JM region of EphrinB2 as deletion mutations in this region resisted the down fall in ligand expression. EphrinB2 and ADAM10 were coimmunoprecipitated in the HT29 colon cancer cell, confirming their specific association beyond the embryonic stage and in disease state. To study cellular adhesion as a response of Eph/Ephrin signalling, canine kidney and intestinal cell line were used to generate green or red fluorescently tagged E-cadherin constructs (Solanas et al. 2011). These fluorescently tagged cell populations mixed together in co-culture. However, when EphB3 and EphrinB1 expression was introduced, cells separated into two distinct cell populations and remained separated even at confluence. E-cadherin was found to be shed from Eph and Ephrin expressing co-cultures. Inhibiting MMPs resulted in E-cadherin mediated adhesion to be reinstated between Eph and Ephrin cells and promoted cell mixing of the two populations of cells.

After cleaving of the ectodomain of EphrinB2, the remaining intracellular 'carboxyl-terminal' fragment has been found to be processed by the MMP PS1/γ-secretase (Georgakopoulos et al. 2006). A small peptide, produced because of this cleavage, binds to intracellular protein Src. The protein Csk, which usually binds to Src and inhibits its autophosphorylation, is prevented from doing so when the cleaved EphrinB2 peptide binds to Src. The autophosphorylation and activation of

Src, in turn causes phosphorylation of EphrinB2, which inhibits its further processing by γ-secretase. These findings consequently also shed light on how the EphrinB ligands become phosphorylated upon receptor stimulation. This cleavage and phosphorylation dependent loop may act as a feedback mechanism to limit the production of EphrinB2 peptide and in turn control extend of signalling through EphB-EphrinB binding (Georgakopoulos et al. 2006). In general, *cis* cleavage of EphrinB ligands appear to be a regulatory measure, perhaps to attenuate signalling by reducing the number of Ephrins available for Eph binding. On the other hand, *trans* cleaving appears to occur before trans-endocytosis of Eph/Ephrin complexes upon activation and promotes downstream signalling.

Other experiments done with a mouse embryonic fibroblast cell line, stably expressing either EphB receptor or EphrinB ligand, proposed a different mechanism of removal for the receptor/ligand complexes from the cell surface. These complexes are reported to be removed through endocytosis and associated pathways (Irie et al. 2005; Nievergall et al. 2010). Upon stimulation of EphB or EphrinB with its respective ligand or receptor antibody, clusters consisting of the full-length proteins were observed within the cell rather than the surface (Zimmer et al. 2003). When cytoplasmic domain truncated receptor or ligand expressing cells were stimulated using full length counterparts, clustering was not affected as this can occur independent of the intracellular domains. However, in this case endocytosis occurs in opposite direction toward the mutant carrying cell. It was also observed that forward signalling through EphB2 receptor mediates endocytosis and cellular retraction in HeLa cells, whereas, reverse signalling with EphrinB1 results in only endocytosis. These results highlight that cellular adhesion is mediated

through reverse Ephrin signalling, while, cellular repulsion occurs with Eph receptor forward signalling (Zimmer et al. 2003; Pasquale 2008). Furthermore, the receptor ligand complexes may continue to signal after being internalised as they were still phosphorylated. The adhesive and repulsive responses of cells lead to one of the most well characterised role of Eph/Ephrin interactions, which is cell sorting in populations that express different levels of the receptors and ligands (Lisabeth et al. 2013; Park et al. 2015; Perez-White et al. 2014). Similar results were obtained in fibroblasts, where EphB4 expressing cells segregated away from the EphrinB2 expressing cells (Marston et al. 2003). For cellular repulsion to occur, the receptor/ligand complexes must be removed from the membrane, for the cells to move apart. In investigating endocytic pathways as a possible mechanism of receptor/ligand removal, it was observed by both studies (Marston et al. 2003; Zimmer et al 2003), that EphB4 clusters could be co-localised with early endosome marker-1 (EEM-1). As such, it is suggested that Eph/Ephrin internalisation could be occurring through other means like micropinocytosis. Also, in these studies, full length of the receptor/ligand clusters transcytosed into mainly the Eph receptor expressing cell. In addition to this, Marston and associates (2003) also showed that sections of the ligand still attached to the membrane were found internalised in vesicles. Full length EphrinB1 ligand has also been shown to be internalised through this process (Parker et al. 2004). When stimulated with EphB1, EphrinB1 ligands with fluorescently tagged cytoplasmic tails, were found to co-localise with early endosome antigen-1 (EEA-1) endosomal marker. This internalisation of EphrinB1 was found to be mediated by a clathrin-mediated pathway as blocking this pathway prevented internalisation of the receptor/ligand complexes. Therefore, these studies demonstrate that cleaving may not be the only means of removing

receptor/ligand complexes, EphrinB ligands can also be engulfed into the neighbouring cell in a phagocytosis-like way with plasma membrane still attached.

One of the key players in regulating endocytosis of Ephs and Ephrins is the Rho and Rac family of GTPases. Lamellipodia and filopodia were found to only assemble upon EphB4 forward signalling (Marston et al. 2003). Phosphorylated EphB4 were shown to co-localise with lamella, suggesting a direct contact may prompt the development these filopodia. Blocking Rac mediated actin polymerisation was observed to inhibit endocytosis of EphB4 and hinder cellular retraction. Similar results are also obtained with EphB2 and EphA expressing cells, where inhibition of Rac proteins affects endocytosis and contact mediated repulsion of Eph and Ephrin expressing cells (Cowan et al. 2005; Gaitanos et al. 2016; Yoo et al. 2011). It appears that endocytosis and repulsion are not mutually exclusive. Eph receptor phosphorylation activates Rac mediated cytoskeleton reorganisation, with endocytosis the receptor/ligand in the cell, contact sites are reduced between the two cells and they move away from each other hence cellular repulsion. Perhaps one of the most recent and exciting discovery about Ephs and Ephrins proposes that, the signalling between these receptors and ligands may not be limited to cellular surfaces. Recently, exosome release containing full length Eph receptors and Ephrin ligands from different type of cells such as primary neuron, glioblastoma and human embryonic kidney cells has been revealed (Gong et al. 2016). The exosomes carrying EphB2 receptor are taken up by EphrinB1 expressing cells and are shown to cause phosphorylation and activation of the ligand, as membrane tethered receptors do. Furthermore, this type of signalling is demonstrated to have real biological consequences, as ligand stimulation leads to growth cone collapse in

neuronal cells. Upon clustering of EphB2 and EphrinB1 on opposing cellular surfaces, EphB2 is suggested to be sorted and eventually packaged into exosomes in the correct structural form that can then bind to EphrinB1 at a distant site.

Earlier work had confirmed that cell intermingling is greatly reduced in bidirectional signalling but not in unidirectional signalling (Mellitzer et al. 1999). This suggests that when Ephs and Ephrins are highly expressed on opposing cells, the two populations do not mix. Truncated EphB2 and EphrinB2, lacking intracellular domains and unable to transmit downstream signalling, were used in conjunction with full length receptors and ligands to test the effects of uni and bidirectional signalling. When truncated versions of the ligand or receptor were exposed to cells bearing the full length, unidirectional signalling was shown to lead to extensive mixing of the two cell types. In comparison, unmodified cell populations with the full-length molecules cause cellular segregation and reduced intermingling. Gap junction regulation was investigated as mediation of cytoplasmic repulsion of cells (Klein 2004; Mellitzer et al. 1999). The presence of gap junctions between cells were detected by luciferase assays where Lucifer yellow diffuses through junctions formed between two cells. In embryonic cells, Lucifer yellow diffusion could not be detected between the EphB2 and EphA2 expressing cells against EphrinB2 populations, suggesting no gap junctions had formed. Furthermore, when full length EphB2 or EphrinB2 were co-cultured with truncated constructs of the ligand or receptor (respectively), the activation of unidirectional signalling lead to cell mixing between two populations, however, gap junctions still failed to form. Consequently, while bidirectional signalling is proposed to restrict cell intermingling, unidirectional signalling can still prevent

cell contact through gap junction. In mice embryos, EphrinB and Eph expressing cells have been shown to form gap junctions at compartment boundaries, as a means of cell-cell adhesion and communication route between these populations (Davy et al. 2006). In embryos harbouring EphrinB1 heterozygote mutations, these junctions did not form and resulted in the segregation and compartmentalisation of Eph and Ephrin expressing cells. In *Xenopus* oocytes, Eph and Ephrin mediated signalling leads to separation of presomitic mesoderm and notochord layers, where EphB4 and EphrinB2 are expressed in complementary patterns, respectively (Fagotto et al. 2013). Segregation of the different cell populations and boundary formation in the embryonic structures were severely disrupted in experiments where EphB4, EphrinB2 and EphA4 expressions were reduced. These effects were reportedly enhanced, when high expressing tissues were specifically targeted removing the inhibitory constraints these receptors and EphrinB2 ligand pose on developing embryological cells. With the overlapping of cells belonging different layers, cadherin clusters were also noted to be abundant between the different cell types. Hence, Eph and Ephrins were found to be modulators of notochord separation and boundary formation (Fagotto et al. 2013).

1.5.1 Common selection of downstream signalling targets of Eph and Ephrins

The Eph receptors, having multiple active domains, can interact with many downstream pathways and molecules to propagate their effect. Even though the signalling pathway utilised by these receptors can be cell-type and context dependent, there are some commonly reported downstream targets, across not just different cell types but also a range of species.

Perhaps the most commonly reported effectors of Eph receptors are guanosine-5'-triphosphate (GTPases) proteins like Rho, Rac and Cdc42 (Bos et al. 2007). The cycle between GDP inactive to GTP active states of these proteins are regulated by guanine nucleotide exchange factors (GEFs) (Bos et al. 2007). There are 5 types of specialised GEFs called 'eph-interacting exchange factor' or Ephexins found in humans. Depending on species and cell type Ephexins can interact with both A and B type Eph receptors to activate one or all RhoA, Rac or Cdc42 GTPases (Margolis et al. 2010; Schmucker et al. 2001; Shamah et al. 2001; Sardana et al. 2018). In its active state RhoA causes the formation of stress fibers through myosin filament assembly, actin polymerisation and changes in focal adhesion, which generally results in contractility bringing about cellular repulsion and retraction because of Eph signalling (Lisabeth et al. 2013; Tojkander et al. 2012). Similarly, Eph receptor interaction with Rac and Cdc42 GTPases cause the formation of lamellipodia and filopodia (respectively) and facilitates directional cell contraction and migration (Pasquale et al. 2008; Ridley et al. 2015). These GTPases can function differently depending on cell type to bring about these responses.

In neuronal studies, ligand stimulated Eph receptor kinase activation and/or Eph receptor overexpression leads to selective regulation of RhoA by Ephexins, rather than Rac1 and Cdc42 proteins, resulting in a stress fiber phenotype and growth cone collapse (Murai et al. 2003; Sahin et al. 2005; Shamah et al. 2001). In the absence of Ephrin simulation, Ephexins are inactive, allowing for axonal outgrowth (Sahin et al. 2005). Whereas, in human vascular endothelial cells EphB receptor mediated contact inhibition and retraction rely on both RhoA and Cdc42 proteins, while Rac is largely responsible for Eph-Ephrin complex internalisation and attenuation of signalling (Groeger et al. 2007; Marston et al. 2003). On the other hand, patterning

of the mesoderm and ectoderm layers results in RhoA and Rac induced repulsion and separation of the layers downstream of EphB4/EphrinB2 interaction (Rohani et al. 2011). In order facilitate repulsion and migration, Eph/Ephrin signalling also effects cell-cell and cell-matrix adhesion molecules like cadherins and integrins, through several means such as proteolytic cleavage, destabilisation through a secondary effector or by causing structural changes. In co-culture experiments with cells expressing EphB receptors or EphrinB ligands, E-cadherin mediated cell adhesion was absent between cells with opposing receptor/ligand expression (Solanas et al. 2010). ADAM metalloproteases have been shown to localise with Eph receptors to cleave both Ephrins and E-cadherins at the site of receptor/ligand complexes, allowing for cell detachment and sorting. Furthermore, Eph kinase activation can dephosphorylate focal adhesion kinase and cause inactivating conformational changes integrins, leading to decreased cell adhesion to matrix (Miao et al. 2000; Zou et al. 1999).

Other commonly reported and important targets that enable bidirectional signalling are the Src family of non-receptor kinases. Src proteins are recruited to the tyrosine domains of both Ephs and Ephrins upon receptor/ligand interaction (Knöll et al. 2004; Palmer et al. 2002). The Src proteins serve the Eph and Ephrins in two ways, firstly, since the EphrinB ligands lack a catalytic domain they rely on Src proteins to phosphorylate them to become active (Lisabeth et al. 2013). Secondly, Src proteins are identified as essential mediators in recruiting and docking multiple signalling complexes like mitogen-activated protein kinases (MAPK), extracellular signal-regulated kinases (Erk), Phosphoinositide 3-kinases (PI3K), RhoGEFs and integrin adherents to the phosphorylated Eph kinase domain (Palmer et al. 2002; Vindis et al. 2003; Zisch et al. 1998). Inhibiting Src was shown to greatly impact

several cell functions like reducing cell migration, retraction and proliferation in a variety of settings (Vindis et al. 2003; Zisch et al. 1998).

However, as previously described, not all Eph signalling is reliant on ligand stimulation. In glioblastoma cells, EphA2 receptor was found to be phosphorylated in response to endothelial growth factor, in a ligand-independent manner, which signals through the Erk pathway to induce cell proliferation (Hamaoka et al. 2016). Other studies have demonstrated the potential for ligand independent Eph receptor phosphorylation by protein kinase B (Akt) to induce migration, invasion and promote cell survival (Miao et al. 2009; Stahl et al. 2011). Nonetheless, Ephrin induced Eph receptor signalling can also act through these pathways to increase the expression and function of cell cycle progression proteins, like cyclinD1, leading to rapid cell growth (Kang et al. 2017). In endothelial cells, EphrinB2 induced EphB4 activation led to the recruitment of PI3K, which in turn phosphorylated Akt and enhanced MAPK signalling for cell proliferation. In addition, the activation of this cascade also prompted secretion of MMP proteinases causing matrix remodelling and cell migration (Steinle et al. 2002). In mouse embryonic palate cells, Ephrin stimulation of Eph receptors had no effect on the phosphorylation of Akt, rather, Eph activation strongly induced the MAPK/Erk transducing pathways to support cell growth (Bush et al. 2010). As an alternate pathway for regulating cell survival and growth, Eph and Ephrin activity is also shown to positively regulate Janus Kinases (JAK), which then phosphorylate their downstream targets STAT proteins and increase their transcriptional activity (Bong et al. 2007; Lai et al. 2004). Some of these targets and their relation to EphB4 in physiological and cancer settings will be discussed in the following sections.

1.6 The EphB4 receptor in vascularisation during development and in cancer

The EphB4 receptor along with its ligand EphrinB2 has been most well studied and recognised for their role in vasculogenesis and angiogenesis. The differentiation and development of blood vessels is termed vasculogenesis (Flamme et al. 1997; Risau et al. 1988). The branching and expansion of existing vessels is known as angiogenesis (Risau 1997). Ultimately, blood vessels are made of two layers, of an endothelial cell lumen, surrounded by smooth muscle cells and pericytes, which are recruited to the site of vessel formation. It appears that beyond the first formation stage, the time when the vessels differentiate and become of venous or arteriole lineage is also very important.

Early work established that EphB4 and EphrinB2 are needed for vascular development and differentiation (Wang et al. 1998). In investigating EphrinB2 expression in vasculature, it was discovered that it is strictly confined to arteries and could not be found in veins. Since EphrinB2 has several Eph receptors it can interact with, upon screening of a few, it was revealed that endothelial cells of venous lineage express the EphB4 receptor. These expressional differences could be observed as early as embryonic day 9 (E9). During E9 and E9.5, expressional differences of EphrinB2 and EphB4 in arterial and venous capillaries segregate and mark them as molecularly distinct, before angiogenesis begins the sprouting and remodelling of existing vasculature. Difference in expression of EphB4 and EphrinB2, suggests their *cis* and *trans* interactions could be behind the arteriole and venous segregation roles (Yancopoulos et al. 1998). It is anticipated that during the initial stages of vascular development EphB4 and EphrinB2 may be engaged in *cis*,

while their subsequent *trans* interaction would cause them to segregate and limit cell intermixing. Loss of *EPHRINB2* gene function in embryonic stem cells arrests branching of arterial networks and proper assembly of EphB4 expressing vessels (Wang et al. 1998). Similar results are also observed with loss of *EPHB4* gene function, which disrupts cardiac development and angiogenesis, causing necrosis early in embryonic development (Gerety et al. 1999). Hence, EphB4 may be implicated in vasculogenesis as well as angiogenesis. Furthermore, expression of other Eph receptors and ligands in developing vasculature, could not compensate for the fatal outcomes of *EPHB4* and *EPHRINB2* gene loss (Adams et al. 1999; Gerety et al. 1999). It is suggested that, vessels with overlapping expression of EphrinB1 were not able to rescue the phenotype caused by the loss of *EPHRINB2*, signifying that either EphrinB1 it plays a different or less potent role in vascular development (Adams et al. 1999). Also, the highly specific nature of EphB4 receptor to EphrinB2 likely contributes to a unique role in this system (Gerety et al. 1999). Ultimately, interfering with EphB4 and EphrinB2 causes failure in angiogenic sprouting and leads to embryonic lethality.

The *trans* interaction between EphB4 and EphrinB2, not just in endothelial cells but also in surrounding tissues that encounter vasculature, is demonstrated to aid proper development and branching. In co-culture experiments, EphrinB2 and EphB4 overexpressing stromal cell lines were generated and cultured with paraaortic splanchnopleural mesoderm explants (Zhang et al. 2001). EphrinB2 stromal cells inhibited the expansion of EphB4 positive endothelial cells, supporting arteriole formation (Hamada et al. 2003; Zhang et al. 2001). Similarly, the EphB4 overexpressing stromal cells inhibited EphrinB2 endothelial cells, to

sustain venous development (Zhang et al. 2001). Hence, EphB4 and EphrinB2 expression in tissues aids the expansion of like expressing endothelial cells. In the *Xenopus* embryos, EphrinB expression was found to be abundant in somites around the EphB4 expressing endothelial cells, guiding venous growth and migration through inhibitory regulation (Helbling et al. 2000). The expression of EphrinB ligands prevent cell intermixing of EphB4 positive endothelial cells stunting the directional growth of veins (Helbling et al. 2000). The overexpression of truncated EphB4 receptor, unable to transmit downstream signalling, caused abnormal penetration of the intersomitic veins into somatic tissue. Maturation of vessels are also regulated by expressional differences, since endothelial cells recruit smooth muscle cells with appropriately matching expression of EphB4 or EphrinB2 (Zhang et al. 2001). Bidirectional signalling occurring between EphB4 and EphrinB2 and contributing to the developing vascular system have also been studied in zebrafish (Herbert et al. 2009). In these animal models, morpholino (MO) oligonucleotides are injected into embryos and this antisense oligonucleotide reduces the level of the protein of interest (Tandon et al. 2012). When EphB4 or EphrinB2 MOs were injected into developing zebrafish, venous and arterial vessel dissemination was disrupted with the inhibition in migration of angioblasts. Once again highlighted by this study the bidirectional interaction between this receptor and ligand controls the pathways taken for angiogenic sprouting through cellular repulsion and reduced intermingling as discussed by previous studies.

An investigation with mouse embryonic stem cells revealed that, mechanic strain causes a rise in reactive oxygen species and nitric oxide may be the cause in determining EphB4 and EphrinB2 expression in vascular cells (Sharifpanah et al.

2016). Under stress, intracellular calcium signalling enhanced EphrinB2 expression and decreased that of EphB4. Inhibiting calcium partially reversed the downregulation of EphB4 expression, however, EphrinB2 was not affected and remained elevated. Under mechanical strain, vascular branching was inhibited with the knockdown EphrinB2. As a result, mechanical stress is proposed to commit these embryonic cells to an arterial lineage rather than venous, since, EphB4 expression is suppressed (Sharifpanah et al. 2016).

The overexpression of EphB4 has also been found to enhance the vascularisation of various cancers, which is suggested to enhance tumour viability, survival and growth (Alam et al. 2009; Heroult et al. 2010; Kumar et al. 2006; Krasnoperov et al. 2010; Noren et al. 2007; Salvucci et al. 2006). This will be discussed further in **section 1.8**. Studying EphB4 and EphrinB2 in vascular system proves to be difficult due to high mortality rate of animals when expression is interfered with, other well-defined roles of Eph and Ephrin interaction occur within the intestinal tract.

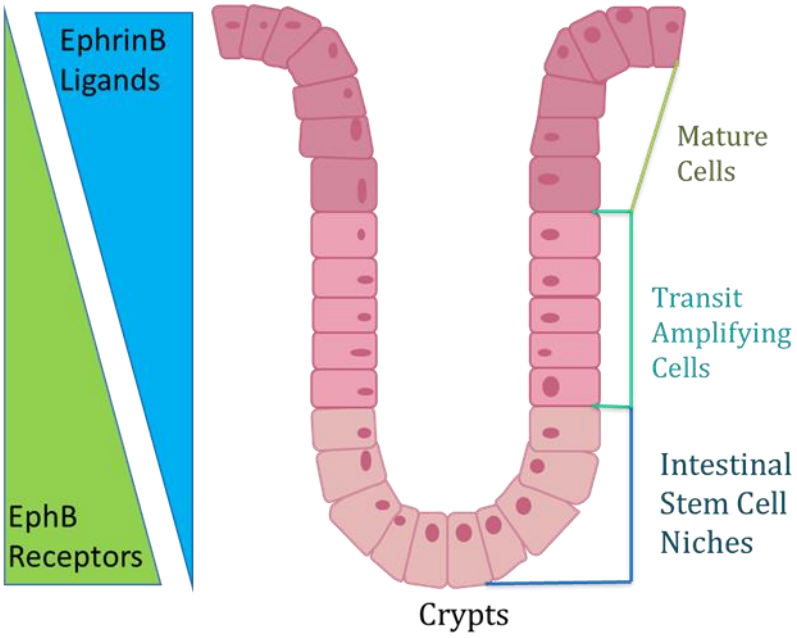
1.7 Eph and Ephrin expression in the intestinal tract and in CRC

In the development of healthy colon, simple epithelial cells aggregate and fold to form pit-like projections known as crypts of Lieberkuhn. Intestinal stem cells (ISCs) occupy the bottom most compartments of these crypts (Genander et al. 2010; Merlos-Suárez & Batlle 2008; Solanas et al. 2011; Willis 2008). The ISCs generate multipotent transit-amplifying cells that then give rise to three main functional cell types found in the large intestines, which are mucous secreting goblet cells, absorptive enterocytes and rare enteroendocrine cells (Cheng et al. 1974). Epithelial

cells of the bowel migrate in a base-to-axis manner and lateral inhibition does not allow for the terminally differentiated cells to migrate back and infiltrate the crypts. Once near the surface, mature cells fulfil their functional roles and are eventually destroyed by apoptosis and shed out into the lumen (Genander et al. 2010; Solanas et al. 2011; Willis 2008; Yeung et al. 2011).

Compartmentalization and segregation of cells in the colonic epithelium is partly achieved with the expression of Eph receptors and Ephrin ligands. Although both A and B types of Eph receptors and Ephrins are expressed within the bowel, EphB receptors and EphrinB ligands play a predominant role in the cellular compartmentalization. EphB receptors control the adhesion and assembly of neighbouring stem cells that also express these receptors (**Figure 1.4**) (Merlos-Suárez et al. 2008). However, as cells mature, they lose EphB receptor expression gradually and begin to express EphrinB ligands. The repulsive activity that results from the interaction of the Eph receptors and Ephrin ligands, ultimately leads to the segregation of the cell populations and forms boundaries between the proliferative compartment in the basal two thirds of the crypt and the differentiated cells above it. This is particularly important since the relatively short lifespan of the intestinal epithelial cells (3-5 days) demands the need for constant and efficient renewal along with appropriate cell positioning to support the normal and vital functional abilities of the colonic cells (Merlos-Suárez & Batlle 2008; Solanas et al. 2011). The strong adhesive forces created by the interactions of Eph and Ephrin expressing cellular domains seal off the internal structures from the toxic luminal environment and serve as protective elements (Merlos-Suárez et al. 2008; Solanas et al. 2011).

Figure 1.4. Schematic representation of counter-gradient EphB receptor and EphrinB ligand expression in the colonic crypts. EphB receptors (EphB1, EphB2, EphB3, EphB3) are predominantly expressed in the bottom compartment of the colonic crypts and EphrinB ligands (EphrinB1, EphrinB2) are expressed by the mature epithelium (adapted from Genander et al. 2010).


EphrinB
Ligands
EphB
Receptors
Mature
Cells
Transit
Amplifying
Cells
Intestinal
Stem Cell
Niches
Crypts

As previously mentioned (**section 1.3**), Wnt signalling is one of the predominant pathways that regulate cell proliferation and differentiation in the colonic crypts and one of the most commonly mutated in the onset of CRC. In an early pioneering study, EphB2 and EphB3 expression was found to be confined to the bottom compartments of the colonic crypts, which are characterised by highly proliferating multipotent progenitor cells (Batlle et al. 2002). Within this compartment Wnt signalling downstream target, β-catenin, also its transcriptional factor, Tcf-4, are also abundantly expressed. When Tcf-4 expression is silenced in mice, the expression of EphB2 and EphB3 become undetectable, suggesting their expression is maintained within the stem cell compartments (Batlle et al. 2002). Also, in the colonic crypts, EphrinB1 was predominantly expressed inversely in the non-proliferative regions, away from the EphB2 and EphB3 dominated compartments. However, co-expression of these receptors and EphrinB1 was observed in the central section of the villus. To investigate the biological relevance of this expression gradient, the intestines of EphB2 and EphB3 null animals were examined. Although morphologically similar, upon closer examination the double mutant animals lacking EphB2 and EphB3 expression were found to be absent of a definitive border between the proliferative stem cell compartment and the differentiated cells. As such, the repulsive and adhesive interactions of Ephs and Ephrins also maintain the structural integrity of the intestinal tissue by separating out the different cell types. In the adult intestines of mice, similar expression patterns as the neonates were observed, with the addition of EphrinB2 expression which was also detected (Batlle et al. 2002). This study was also one of the first to put forward the notion of malignancy compartmentalisation through Eph-Ephrin restriction, by demonstrating that EphB2 positive polyps developed by the animals

carrying an *APC* mutation were surrounded by EphrinB expressing normal adjacent mucosa.

A role of Eph and Ephrin in regulating proliferation in the intestinal tract was studied in animals with individual or combination mutations of EphB2 and EphB3 also kinase inactive forms of these receptors were investigated (Holmberg et al. 2006). In the colonic crypts, animals bearing a double mutation of the receptors had a reported 50% reduction in the proliferative cells of the stem cell niches. Interestingly though, this reduction did not occur in single mutation animals where the observed effect was possibly rescued with the expression of either one of the Eph receptors. The reduction in the proliferative compartment colon was also evident in the kinase inactive forms of receptors suggesting that proliferation is controlled through kinase dependent Eph receptor signalling. In order to isolate the functional relevance of kinase activation, Eph receptor signalling was inhibited using unclustered monomeric ligand Fc which was injected into animals at high concentrations along with controls. At day 3 after injection, displacement of cells, a reduction in proliferative compartments and shortening in the length of the small intestines were detected. In addition, a gain of function mutation introduced to EphB2 increased the level of proliferation in the crypts without affecting cell positioning. These results confirmed that Eph receptor signalling through kinase activation regulates proliferation in the intestinal tract independently of their cell positioning effects (Holmberg et al. 2006).

In another study, while investigating the potential role of Smad3 protein in the development of the intestinal crypts, the authors came across its association with

Eph receptors amongst various other findings (Furukawa et al. 2011). In isolated colonic epithelial cells, there was a significant increase of proliferation in Smad3 deficient mice. This rate of proliferation could not be repressed even with the addition of a known proliferation inhibitor. Furthermore, the expression of some cell cycle inhibitors had reduced, while the cell cycle promoting c-Myc protein was upregulated in the Smad3 deficient mice. Also, in these mice, the proliferation marker Ki67 expression was found to be expressed all over the crypts suggesting that the differentiated epithelial cells and the progenitor cells were no longer compartmentalised. As such, Smad3 was found to negatively regulate cell proliferation in colonic cells by controlling cell cycle related proteins. The expression of EphB2 and EphB3, as expected, was found in the colonic crypts of wild-type mice (Furukawa et al. 2011). Their expression was greatly reduced and only weakly detectable in western blots, while no expression could be detected through immunostaining in Smad3 deficient animals. The phenotype of cellular intermixing in the colonic crypts resembled the EphB2 and EphB3 double mutant animals in the previously discussed study. Therefore, Smad3 is proposed to be necessary for the expression of EphB2 and EphB3 in the colonic crypts, although more thorough investigation is needed.

Increased rate of proliferation is often one of the first mutational advantages conferred to cancer cells, allowing for unregulated cellular growth. As it has been discussed in this review, there are several factors regulating the progenitor compartments of the colon and a deregulation in function or expression of these factors can lead to the onset of CRC. To investigate the role of β-catenin and its transcriptional factor target, T-cell factor (Tcf), in the initiation of CRC, inducible

truncated forms of Tcf were expressed in two CRC cell lines (van de Wetering et al. 2002). This form of Tcf behaves as a dominant negative construct as it cannot bind to β-catenin and acts to inhibit its effect. When expression is induced in CRC cell lines, the cells undergo rapid cell cycle arrest. Furthermore, through gene arrays it was determined that the truncated Tcf inhibits β-catenin/Tcf activity, more than 2-fold increase in 115 genes associated with cell differentiation occurs. This provided evidence that under normal circumstances, this complex regulates cell proliferation and maintains their undifferentiated state. One of the genes that exhibit great level of upregulation was the cyclin dependent kinase inhibitor p21, which is a marker for the differentiated cells in the colon (van de Wetering et al. 2002). Using an antisense cDNA, p21 was downregulated in the Tcf mutant cells and as a result the cell cycle arrest previously observed in these cells had decreased. This study established that the β-catenin/Tcf complex regulates cells between the state of proliferation and differentiation in the adult gut and in CRC cells. Upon immunostaining neoplastic tissue, it was revealed that β-catenin accumulation in cancer cells results in an increase in the upregulation of target genes like EphB2. Due to this Eph receptor involvement in CRC development and progression have been investigated over the years.

β-catenin and Tcf activation have similar effects on cancer cells, as they promote the proliferation and dedifferentiation of cells, their target genes would expect to be upregulated in lesions (Batlle et al. 2005). Interestingly though, EphB2 messenger RNA expression was found to be downregulated in most CRC cell lines and in samples of lymph node and liver metastasis from CRC patient samples. In the tissues, EphB2 expression was also seen in the bottom compartments of the crypts

of normal tissue. The tumour samples and liver metastasis showed large areas with lost or reduced EphB2 expression despite an obvious accumulation of β-catenin in the nucleus. Therefore, it was proposed that EphB2 silencing occurs in malignancies. To determine when the silencing takes place, tumour samples with different Duke staging was examined for EphB2 expression. EphB2 receptor expression was found to decrease with increasing stage and malignancy of the tumour, whereby, only a small subset of stage IV tumours was positive for its expression. EphB3 is also thought to follow a similar pattern as the mRNA levels were also downregulated. In inspecting other Eph receptors, it was found that EphB4 was also a target of Tcf and its expression was downregulated with Wnt cascade inhibition (Batlle et al. 2005). While EphB4 was also found to be expressed in the colonic crypts and early CRC lesions, its expression is also reported to be lost in advanced stages of the disease. Its decrease is said to overlap with that of EphB2, hence, they are suggested to be uniformly silenced in cancer progression. A cause for this was investigated by using *APC* mutant animals that express dominant negative EphB2 and EphB3 in the intestinal epithelium. The tumours that arose in these animals were more aggressive and invasive and so the silencing of these receptors may be essential in the progression of CRC.

EphB receptor involvement in CRC has also been investigated with introducing expression into negative cell lines. The fluorescently tagged cell lines were then co-cultured and mixing occurred between EphB3 and EphB2 expressing cell lines and unmodified cell lines that do not express these receptors (Cortina et al. 2007). The intermingling of these cells was reduced when they were co-cultured with EphrinB1 expressing cells. Quantifying the size of the clusters showed that in the presence of

EphrinB ligand, EphB expressing cells formed large colonies approximately 450 cells in size, in comparison to 10-fold less in control experiments. The cells continued to grow in this manner without spreading out and mixing suggesting continuous EphrinB restriction is imposed. Similar results were also obtained with EphB4 and EphrinB2 expressing cells, while, EphrinB1 and EphB4 co-cultures failed to mount a similar extend of compartmentalisation. In testing cell-cell and cell-matrix adhesion, cells were grown on laminin coated surface which showed protrusion and cellular spreading. When soluble EphrinB1 was added, EphB expressing cells contracted and ultimately formed clusters. From these results, it is proposed that EphB signalling may result in tumour compartmentalisation through EphrinB stimulation *in vivo*. When investigated it was found that EphB and EphrinB expression is complementary in mice and human CRC (Cortina et al. 2007). In the small intestine of animals, the growing tumours were surrounded by EphrinB expressing villus cells and a layer of stroma separated the two populations. In the colon, the absence of villi leads to the direct contact between cancer cells and normal EphrinB expressing epithelium and reduced tumour growth. To research the restriction imposed on tumours by EphrinB expression, reduced EphrinB expression in intestinal tract was engineered in *APC* mutant mice. The tumours of these animals fused with the normal epithelium and formed villus like structures that replaced the normal crypt-villus units. These tumours were not enclosed by normal mucosa suggesting the Eph/Ephrin interaction induced repulsion and compartmentalisation of the tumours had been lost. As a result, these tumours were reported to grow at an acceleration rate. Although, some tumours were observed to be restricted suggesting a role for EphrinB2 mediated compartmentalisation in these mutant animals. In conclusion, the repulsive interaction between EphB receptors

and EphrinB ligands in colonic tumours is suggested to suppress tumour progression and prevents cancer invasion and metastasis by promoting strong cellular adhesion through E-cadherin (Cortina et al. 2007).

A study reviewing information available on the Cancer Genome Atlas suggests that Eph receptor genes located on the same chromosomes can be deleted or amplified together (Al-Ejeh et al. 2014). As such Eph receptors that are likely to be coexpressed were grouped as 'cluster 1' and cluster 2'. It was then noted that the Eph receptors in cluster 1 tended to be pro-oncogenic according to literature and those in cluster 2 tended to be related with better prognosis. EphB4 was grouped along with EphB2 and several EphA genes as cluster 2. However, the controversial findings in EphB4 related cancer literature is addressed as being 'disease setting and tissue specific'. Also mentioned is that cluster 1 genes show an association with DNA regulation and repair pathways while cluster 2 genes were involved in pathways in cell cycle and survival. These distinct patterns of molecular involvement suggest that while cluster 2 genes are predominantly expressed in early stages of tumour growth, cluster 2 genes lead the epithelial to mesenchymal transition by deregulating gene repair (Al-Ejeh et al. 2014). The EphB4 receptor has yielded highly contradictory results in CRC and both tumour suppressing and promoting roles have been suggested.

1.8 The EphB4 receptor in cancer

In the adult, EphB4 is expressed in venous vessels while EphrinB2 is expressed in the arteries and together they control the remodelling and branching of vasculature. EphB4 and EphrinB2, like many of their relatives, are also overexpressed in several different types of cancer. Vascularisation is vital for tumour growth; a pro-tumourigenic characteristic of EphB4 is thought to be via reverse signalling upon interaction with EphrinB2 which induces angiogenesis through the formation of extracellular matrix-dependent capillary-like structures (Salvucci et al. 2006). EphB4/EphrinB2 interaction also enhances endothelial progenitor cell migration, mediated by stromal-derived factor-1 (SDF-1) (Salvucci et al. 2006). In terms of tumour progression this could mean that potentially carcinogenic cells in which EphB4 receptors are highly expressed can gain the ability to grow their own blood supply and become metastatic (Heroult et al. 2010; Krasnoperov et al. 2010; Noren et al. 2007; Salvucci et al. 2006). Aside from the angiogenic effects of EphB4 and EphrinB2 interactions another suspected mechanism of increased cancer cell malignancy arises from the typical regulatory roles they play in cytoplasmic dynamics. It is proposed that in the presence of irregular ratios of receptor and ligand, where one exceeds the levels of the other, their interactions result in cellular repulsion with an increase in cellular motility and invasive ability conferred to cancer cells (Dodelet et al. 2000). Several cancers such as kidney, bladder, prostate, uterine, cervical and breast showed an upregulation in EphB4 levels (**Table 1.1**). In these cancers an increase in tumour vascularization and malignancy was proposed to be related to high expression of EphB4.

Table 1.1 The role of increased expression of EphB4 receptor in cancer

Cancer Type	Contribution to Cancer	References
Breast Cancer	Poor prognosis Tumour suppression	(Brantley-Sieders et al. 2011) (Kumar et al. 2006) (Noren et al. 2006)
Bladder Cancer	Enhanced malignancy	(Xia et al. 2008)
Gastric Cancer	Promote carcinogenesis	(Li et al. 2010)
Head and Neck Squamous Cell Carcinoma	Poor prognosis, increased malignancy	(Masood et al. 2006) (Sinha et al. 2003) (Yavrouian et al. 2008)
Lung Cancer	Progression of cancer	(Zhu et al. 2007)
Ovarian and Uterine Cancer	Poor prognosis	(Alam et al. 2008; 2009) (Kumar et al. 2007) (Spannuth et al. 2010)
Prostate Cancer	Poor prognosis	(Lee et al. 2005) (Ozgur et al. 2011)
Urogenital Cancer	Increased tumour vascularization	(Ozgur et al. 2011)

As it is well documented, that Eph and Ephrin activation in cells can be achieved through treatment with clustered versions of the ligand or receptor, monomeric EphB4 is suggested to inhibit reverse signalling through EphrinB2 (Martiny-Baron et al. 2004). As a consequence of this inhibition, cellular sprouting, proliferation and adhesion is reduced in melanoma cancer cell line. Furthermore, in animals treated with soluble EphB4, there is also a fall in tumour vascular density. Another study conducted using the same soluble EphB4 approach, also found that treating epithelial tumours reduced proliferation and increased apoptosis rate (Kertesz et al. 2006). Treatment of cells is reported to block EphrinB2 and EphB4 phosphorylation and activation, as a result, migration and organisation of endothelial cells into tubular vessels is reduced leading to poor vascularisation of tumour tissue. This study also found a decrease in cell-cell and cell-matrix adhesion with treatment and this is proposed to be a reason behind failure of endothelial cell aggregation into vascular networks. Studies in CRC cells also show similar results with EphB4 and EphrinB2 inhibition, which will be discussed in more detail in **section 1.8.1**. When EphB4 is targeted with siRNA in breast cancer cell lines, the decrease in expression leads to more cell-substrate adhesion and enhanced migration (Noren et al. 2009). Inhibition of EphB4-EphrinB2 interaction using the TNYL-RAW peptide and truncated EphB4 constructs unable to bind to a ligand, did not alter integrin mediated adhesion of cells. As such, EphB4 expression in breast cancer cell line reduced integrin-mediated substrate adhesion even in the absence of EphrinB2 stimulation. This is proposed to occur as a result of *cis* clustering of EphB4 in the presence of high expression of the receptor, which in turn can lead to heightened kinase activity.

Many studies are in strong agreement with the reports of pro-tumourigenic characteristics of EphB4 (**Table 1.1**) (Alam et al. 2009; Kumar et al. 2006; Ozgur et al. 2011). However, EphB4 receptors contribution towards CRC yields contradictory findings. Although there is mounting evidence suggesting a pro-tumourigenic role for EphB4 in CRC (**Table 1.2**), there are also reports which propose that it aids the clonogenic transition of normal intestinal cells to adenomas and further provides mutational advantages toward cancer cell survival (**Table 1.3**) (Davalos et al. 2006; Dopeso et al. 2009; Heroult et al. 2010; Kumar et al. 2009).

An increase in the levels of mitogenic and growth factors as well as extracellular matrix remodelling molecules in tumour tissues have been observed when EphB4 levels are low or dysfunctional (**Table 1.3**) (Davalos et al. 2006; Dopeso et al. 2009). This observation would mean that tumour cells could proliferate at higher rates and gain the ability to migrate and invade through the surrounding structures. Also, an increase in expression of EphB4 receptors has been shown to correlate positively with the survival of CRC patients (Davalos et al. 2006; Dopeso et al. 2009). However, other findings indicate that EphB4 receptors are preferentially expressed in the late and metastatic stages of CRC, presumably supporting the pro-tumourigenic roles they play in other cancers (**Table 1.1, 1.2**) (Kumar et al. 2009). Therefore, the function and influence of the EphB4 receptor may be different depending on tumour and cellular context.

Table 1.2 Pro-tumourigenic findings of EphB4 in CRC

EphB4 is overexpressed in late and metastatic stages	(Kumar et al. 2009) (Stephenson et al. 2001) (Liu et al. 2002) (Stammes et al. 2017)
High EphB4 levels increased migratory and proliferative abilities of cells	(Heroult et al 2010) (McCall 2016)
Inhibition of EphB4 arrests vessels development in tumours	(Djokovic et al. 2010) (Krasnoperov et al. 2010) (Guijarro-Muñoz et al. 2013)
EphB4 overexpressing tumours grow faster	(Lv et al. 2016)

Table 1.3 Anti-tumourigenic findings of EphB4 in CRC

Low levels of EphB4 associated with the disseminated state of cancer	(Davalos et al. 2006) (Doleman et al. 2010)
High EphB4 expression can compartmentalise tumour and inhibit metastasis	(Batlle et al. 2005)
High EphB4 levels decrease invasive potential of cells	(Dopeso et al. 2009)
EphB4 expression does not affect level of vascularisation in tumours	(Dopeso et al. 2009)

1.8.1 EphB4 receptor as a pro-tumourigenic factor in CRC

A study of EphB4 and EphB2 receptors and their possible role in colorectal cancers showed that in the samples tested, the EphB2 and EphB4 expression from at the early stages of cancer tended to be similar (Kumar et al. 2009). However, as the cancer progressed, the expression of these two receptors was observed to differ, with EphB4 being overexpressed and EphB2 receptor expression being suppressed. As previously stated, the Wnt signalling pathway is responsible for maintaining stem cell populations and proliferative compartments within the colonic epithelium. It is suggested that this difference may arise in the progression of CRC due to the switch between two different Wnt co-activators of transcription in malignant cells. It is proposed that in stem cells an undifferentiated state is maintained when gene expression is initiated by co-activator 'Cyclic AMP response element-Binding Protein' (CBP), which also allows for rapid proliferation and growth of cells. On the other hand, co-activator p300 is used by cells committed to terminal differentiation. Hence, a switch from co-activator p300 to CBP would provide the tumour cells with the ability to maintain an undifferentiated state while proliferating and self-renewing at higher frequencies. Small interfering RNA (siRNA) used to target each of the co-activators lead to downregulation of EphB4 with CBP silencing, while reduced EphB2 expression was observed with p300 knockdown. This was consistent with the hypothesis that EphB4 expression is upregulated in latent stages of CRC due to CBP transcriptional influence while EphB2 is downregulated. EphB4 expression, either full length or the cytoplasmic truncated protein, is also found to desensitise colon cancer cells to 'Tumour Necrosis Factor-related apoptosis-inducing ligand' (TRAIL) mediated cellular death. The mechanisms of EphB4-mediated escape from apoptosis are not understood. The

reduction of EphB4 expression had anti-angiogenic effects that inhibited tumour growth, while overexpression tended to assist the tumours in growing larger (Kumar et al. 2009). Furthermore, immunofluorescence and immunoblotting examinations of human primary tumour samples showed that 67 out of 90 revealed an increase in EphB4 levels in comparison to samples of the adjacent "normal" tissue. EphB4 expression was also noted to be present in mesenteric lymph node metastasis. It was concluded that EphB4 expression levels correlate positively with the stage and grade of cancer (Kumar et al. 2009). These findings are in good agreement with studies of EphB4 receptors in other cancer types (**Table 1.1**).

Other studies have also examined EphB4 expression levels in CRC samples and cell lines. An Australian study (Stephenson et al. 2001) conducted complementary DNA (cDNA) array experiments on colon tumour and normal samples. Investigations of 588 genes, that were chosen based on their roles in cancer progression, showed that EphB4 was amongst the most significantly upregulated genes in the tumours compared to normal samples. It is reported that upregulation of EphB4 was evident in 82% of 62 tumour samples in comparison to adjacent normal tissue. This result was further supported by real time-PCR conducted on 10 CRC cell lines, where EphB4 expression was confirmed to be higher when compared to CK19 colonic epithelial cells. Moreover, real time-PCR was conducted on five primary tumours and one liver metastasis samples. Upon observation EphB4 upregulation is evident in all of the primary tumour samples. However, the liver metastasis sample showed a decrease in EphB4 expression when compared to the primary tumour of the colon and also to normal liver sample, yet this result was overlooked by the researchers. Similar results were observed in another study, where analysis of EphB and EphrinB expression in a small group of

CRC cell lines and carcinoma samples also showed an overexpression of EphB4 and EphrinB2 (Liu et al. 2002). Northern blots were conducted on CRC, pancreatic and renal carcinoma cell lines for a comparison. An abundant expression of this receptor and ligand were found in CRC cell lines, while EphrinB2 expression was negligible in pancreatic and renal cancer cells. A more recent evaluation of EphB4 in tumour samples from CRC patients also revealed an upregulation in comparison to adjacent healthy tissue (Stammes et al. 2017). Similar results were observed for the EphA2 receptor, however, the high level of expression of this receptor was observed in some healthy tissue of the gut. However, unlike Kumar and associates (2009) a strong positive correlation between EphB4 expression and the stage and grade of cancer could not be established (Stammes et al. 2017). Nonetheless, it is suggested that the overexpression of EphB4 may help in imaging of tumours in CRC patients and may help identify gut mucosa from tumour tissue.

A study by Guijarro-Muñoz and associates (2013) highlighted the importance of developing not only prognostic biomarkers but also predictive ones, whereby, the degree of benefit a patient may receive from a specific type of treatment can be evaluated using biomarkers. Patients diagnosed with metastatic CRC received an anti-VEGF (vascular endothelial growth factor) monoclonal antibody, Bevacizumab, and chemotherapy treatment. A strong point of the experimental design was that all patients were at the same stage of the disease and after the treatment they were appropriately categorized as responders (complete and partial) and non-responders (stable or progressive state) according to tomographic evaluations of their tumours. Amongst the 13 analysed samples, 7 had low EphB4 expression and the remaining had high EphB4 expression. Within the low expressing group, all 7 patients had responded to the bevacizumab treatment with a

71% survival rate in comparison to only 2 patients out of 6 in the high EphB4 expressing group responding to the treatment with all of them dying before the end of the study (Guijarro-Muñoz et al. 2013). It was also indicated that there were no significant differences in clinicopathological features of patients in the responders and non-responders group which eliminated factors such as age and gender contributing to the outcome of results. The authors concluded that EphB4 expression is a good prognostic and predictive biomarker for CRC patients undergoing bevacizumab treatment. This study is of great significance since the results obtained highlight the importance and relevance of identifying specific molecular targets for CRC treatments in order to increase the likelihood of patient's response to these treatments and better survival rates.

In a recent study, EphB4 is found to associate with signalling modulators. Kinase suppressor of Ras1 (KSR1) acts as a scaffold for these modulators and sustains optimal signalling efficacy (McCall et al. 2016). It has been found that this protein is crucial for the survival of CRC cells while unessential in normal epithelium. In investigating other targets that appear to be essential for CRC cell survival, EphB4 was also found to be indispensable. In most CRC cell lines that were examined EphB4 is reported to be overexpressed. Similarly, in human CRCs EphB4 mRNA expression was found to be increased in majority of the carcinoma samples in comparison to normal mucosa. When EphB4 expression was targeted with RNAi-mediated depletion, KSR1 expression was also found to decrease. Interestingly, KRS1 depletion did not affect EphB4 mRNA levels, rather, resulted in the reduction of protein levels. Targeting EphB4, however, did not alter mRNA or protein levels of KRS1, so it appears that KRS1 regulates EphB4 expression post-transcriptionally. The decrease in expression of these proteins were shown to slow

the rate of proliferation of cancer cells, while enhancing the expression of apoptotic indicators in comparison to colonic epithelial knockdown cells. In the CRC cells, kinase inhibition also leads to reduced cellular viability through induction of apoptosis. Post-transcriptionally EphB4 is proposed to be inclined for proteasome degradation, since proteasome inhibitors rescues the expression of EphB4 in cancer cells. Lysosomal inhibitors resulted in the increase expression of EphB4 even when KRS1 was depleted. This suggests that, KRS1 under normal circumstances may suppress lysosomal degradation of EphB4, stabilising its expression.

Two highly specific antibodies raised against the first and second FN repeats (respectively) of EphB4 were used in experiments as inhibitors of EphB4 and EphrinB2 signalling (Krasnoperov et al. 2010). Treatment of endothelial cells with these antibodies prevents tube like formations. Furthermore, in animals bearing head and neck squamous cancer, prostate cancer and CRC cell line xenografts treatment yields smaller tumours that grow much slower than controls. There was also a reduction in vascular density and perfusion in treated animals. This was noted to increase levels of hypoxia in these tumours. Investigation of the HT29 CRC cell line induced tumours revealed that upon treatment of animals with one of the antibodies, there was a marked reduction in EphB4 levels. *In vitro*, it was discovered that EphB4 expression decreases upon internalisation of the receptor when bound by the antibody. When HT29 tumours were treated with an anti-vascularisation agent, Avastin (bevacizumab), in combination the two EphB4 specific antibodies up to 80% reduction in tumour size compared to control tumours and tumour regression was noted (Krasnoperov et al. 2010). Another commercially available antibody, H200 (Santa Cruz), raised against the CRD and a portion of FN repeats of EphB4, could potentially functionally inhibit EphB4 (Stephenson et al.

2015). The H200 antibody drastically reduced cellular viability through induction of apoptosis in the colorectal cancer cell line SW480 in comparison to a control. Testing on other cancer cell lines like bladder, prostate, breast cancer and osteosarcoma revealed altering outcomes, with a reduction in viability in some and no observed effects in other cells. Colony formation assays conducted with a breast cancer cell line, revealed a considerable amount of cellular death and the surviving colonies had a lack of cellular projections with H200 antibody treatment. A reduction in protein levels of EphB4 with antibody treatment is also evident, this is proposed to occur as a result of internalisation and degradation of EphB4 as it would with EphrinB2 stimulation. The mode of inhibition and the reasons behind why this antibody would cause cellular death in some cancer cell lines and not the others stand to be investigated.

Recently, the effects of EphB4 expression was investigated in 200 samples of primary CRCs and 50 matched adjacent samples by immunohistochemistry (Lv et al. 2016). In these samples, EphB4 receptor expression was noted to be in the cell membrane, while EphrinB2 is reportedly in the cytoplasm. However, EphB4 expression is found to be significantly higher in malignant samples than normal mucosa, while EphrinB2 expression remains constant and the expression of the two are not correlated. High EphB4 expression was associated with invasion depth, lymph node metastasis and distant metastasis. To investigate effects of expression in xenografts, stably transfected SW480 CRC cell line was used in subcutaneous models. EphB4 overexpression lead to quicker growth as well as heavier weight in tumours, compared to the low EphB4 expressing controls. Once again, no expressional differences in EphrinB2 were reported across experimental groups. This study reports an increased area of necrosis and invasion into surrounding

muscle and vascular layers with EphB4 overexpressing tumours. This suggests that with EphB4 overexpression the cancer cells become more aggressive and invasive. This study also reports that EphB4 overexpressing tumours had increased angiogenesis, which seems rather inconsistent with the results that these tumours also had high level of necrosis. EphB4 overexpression is also found to decrease E-cadherin mediated tumour cell adhesion.

1.8.2 EphB4 receptor as a tumour suppressor in CRC

Eicosapentaenoic acid treatment of the HT29 CRC cell line, was shown to upregulate EphB4, which was identified amongst many other genes that were screened (Doleman et al. 2010). It is postulated that this increase in expression may help compartmentalise tumours and prevent metastasis of CRC. However, this assumption was based on other studies and it was not actually investigated in this one. The mounting amount of evidence suggests a pro-tumourigenic role of EphB4; enhanced expression with Eicosapentaenoic acid treatment could result in a more aggressive turn for cancer cells. Nonetheless, EphB4 receptor's tumour promoting tendencies, discussed in the previous studies, are contradicted by the study of Davalos et al. (2006). Unlike Kumar and associates (2009), the authors (Davalos et al. 2006) suggested that although the EphB4 gene is a direct target of TCF/β-catenin complexes, EphB4 expression tends to be frequently lost in advanced stages of CRC. EphB silencing, particularly EphB2 and EphB3, with the progression to malignancy in CRC have also been demonstrated in various other studies (Clevers et al. 2006; Herath et al. 2012; Senior et al. 2010; Sheng et al. 2008). Histochemical studies were conducted on 125 tumour samples obtained from patients and the EphB4 levels were graded on a scale of 0-4. The results showed that low EphB4

levels were associated with poor prognosis and shorter survival rates, and this was based on the 'disease-free survival' time measured as 'time to recurrence' of cancer. It was observed that patients with low EphB4 expression had a median survival time of 1.8 years and the patients with high EphB4 expression had a median survival time of over 9 years. Although the clinical information of the patients was handled relatively well as supporting evidence for the findings of Davalos et al. (2006), the treatment regimens were not considered which could have ultimately influenced the obtained results. In contrast to the findings of Kumar et al. (2009), these authors (Davalos et al. 2006) observed that the EphB4 expression levels in 16 regional lymph node tissue samples were lower than in the primary tumour samples suggesting that EphB4 is silenced in the disseminative state of the cancer. The exact cause of the contradictory findings presented in these two papers cannot be determined, however, possible causes may include a difference in tissue processing and antibody used to detect the levels of EphB4 which could have influenced the outcome. Davalos et al. (2006) investigated the possible mechanism behind the inactivation and silencing of EphB4 receptors. Of the 112 tumour samples tested 54 were found to have hypermethylation in the promoter region of the *EPHB4* gene. In order to test the effects of promoter methylation, a cell line that showed *EPHB4* promoter methylation (SW480) was treated with a DNA methyltransferase inhibitor for 72 hours, as a result of which the promoter region was found to be demethylated and upregulation of EphB4 mRNA was observed. However, no experiments such as proliferative or migratory assays, were then conducted with the treated cell line to determine the functional relevance of the reactivation of EphB4 expression. This result has recently been opposed by another study, where CRC patient samples and various CRC cell lines were investigated for methylation in promoter sites of

EphB4, EphB2 and EphB3 (Wu et al. 2008). All examined samples and all but one CRC cell line were found to be unmethylated in the EphB4 promoter region. The methylation in the positive cell line was found to be upstream of the transcription initiation point. It was therefore suggested that, DNA methylation of these the *EPHB* genes appeared to be an unlikely event in CRC and the loss in expression is probably a result of post-transcriptional modification. Epithelial and ovarian cancer cell lines were also studied and once again EphB4 methylation was not observed. Eph receptor epigenetic silencing in CRC have been hypothesized and mentioned in other studies (Herath et al. 2012; Sheng et al. 2008).

Dopeso et al. (2009) also confirmed the likely tumour suppressing activities of EphB4 receptors in CRCs. To test the proliferative potential of CRC in regard to EphB4 expression and signalling, dominant negative constructs of EphB4 with a 'truncated' intracellular domains were transfected into a high EphB4-expressing CRC cell line (HT29). The truncated construct of EphB4 is able to bind ligands and become a part of multimeric clusters but cannot transmit signal. The controls for these cells were empty vectors which should not alter the cellular characteristics in any way. These cell lines were then introduced into six athymic mice. The tumour sizes obtained from the animals injected with the cell line expressing the truncated EphB4 receptor were shown to be larger than tumours from animals injected with the cell line with empty vector controls. The researchers also used a low expressing cell line (SW837) and upregulated the EphB4 levels by transfecting it with a vector expressing full length EphB4. When introduced into the animals the upregulation of EphB4 slowed tumour growth and resulted in a 2.5-fold decrease in the invasive potential of these cells.

To investigate the ongoing debate about the angiogenic effects of EphB4 and EphrinB2 interactions, immunostaining experiments were conducted targeting endothelial markers ‘CD34’ and ‘CD105’ with human colorectal cancer xenografts with low and high EphB4 expression in athymic mice (Dopeso et al. 2009). According to the authors, no significant difference was found with vascularization of tumour samples obtained from these xenografts. The method employed to analyse this result was not clear.

Moreover, the authors demonstrated the effect of mutation in alleles coding for EphB4 receptors (Dopeso et al. 2009). Mice harbouring heterozygous *APC* and *EPHB4* mutations were noted to develop several spontaneous tumours in the gastrointestinal tract (Dopeso et al. 2009). Also, the lifespans of these animals were 199 days on average which was 25% shorter than lifespans of *APC* min/+ *EPHB4* +/+ littermates. Hence the conclusion reached was that inactivation of *EPHB4* alleles in animals accelerates tumour progression. However, the likely elements that contributed to the shortened lifespan of these animals and if it was due to the higher tumour burden was not made clear. Also, another factor that should be considered is that these animals were a breed that the researchers attained by crossing mice that were carrying *APC* min/+ mutations with mice that were *EPHB4* +/-. Hence the mutations in these genes would affect cellular systems in the entire animal not just the intestine; as a consequence, this could have influenced the health and lifespan of these animals.

To understand the process of *EPHB4* mutations accelerate tumour progression, the number, size and location of tumours were studied in animals sacrificed at 18 weeks. Those animals bearing heterozygous mutations for *EPHB4* and *APC* had

larger tumours in the small intestine and a higher frequency of tumours in the large intestine. This result was displayed as supporting evidence to the observation that overall EphB4 expressional abnormalities resulted in shorter survival rates. However, the authors fail to mention that animals with functional *EPHB4* alleles yielded larger tumours in the large intestine while both the functional *EPHB4* alleles and heterozygous mutations of *EPHB4* resulted in similar frequency of tumours within the small intestine (Dopeso et al. 2009). Thus, one criticism of this paper is that results which support their hypothesis have been selected for discussion. Nonetheless, mice harbouring *APC* mutations have previously been reported to develop adenomas in the small intestine, with high level of β-catenin expression (Batlle et al. 2005). This is suggested to enhance EphB4, EphB3 and EphB2 expression in these lesions. However, the lesions remain confined and do not progress or metastasise. Hence, EphB expression in CRC adenomas was suggested to suppress oncogenic transition.

One proposed mechanism behind the switch from tumour suppressive to enhancing properties of EphB4 is the presence or absence of EphrinB2 stimulation (Rutkowski et al. 2012). In the absence of ligand stimulation, an unopposed overexpression of EphB4 was found to enhance the migration, invasion and anchorage independent growth of a non-tumourigenic mammary cell line, hence transforming it into a malignant phenotype. EphrinB2 stimulation of these cells suppressed these properties, potentially as a result of reduction and degradation of EphB4. It is worth noting that endogenous Ephrin expression in these cells did not yield the same results, rather, the addition of soluble EphB4 and EphrinB2 is proposed to inhibit trans interaction and activation of the receptor.

1.9 Summary

Colorectal cancer (CRC) is one of the most common causes of cancer related mortalities in Australia (AIHW 2017). Every year approximately 16,000 people are diagnosed with CRC and more than 10-20% of them are expected to die from this disease (AIHW 2012; NHMRC 2005). An exact cause for CRC development has not been identified, however, genetic predispositions along with a diet high in saturated fats, excessive alcohol consumption, tobacco smoking and sedentary life style are thought to be some of the potential contributing factors (Cancer Council 2015).

CRC is treated through surgical intervention to resect the primary tumour. However, due to the asymptomatic nature of CRC, some cases are not diagnosed until the cancer has spread to a secondary location, which is primarily to the liver. Metastatic spread of cancers decreases survival chance and is the leading cause of patient mortality. The very few symptoms that do present in the early stages of CRC are often vague and overlooked by individuals and they can range from abdominal pain/discomfort, change in bowel habits, tiredness and at times bloody stools (Cancer Council 2015). A problem with these types of symptoms is that, they are often experienced by elderly individuals and are perceived as age related events. Although preventative and diagnostic measures are currently being tested and tried in order to reduce the incidence, mortality remains high due to a lack of specific treatment methods that could combat CRC recurrence.

These problems raise the need to identify molecular targets for which specific and aggressive therapeutic interventions may be designed and developed. One such

molecular target is the erythropoietin-producing hepatoma (Eph) family of receptor tyrosine kinases and their corresponding 'Eph receptor interacting' (Ephrin) ligands.

Ephs are the largest family of receptor tyrosine kinases (Bush et al. 2012; Egea et al. 2007; Himanen et al. 2012). They comprise of A and B types that interact with and are activated by similar subclass of their cell-bound Ephrin ligands. Eph and Ephrin interactions lead to bidirectional signalling, where both the cell carrying the receptor and the cell carrying the ligand can be activated (Krasnoperov et al. 2010; Herath et al. 2010). A primary role of Ephs and Ephrins is to control and regulate cellular mobility and migration. For this reason, their involvements in the metastatic progression of cancers have been widely investigated. Some members, i.e. the EphA2 receptor, are overexpressed in breast, liver and prostate cancers and are associated with enhanced malignancy and poor prognosis (Pasquale 2010; Surawska et al. 2004). On the other hand, the expression of the EphB2 receptor is suggested to restrict tumour growth and hinder the metastatic progression of CRC (Senior et al. 2010). However, it is the EphB4 receptor and its corresponding EphrinB2 ligand that have yielded highly contradictory results in CRC. Studies on this topic have been inconsistent and so it is not yet clear whether they function as tumour suppressors or whether they aid the survival of CRC cells (Batlle et al. 2005; Davalos et al. 2006; Dopeso et al. 2009; Kumar et al. 2009; Stephenson et al. 2001).

The controversy has arisen with the expression of EphB4 receptors in CRC. Some studies suggest that EphB4 is upregulated in late and metastatic stages of CRC (Kumar et al. 2009; Stephenson et al. 2001) while others argue that EphB4 expression is often silenced (Batlle et al. 2005; Davalos et al. 2006; Dopeso et al.

2009). It has been reported that high EphB4 expression enhances migratory abilities in CRC cells, leading to increased rate of metastasis (Kumar et al. 2009). While in opposing findings, it has been stated that EphB4 expression in CRC may restrict tumour expansion and dissemination (Batlle et al. 2005; Davalos et al. 2006). Many factors may have contributed to these conflicting results including different antibodies that were used, the way tissues were processed and also that the samples were sourced from different countries.

Possible genetic and epigenetic mechanisms underlying the observed expressional trends have also been investigated. In one study, EphB4 was found to be a target gene for the transcriptional factor Cyclic AMP response element-binding protein (CBP) (Kumar et al. 2009). CBP regulated transcription maintains an undifferentiated state in colonic stem cells and result in their continuous proliferation. Hence when CBP is preferentially activated in CRC, it is thought to aid CRC progression through expression of oncogenic genes like *EPHB4*. However, in other studies hypermethylation of *EPHB4* gene promoters was present in approximately half of the tumours that were tested particularly in secondary and metastatic lesions, which supported the hypothesis of *EPHB4* silencing in late stages of CRC (Batlle et al. 2005; Davalos et al. 2006). However, the researchers do not consider post-transcriptional regulation could also influence EphB4 expression, which is not taken into consideration in these studies.

Further conflicting data exist regarding the implications EphB4 receptor expression has on CRC cells and disease progression. A key aspect of cancer progression is the ability of a tumour to undergo vascularisation in order to support continuous growth. Ultimately the cancer cells then use these blood vessels as portal gateways

into the systemic circulation and other organs in the body. The EphB4 receptor along with its ligand EphrinB2 regulates vascularisation and angiogenesis in tissues (Krasnoperov et al. 2010). Hence the upregulation of EphB4, observed in some studies, is thought to accelerate CRC tumour growth due to more blood vessels supplying the cancerous tissue (Djokovic et al. 2010; Krasnoperov et al. 2010; Kumar et al. 2009; Stephenson et al. 2001). Inhibition of EphB4 receptor signalling was shown to arrest endothelial cell migration, vessel formation and branching suggesting it could be used as a part of anti-angiogenic therapy (Djokovic et al. 2010; Krasnoperov et al. 2010; Kumar et al. 2009; Stephenson et al. 2001). Therefore, targeting the EphB4 receptor has been theorised as a possible way to stop the two main events in cancer progression; the vascularisation of tumours and ultimately the metastatic spread of cancers.

However, in other studies EphB4 expression was found not to cause significant changes in tumour vasculature (Davalos et al. 2006; Dopeso et al. 2009). These studies demonstrate that the silencing of EphB4 expression and signalling leads to an increase in mitogenic and vascular growth factors, as well as extracellular matrix remodelling molecules in tumour tissues. Hence, EphB4 expression is thought to suppress the clonogenic potential of CRC cells by influencing the constitutes within the tumour microenvironment, that would otherwise lead to the attainment of an aggressive cancer state (Davalos et al. 2006; Dopeso et al. 2009).

In addition, EphB4 receptor involvement in assisting apoptotic insensitivity of CRC cells hence aiding cell-autonomy has also been proposed (Kumar et al. 2009). Whereas in other studies, EphB4 expression was found to reduce cancer cell

viability and proliferation resulting in smaller tumour sizes (Davalos et al. 2006; Dopeso et al. 2009).

In conclusion, although it is hard to determine the exact causes behind the inconsistent findings of previous studies, a large array of experimental methods must be employed to elucidate the exact role of EphB4 receptors in CRC.

1.10 Aims of the project

The overall aim of this study is to elucidate the influence of EphB4 receptor expression and function on the development and progression of CRC. To achieve this, we will be using modified derivatives of human and mouse CRC cell lines. The detailed **aims** are:

- To generate EphB4 overexpressing and knockout cells and to characterise the morphological and phenotypical changes in these modified cells, in comparison to control cell lines.
- To characterise changes in cancer cell behaviour that may be conferred through EphB4 expression and knockout. *In vitro* experiments will be utilised to study proliferative aptitude, migratory and invasive abilities of CRC cells.
- To evaluate the ability of high, low and knock down of EphB4 receptor expression to regulate and influence morphological changes, rate of growth, vascularization and tumour-stromal interactions in *in vivo* animal models of subcutaneous tumours.

- To study the time course and rate of metastasis of CRC cells to the liver using *in vivo* orthotopic and intra-splenic animal models.

- To analyse the impact of *EPHB4* and *EPHRINB2* expression on the survival and disease-free outcomes of CRC patients.

CHAPTER 2

Generation and Characterisation of EphB4 Overexpression and Knockout Constructs of CRC Cell Lines

2.1 Introduction

Investigating EphB4 expression and its influence on cells has yielded varying results across multiple cancer types (Alam et al. 2009; Kumar et al. 2006; Ozgur et al. 2011). Its expression is largely accepted to be oncogenic. However, whilst patient samples are used to support *in vitro* and *in vivo* findings, most often only one or two cell lines with similar properties are used to conduct functional and molecular analysis in some cancer studies. This leaves in question the possibility of EphB4 expression having diverse outcomes for cells of the same cancer type but with different morphological and mutational characteristics. In some of the CRC studies that have contributed toward controversial findings, the type of EphB4 modification introduced, and the cell lines used are different. Furthermore, in depth functional analysis of EphB4 modifications are only completed by few of these studies. For example, Davalos et al. (2006) study primarily concentrates on identifying level of *EPHB4* expression in patient tissues, as well as transfecting two low EphB4 expressing cell lines (SW837, KM12) with an expression vector and another two (HT29, LIM2405) with the dominant negative protein of EphB4. These cells are then used in a colony formation assay, and it was found that EphB4 can suppress clonogenic potential of these cancer cells. However, no further experiments are conducted with the modified cells and it is not clear how EphB4 modification may affect other cellular functions. Another study uses the low expressing SW837 cell line to overexpress EphB4 and HT29 cells to knocked down the expression of EphB4 (Dopeso et al. 2009). These cells are used in various *in vitro* and *in vivo* assays and it is concluded that EphB4 has tumour suppressing influence of these CRC cell lines. However, in opposing findings by another CRC

study, EphB4 silencing in the HT29 and COLO205 induced xenografts negatively impacted growth and metastasis (Kumar et al. 2009). These conflicting findings may result from differences in the level of EphB4 expression knockdown that was achieved in each study. Nonetheless, the influence of EphB4 expression and silencing in the HT29 cell line remains unresolved. In Lv et al. (2016) study, EphB4 overexpression and knockdown constructs of the SW480 cell line were used in subcutaneous experiments, with the outcome of EphB4 expression positively influencing tumour growth through enhanced vasculature. However, no other functional assays are undertaken using the cells. In another study, EphB4 expression was evaluated across 10 CRC cell lines using PCR and western blotting (Stephenson et al. 2001). EphB4 levels were found to be elevated in CRC cell lines, in line with the expression trend observed in patient tumour samples. Nonetheless, biological implications of enhanced EphB4 expression in the cell lines was not studied (Stephenson et al. 2001). Furthermore, McCall et al. (2016) study characterising survival pathways in CRC cells, identified that EphB4 and kinase suppressor of Ras-1 can promote survival in association with downstream targets like Myc and transcriptional factor Peroxisome Proliferator-Activated Receptor Gamma Coactivator-1β. Although this study was thorough in their investigation, they mainly used two CRC cell lines (HCT116 and Caco2), which are both epithelial colon cancer cell lines derived from male patients. As such, these outcomes leave in question the possibility of EphB4 expression having alternate cellular consequences for CRC cells with different characteristics. To investigate if EphB4 expression influence is cell-type dependent, we have chosen cell lines with different phenotypical or morphological characteristics to conduct our research. These are the human SW480, LIM2405, HT29 and mouse CT26 CRC cell lines.

The SW480 cell line was isolated from a primary Dukes Type B tumour from a male patient. It features some of the most commonly mutated genes in CRC, such as *KRAS* and *p53* (Ahmed et al. 2013). It is suggested to possess cancer stem cell like activity with a high capacity of self-renewal and proliferation (Takaya et al. 2016; Xiong et al. 2014). These cells possess an epithelial phenotype in culture; they grow in colonies which spread out to form a uniform monolayer at confluence. The LIM2405 cell line was derived from a poorly differentiated primary caecum tumour from a male patient. It also has the *APC* and *BRAF* mutations and microsatellite instabilities. Furthermore, endothelial growth factor receptor (EGFR), RhoA and cdc42 proteins are upregulated in LIM2405 compared to other types of LIM cell lines (Fanayan et al. 2013). In culture, these cells produce cellular projections, they proliferate in an outward manner forming a monolayer when at confluence, however, the cells can begin to form tight colonies if not passaged. The HT29 cell line was isolated from the primary Dukes Type C tumour from a female patient. According to the Dukes staging this tumour had invaded through the bowel layers into at least one nearby lymph node. This cell line expresses oncogenes *c-myc* and various *Ras* family of proteins and is positive for the commonly found *APC* and *p53* mutant genes. It has an epithelial morphology and these cells do not demonstrate an outward growth rather they form large multi-cellular colonies. An important feature of these cells is that they are a pluripotent population, which remains undifferentiated under standard culture conditions and differentiation can be initiated with inducers (Martínez-Maqueda et al. 2015). Furthermore, karyotype studies have shown that the HT29 cell line remains highly stable during culture even up to one hundred passages. The CT26 cell line was derived from chemically-induced colon tumours from the Balb/c mouse strain. It is fibroblastic in both

phenotype and in morphology, and these cells produce large cell projections and form clusters and domes at confluence. Although the CT26 cell line does not have *APC* and *p53* mutations like the other cell lines used in this study, it is positive for *KRAS* mutations (Castle et al 2014). Genes like *myc* and other *ras* family proteins are overexpressed and no *EGFR* expression is present. Furthermore, some mature colonic cell markers are absent from the CT26 cells, suggesting that they are poorly differentiated (Castle et al 2014).

EphB4 receptor expression is reported to be high in aggressive forms of cancer and cell lines (Heroult et al. 2010; Krasnoperov et al. 2010; Noren et al. 2007; Salvucci et al. 2006). Its expression has also been suggested to be upregulated in metastatic stages, allowing cancer cells an advantage through enhanced migratory abilities (Kumar et al. 2007). Furthermore, EphB4 receptor is suggested to help maintain an undifferentiated state in cancer cells aiding more rapid renewal and survival (Mertens-Walker et al. 2015). To investigate the functional consequences of upregulating EphB4 in CRC, we have used expressional vector systems to overexpress the receptor in the SW480, LIM2405 and CT26 cell lines.

Inhibitors against EphB4 are being designed and tested (Herington et al. 2014; Lamminmaki et al. 2015; Xiong et al. 2011). Targeting EphB4 receptor seems to reduce cellular viability, tumour size and burden, often side effects can be observed, and inhibition is transient (Djokovic et al. 2010; Stephenson et al. 2015; Xia et al. 2006). Hence, it is important to explore the consequence of EphB4 silencing in CRC. The CRISPR-Cas9 permits permanent mutation at the genomic level and can be used to ablate gene expression (Jiang et al. 2017; Wang et al. 2016). This system comprises of two molecules, the first, a sequence specific guideRNA, which helps

the second molecule, a Cas9 nuclease, recognise a target sequence in target DNA (Zhang et al. 2017). The Cas9 molecule then introduces a double strand break into the target sequence. This break in DNA is repaired by non-homologous end joining, a process that often causes small deletions, which can cause mutational silencing of the target gene (Singh et al. 2017). To the best of our knowledge, to this date EphB4 has not yet been targeted in cancer studies using the CRISPR-Cas9 gene editing technology. To decipher the effect and functional relevance of EphB4 silencing, we used knockout constructs in HT29 CRC cells.

2.2 Methods

2.2.1 Preparation of vectors

2.2.1.1 pMono-neo-mcs expression vector

In this study, the pMono-neo-mcs vector system (Invivogen, California, United States) was used as an empty vector, without a gene insert, or as vector containing full-length cDNA corresponding exactly to the published sequence (NM-0044444) of EphB4 (**Figure 2.1A**). Firstly, the EphB4 cDNA was excised from the existing vector by digestion with NotI enzyme, followed by a reaction with T4 DNA polymerase in the presence of all 4 deoxynucleotides to create a "blunt" end, then by digestion with BamHI enzyme (**Figure 2.1B**). The digest was run on a 0.8% agarose gel and the band corresponding to the EphB4 cDNA excised and purified using UltraClean (MO BIO Laboratories, California, United States). Secondly, the pMono vector was prepared by digestion with SalI enzyme, followed by reaction with T4 DNA polymerase in the presence of all 4 deoxynucleotides to create a "blunt" end, then by digestion with BamHI and purified using UltraClean (MO BIO

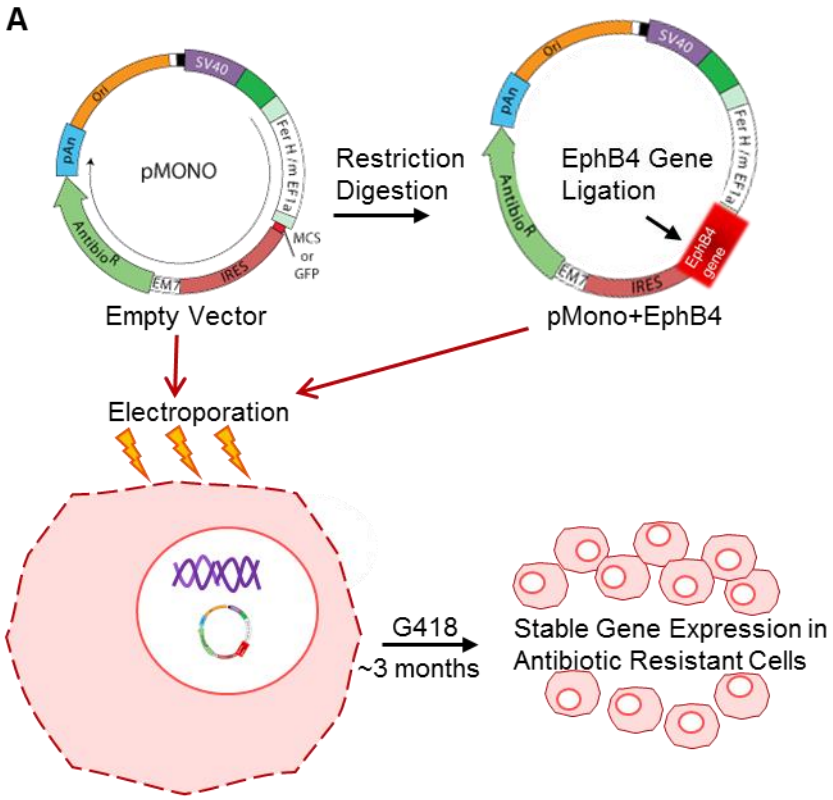
Laboratories). The fragments of EphB4 and pMono were ligated with DNA ligase enzyme, at room temperature for 3 hours. The ligated vector and gene solution were added into competent cells (JM109), which were subjected to cold and heat shock for the plasmids to be taken into cells. These cells could grow in media (10mL of Luria Bertoni broth, 100 µL of 2M glucose solution, and 50 µL of magnesium chloride solution) for 1 hour before being plated. The solution was then plated on the kanamycin plates and incubated at 37°C overnight. The visible colonies were picked into kanamycin media and placed in the air shaker at 245rpm at 37°C overnight.

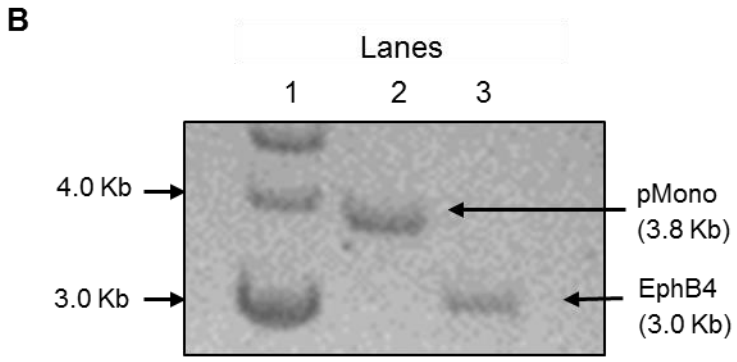
2.2.1.2 Miniprep and maxiprep of pMono+EphB4

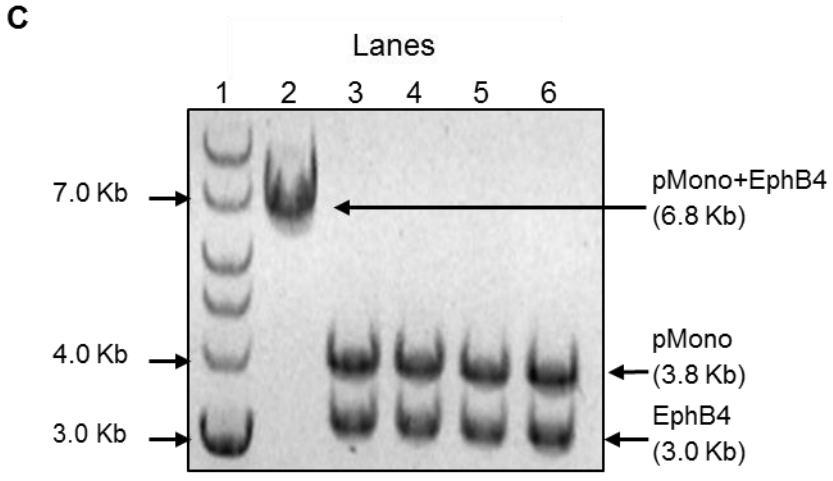
The bacterial cultures of the picked colonies were centrifuged to pellet the cells, then the supernatant was aspirated and removed. Miniprep solution 1 containing 50mM of glucose, 0.25mM of Tris-HCl and 10mM of EDTA was added to tubes and the pellet was resuspended. Then miniprep solution 2 (10mL of water, 100 µL of 10M sodium hydroxide and 1 mL of sodium dodecyl sulfate) was also added and incubated on ice for 5 minutes. Finally, miniprep solution 3 containing 3M of potassium acetate and 5M of glacial acetic acid was added and the solution was left on ice for a further 5 minutes. Once centrifuged, the supernatant was removed and added into tubes containing isopropanol and centrifuged again to pellet the DNA. Finally, 70% ethanol was added to the pellet and removed without resuspension. The pellet was then suspended in Tris-EDTA buffer with RNAseA enzyme, which was incubated at room temperature for approximately 1 hour. The pMono+EphB4 vector was then digested with NcoI+MluI enzymes and ran on 0.8% gels using electrophoresis (100V for 1 hour) (**Figure 2.1C**). Plasmids containing the EphB4

fragments were then cultured and purified on a large scale using Maxiprep kit (ThermoFisher, Scorseby, Australia) according to manufacturer's instructions.

Figure 2.1. Preparation of Empty Vector and EphB4 overexpressing cell lines using pMono-neo-mcs expression plasmid. **A.** Flow diagram of vector preparation, transfection of cells and obtaining stable gene expression (vector image adapted from Invivogen). **B.** Visualisation of molecular weight markers (lane 1), pMono vector (lane 2) and fragment EphB4 (lane 3) before ligation of DNA and vector. **C.** Using MluI+NcoI enzymes, the fragment of interest (EphB4=3.0 Kb) is liberated from the vector (3.8 Kb). lane 1: molecular weight markers, lane 2: uncut pMono-EphB4 plasmid (6.8 Kb), lanes 3-6: pMono DNA (3.8 Kb) and EphB4 fragment (3.0 Kb).


A
pMONO
Ori
SV40
pAn
Fet H /m EF1a
MCS or GFP
AntibioR
EM7
IRES
Empty Vector
Restriction Digestion
EphB4 Gene Ligation
EphB4 gene
pMono+EphB4
Electroporation
G418
~3 months
Stable Gene Expression in Antibiotic Resistant Cells


B
Lanes
1
2
3
4.0 Kb
3.0 Kb
pMono
(3.8 Kb)
EphB4
(3.0 Kb)


C
Lanes
1
2
3
4
5
6
7.0 Kb
4.0 Kb
3.0 Kb
pMono+EphB4
(6.8 Kb)
pMono
(3.8 Kb)
EphB4
(3.0 Kb)

2.2.1.3 Preparing CRISPR-Cas9 vector

The dual nikase CRISPR-Cas9 vectors, official name PX335, were received as stab cultures from Addgene (Massachusetts, United States) with Institutional Biosafety Committee clearance. The samples were cultured on a large scale in bacterial media and maxipreped (ThermoFisher) and DNA concentration quantified using Nanodrop (ThermoFisher). The vectors were then digested with BbsI enzyme, run on 0.7% electrophoresis gel and fragments of interests were cut and purified using UltraClean (MO BIO Laboratories). The Optimized CRISPR design program (http://crispr.mit.edu/) was used to identify a pair of potential "nick" sites as close as feasible to the site of protein initiation (ATG) of the hEphB4 genomic sequence (**Figure 2.2A**). Oligomers for target sequence of *EPHB4*, namely B4N1 and B4N2 were obtained from Geneworks (Thebarton, Australia) (**Figure 2.2B**) (**Table 2.1**).

Table 2.1 CRISPR-Cas9 *EPHB4* target DNA sequence

***EPHB4* Target Sequences**	
B4N1	5'-CACCGGGAGCGCCCAGCCCGAGGC-3'
	3'-AAACGCCTCGGGCTGGGCGCTCCC-5'
B4N2	5'-CACCGTTCTACTATGAGAGCGATG-3'
	3'-AAACCATCGCTCTCATAGTAGAAC-5'

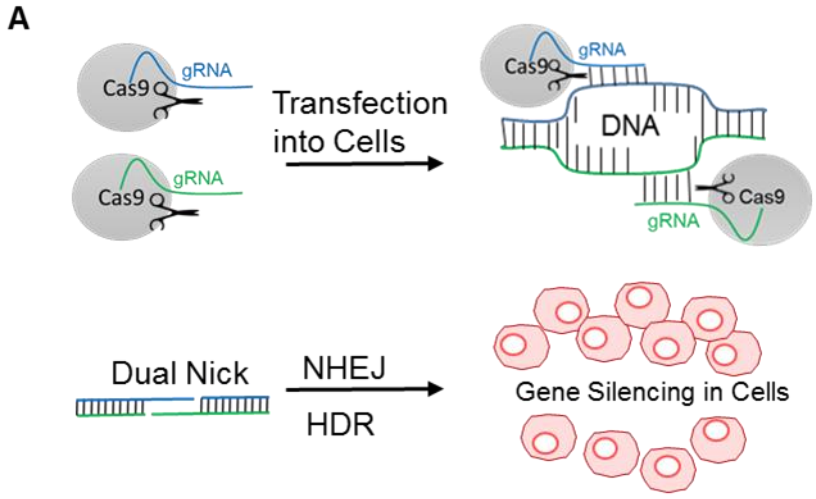
The pairs of complimentary oligonucleotides were annealed together by heating to 95°C, 1µg of each in a 50µL reaction with annealing buffer, then allowed to cool slowly to room temperature (the oligonucleotides are designed to create BbsI ends in the correct orientation when annealed together) (**Figure 2.2B**). Each oligo (B4N1 and B4N2) was ligated with the CRISPR-Cas9 vector PX335 (PX335+B4N1, PX335+B4N2). The ligation mixture was added to LI22A competent cells (Promega, Wisconsin, United States) then plated on agar and incubated overnight

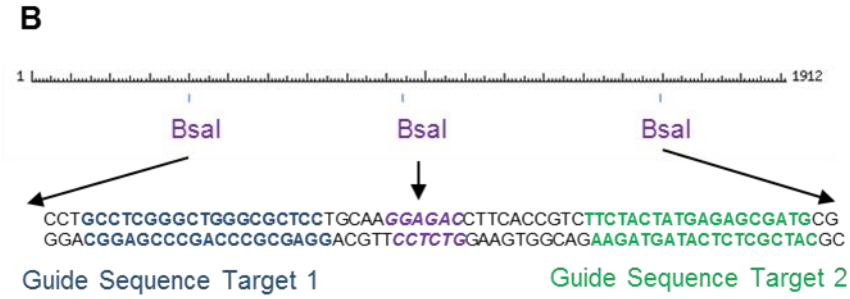
at 37°C. Colonies were picked into LB media and grown overnight. Samples were minipreped as described above (**Methods section 2.2.1.1**) then digested with SacI+BbsI enzymes to confirm the presence of an insert (**Figure 2.2B**). Cultures positive for B4N1 or B4N2 fragment were grown to a large scale overnight and maxipreped.

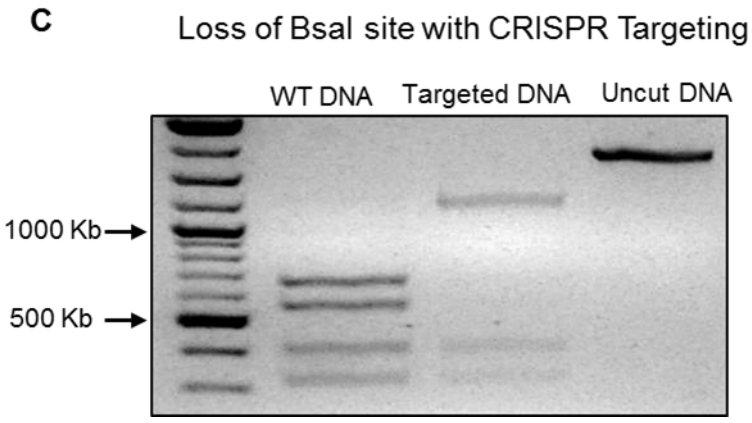
2.2.1.4 Transfecting CRC cells with pMono and CRISPR-Cas9 vectors

The concentrations of final plasmid solutions were obtained using the Nanodrop 2000 (ThermoFisher) and sent to Australian Genomic Research Facility (Brisbane, Australia) to confirm sequence of plasmids (pMono+EphB4, PX335+B4N1, PX335+B4N2). Since the CRISPR-Cas9 vector purchased at the time did not contain any selectable markers, we transfected a very low concentration of the empty pSelect-puro vector (Invivogen). This vector confers puromycin resistance to mammalian cells and it was transfected at a much lower concentration than CRISPR-Cas9, to maximise the selection for cells that have also taken up the CRISPR-Cas9 vectors. For the transfections, cells were trypsinised and counted then suspended in Gene Pulser Electroporation Buffer (Biorad, California, United States). Cell suspension was placed directly within the electrodes of a cuvette alongside DNA and placed within the Gene Pulser Electroporator (Biorad). The cells were then very swiftly placed within 6 well plates containing media for culturing. Cells were cloned by serial dilution in 96 well plates, then single colonies were picked and expanded. Successful targeting was identified by the loss of a BsaI enzyme recognition site (**Figure 2.2B**). As such, when the WT gene was digested with this enzyme, four fragments of DNA were obtained as opposed to mutant DNA which had three fragments (**Figure 2.2C**).

Figure 2.2. Knocking out EphB4 expression using the CRISPR-Cas9 system. A. Flow diagram of modified CRISPR-Cas9 system creating dual nicks in DNA leading to gene silencing through non-homologous end joining (NHEJ) or homology directed (HDR) repair system. **B.** The *EPHB4* gene has 3 recognition sites for the enzyme BsaI, one of these sites is lost through targeting (purple), guide sequence sites are highlighted in blue and green. **C.** *EPHB4* gene is digested with BsaI, WT gene showing four fragments, the successfully targeted gene showing three fragments.


A
gRNA
Cas9
Transfection
into Cells
DNA
Cas9
gRNA
gRNA
Cas9
Dual Nick
NHEJ
HDR
Gene Silencing in Cells


B
1
1912
BsaI
BsaI
BsaI
CCTGCCTCGGGCTGGGCGCTCCTGCAAGGAGACCTTCACCGTCTTCTACTATGAGAGCGATGCG
GGACGGAGCCCGACCCGCGAGGACGTTCCTCTGGAAGTGGCAGAAGATGATACTCTCGCTACGC
Guide Sequence Target 1
Guide Sequence Target 2


C
Loss of BsaI site with CRISPR Targeting
WT DNA
Targeted DNA
Uncut DNA
1000 Kb
500 Kb

2.2.2 Cell culture

The various cell lines were maintained in humidified incubators in an atmosphere of 5% carbon dioxide and at 37°C. The cells were fed with Roswell Park Memorial Institute (RPMI)-1640 medium (Sigma-Aldrich, Missouri, United States) containing 10% foetal-calf serum (ThermoFisher), which was refreshed every 3 days and cells were passaged when they reached near confluence. Transfected cell lines were maintained in the presence of Geneticin selection (50µg/mL) (Invitrogen, California, United States) for pMono transfected cells and Puromycin (5 µg/mL) (ThermoFisher) for CRISPR-Cas9 transfected cells. Protein was extracted from these cells at frequent intervals and analysed by western blots to ensure consistent protein expression levels were maintained across all experiments.

2.2.3 Stimulation of cells with EphrinB2-Fc

Vials of EphrinB2-Fc and anti-human IgG were generously gifted by Dr. Peter Janes from Monash University, Australia. EphrinB2-Fc (1.5µg/mL) was clustered with anti-human IgG (0.75µg/mL) in media at 37ºC for 15 minutes. Culture medium was then removed from the cells, clustered EphrinB2-Fc was added, and cells were stimulated for 15 minutes. Morphology images were taken on the Nikon IX53 microscope before and after stimulation at the same area within the wells. Experiments were performed on one well at a time, to minimise the effect of having cells out of the incubator at room temperature.

2.2.4 Immunofluorescent labelling of EphB4

Cells were seeded and grown on autoclaved coverslips within 6 well plates in duplicates, where one well was stimulated with clustered EphrinB2-Fc (as

described above in **Methods section 2.2.3**). The medium was removed, and wells were washed with ice-cold PBS before 100% methanol was added to fix the cells, on ice for 5 minutes. The cells were washed three x5 minutes with PBS then treated with 0.01% TritonX and PBS for 30 minutes to puncture cell and nuclear membranes, then, blocked with 5% horse serum in PBS. Primary anti-EphB4 rabbit polyclonal antibody (1:250, H200) (Santa Cruz Biotechnology, Texas, United States) was applied overnight at 4ºC. Cells were washed 3x 5minutes with 1% Tween20 and PBS, then secondary anti-rabbit Alexa 594 antibody (1:500) (Jackson Immunoresearch Laboratories, West Baltimore, United States) was applied for 1 hour and anti-nuclear marker 4′,6-diamidino-2-phenylindole (DAPI) was applied for 15 minutes (1:10000, ThermoFisher). After 3x5 minutes washes, coverslips were mounted using fluorescent mounting medium (DAKO, Agilent Technologies, Australia). All images were taken on Nikon Eclipse Ti laser scanning microscope. Fluorophores were visualized using excitation filters for Alexa 594 Red (excitation wavelength 559nm) and DAPI blue (excitation wavelength 358nm).

To determine EphB4 localisation before and after EphrinB2-Fc stimulation, Imagej (FIJI) 'Colocalisation' plugin (https://imagej.net/Coloc_2) was used to analyse 4 images/construct/condition (20x magnification and ~20 cells/field). The results are reported on statistical correlation between the intensities of two fluorescent dyes based on Mander's equation. A result of 1 in Manders' coefficient (MC) suggests perfect correlation and colocalization of fluorescence, 0 suggests no correlation and random placement, while, -1 stands for perfect inverse correlation. An automated threshold separates signal from background and the coefficient is calculated by total red/blue intensity colocalised over the threshold, divided by total red/blue intensity over the threshold (Manders et al. 1993). Furthermore, Costses' statistical

significance test was used to evaluate the probability of false positive overlapping. Results are reported as mean MC±SD.

2.2.5 Assessing the growth of fluorescent EphB4 and EphrinB2 expression knockout cells in culture

Dr. Paul Senior has generate the EphrinB2 knockout and fluorescent protein expressing cells (WT GFP, WT RFP, EphB4 KO GFP and B2 KO RFP) cells used in these experiments. EphrinB2 expression was targeted using CRISPR-Cas9 based on target sequences in **Table 2.2**.

Table 2.2 CRISPR-Cas9 *EPHRINB2* target DNA sequence

***EPHRINB2* Target Sequences**	
B2N1	5'-CACCGAGTTTTAGAGTCCACTTTG-3'
	3'-AAACCAAAGTGGACTCTAAAACTC-5'
B2N2	5'-CACCGGAATATTATAAAGTTTATA-3'
	3'-AAACTATAAACTTTATAATATTCC-5'

The pMono plasmid containing a gene for green fluorescent protein (GFP) expression was transfected into HT29 WT cells (WTGFP) and EphB4 knockout cells (B4KOGFP) (**Table 2.1**). Similarly, pMono plasmid with red fluorescent protein (RFP) gene was transfected into HT29 WT (WTRFP) cells and EphrinB2 knockout (B2KORFP) (**Table 2.1**). The cells were cloned through serial dilution in 96 well format and single clones were picked using the inverted fluorescent microscope then expanded.

After trypsinisation, $1x10^3$ cells/construct were thoroughly pipetted to obtain single cell suspensions and seeded on autoclaved coverslips in 6 well plates. Each experiment was set up in three wells and each day cells were fixed with ice-cold

4% paraformaldehyde for 10 minutes before being washed with 1% Tween20 and PBS and mounted using mounting medium (DAKO). Experiments were repeated three times. Images were taken using Nikon Eclipse Ti laser scanning microscope.

For analysis confocal images (20x magnification) were 'split' into their respective channels in ImageJ (FIJI) program. In a blinded manner, using the 'cell count' plugin, cells in 10 random colonies in 5 images (per experimental condition) were counted. Scoring criteria was applied to the cell numbers whereby, 1-10 cells was scored as 1, 10-20 cells=2, 20-50 cells=3, 50+ cells=4. These scores were then converted to percentages based on how many times they appear in the initial count of 10 colonies. Data was displayed as mean %±SD.

2.2.6 Protein extraction

Cell culture flasks were removed of media and washed with PBS and radioimmunoprecipitation lysis buffer (ThermoFisher) was placed over the cellular monolayer. Cell scrapers were used to liberate the cells and the cell suspension was removed into a microfuge tube. The microfuge tube was shaken at 4°C for 10 minutes to ensure complete lysis of cells, after which it was centrifuged at 1200rpm for 20 minutes at 4°C. Bicinchoninic acid assay kit (ThermoFisher) was used to determine the concentrations of proteins according to manufacturer's instructions. Using the VarioSkan spectrophotometer the 96 well plates were exposed to wavelength of 562 nm. The known concentration standards were used to plot a standard curve which was used to calculate the protein concentration of the samples (µg/mL).

2.2.7 Western blot analysis

The protein samples were diluted using distilled water to a concentration of 5µg/mL in a total volume of 11µL. Loading dye containing 15% β-mercaptoethanol was added to the protein samples at a volume of 4µL and incubated at 95°C for 5 minutes. Using mini-Protean TGX (4-20%) stain free precast gel (Bio-Rad) and Tris-glycine running buffer (Bio-Rad) the western blot apparatus was assembled. The samples were loaded into wells together with Precision Plus Protein unstained molecular weight standard (Bio-Rad). Gels were run at 200 Volts until the loading dye reached the bottom of the gel (~30 minutes). The separated proteins were visualised using a Gel-Doc EZ (Bio-Rad). The Trans-Blot transfer pack (Bio-Rad) was then used to transfer the proteins from the gel onto the Polyvinylidene difluoride membranes which were rinsed briefly under distilled water. Efficiency of transfer was assessed by visualising the membranes using the Gel-Doc EZ system (Bio-Rad).

The membrane was then treated with 5% skim milk blocking solution at 80rpm room temperature for 2 hours. After this the membrane was treated with the primary anti-EphB4 rabbit polyclonal antibody (1:1000, H-200) (Santa Cruz Biotechnology), goat anti-human/mouse EphB2 polyclonal antibody (1:1000, AF467) (R&D systems, Minneapolis, United States), anti-phosphorylated Erk1/2 rabbit (1:1000) (Cell Signalling, Massachusetts, United States), anti-total Erk1/2 rabbit (1:1000) (Cell Signalling), anti-RhoA rabbit (1:1000) (Cell Signalling), anti-phopsho Creb rabbit (1:1000) (Cell Signalling), anti-Gapdh (1:2000) (Santa Cruz Biotechnology) antibodies for 1 hour. The primary antibody solution was discarded, and the membrane rinsed and washed with 1% Tween20 and PBS. The membrane

was then placed in a solution consisting of recognition antibody directed against the markers (1:3000, Precision Protein StrepTactin-Horse Radish Peroxidase conjugate) (Bio-Rad) and secondary goat pAB to rabbit IgG (1:1000) (Abcam, Cambridge, United Kingdom) antibodies and incubated at room temperature for 1 hour.

The staining was visualised by chemiluminescent imaging using the Clarity ECL blotting Kit (Biorad); the membrane was gently shaken in a mixture of the equal volumes of the luminol and peroxidase solution for 5 minutes then imaged using VersaDoc imaging system (Bio-Rad). Quantitation was performed using ImageLab 4.1 software (Bio-Rad).

2.3 Results

In this study, the SW480, LIM2405, CT26 and HT29 cell lines were stably transfected. The vector systems used, and the type of modifications made are listed in **Table 2.3**.

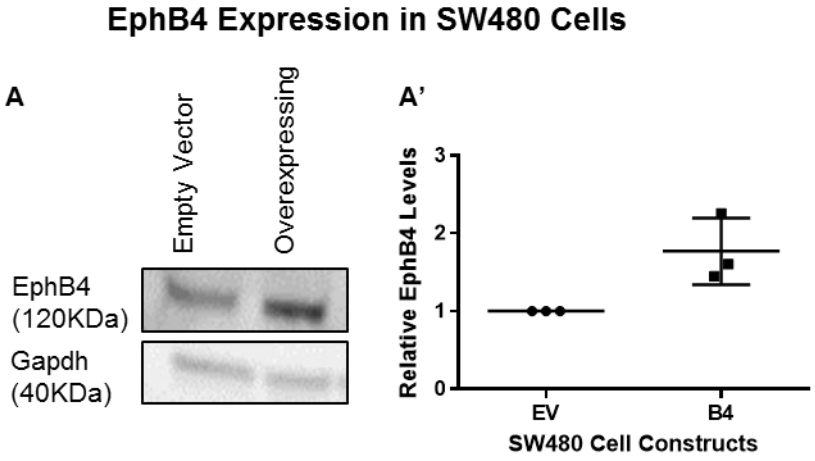
Table 2.3 Cell lines used and type of modifications

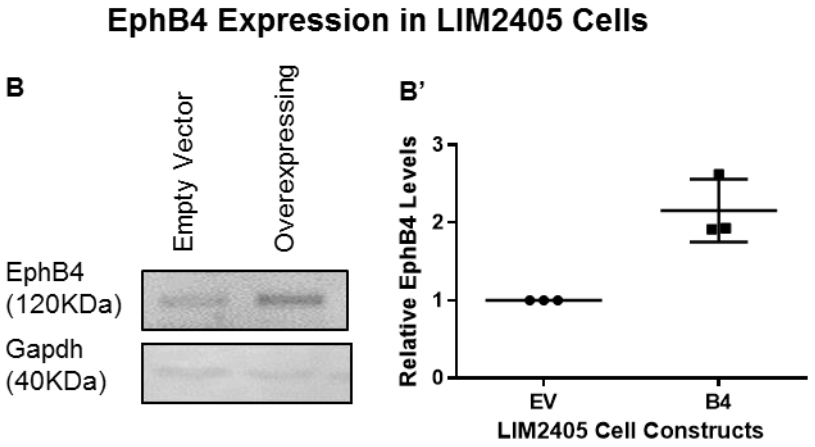
Cell Line	Expression Vector system-Gene	Name used in text	Modification Type
SW480 (Human)	pMono-neo-mcs-EphB4	B4	EphB4 overexpression
	pMono-neo-mcs	EV	Empty vector control
LIM2405 (Human)	pMono-neo-mcs-EphB4	B4	EphB4 overexpression
	pMono-neo-mcs	EV	Empty vector control
CT26 (Mouse)	pMono-neo-mcs-mEphB4	B4	EphB4 overexpression
	pMono-neo-mcs	EV	Empty vector control
HT29 (Human)	CRISPR-Cas9-EphB4	KO	EphB4 targeted expression knockout
	Wild-type	WT	Unmodified control cells
	Wild-type cells with GFP plasmid	WTGFP	Wild-type cells expressing green fluorescent protein
	Wild-type cells with RFP plasmid	WTRFP	Wild-type cells expressing red fluorescent protein
	CRISPR-Cas9-EphB4 EGFP plasmid	B4KOGFP	EphB4 knockout cells expressing green fluorescent protein
	CRISPR-Cas9-EphrinB2 RFP plasmid	B2KORFP	EphrinB2 knockout cells expressing red fluorescent protein

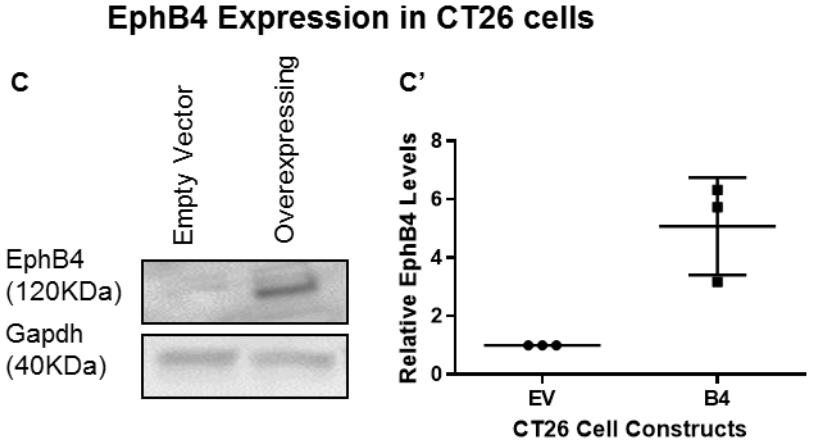
2.3.1 Upregulating EphB4 expression using expression vector systems

The pMono-neo-mcs (pMono) vector was used to upregulate EphB4 expression in SW480, LIM2405 and CT26 cell lines (**Figure 2.1**). Once inside, plasmids use host cell replication mechanisms to stably express the gene of interest EphB4 (Carter et al. 2015). As it is not possible to control the number of plasmids delivered into cells and consequently how much expression of the gene is produced, the cells were cloned, and through western blot analysis, clones that express appropriate level of EphB4 were chosen. These cells are referred to as empty vector (EV) and EphB4 overexpressing (B4). Level of EphB4 overexpression was calculated based on the EV control of each cell line. According to western blot results, the level of EphB4 overexpression in the B4 constructs for the LIM2405 and SW480 cells were approximately two times higher, relative to that of EV protein expression (**Figure 2.3A-A', B-B'**). In the CT26 cell line, which expresses very low levels of endogenous EphB4, relative levels of EphB4 overexpression was approximately 5 times that of EV cells (**Figure 2.3C-C'**).

Figure 2.3. Western blot analysis of the EphB4 protein band at 120kDa of the SW480, LIM2405 and CT26 cell constructs. A. Protein harvested from SW480 constructs showing the EphB4 protein band and Gapdh loading control. **A'.** Quantified EphB4 expression in SW480 EV and B4 cells (N=3/cell construct), expression relative to EV cells and normalised to Gapdh loading control. **B.** Protein harvested from LIM2405 constructs showing the EphB4 protein band and Gapdh loading control. **B'.** Quantified EphB4 expression in LIM2405 EV and B4 cells (N=3/cell construct), expression relative to EV and normalised to Gapdh loading control. **C.** Protein harvested from CT26 cell constructs showing the EphB4 protein and Gapdh loading control. **C'.** Quantified EphB4 expression in CT26 EV and B4 cells (N=3/cell construct), expression relative to EV and normalised to Gapdh loading control.


EphB4 Expression in SW480 Cells
A
Empty Vector
Overexpressing
EphB4
(120KDa)
Gapdh
(40KDa)
A'
Relative EphB4 Levels
3
2
1
0
EV
B4
SW480 Cell Constructs

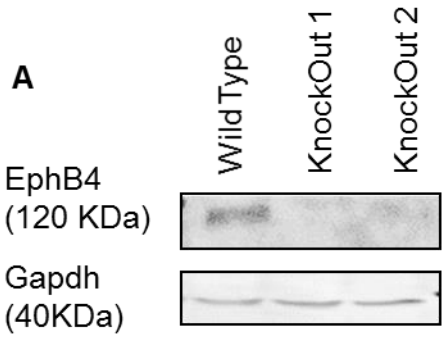

EphB4 Expression in LIM2405 Cells
B
Empty Vector
Overexpressing
EphB4
(120KDa)
Gapdh
(40KDa)
B'
Relative EphB4 Levels
3
2
1
0
EV
B4
LIM2405 Cell Constructs

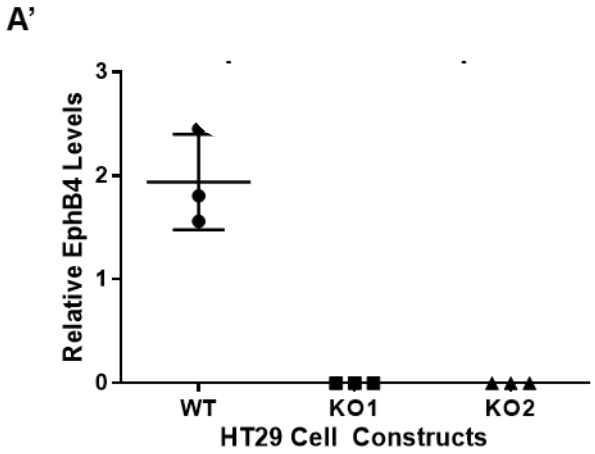

EphB4 Expression in CT26 cells
C
Empty Vector
Overexpressing
EphB4
(120KDa)
Gapdh
(40KDa)
C'
Relative EphB4 Levels
8
6
4
2
0
EV
B4
CT26 Cell Constructs

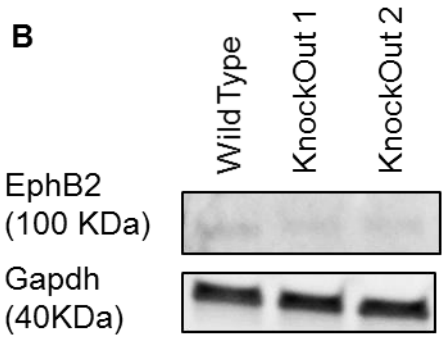
2.3.2 Knocking out EphB4 expression using CRISPR-Cas9 system

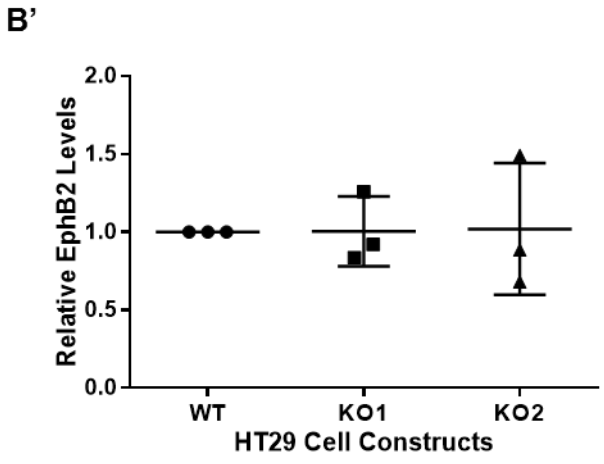
The CRISPR-Cas9 system was used to target EphB4 expression in the HT29 cell line (**Figure2.2**). The ultimate aim of these experiments was to ensure no EphB4 protein is produced. Through western blot analysis, it was observed that the two knockout constructs (KO1 and KO2) do not express the EphB4 protein (**Figure 2.4A**). One possible consequence of completely removing EphB4 receptor expression could be compensation through the upregulation of another receptor. The EphB4 receptor (as described in detail in **Chapter 1**) is unique in both biochemical structure, ligand interaction and function. For this reason, the loss of expression is not likely to be compensated by the upregulation of another receptor. However, if it had to be compared to another Eph receptor, due to their similarity in strong binding affinities toward the EphrinB2 ligand, EphB4 would be more closely related to the EphB2 receptor (Chrencik et al 2006*a*). Western blot analysis, confirmed that the EphB2 receptor is expressed at low levels within the HT29 cell line and its expression remained unchanged across the knockout constructs (**Figure 2.4B**).

Figure 2.4. Western blot analysis of the EphB4 (120kDa) and EphB2 (100kDa) protein band in HT29 cell constructs. **A.** Protein harvested from HT29 constructs showing the EphB4 protein band and Gapdh loading control. **A'.** Quantified EphB4 expression in HT29 WT, KO1 and KO2 cells (N=3/cell construct), expression relative to WT cells and normalised to Gapdh loading control. **B.** Protein harvested from HT29 showing the EphB2 protein band and Gapdh loading control. **B'.** Quantified EphB2 expression in HT29 WT, KO1 and KO2 cells (N=3/cell construct), expression normalised to Gapdh loading control.


A
Wild Type
KnockOut 1
KnockOut 2
EphB4
(120 KDa)
Gapdh
(40KDa)


A'
Relative EphB4 Levels
3
2
1
0
WT
KO1
KO2
HT29 Cell Constructs


B
Wild Type
KnockOut 1
KnockOut 2
EphB2
(100 KDa)
Gapdh
(40KDa)


B'
Relative EphB2 Levels
2.0
1.5
1.0
0.5
0.0
WT
KO1
KO2
HT29 Cell Constructs

2.3.3 Differences in morphology and EphB4 localisation within CRC cells upon stimulation with EphrinB2

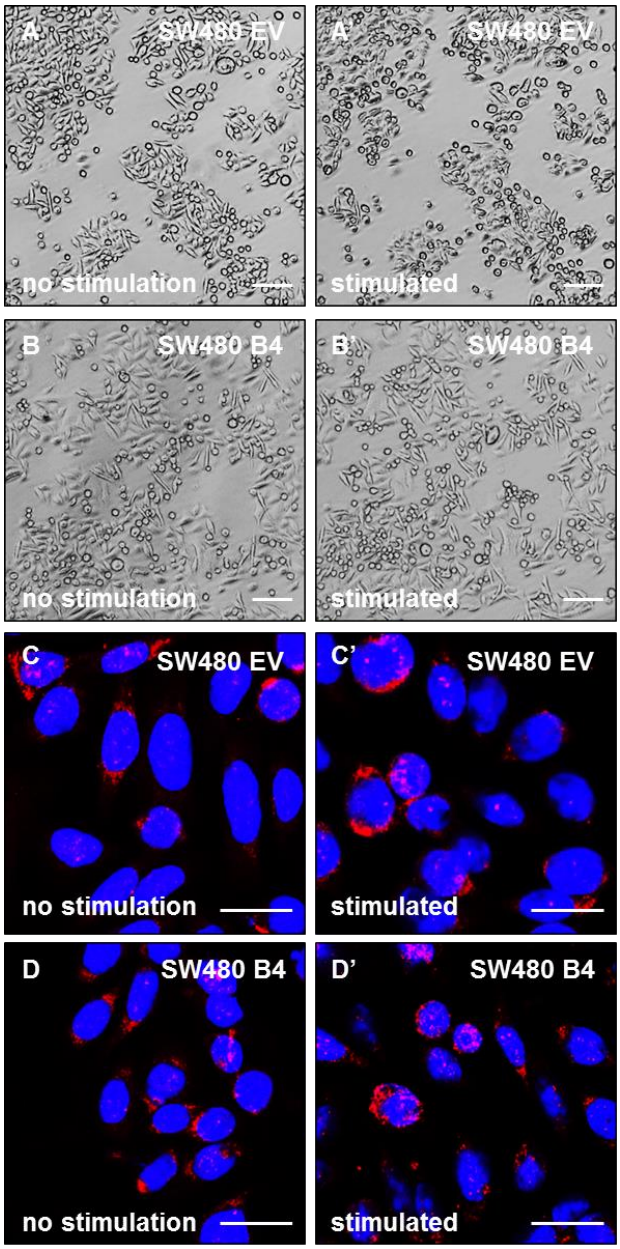
In order to test the functionality of the overexpressed EphB4 receptors, clustered EphrinB2-Fc was used to stimulate these cells. It was observed that before stimulation there were no notable differences in the morphological structure between the modified SW480 EV and B4 cells (**Figure 2.5A, B**). With the addition of EphrinB2-Fc, cellular rounding became evident in the EV cells (**Figure 2.5A'**), which was more prominent within the B4 culture (**Figure 2.5B'**). The localisation of EphB4 protein within the SW480 cells, appears to be confined to the leading and following ends of the cell (**Figure 2.5C, D**). Upon stimulation, EphB4 protein could be observed within the nucleus (**Figure 2.5C', D'**). The level of EphB4 colocalization in the in the nucleus was quantified using Manders' coefficient (MC), which reports on the statistical correlation of overlapping in fluorescent intensities. This analysis revealed that, although some EphB4 is present in the nucleus of both SW480 EV (0.09±0.03) and B4 cells (0.15±0.09), post-stimulation this correlation significantly increases (EV=0.53±0.21, B4=0.68±0.06) (*P<0.05 for EV, **P<0.01 for B4 not stimulated vs stimulated) (**Figure 2.5E**). As the SW480 cells are small with epithelial phenotype, the effects of stimulation were less prominent compared to changes observed in LIM2405 and CT26 cell lines.

The LIM2405 EV cells have cellular projections and exhibited shortening of these projections after EprhinB2-Fc stimulation (**Figure 2.6A-A'**). The B4 cells, which also possess these projections, were once again affected by stimulation to a larger degree, as cells underwent complete rounding, and some began to detach completely (**Figure 2.6B-B'**). In these cells, EphB4 was once again found at the

edge of cell projections and at the site of cell-cell contact (**Figure 2.6C, D**). Although, some EphB4 could be detected in the nucleus prior to stimulation (MC=0.22±0.09 for EV and B4=0.38±0.05), its localisation became significantly more nuclear after stimulation (MC=0.67±0.04 for EV and B4=0.80±0.09) (***P<0.001 for EV, **P<0.01 for B4 not stimulated vs stimulated) (**Figure 2.6C', D', E**). The CT26 cell line underwent similar changes to that of LIM2405 (**Figure 2.7A, B**), with B4 cells displaying more cellular rounding than that of EV cells upon stimulation (**Figure 2.7A', B'**). In these cells similar patterns of EphB4 localisation in the nucleus could be observed pre (MC=0.09±0.03 for EV and B4=0.21±0.12) and post stimulation (MC=0.80±0.11 for EV and B4=0.80±0.09) (**P<0.01 for both) (**Figure 2.7C-C', D-D', E**).

Treating the HT29 WT, KO1 and KO2 cells with EphrinB2-Fc appears to stimulate the cells into forming tight colonies with indistinguishable cell borders, and they appear as though they have fused together (**Figure 2.8**). This effect is more pronounced within the WT cells (**Figure 2.8A-A'**) and reduced in the KO1 (**Figure 2.8B-B'**) and KO2 (**Figure 2.8C-C'**) constructs.

Figure 2.5. EphrinB2-Fc stimulation causes rounding of SW480 cells and EphB4 localisation becomes more nuclear. **A-A'.** EV cells pre and post-stimulation (respectively). **B-B'.** B4 cells pre and post-stimulation (respectively). **C-C'.** Visualisation of EphB4 (H200, red) and the nucleus (DAPI, blue) in EV cells pre and post-stimulation (respectively). **D-D'.** Visualisation of EphB4 (H200, red) and the nucleus (DAPI, blue) in B4 cells pre and post-stimulation (respectively). (Scale bars for A-B' = 100μm) (Scale bars for C-D' = 400μm). **E.** Quantitative analysis of EphB4 localization in the nuclei of SW480 cells, presented as Manders' colocalization coefficient based on 4 images/construct/condition. **P*<0.05, ***P*<0.01.


A
SW480 EV
no stimulation
A'
SW480 EV
stimulated
B
SW480 B4
no stimulation
B'
SW480 B4
stimulated
C
SW480 EV
no stimulation
C'
SW480 EV
stimulated
D
SW480 B4
no stimulation
D'
SW480 B4
stimulated

E

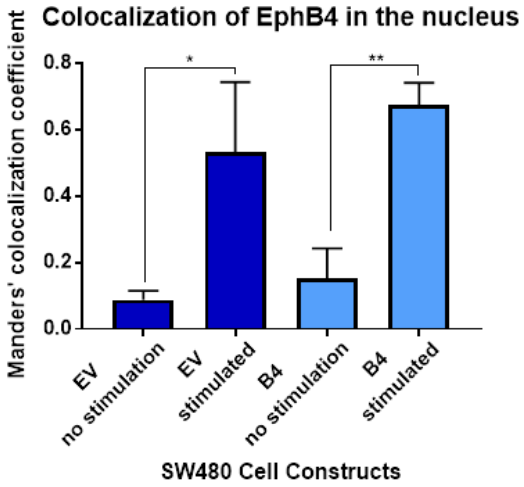
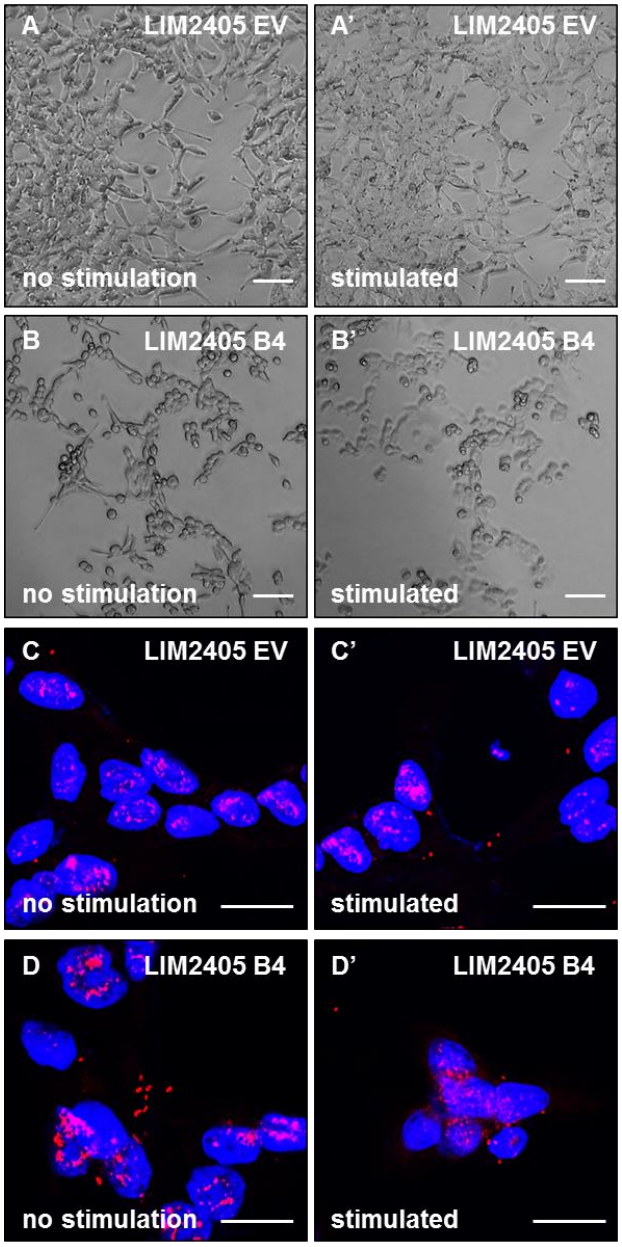

Colocalization of EphB4 in the nucleus
Manders' colocalization coefficient
0.0
0.2
0.4
0.6
0.8
*
**
EV no stimulation
EV stimulated
B4 no stimulation
B4 stimulated
SW480 Cell Constructs

Figure 2.6. EphrinB2-Fc stimulation shortens cellular extensions of LIM2405 cells and EphB4 localises in the nucleus and projections. A-A'. EV cells pre and post-stimulation (respectively). **B-B'.** B4 cells pre and post-stimulation (respectively). **C-C'.** Visualisation of EphB4 (H200, red) and the nucleus (DAPI, blue) in EV cells pre and post-stimulation (respectively). **D-D'.** Visualisation of EphB4 (H200, red) and the nucleus (DAPI, blue) in B4 cells pre and post-stimulation (respectively). (Scale bars for A-B' = 100μm) (Scale bars for C-D' = 400μm). **E.** Quantitative analysis of EphB4 localization in the nuclei of LIM2405 cells, presented as Manders' colocalization coefficient based on 4 images/construct/condition. $^{**}P<0.01$, $^{***}P<0.001$.


A
LIM2405 EV
no stimulation
A'
LIM2405 EV
stimulated
B
LIM2405 B4
no stimulation
B'
LIM2405 B4
stimulated
C
LIM2405 EV
no stimulation
C'
LIM2405 EV
stimulated
D
LIM2405 B4
no stimulation
D'
LIM2405 B4
stimulated

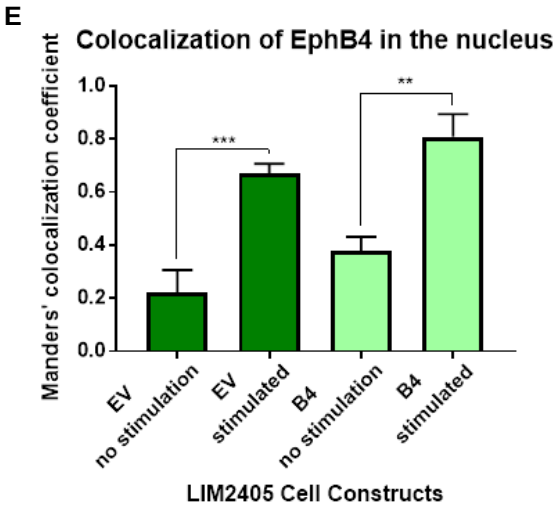
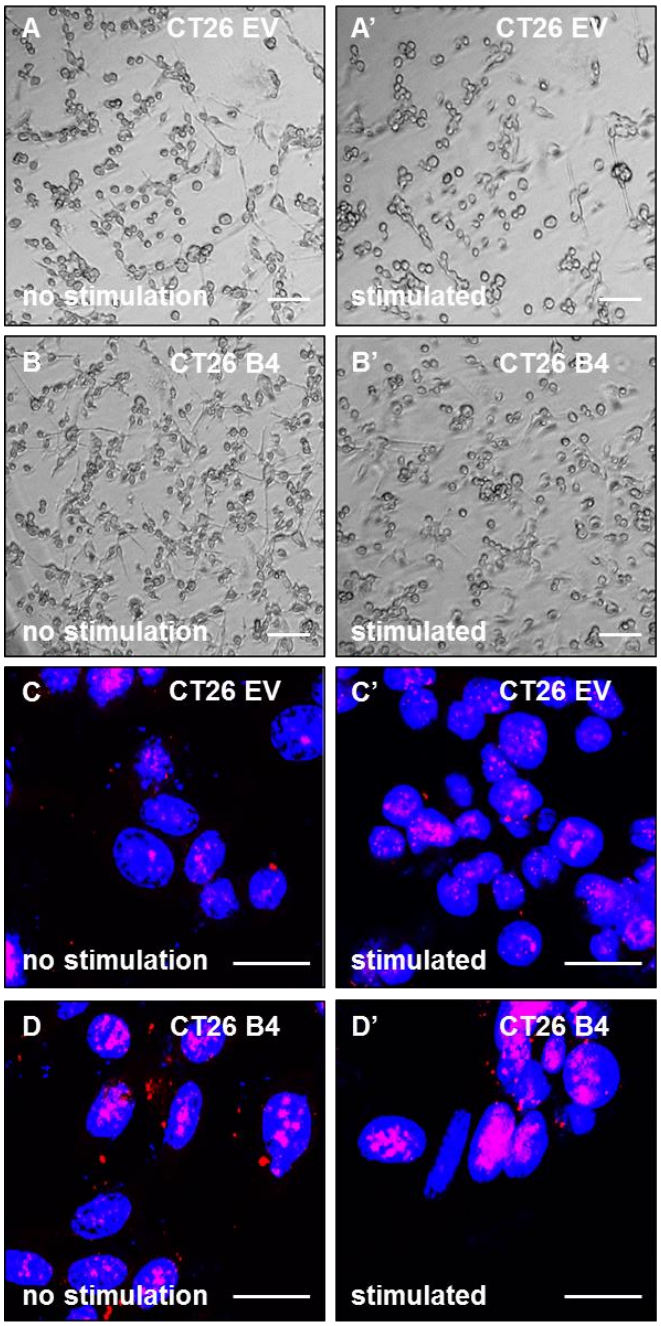

E
Colocalization of EphB4 in the nucleus
Manders' colocalization coefficient
1.0
0.8
0.6
0.4
0.2
0.0
***
**
EV no stimulation
EV stimulated
B4 no stimulation
B4 stimulated
LIM2405 Cell Constructs

Figure 2.7. EphrinB2-Fc stimulation causes retraction and rounding of CT26 cells and changes EphB4 localisation. A-A'. EV cells pre and post-stimulation (respectively). **B-B'.** B4 cells pre and post-stimulation (respectively). **C-C'.** Visualisation of EphB4 (H200, red) and the nucleus (DAPI, blue) in EV cells pre and post-stimulation (respectively). **D-D'.** Visualisation of EphB4 (H200, red) and the nucleus (DAPI, blue) in B4 cells pre and post-stimulation (respectively). (Scale bars for A-B' = 100μm) (Scale bars for C-D' = 400μm). **E.** Quantitative analysis of EphB4 localization in the nuclei of CT26 cells, presented as Manders' colocalization coefficient based on 4 images/construct/condition. $^{**}P<0.01$.


A
CT26 EV
no stimulation
A'
CT26 EV
stimulated
B
CT26 B4
no stimulation
B'
CT26 B4
stimulated
C
CT26 EV
no stimulation
C'
CT26 EV
stimulated
D
CT26 B4
no stimulation
D'
CT26 B4
stimulated

E

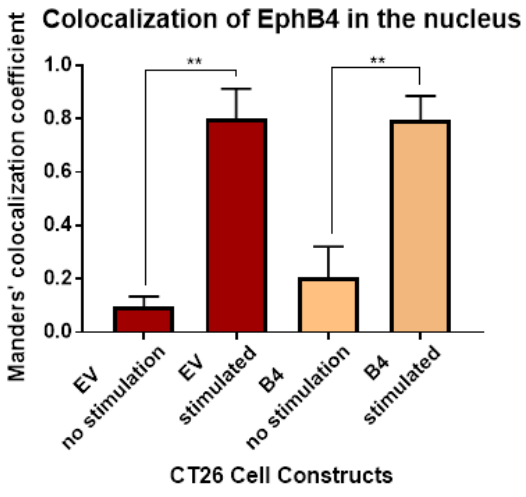
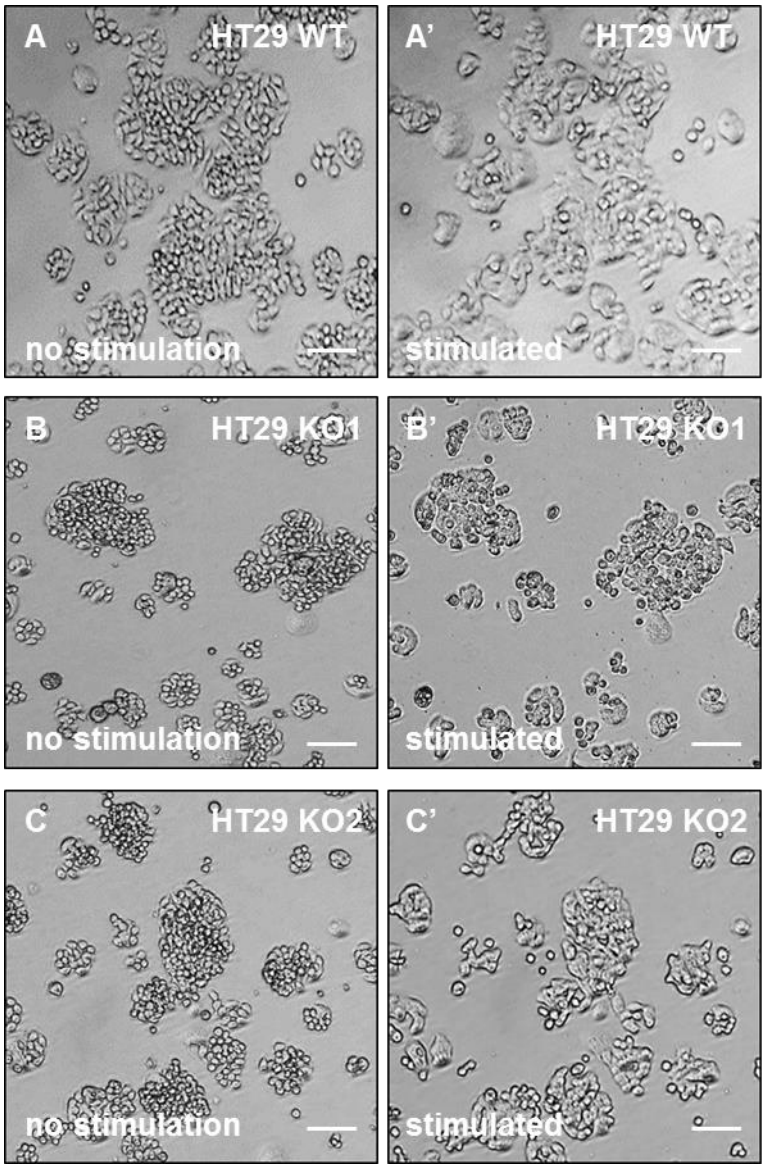

Colocalization of EphB4 in the nucleus
Manders' colocalization coefficient
1.0
0.8
0.6
0.4
0.2
0.0
**
**
EV
no stimulation
EV
stimulated
B4
no stimulation
B4
stimulated
CT26 Cell Constructs

Figure 2.8. EphrinB2-Fc stimulation leads to cell clusters with indistinguishable cell borders predominantly in HT29 WT constructs. A-A'. WT cells pre and post-stimulation (respectively). **B-B'.** KO1 cells pre and post-stimulation (respectively). **C-C'.** KO2 cells pre and post-stimulation (respectively). (Scale bars for all = 100μm).


A
HT29 WT
no stimulation
A'
HT29 WT
stimulated
B
HT29 KO1
no stimulation
B'
HT29 KO1
stimulated
C
HT29 KO2
no stimulation
C'
HT29 KO2
stimulated

2.3.4. Mixing EphB4 and EphrinB2 expressing cells in culture leads to segregation of cell populations

In this study, green and red fluorescent protein expressing HT29 WT (WT GFP, WT RFP), EphB4 knockout (B4 KO GFP) and EphrinB2 knockout (B2 KO RFP) cells were mixed in culture at low density, to observe cellular outcomes and growth differences. Initially at day 1 after seeding, all cells were mixed coherently amongst each other (**Figure 2.9A, B, C, and D**). At day 5 when the cultures reached near confluence, the WT cells that are tagged with green or red fluorescent protein showed uniform growth and appeared to be still evenly dispersed amongst each other (**Figure 2.9A**'). This growth pattern was slightly altered when WT cells were mixed with B2 KO RFP and B4 KO GFP cells, as some population segregation could be observed (**Figure 2.9B', C'**). However, greatest level of separation was observed when the same experiments were conducted by mixing B2 KO RFP with B4 KO GFP cells. At day 5, the cells had almost entirely segregated into distinctive B4 KO GFP and B2 KO RFP colonies (**Figure 2.9D'**). Quantification of cell cluster sizes at day 5 cultures, revealed that at confluence approximately 80% of WT RFP and GFP cells tended have single cells or small clusters (1-20 cells) and 20% of medium size colonies (20-50 cells) (**Figure 2.10A**). Mixing WT GFP cells with B2 KO RFP cells yielded similar size colonies (**Figure 2.10B**). Co-culture experiments with B4 KO GFP cells resulted in the greatest differences in cluster sizes, whereby, when mixed with WT RFP cells more than 60% of the clusters comprised of 20 or more cells (**Figure 2.10C**). Similarly, when B4 KO GFP cells were cultured with B2 KO RFP cells, there were no single cell or small clusters observed, rather large homogenous colonies, 50% of which had 20 or more cells (**Figure 2.10D**).

Figure 2.9. Differential EphB4 and EphrinB2 expression leads to segregation of cell populations. A-A'. WT RFP and GFP cells 1 and 5 days after being mixed and seeded. **B-B'.** WT GFP and B2 KO RFP cells 1 and 5 days after being mixed and seeded. **C-C'.** WT RFP and B4 KO GFP cells 1 and 5 days after being mixed and seeded. **D-D'.** B2 KO RFP and B4 KO GFP cells 1 and 5 days after being mixed and seeded. (Scale bars for A, B, C, D = 200μm) (Scale bars for A', B', C', D' = 100μm).

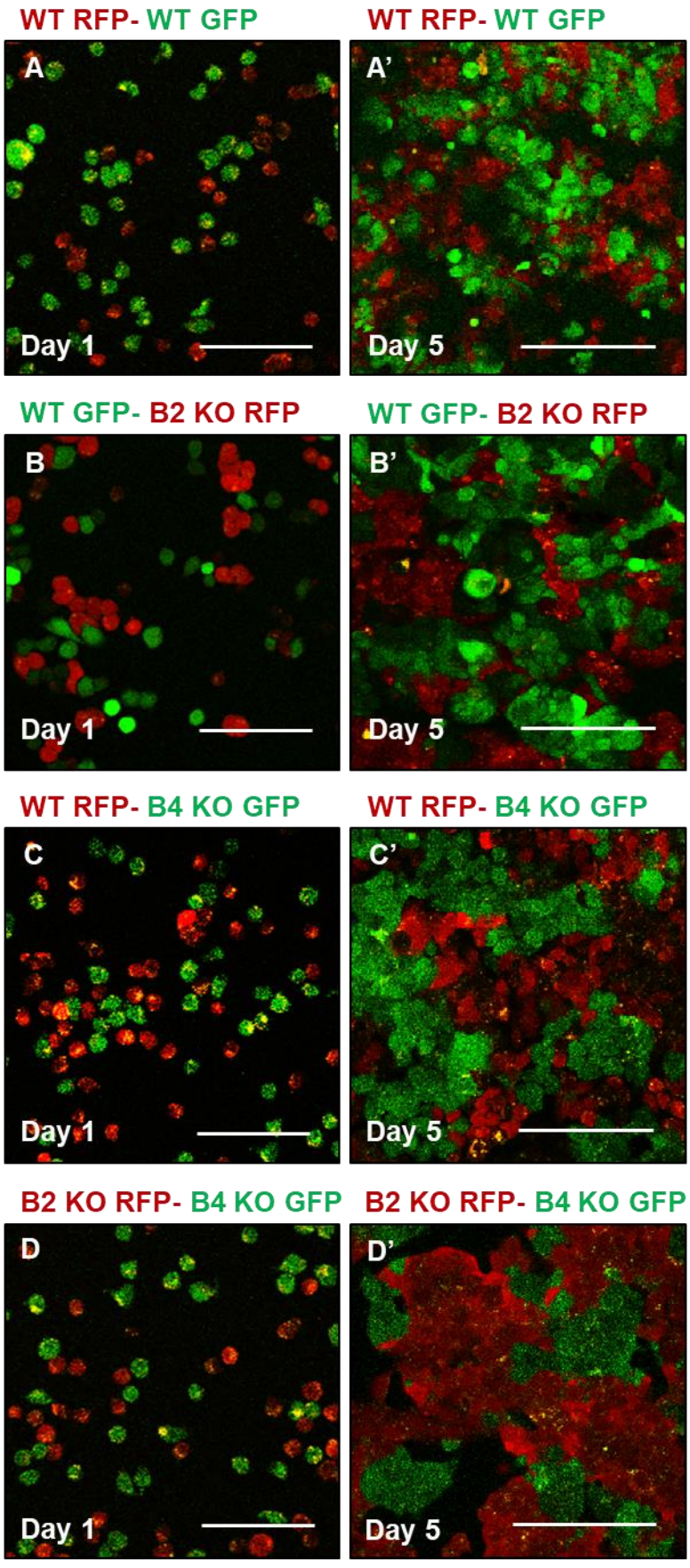
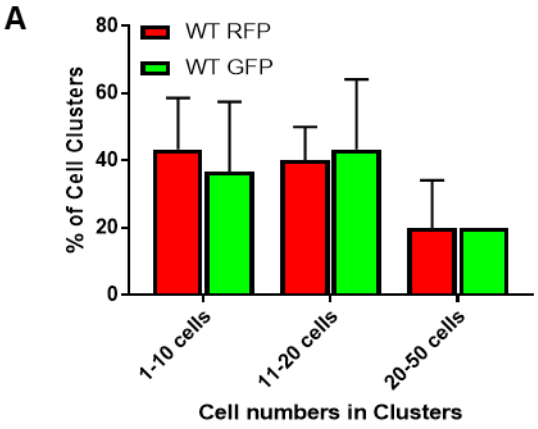
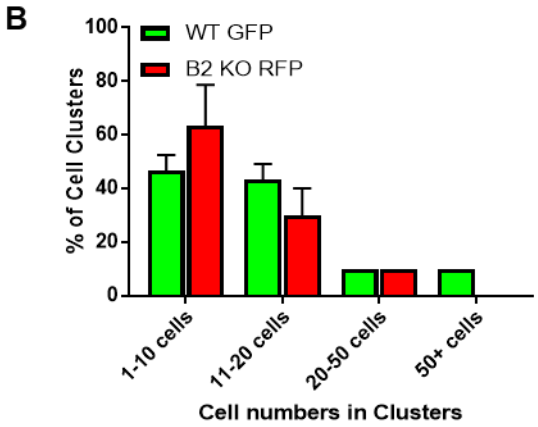
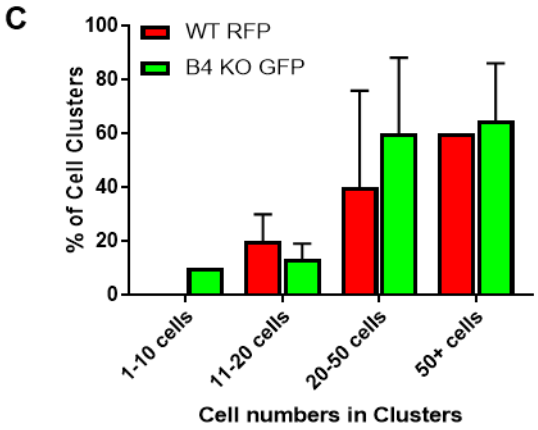
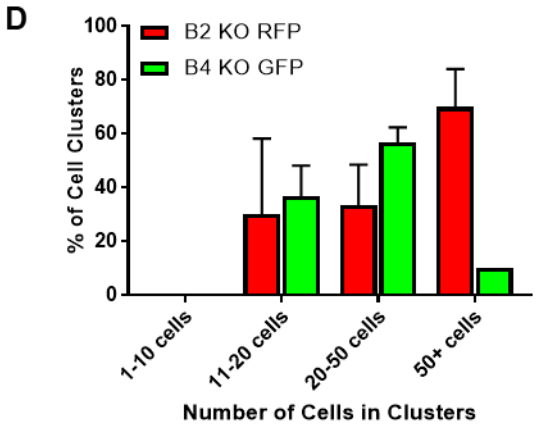

WT RFP- WT GFP
WT RFP- WT GFP
A
A'
Day 1
Day 5
WT GFP- B2 KO RFP
WT GFP- B2 KO RFP
B
B'
Day 1
Day 5
WT RFP- B4 KO GFP
WT RFP- B4 KO GFP
C
C'
Day 1
Day 5
B2 KO RFP- B4 KO GFP
B2 KO RFP- B4 KO GFP
D
D'
Day 1
Day 5

Figure 2.10. EphB4 knockout and EphrinB2 knockout cells in co-culture experiments lead to the formation of larger homogenous clusters and fewer mixing of single cells. A. Percentage of approximate cell cluster sizes in WT RFP and GFP mixing experiments. **B**. Percentage of approximate cell cluster sizes in WT GFP and B2 KO GFP mixing experiments. **C.** Percentage of approximate cell cluster sizes in WT RFP and B4 KO GFP mixing experiments. **D.** Percentage of approximate cell cluster sizes in B2 KO RFP and B4 KO GFP mixing experiments.


A
WT RFP
WT GFP
% of Cell Clusters
80
60
40
20
0
1-10 cells
11-20 cells
20-50 cells
Cell numbers in Clusters


B
WT GFP
B2 KO RFP
% of Cell Clusters
100
80
60
40
20
0
1-10 cells
11-20 cells
20-50 cells
50+ cells
Cell numbers in Clusters

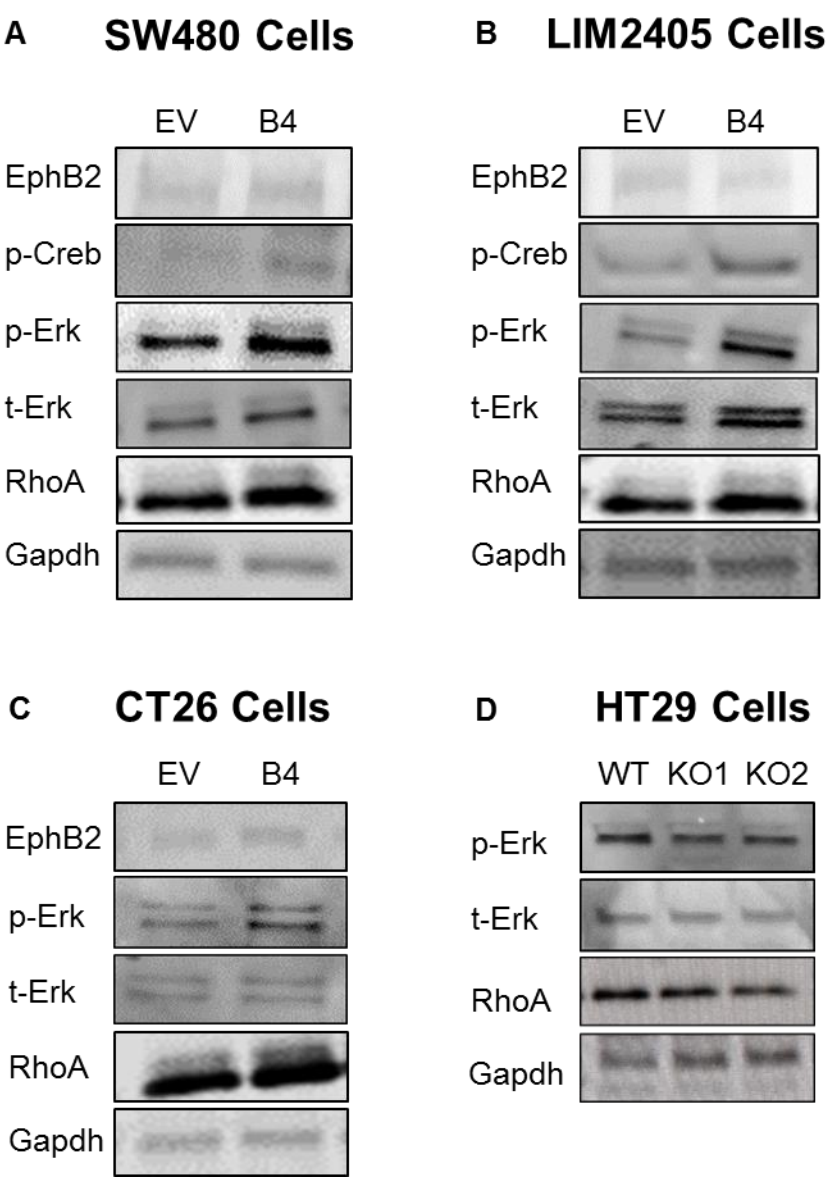

C
WT RFP
B4 KO GFP
% of Cell Clusters
100
80
60
40
20
0
1-10 cells
11-20 cells
20-50 cells
50+ cells
Cell numbers in Clusters


D
B2 KO RFP
B4 KO GFP
% of Cell Clusters
100
80
60
40
20
0
1-10 cells
11-20 cells
20-50 cells
50+ cells
Number of Cells in Clusters

2.3.5 EphB4 modification causes changes to Erk 1/2, Creb and RhoA protein levels

In order to determine possible molecular consequences of EphB4 overexpression and knockout, several protein targets were investigated through western blot analysis. Cell extractions were probed for the phosphorylated and total targets of Akt, Stat3, EGFR, cortactin, E-cadherin, ADAM 2 and 9 proteins (data not shown). In these targets no expression differences were observed between that of EphB4 overexpressing, knockout and control constructs. Similarly, EphB2 protein expression also remained unchanged between EV and B4 constructs of the SW480, LIM2405 and CT26 cell lines (**Figure 2.11A, B, C**). However, phosphorylated Erk1/2 and RhoA protein levels tended to be higher in high EphB4 expressing (B4) cells, in comparison to controls (EV) of the SW480, LIM2405 and CT26 cells (**Figure 2.11A, B, C**). As well as, phosphorylated Creb protein being more highly expressed in the SW480 and LIM2405 cell lines (**Figure 2.11A, B**). The HT29 unmodified WT cells have slightly higher levels phosphorylated Erk1/2 and RhoA protein expression in comparison to the knockout (KO1, KO2) constructs (**Figure 2.11D**).

Figure 2.11. Erk, RhoA and Creb protein levels are upregulated in EphB4 overexpressing cells, whilst Erk and RhoA proteins are reduced in EphB4 knockout cells. A. Representative western blot images of protein target expression of SW480 constructs and Gapdh loading controls. **B.** Representative western blot images of protein target expression of LIM2405 constructs and Gapdh loading controls. **C.** Representative western blot images of protein target expression of CT26 constructs and Gapdh loading controls. **D.** Representative western blot images of protein target expression of HT29 constructs and Gapdh loading controls.


A
SW480 Cells
EV
B4
EphB2
p-Creb
p-Erk
t-Erk
RhoA
Gapdh
B
LIM2405 Cells
EV
B4
EphB2
p-Creb
p-Erk
t-Erk
RhoA
Gapdh
C
CT26 Cells
EV
B4
EphB2
p-Erk
t-Erk
RhoA
Gapdh
D
HT29 Cells
WT
KO1
KO2
p-Erk
t-Erk
RhoA
Gapdh

2.4 Discussion

2.4.1 EphB4 expression modification of CRC cell lines

EphB4 receptor expression is suggested to be enhanced during late and metastatic stages of various cancer types (**Chapter 1, Table 1.1**). However, due to the contradictory results obtained in CRC and in some other cancer types, it appears that EphB4 expression may have diverse consequences in different cell types and tumour microenvironments. Expressional studies using multiple CRC cell lines will contribute to better understanding of the type of influence EphB4 overexpression and knockout has on CRC cells. Additionally, this will also determine if the influence remains constant across multiple cell lines with different morphological, phenotypical and mutational characteristics.

2.4.1.1 Upregulating EphB4 expression in CRC cell lines

The SW480, LIM2405 and CT26 cell lines express low levels of EphB4 and for this reason they were used in overexpression studies. The upregulation of certain genes is a necessity in tumour development and progression (Santarius et al. 2010). For this reason, expression vector systems are beneficial for investigating biological consequences of gene overexpression in cancer cells (Prelich et al. 2012). In this study, a plasmid-based expression vector was used to stably transfect the cells. Plasmids are circular DNA molecules that have strong viral promoters (Khan et al. 2013). Plasmids contain an 'origin of replication' sequence that permits autonomous replication and is inherited by daughter cells during mitosis (Rosano et al. 2014). This feature of the plasmid allows stable, hence continuous, expression of genes, rather than transient expression which can be lost as a result cellular

division (Kim et al. 2010). This permits long term experimentation using modified cells with minimal treatment and interference needed to maintain the culture. However, one shortcoming could be that upregulated expression of some genes can be toxic to the cells. Although, some plasmids have internal controls that limit the number of replications, the vector used in this study (pMono-neo-mcs) does not possess such controls (Kim et al. 2010). For this reason, the number of plasmids introduced into cells during electroporation and ultimately level of gene expression need to be taken into consideration (del Solar et al. 2000; Laurenti et al. 2013). Therefore, morphological experiments were conducted to ensure that overexpression of EphB4 itself does not have adverse effects on cells. In our study, overexpression is approximately two times that of controls, hence, this is not supra-physiological levels of the receptor introduced into the cells. No obvious morphological or adverse differences were observed between the control EV and B4 overexpressing cells. Nonetheless with EphB4 overexpression, amount of detached and floating viable cells, normally seen with SW480 cell line, appear to be increased (data not shown). This may be due to increased proliferation resulting in cell detachment when culture is near confluence. As mentioned in the **section 2.1**, the SW480 cell line is thought to possess rapid self-renewal and proliferation characteristics (Takaya et al. 2016; Xiong et al. 2014). The potential of EphB4 overexpression enhancing the proliferative capacity of these cells will be investigated in more detail in the following chapters.

2.4.1.2. CRISPR-Cas9 targeting of EphB4

The HT29 cell line was found to express moderate level of EphB4 and it was chosen to establish knockout constructs. To achieve gene knockout, the CRISPR-Cas9

system was used in our study. As of recent times, the use of this system in CRC studies is becoming more common (Cortina et al. 2017; Matano et al. 2015; Zheng et al. 2017). To the best of our knowledge, our study is the first to target EphB4 receptor expression using the CRISPR-Cas9 system. This system was discovered in bacterial cells and function as an adaptive defence mechanism against invading plasmids and bacteriophages (Barrangou et al. 2007; Deveau et al. 2010; Jansen et al. 2002). There are three types of CRISPR-Cas systems discovered. The CRISPR-Cas9 is a type II system, which differs from other types in the way it processes precursor CRISPR RNA (precrRNA), and how it interferes with target DNA (Thurtle-Schmidt et al. 2018). Interference with DNA is achieved in stages, in the adaptive phase, when bacterial cell encounters foreign DNA, portion of the foreign sequence is captured and incorporated as 'protospacers' within specialised loci (Horvath et al. 2010). Each protospacer is then separated by conserved repeat sequences, giving rise to the name clustered, regularly interspaced, short palindromic repeats (CRISPR) (Wiedenheft et al. 2012). In the interference phase, a long precrRNA is transcribed, which has several protospacer and repeat sequences. In the type II CRISPR-Cas system, precrRNA processing requires the transcription of another non-coding RNA, the trans-activating RNA (tracrRNA), which is partly complementary in sequence to precrRNA (Chylinski et al. 2013; Deltcheva et al. 2011). The tracrRNA acts as a scaffolding backbone, as it pairs and complexes with precrRNA and together they are loaded into Cas9 nucleases. This promotes cleavage of the complex by RNAse III enzyme (Deltcheva et al. 2011). Further processing of precrRNA gives rise to mature crRNA, with one unique protospacer sequence, which guides Cas9 to target DNA sequence.

Recognition is first initiated several base pairs upstream of target, in the protospacer motif (PAM) sequence (Mojica et al. 2009; Redding et al. 2015). The PAM sequence preference can vary between different Cas proteins and different bacterial species, the Cas9 system largely recognises PAM that is any nucleotide followed by two guanine residues (5'-NGG-3'). The PAM recognition causes unwinding in target DNA, which allows for it to complex with guideRNA of CRISPR-Cas9 molecule by Watson-Crick base pairing (Jackson et al. 2017; Sternberg et al. 2014). The Cas9 nuclease also undergoes activating conformational change upon PAM sequence binding (Sternberg et al. 2014). Without this sequence, Cas9 nuclease cannot be activated, as such no cleavage can occur. This helps protect the bacterial cells from auto-immunity, as the CRISPR arrays do not contain any PAM sequences. The presence of a PAM sequence is also essential when designing CRISPR-Cas9 constructs for gene modification experiments.

The active Cas9 nuclease has two functional nuclease domains, HNH and RuvC (Gasiunas et al. 2012; Nishimasu et al. 2014). In an inactive conformation, the HNH domain is situated away from the target DNA, upon PAM recognition conformational change brings it closer to the guideRNA: target DNA complex, allowing for it to cleave the strand of DNA complementary to guideRNA (Nishimasu et al. 2014). The non-complementary strand is located near and is cleaved by the RuvC nuclease compartment. This causes a double stranded blunt ended break, three base pairs upstream of PAM sequence, destroying foreign DNA (Gasiunas et al. 2012; Nishimasu et al. 2014; Jinek et al. 2012). The CRISPR-Cas9 system has since been modified for use in mammalian cells (Cong et al. 2013; Mali et al. 2013; Jinek et al. 2012). The underlying principle remains the same, where the single guide crRNA is a user-defined target sequence, which anneals with the

scaffold RNA in the Cas9 molecule. In a similar manner to the excision that takes place in bacterial cells, the Cas9 nuclease cleaves human genome causing a double stranded break (Singh et al. 2017). This break is repaired through two main pathways, non-homologous end joining (NHEJ) and homology directed repair (HDR) (Khanna et al. 2001; Pawelczak et al. 2018). The NHEJ is an error-prone system that results in insertions/deletions (indel) or frameshift mutations at the site of break, which can lead to loss-of-function and silencing in genes with premature stop codons. Alternatively, HDR fundamentally relies on homologous recombination with donor DNA, and can be used for gene knock-in experiments.

However, the double strand break system has been widely criticized previously, as the guide-RNA sequence is very short (20 base pairs long) and therefore the chances of having this exact sequence repeating in the genome is highly likely. Mostly because the CRISPR-Cas9 system has been found to tolerate up to 3 base pair mismatches in the target sequence, particularly at the 5' end of the spacer (Mali et al. 2013). Mismatch tolerance may benefit bacterial immunity by allowing greater degree of recognition of bacteriophages, however, it can lead to off-target effects in human gene modification (Fu et al. 2014). Increasing the length of the guide sequence was determined to be insignificant in reducing off-target effects, rather, truncating it at the 5' end of spacer was reported reduce unwanted targeting (Fu et al. 2014). Alternatively, independently introduced mutations in either the RuvC or HNH domains of Cas9 convert the nuclease to a nikase, which instead of a double stranded break in DNA, causes a single strand nick (Ran et al. 2013; Sander et al. 2014). This was shown to drastically reduce off-target effects, particularly when two Cas9 nikase molecules were engineered to target opposite strands of target gene, within close proximity to each other (Ran et al. 2013; Zhang et al. 2017). The

dual nick system has reduced off-target effects as having the same complimentary sequences in the genome is less likely. When using dual nikases, due to the close proximity of the nicks introduced on opposite strands of the DNA, this is recognised as a double stranded break with overhangs on each strand. The overhangs created by the nicks can be ligated with donor DNA through HDR mediated repair for knock-in experiments. Alternatively, in the absence of donor DNA, NHEJ repair can lead to indel mutations or genomic microdeletions in the region (Ran et al. 2013; Zhang et al. 2017). In this study, we have used the dual CRISPR-Cas9 nikase system to target EphB4 gene sequence, close to the translational initiation codon. This caused deletions in sequences recognised by the BsaI enzyme in the target region, and a lack of detectable protein band of EphB4 confirmed successful targeting.

2.4.1.3 Gene modification and possible cell consequences

Previously, we have attempted to produce EphB4 knockdown constructs with short interfering RNA in the HT29 cell line (data not shown). However, there was poor targeting efficiency with any of the methods we tried. Typically, our targeting was in <5% of the cells, so seeing a biological effect in the population was unlikely. For this reason, we have opted for the use of complete knockout cells, to determine the role of EphB4 expression in CRC cells. Gene targeting can be challenging in cancer research, since these cells are highly robust due to cellular trait and genetic heterozygosity, which allows for rapid adaptation and survival (Tian et al. 2011). As such, one possible consequence of gene knockout, which is less likely to occur in knockdown studies, is compensation through upregulation of a related gene with a similar function (El-Brolosy et al. 2017). EphB2 shares high level of sequence

similarity and has some overlapping functions with EphB4. Furthermore, while EphB4 is highly specific to the EphrinB2 ligand and does not interact with other Ephrins, EphrinB2 can bind to the EphB2 receptor with a similar affinity to that of EphB4 (Chrencik et al. 2006*a*; Noberini et al. 2012). Previously, it has been suggested that EphB4 is preferentially expressed over the EphB2 receptor in progressive CRC (Kumar et al. 2009). EphB2 expression silencing was reported to coincide with EphB4 upregulation. For this reason, we investigated the possibility of EphB4 knockout impacting EphB2 protein expression in the HT29 cells. It was determined through western blots, that this cell line expressed very low levels of EphB2, the expression of which remained unchanged within EphB4 knockout cells. Hence, these results suggest that EphB2 receptor expression is not upregulated to compensate for EphB4 receptor knockout and loss of function in these cells. Similarly, overexpressing EphB4 in the SW480, LIM2405 and CT26 cell lines, did not result in differences in EphB2 protein levels. Although, EphB4 knockout was not found to have direct adverse effects on cells, the tight colonies formed at confluence with indistinguishable individual cell borders of the unmodified WT cells, appear to be slightly reduced within the knockout constructs. Like other Eph receptors, EphB4 regulating cell-cell and cell-substrate interactions in cells have been reported (Cortina et al. 2007; Lv et al. 2016). In the embryonic stage, inhibition of EphB4 signalling is shown to disrupt cadherin-mediated adhesion of cells causing overlapping of different layers (Fagotto et al. 2013). Its expression and function proved to be one of the vital factors in governing homotypic cell-cell contact and separation. Perhaps knocking out EphB4 also reduces the adhesive forces between our modified CRC cells. Potential changes to cell adhesion could have functional consequences like enhanced motility. In breast cancer cells, EphB4

receptor expression is reported to reduce level of integrin-β1 when not stimulated by EphrinB2 (Noren et al. 2009). This is suggested to enhance rate of migration in these cells. In a prostate cancer cell line, EphB4 expression correlates with integrin-β8, and are to be co-regulated together to enhance migratory abilities of cells (Mertens-Walker et al. 2015). Whether, the reduction in cell-cell contacts observed in our study leads to changes in migratory abilities will be investigated in the following chapter. Furthermore, knocking down EphB4 receptors was shown to cause up to a 69% reduction in viability of some CRC cell lines (McCall et al. 2016). Whilst we did not observe any obvious reductions in cellular viability with EphB4 knockout, these constructs tend to grow slower *in vitro*, this will be assessed further in **Chapter 3** using proliferation assays.

2.4.2 Cellular outcomes of EphB4 stimulation

The Eph receptors can form oligomer clusters through interaction with their cell bound Ephrin ligands (Himanen et al. 2001; Janes et al. 2012). It is accepted that heterodimeric formations can activate other types of receptor tyrosine kinases, Eph receptors need to form oligomer clusters to function (Schaupp et al. 2014). Various factors can influence the activating or inhibiting outcome of Eph and Ephrin interaction, such as the size of the oligomer clusters formed, the ratio of receptor to ligand constituents within clusters, type of interaction occurring in *trans* or *cis* and which cell the cluster is endocytosed into (**Chapter 1, section 1.3.1**). Even mutant, hence dysfunctional, EphB4 receptor overexpression was shown to impact cancer cell behaviour (Dopeso et al. 2009). In order to demonstrate the functionality and cellular consequences of EphB4 receptor stimulation, we have used clustered EphrinB2-Fc to stimulate our cells.

Clustering the ectodomain of EphrinB2 with human IgG induces the oligomerisation of EphB4, in the way it would if the ligand was expressed on the cell surface (Davis et al. 1994). As a result of stimulation, cellular rounding and retraction occurred across all of our cell lines. As expected, these effects were most pronounced in EphB4 overexpressing constructs and are less apparent in control cells and EphB4 knockout constructs. Similar results were reported with an EphB4 overexpressing prostate cancer cell line, where EphrinB2-Fc stimulation reduced cell spreading (Rutkowski et al. 2012). On the other hand, the control cells were less affected and mostly maintained their fibroblast-like phenotype even after stimulation. This is a well characterised outcome of Eph receptor activation, hence forward signalling, which leads to cellular retraction and repulsion upon encountering its Ephrin ligand (Aharon et al. 2014; Zimmer et al. 2003). This effect is of importance in facilitating cell population segregation and sorting in organ systems (Cowan et al. 2005; Gaitanos et al. 2016; Yoo et al. 2011).

This phenomenon was further demonstrated in our study, with the mixing of fluorescent EphrinB2 and EphB4 knockout cells in culture. In most cells, Eph receptors and Ephrin ligands will generally be coexpressed in distinct cellular domains (Bruckner et al. 1999; Chavent et al. 2016; Marquardt et al. 2005). As such in the HT29 cell line expresses both EphB4 and EphrinB2, and engineering knockout constructs creates expressionally different cell populations, whereby, EphB4 receptor knockout cells are left with EphrinB2 expression, and EphrinB2 knockout cells have EphB4 expression. These results suggest that the mixing of EphB4 and EphrinB2 knockout cells lead to distinct colonisation amongst ‘like’ expressing cells. Similar results were obtained with fibroblasts expressing EphB4

and EphrinB2, where EphB4 expressing cells segregated away from the EphrinB2 expressing cells (Marston et al. 2003).

2.4.3 Molecular outcomes of EphB4 expression modification

Upon Eph receptor stimulation, Rho and Rac family of GTPases are some of the key players in regulating cytoplasmic dynamics that lead to changes in morphology that mediating cell migration and segregation. Cellular motility and contractility are mediated through the formation of lamellipodia and filopodia. These projections were found to assemble upon EphB4 forward signalling (Marston et al. 2003). Phosphorylated EphB4 receptors were shown to co-localise with lamellipodia, suggesting a direct contact may prompt the development of filopodia. Blocking Rho and Rac mediated actin polymerisation inhibited endocytosis of EphB4 and as a result hindered cellular retraction (Marston et al. 2003). In our study, EphB4 overexpressing cells tended to have increased level of RhoA protein, whilst, EphB4 knockout slightly reduced its expression. As previously mentioned, the LIM2405 cell line has been characterised to overexpress RhoA (Fanayan et al. 2013). In this study, the upregulation of EphB4 increased RhoA expression, as such indicating a potential for enhanced migratory abilities of cells.

We also report the localisation of EphB4 in the leading and following ends of cells, also at the site of cellular interaction and within the nucleus. Upon stimulation, EphB4 is removed from cellular projections and becomes distributed more heavily in and around the nucleus. Another study also reported the nuclear localisation of EphB4 within prostate cancer cells, using the same antibody as the one in our study (Mertens-Walker et al. 2015). This antibody recognises the ectodomain of the receptor, however, through nuclear fractionation it was confirmed that it was the

full length of the receptor in the nucleus (Mertens-Walker et al. 2015). It is tempting to contemplate if EphB4 could bind to transcriptional factors to regulate gene expression.

The cyclic AMP-responsive element binding protein (Creb) is a basic leucine zipper (bZIP)-containing transcription factor that regulates wide variety of genes associated with cell survival and growth (Steven et al. 2016). When activated it induces transcription in association with coactivator protein Creb-binding protein (CBP). Creb is integrated into several signalling pathways and becomes phosphorylated by multiple upstream targets (Sakamoto et al. 2009). One way is through the Wnt signalling pathway. As reviewed in **Chapter 1** (**section 1.1.1**), this pathway is one of the key regulators of cell proliferation in the colonic crypts (Song et al. 2015). Signalling initiation, with the binding of a Wnt ligand to a frizzled receptor, inhibits the formation of a destruction complex, which would otherwise degrade the β-catenin protein. This causes β-catenin to be translocated to the nucleus, where it binds and interacts with several transcriptional co-activators, including CBP (Hecht et al. 2000; Takemaru et al. 2000). CBP activity is proposed to help maintain a dedifferentiated state in cells that allow for rapid proliferation (Kumar et al 2009; Teo et al 2005). As the Wnt pathway is constitutively activated in CRC cells, expression of CBP was reported to be higher in progressive stages of the disease (Kumar et al. 2009). EphB4 expression was found to be regulated by transcriptional coactivator CBP in the SW480 cell line, as siRNA targeting CBP specifically reduced EphB4 expression (Kumar et al. 2009). Inhibitors against CBP increased apoptotic activity in this cell line (Katayoon et al. 2004). In our study, we found higher levels of phosphorylated Creb protein in the EphB4 overexpressing SW80 and LIM2405 cells. This is in line with previous findings that confirm their

association, perhaps, EphB4 signalling can also mediate gene transcription through Creb. Another possible mediator of Creb phosphorylation and activation is Erk 1/2 proteins (Lu et al. 2006). This is suggested to promote cell survival through inhibition of apoptosis.

The Erk 1/2 proteins are involved in the MAPK signalling pathways and can regulate cell survival and proliferation, in addition to acting on cytoskeletal proteins for aiding cellular migration (Matallanas et al. 2011; Roskoski 2012). EphB4 regulation of Erk is suggested to be cell-type dependent, whereby EphB4 activation was shown to enhance Erk phosphorylation in breast cancer cells, whilst inhibiting it human umbilical vein endothelial cells (Xiao et al. 2012). In other studies, stimulation of EphB4 was shown to increase vascular endothelial growth factor transcription and secretion at the site of angiogenesis to mediate migration, proliferation and branching of endothelial cells through downstream interaction with Erk (Das et al. 2010; You et al. 2017). Furthermore, EphB4 was found to be overexpressed in cisplatin-resistant melanoma xenografts in comparison to cisplatin sensitive xenografts. In these tumours, Erk 1/2 expression was found to correlate with EphB4, for this reason, their interaction is assumed to contribute to cancer cell resistance to chemotherapy (Yang et al. 2015). In CRC studies, using cell lines that are different to those in our study, transient knockdown of EphB4 reduced Erk protein phosphorylation (McCall et al. 2016). In our study, we have observed that stable knockout of EphB4 expression also tends to reduce Erk phosphorylation.

In essence these results demonstrate that the EphB4 overexpression has functional impact on cells. Also, the level of response exhibited with overexpressing and

knockout cells differ from control cells, which will allow us to characterise the effects of EphB4 modification on cell behaviour, discussed in **Chapter 3**.

2.5 Conclusion

In conclusion, successful overexpression of EphB4 protein was achieved in three CRC cell lines and its expression was knocked out using CRISPR-Cas9 system, which is a novel approach that has not been attempted previously. It was found that whilst modifying EphB4 expression in these CRC cells has not produced significant morphological differences, it has affected the level of cellular response to EphrinB2-Fc stimulation. The EphB4 overexpressing cells undergo greater degree of cell rounding, retraction and clustering. Furthermore, EphB4 receptor localisation within the nucleus was also observed in the CRC cells used in this study. In addition, Creb and Erk phosphorylation also appeared to be enhanced in overexpressing cells along with RhoA, which is reduced in knockout constructs.

CHAPTER 3

The Effect of EphB4 Overexpression and Knockout on Colorectal Cancer Cell Behaviour

3.1 Introduction

In Chapter 2, we generated EphB4 receptor overexpressing constructs of the SW480, LIM2405 and CT26 cell lines and obtained EphB4 expression knockout HT29 cells. These cell lines were characterised to be responsive to EphrinB2 treatment. However, EphB4 overexpressing cells responded considerably more to EphrinB2, which caused extensive rounding and clustering in these cells. Furthermore, modifying EphB4 expression, with overexpression or knockout constructs, was found to alter the protein levels of some signalling targets. EphB4 overexpressing cells tended to have higher protein levels of Erk1/2, RhoA and Creb, whilst EphB4 knockout cells had reduced Erk and RhoA proteins. These proteins can affect a variety of cellular outcomes; however, their primary functions involve regulation of cell proliferation, migration and invasion.

EphB4 overexpression is largely reported to enhance the proliferative capacity of a wide variety of cancer cells. In gastric cancer patient samples, *EPHB4* was found to be upregulated as part of an oncogenomic network of signature genes that regulate cell cycle, metabolism and proliferation (Liersch-Löhn et al. 2016). Immunohistochemical analysis showed it was predominantly expressed in the late stage of the disease. Similarly, in 94 breast cancer specimens, EphB4 protein levels were found to associate with increasing grade and stage of tumours (Wu et al. 2004). In breast cancer cell lines, using S-phase of the cell cycle as a proliferative index, EphB4 upregulation was determined to cause higher S-phase fraction and DNA aneuploidy. In sarcoma cell lines, 25-48% knockdown of EphB4 led to up to 60% reduction in proliferation, which was confirmed through both colourimetric and DNA-synthesis based proliferation assays (Becerikli et al. 2015). In addition,

EphrinB2 treatment can also inhibit proliferation. Stimulating EphB4 expressing breast cancer cell line with EphrinB2 in 3D culture models resulted in smaller spheroids (Noren et al. 2006). Fragmentation of DNA and induction of apoptosis were found to be prevalent in these cells. As such, EphB4 activation through EphrinB2 stimulation was found to suppress oncogenic potential of the cell lines that were tested (Noren et al. 2006). In another breast cancer study, prolonged exposure of EphB4 overexpressing cells to EphrinB2-Fc caused a significant reduction in cell viability and growth, as determined by Alamar-blue viability assays (Barneh et al. 2013). Furthermore, injecting EphrinB2-Fc into mice bearing high EphB4 expressing head and neck squamous carcinoma tumour xenografts, inhibited growth and had a negative impact on tumour vessel number, maturation and sprouting (Kimura et al. 2009). EphB4 mRNA levels were found to be upregulated in leukaemia patient samples and also in a leukaemia cell line (Li et al. 2015). In this cell line, knocking down EphB4 arrested cell division and reduced RhoA expression. Reduction in cytoskeletal modelling proteins like RhoA are suggested to be a possible contributing factor to this, since, without sufficient mitotic fibre assembly cells cannot divide.

A primary role of Ephs and Ephrins is to control and regulate cellular mobility and migration (Aharon et al. 2014; Zimmer et al. 2003). The EphB4 receptor involvement in the mobility of cancers have been widely investigated. Melanoma cell lines with highly malignant properties were found to overexpress EphB4, which enhanced RhoA mediated cell migration (Yang et al. 2006). Similarly, a highly migratory and invasive prostate cancer cell line also overexpressed EphB4 and other B type Eph receptors (Astin et al. 2010). In these cells, locomotion was not restricted by cell contact with fibroblast or endothelial cells in co-culture

experiments. Knocking down EphB4 expression was found to restore contact inhibition and reduce rate of migration in prostate cancer cells (Batson et al. 2013; Astin et al. 2010). In invasive bladder cancer cell lines, biological importance of EphB4 was also investigated using a knockdown approach, which decreased the invasive ability of cells *in vitro* (Xia et al. 2006). This result was attributed to a consequent decrease in matrix metalloproteinase 9 (MMP9) expression with the targeting of EphB4.

However, studies on the role of EphB4 in CRC have given contradictory results. Some studies suggest that high EphB4 expression can help maintain undifferentiated and highly proliferative state in cells, and knockdown of the receptor reduces this effect in cells and tumours (Kumar et al. 2009; McCall et al. 2016). Furthermore, it has been reported that high EphB4 expression enhances migratory abilities in CRC cells, leading to increase rate of metastasis (Kumar et al. 2009). In contrary, other CRC studies propose that EphB4 expression is silenced in progressive state of the disease, and EphB4 promoters are hypermethylated in cells, which leads to their uncontrolled proliferation (Davalos et al. 2006; Dopeso et al. 2009). It has been stated that EphB4 expression in CRC may restrict tumour expansion and dissemination (Batlle et al. 2005; Davalos et al. 2006).

In order to gain insight into the effects of EphB4 expression on CRC cell behaviour, we have used the SW480, LIM2405 human and CT26 mouse CRC cell lines to overexpress EphB4, and HT29 cell line to investigate the consequences of EphB4 knockout. We studied the proliferative aptitude, migrational and invasive abilities of CRC cells *in vitro*.

3.2 Methods

3.2.1 WST-1 proliferation assay

Cells were seeded in triplicates into 96 well microplates ($1x10^4$/100µL/well) and incubated under standard conditions. Water soluble tetrazolium (WST-1) (Roche Applied Science, Penzberg, Germany) was added to a set of 3 wells for each cell line, once a day for 4 days and incubated at standard conditions for one hour. The absorbance was read using medium plus WST-1 as the blank on a VarioSkan spectrophotometer (ThermoFisher, Scorseby, Australia) at 440nm (optimal absorbance wavelength of the dye) and again at 690nm (outside the absorbance wavelength of the dye) to compensate for well-well variability. Mean values were calculated for each cellular construct/day, 2-way ANOVA statistical analysis was conducted using Graphpad Prism 6 software.

3.2.1.1 EphrinB2-Fc stimulation proliferation assay

Cells were seeded in duplicates into 96 well microplates at a density of ($1x10^4$/100µL/well) as described above (**Section 3.2.1**). At day 1 after seeding, an initial measurement was taken and then the cells were starved with serum free media (SFM) for 24 hours. The medium was then replaced with 1% foetal calf serum media and clustered EphrinB2-Fc at a concentration of 1.5µg/mL (clustering described in **Chapter 2, section 2.2.3**), under standard culture conditions (**Chapter 2, section 2.2.2**). Proliferation was measured at 1, 3, 5 and 7 days post-treatment, using the WST-1 reagent as described above (**Section 3.2.1**). Cells not treated with EphrinB2-Fc were used as controls. Statistical analysis was conducted using 2-way ANOVA with multiple comparisons on the Graphpad Prism 6 software.

3.2.2 Xcelligence proliferation assay

Medium was added to E-Plate 16 (ACEA Biosciences, San Diego, United States) and allowed to equilibrate 37°C, before a background measure was taken. Cells were seeded in E-plate 16 at a concentration of $1x10^4$/100µL/well and allowed adhere to the plates for 30 minutes at room temperature. The plates were then placed in a Real-Time Cell Analyser System (ACEA Biosciences) in 37°C incubator with 5% carbon dioxide. Electrode impedance measurements were taken with Real-Time Cell Analyser software 1.2.1, every hour for 100-170 hours depending on the cell line used. The electrode impedance measured as 'Cell Index' was plotted as mean for each cellular construct and then analysed for significance using Graphpad Prism 6 software, paired *t*-tests were used to analyse data.

3.2.3 Ki67 cell immunofluorescence

Autoclaved coverslips were placed in 6 well culture plates. Cells were seeded and allowed to grow to approximately 90% confluence under standard culture conditions. At this point media were removed and the wells washed with PBS. Cells were then fixed with methanol on ice for 5 minutes, then washed with 10% Tween in PBS (PBST) for 10 minutes at room temperature. Skim milk 2.5% was used as a block, primary anti-Ki67 polyclonal rabbit antibody (1:1000) (Abcam, Cambridge, United Kingdom) and the anti-rabbit Alexa 488 secondary antibody (1:500) (Jackson Immunoresearch Laboratories, West Baltimore, United States) were diluted in PBST and incubated for 1 hour at room temperature, then washed 3x 5 minutes. Cellular nuclei were visualised using DAPI (4′,6-diamidino-2-phenylindole) (1:5000) (ThermoFisher), which was applied after the primary antibody for 30 minutes at room temperature. Coverslips were then mounted on

slides, using immunofluorescent mounting medium (DAKO, Agilent Technologies, Mulgrave, Australia). Fluorophores were visualized with FITC (Alexa 488 green, excitation wavelength 488nm) and DAPI (blue, excitation wavelength 358nm) filters on the IX81 Nikon microscope. Four random images were taken for each cell construct, where the Alexa 488 image was merged with DAPI. Cells were counted using Image J software as positive if there was Ki67 nuclear presence and nuclei labelled with only DAPI were taken as a negative. Percentage of positive cells was calculated from total (positive plus negative) cell counts. Statistical analysis was conducted using unpaired *t*-tests on the Graphpad Prism 6 software.

3.2.4 Boyden chamber migration and invasion assays

Cells were treated with serum-free medium for 24 hours. Complete culture medium (0.6mL) with 10% foetal calf serum was added to bottom compartment of wells as a chemo-attractant. For migration assays, cell suspension ($1x10^5$ cells/ 200µL) was added directly into 3 TransWell chambers (8µm pore-size)/cell construct (Corning, New York, United States). In addition, for each cell construct one well with 0.6 mL of serum-free medium (SFM) was used to consider random migrations that may occur in the absence of a chemoattractant. Invasion assays were set up in the same manner as migration assays, except 100µL of diluted Matrigel (3mg/mL) (Corning) was used to coat the TransWell chambers prior to seeding cells. For both assays, plates were incubated for approximately 18 hours at standard culture conditions. The plates and the TransWell chambers were then trypsinised and cells were counted using the Neubauer haemocytometer. Unpaired *t*-tests were used to analyse data and the results were graphed as mean ± standard deviations for each cellular construct using Graphpad Prism 6 software.

3.2.5 Xcelligence migration and invasion assay

Cells were treated with SFM for 24 hours. Complete culture medium (165µL) with 10% foetal calf serum was added to the bottom chamber, and 50µL of SFM added to the upper chamber of CIM-plate 16 (ACEA Biosciences). Plates were allowed to equilibrate at 37°C for 1 hour before a background measure was taken. For invasion assays, 50µL of diluted Matrigel (3mg/mL) (Corning) was added to the top chamber; after ensuring even coverage, 30µL of the Matrigel was removed and plates equilibrated for 4 hours at 37°C. For both experiments, a final concentration of $2.5x10^4$ cells/well was suspended in 100µL SFM and set up in triplicates/cellular construct in the upper chamber of CIM-plate 16 plates. One well with SFM in the bottom chamber was used as a control for random migrations. After a 30-minute incubation at room temperature, the plates were then placed in a Real-Time Cell Analyser System (ACEA Biosciences) at standard culture conditions. Electrode impedance measurements were taken every hour for a total duration of 70 hours for SW480 cells and 24 hours for LIM2405 and CT26 cells using the Real-Time Cell Analyser software 1.2.1. The electrode impedance measured as 'Cell Index' was plotted as mean for each cellular construct and then analysed for significance using Graphpad Prism 6 software. Paired *t*-tests and non-parametric tests were used to analyse data.

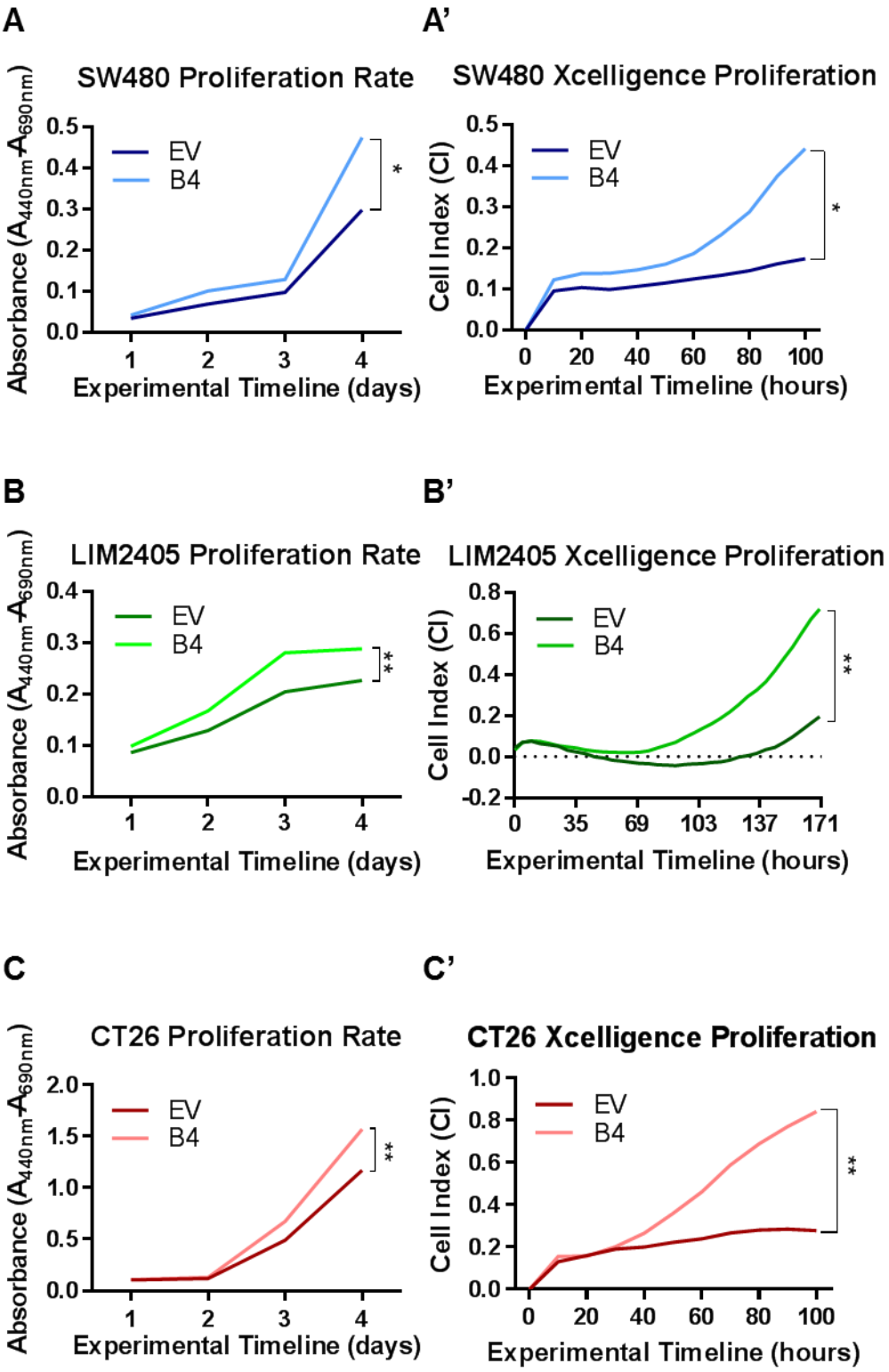
3.3 Results

3.3.1 EphB4 overexpression enhances rate of proliferation in CRC cells

In this study, cell proliferation was investigated by two widely used methods. Firstly, with the use of the colourimetric reagent WST-1, which is a stable tetrazolium salt that is cleaved to a soluble formazan dye in metabolically active cells (Quent et al. 2010). The quantity of formazan dye can be measured using a spectrophotometer, and the absorbance values obtained correlate with the number of viable cells in culture. Secondly, we used the Xcelligence system, which permits real time measurements of proliferation, based on low electrical potentials passing across plates with microelectrode biosensor surfaces (Kho et al. 2015; Sener et al. 2017). Cellular growth and attachment to the microelectrode surfaces, impedes the current of electrons and this is reported as 'Cell Index' values.

In the WST-1 proliferation experiment, the trend of higher absorbance values emerges early (at day 2), with the SW480 and LIM2405 B4 cells, compared to EV controls (**Figure 3.1A, B**). By day 4, the difference in these values reached statistical significance across all three cell lines, SW480 (EV: 0.30nm, B4: 0.45nm, $P<0.05$), LIM2405 (EV: 0.23nm, B4: 0.29nm, $P<0.01$) and CT26 (EV: 1.17nm, B4: 1.57nm, $P<0.01$) (**Figure 3.1A, B, C**). Using the Xcelligence system, B4 constructs were found to proliferate quicker and had significantly higher Cell Index values compared to EV controls for the SW480 (EV: 0.17 Cell Index, B4: 0.44 Cell Index, $P<0.05$ at 100 hours), LIM2405 (EV: 0.20 Cell Index, B4: 0.72 Cell Index, $P<0.01$ at 170 hours) and CT26 (EV: 0.28 Cell Index, B4: 0.84 Cell Index, $P<0.01$ at 100 hours) cell lines (**Figure 3.1A', B', C'**).

Figure 3.1. EphB4 overexpression enhances the rate of proliferation in CRC cells. A. SW480 cell proliferation measured over four days in WST-1 assay and plotted as absorbance (n=3 in duplicates/cell construct). **A'.** SW480 cell proliferation monitored in real time over 96 hours (4 days) using Xcelligence system (n=3/cell construct). **B.** LIM2405 cell proliferation measured over four days in WST-1 assay and plotted as absorbance. **B'.** LIM2405 cell proliferation monitored in real time over 170 hours (7 days) using Xcelligence system (n=3/cell construct). **C.** CT26 cell proliferation measured over four days in WST-1 assay and plotted as absorbance (n=3 in duplicates/cell construct). **C'.** CT26 cell proliferation monitored in real time over 100 hours (4 days) using Xcelligence system (n=3/cell construct).


A
A'
SW480 Proliferation Rate
SW480 Xcelligence Proliferation
Absorbance (A440nm-A690nm)
Cell Index (CI)
EV
B4
Experimental Timeline (days)
Experimental Timeline (hours)
B
B'
LIM2405 Proliferation Rate
LIM2405 Xcelligence Proliferation
C
C'
CT26 Proliferation Rate
CT26 Xcelligence Proliferation

3.3.2 EphrinB2-Fc treatment limits proliferation of EphB4 overexpressing cells

In order to determine the effect of EphrinB2 treatment, proliferation assays were conducted using clustered Fc, on the SW480 and LIM2405 cell lines. The EV and B4 constructs 'treated' with EphrinB2-Fc were compared to cells that did not receive treatment, which are regarded as EV and B4 'controls' in the graphs. Proliferation was measured with WST-1 as described above.

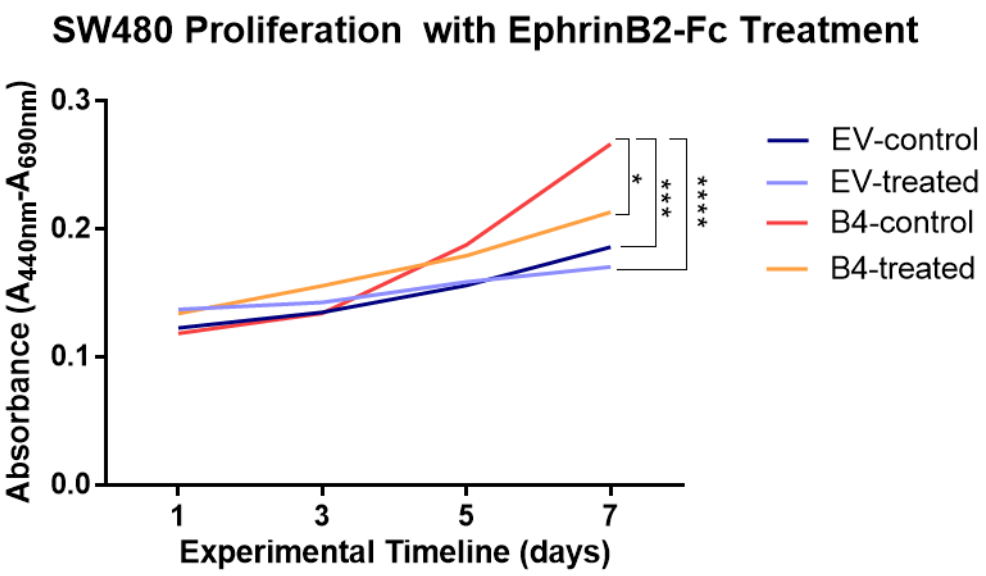
Overall, absorbance readings for treated and untreated B4 constructs were higher than EV counterparts in both cell lines ($P<0.05$, $P<0.01$, $P<0.001$, $P<0.0001$) (**Figure 3.2A, B**). Treating EV cells (SW80 EV-treated: 0.17nm at day 7) (LIM2405 EV-treated: 0.19nm at day 7) did not appear to impact absorbance values obtained, compared to untreated EV controls (SW480 EV-control: 0.18nm at day 7) (LIM2405 EV-control: 0.20nm at day 7) (**Figure 3.2A, B**). On the other hand, treated SW480 and LIM2405 B4 cells had lower absorbance values than untreated B4 controls. The greatest difference for LIM2405 was observed at day 5, where the value for B4 treated cells (0.17nm) was lower than B4 controls (0.22nm) (**Figure 3.2B**). However, by day 7, the B4 cells appear to recover from treatment (0.25nm) and approach untreated B4 controls (0.27nm). In contrast, the impact of EphrinB2-Fc was much longer lasting in the SW480 B4 constructs, as absorbance values for these cells followed a slow upward trend in comparison to untreated controls (**Figure 3.2A**). At day 7, this difference was statistically significant between SW480 B4 treated (0.21nm) and control cells (0.26nm) ($P<0.05$).

The observed effects indicate that, EphrinB2-Fc treatment limits proliferation in cells that overexpress the EphB4 receptor, in comparison to lower expressing

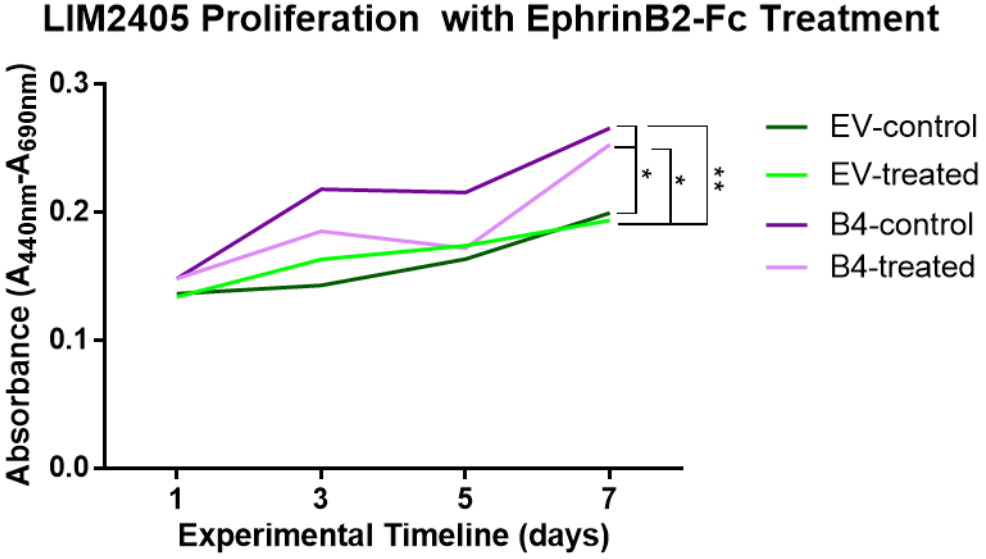
controls. This effect is persistent in the SW480 cell line. The growth rate of SW480 B4 treated cells was significantly slower than B4 untreated cells but not EV cells, highlighting inhibitory effect of EphrinB2 treatment. A similar trend is observed in the LIM2405 cell line, where B4 cells grows slower in the presence of EphrinB2-Fc at comparable levels to EV cells. However, eventually treated B4 cells appear to regain proliferative capacity by overcoming the limiting effect.

Figure 3.2. EphrinB2-Fc treatment limits the rate of proliferation in EphB4 overexpressing cells. A. SW480 EphrinB2-Fc treated and untreated control cell proliferation measured over seven days in WST-1 assay and plotted as absorbance (n=3 in duplicates/cell construct). **B.** LIM2405 EphrinB2-Fc treated and untreated control cell proliferation measured over seven days in WST-1 assay and plotted as absorbance (n=2 in duplicates/cell construct). **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

A


SW480 Proliferation with EphrinB2-Fc Treatment
Absorbance (A440nm-A690nm)
0.3
0.2
0.1
0.0
1
3
5
7
Experimental Timeline (days)
*
***
****
EV-control
EV-treated
B4-control
B4-treated

B

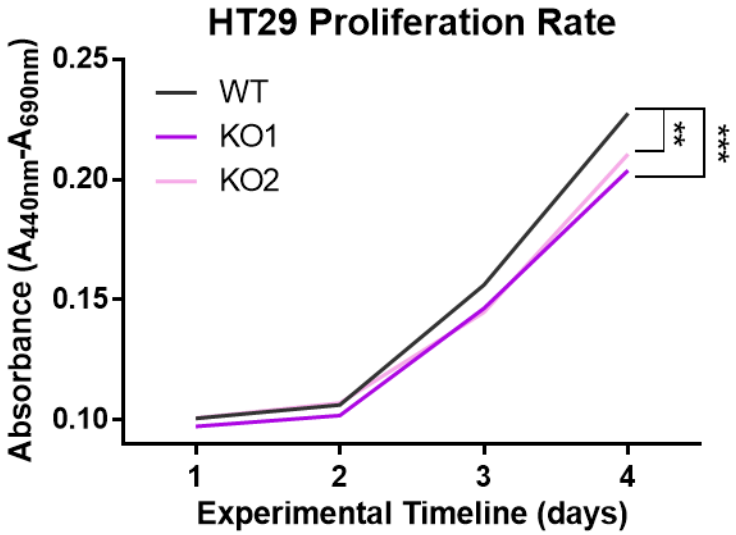

LIM2405 Proliferation with EphrinB2-Fc Treatment
Absorbance (A440nm-A690nm)
0.3
0.2
0.1
0.0
1
3
5
7
Experimental Timeline (days)
*
*
**
EV-control
EV-treated
B4-control
B4-treated

3.3.3 Knocking out EphB4 expression in the HT29 cell line significantly reduces cell proliferation

To determine the effect of EphB4 expression knockout on cell proliferation, WST-1 assays were conducted. The HT29 cell line failed to adhere to the Xcelligence plates despite multiple attempts, hence, this method of assessment was not utilised for this cell line. Initially, absorbance measurements were similar across all HT29 constructs. By day 3, WT growth trend began to diverge from knockout constructs (**Figure 3.3**). On the other hand, the two knockout clones (KO1, KO2) demonstrated similar change in absorbance over time (**Figure 3.3**). At the end of the experiment, absorbance values for KO1 (0.20nm) and KO2 (0.21nm) cells were significantly different compared to EphB4 expressing WT cells (0.23nm) ($P<0.01$ for WT versus KO1, $P<0.001$ for WT versus KO2).

Hence, it appears that knocking out EphB4 expression in the HT29 cell line negatively impacts cell proliferation. This is supported by the fact that both of the knockout clones displayed similar growth patterns, which was reduced compared to WT control cells. Nonetheless, while EphB4 expression is a positive modulator of proliferation, its expression does not appear to be essential for it. This is because, despite lacking EphB4 expression the knockout clones still displayed an upward trend of growth.

Figure 3.3. Knocking out EphB4 expression reduces the rate of proliferation in HT29 cells. HT29 cell proliferation measured over four days in WST-1 assay and plotted as absorbance (n=3 in duplicates/cell construct). $^{**}P<0.01$, $^{***}P<0.001$.

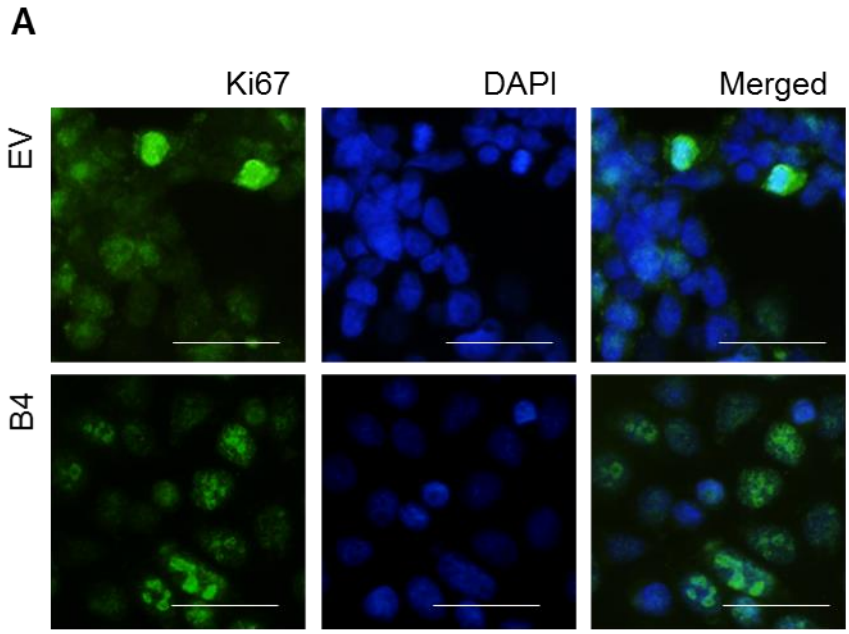

HT29 Proliferation Rate
Absorbance (A440nm-A690nm)
0.25
0.20
0.15
0.10
WT
KO1
KO2
**
***
1
2
3
4
Experimental Timeline (days)

3.3.4 EphB4 overexpressing cells have higher and EphB4 knockout constructs have lower percentage of Ki67 positive cells

Next, in order to visualise and investigate the effect of EphB4 overexpression or knockout on the number of proliferating cells at high confluence, immunofluorescent analysis was conducted using the Ki67 antibody. The CT26 mouse cell line was not labelled with this human specific antibody. Ki67 is regarded as a marker of proliferation (Sobecki et al. 2016). In addition to Ki67 (green), cells were also labelled with DAPI antibody (blue) (**Figure 3.4A**). The DAPI antibody binds to A-T rich regions of DNA, allowing for better visualisation of the cell nuclei (Kapuscinski et al. 1995).

The SW480 B4 cells were found to have higher percentage of Ki67 positive cells (58.2±7.9 % cells), compared to EV cells (36.6±3.9 % cells) (P<0.001) (**Figure 3.4B**). In the LIM2405 cell line, Ki67 labelling was more strongly associated with chromatin in the nuclei of B4 cells (**Figure 3.5A**). Majority of the LIM2405 B4 cells (74.6±5.5 % cells) were still largely positive for the proliferation marker even near confluence, whereas, less than half of the EV cells were labelled with this marker (38.7±11.7 % cells) (P<0.0001) (**Figure 3.5B**). Knocking out of EphB4 in the HT29 cells (KO1: 35.6±4.3 % cells, KO2: 34.1±3.5 % cells) showed a reduction in Ki67 positive cells when compared to WT cells (57.5±17.2 % cells) (P<0.01 for WT versus both knockouts) (**Figure 3.6A, B**). These findings demonstrate that, more than 50% of the EphB4 overexpressing cells are actively dividing, unconstrained by pressure from factors such as limited space and nutrients. On the other hand, knocking out EphB4 expression in the HT29 cells significantly slows proliferation when culture is near confluence.

Figure 3.4. EphB4 overexpressing SW480 cells have greater percentage of Ki67 positive cells. A. SW480 cells stained with Ki67 proliferation marker at approximately 90% confluence (n=3 in duplicates/cellular construct) (Scale bar = 200μm). **B.** Quantification of Ki67 positive cells. ***P<0.001.


A
Ki67
DAPI
Merged
EV
B4

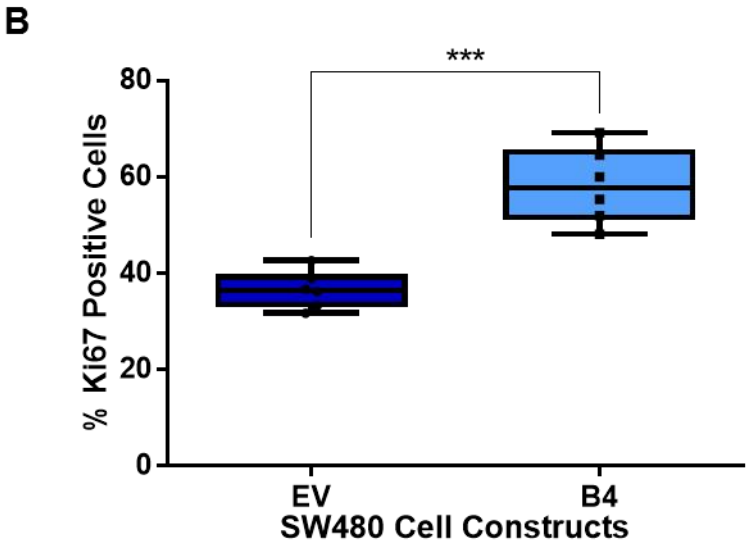

B
***
80
60
40
20
0
% Ki67 Positive Cells
EV
B4
SW480 Cell Constructs

Figure 3.5. EphB4 overexpressing LIM2405 cells have greater percentage of Ki67 positive cells. A. LIM2405 cells stained with Ki67 proliferation marker at approximately 90% confluence (n=3 in duplicates/cellular construct) (Scale bar = 200µm). **B.** Quantification of Ki67 positive cells. ****P<0.0001.

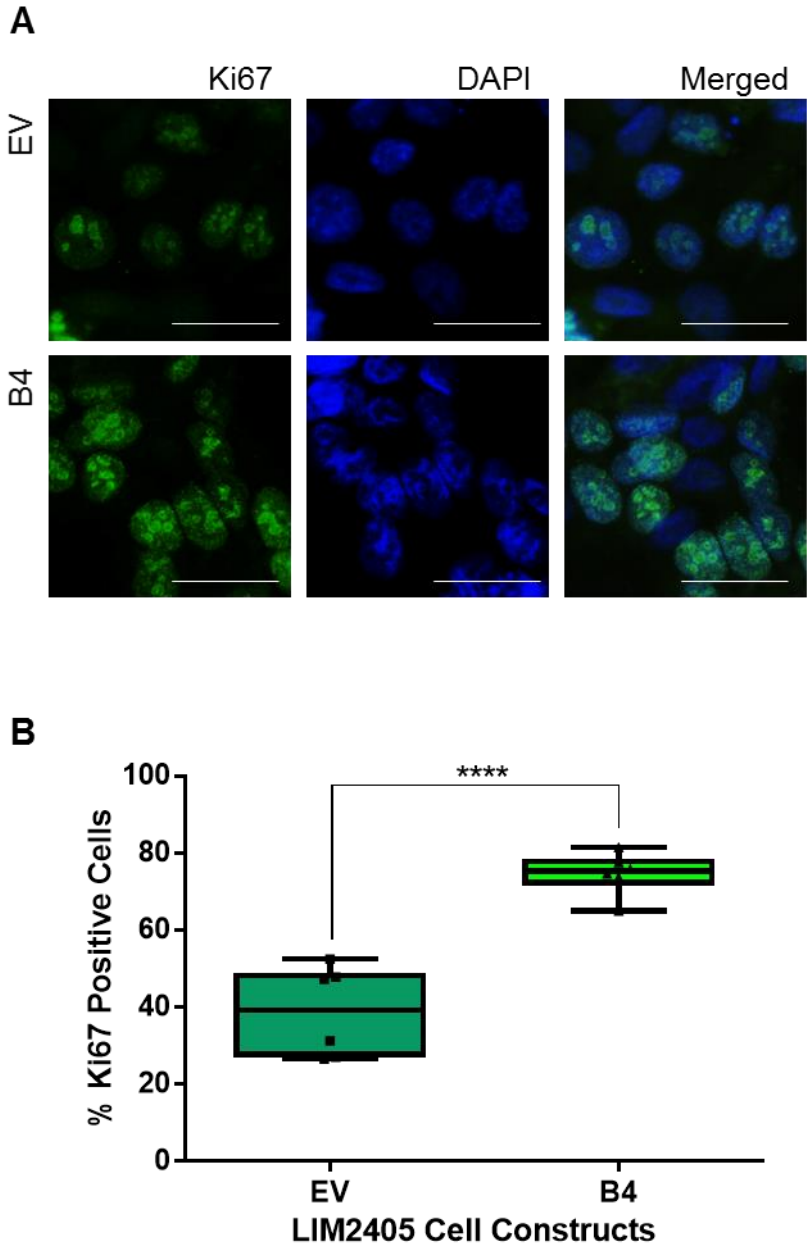
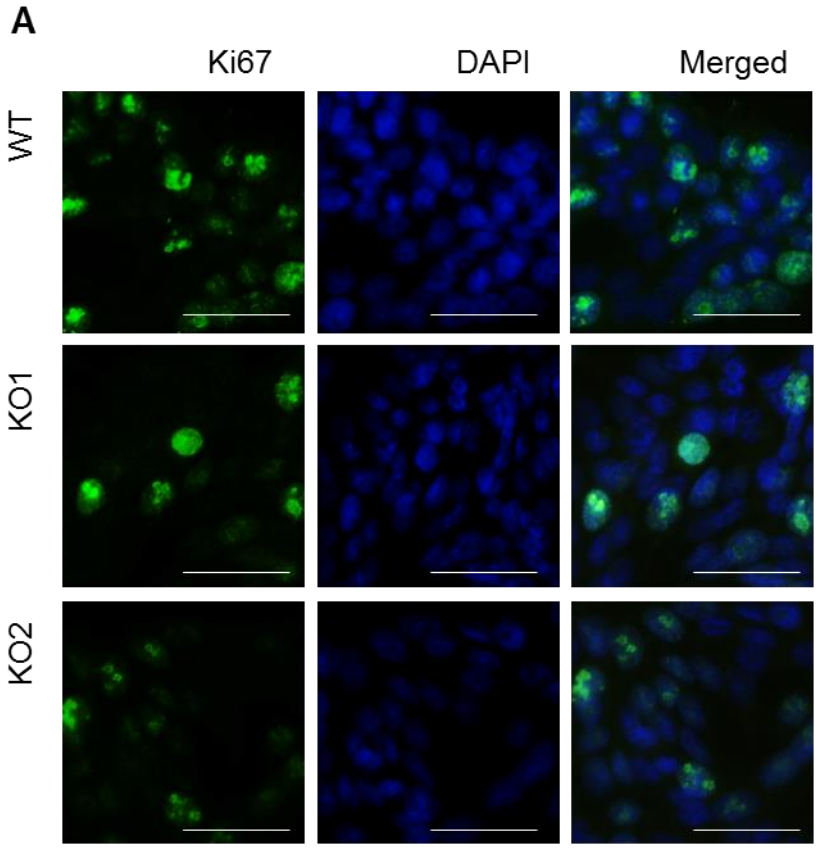
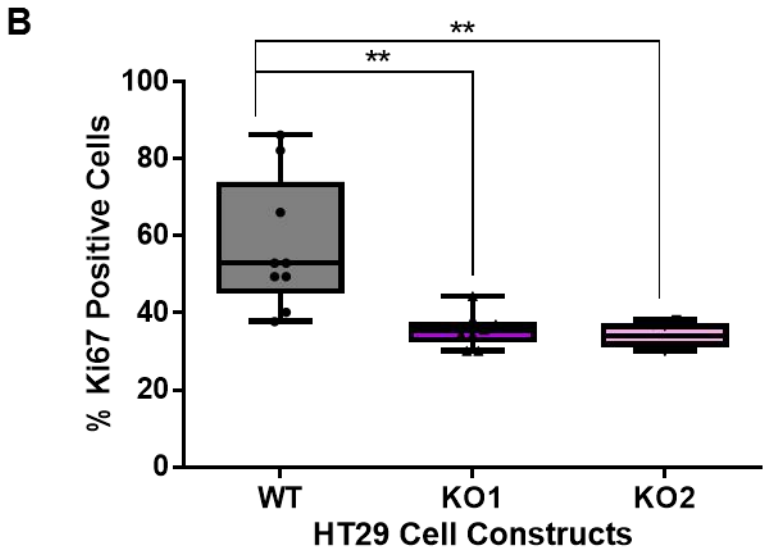

A
Ki67
DAPI
Merged
EV
B4
B
****
100
80
60
40
20
0
% Ki67 Positive Cells
EV
B4
LIM2405 Cell Constructs

Figure 3.6. HT29 EphB4 knockout cells have significantly lower percentage of Ki67 positivity. A. HT29 cells stained with Ki67 proliferation marker at approximately 90% confluence (n=3 in triplicates/cell construct) (Scale bar = 200μm). **B**. Quantification of Ki67 positive cells. ***P*<0.01.


A
Ki67
DAPI
Merged
WT
KO1
KO2


B
**
**
100
80
60
40
20
0
% KI67 Positive Cells
WT
KO1
KO2
HT29 Cell Constructs

3.3.5 EphB4 overexpression increases migration of CRC cells

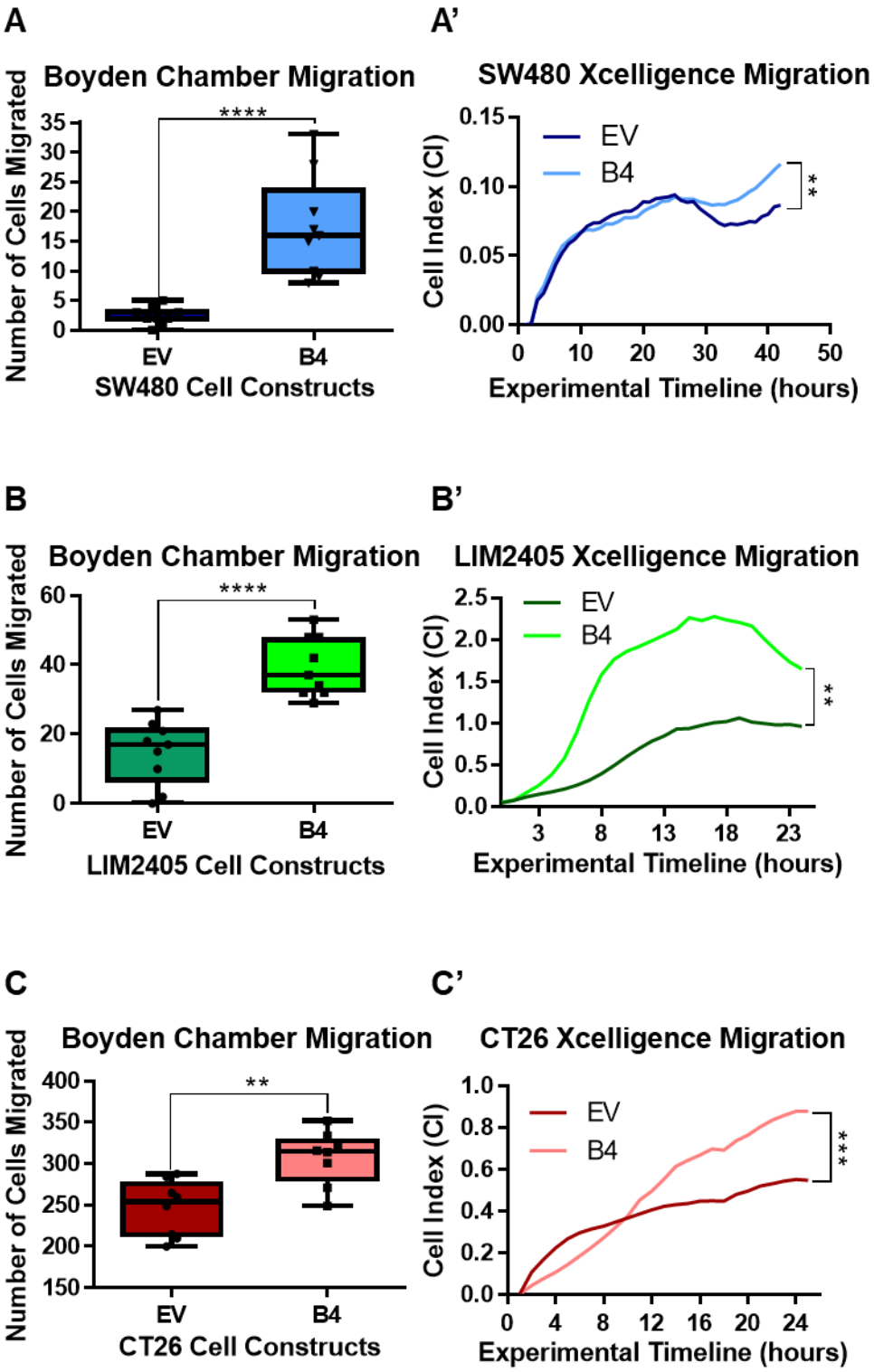
The effects of EphB4 overexpression and knockout on migratory abilities of cells were investigated using both end-point and real-time assays. In Boyden chamber experiments, cells actively migrate through micro-pores in TransWells in response to chemotactic stimuli. These cells are then manually counted and reported as 'number of cells migrated'. Xcelligence plates work on the same principle as Boyden chamber assays, however, as described previously (**Section 3.3.1**), this system allows for real-time monitoring of cell migration rate, which is reported as 'Cell Index'.

The SW480 cell line proved to be the least migratory cell line compared to LIM2405 and CT26 (**Figure 3.7**). The EV controls of this cell line performed very poorly in the Boyden chamber assays (2.4±1.5 cells), compared to B4 cells which had significantly higher number of migrated cells (17.3±8.5 cells) (P<0.0001) (**Figure 3.7A**). Similarly, the SW480 B4 cells also had significantly higher Cell Index measures (0.28±0.01) than EV cells (0.18±0.01) (P<0.01) (**Figure 3.7A'**). Although, this difference only becomes apparent after the 24-hour time point when EV Cell Index begins to decline. In contrast, the LIM2405 cell line is more migratory. The B4 constructs had 39.4 migrated cells (SD±8.6 cells) on average, whereas, EV averaged 14.8 cells (SD±9.2 cells) (P<0.0001) (**Figure 3.7B**). Around 3 hours into the Xcelligence experiment, the rate of migration in the LIM2405 B4 cells begins to occur at a faster rate than EV cells (**Figure 3.7B'**). These cells also migrated significantly more (B4: 2.20±0.22 Cell Index) than EV cells (1.21±0.06 Cell Index) (P<0.01) (**Figure 3.7B'**). The highly migratory cell line CT26 further benefited from EphB4 overexpression, as the number of migrated B4 cells

(315±33.4 cells) was significantly greater compared to EV controls (254.5±34.1 cells) (P<0.01) (**Figure 3.7C**). This was also confirmed in Xcelligence migration assays, as B4 cells (0.84±0.05 Cell Index) had significantly higher Cell Index measures than EV cells (0.53±0.02 Cell Index) (P<0.001) (**Figure 3.7C'**).

Together these data demonstrate that, EphB4 overexpressing cells respond better to chemotactic stimuli and as a result migrate at significantly greater levels when compared to low EphB4 expressing cells. However, level of enhancement appears to be subjective to the inherent migratory abilities of the cell lines. This was demonstrated in Boyden chamber experiments. EphB4 overexpression in SW480 cells with poor mobility causes an approximate 14% increase to the average number of migrated cells. On the other hand, cell lines that are inherently migratory have approximately 38% (LIM2405) and 80% (CT26) enhanced migration with EphB4 overexpression.

Figure 3.7. EphB4 overexpression enhances number of migrated cells. A. Quantification of the number of migrated SW480 cells in Boyden chamber assays at the end of 18 hours (n=3 in triplicates/cell construct). **A'.** SW480 Xcelligence assay depicting the migration measured in real time over 24 hours (n=3/cell construct). **B.** Quantification of the number of migrated LIM2405 cells in Boyden chamber assays at the end of 18 hours (n=3 in triplicates/cell construct). **B'.** LIM2405 Xcelligence assay depicting the migration measured in real time over 24 hours (n=3 in duplicates/cell construct). **C.** Quantification of the number of migrated CT26 cells in Boyden chamber assays at the end of 18 hours (n=3 in triplicates/cell construct). **C'.** CT26 Xcelligence assay depicting the migration measured in real time over 24 hours (n=3/cell construct). $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P< 0.0001$.


A
Boyden Chamber Migration
Number of Cells Migrated
****
EV
B4
SW480 Cell Constructs
A'
SW480 Xcelligence Migration
Cell Index (CI)
EV
B4
**
Experimental Timeline (hours)
B
Boyden Chamber Migration
Number of Cells Migrated
****
EV
B4
LIM2405 Cell Constructs
B'
LIM2405 Xcelligence Migration
Cell Index (CI)
EV
B4
**
Experimental Timeline (hours)
C
Boyden Chamber Migration
Number of Cells Migrated
**
EV
B4
CT26 Cell Constructs
C'
CT26 Xcelligence Migration
Cell Index (CI)
EV
B4
***
Experimental Timeline (hours)

3.3.6 EphB4 overexpression increases invasion of CRC cells

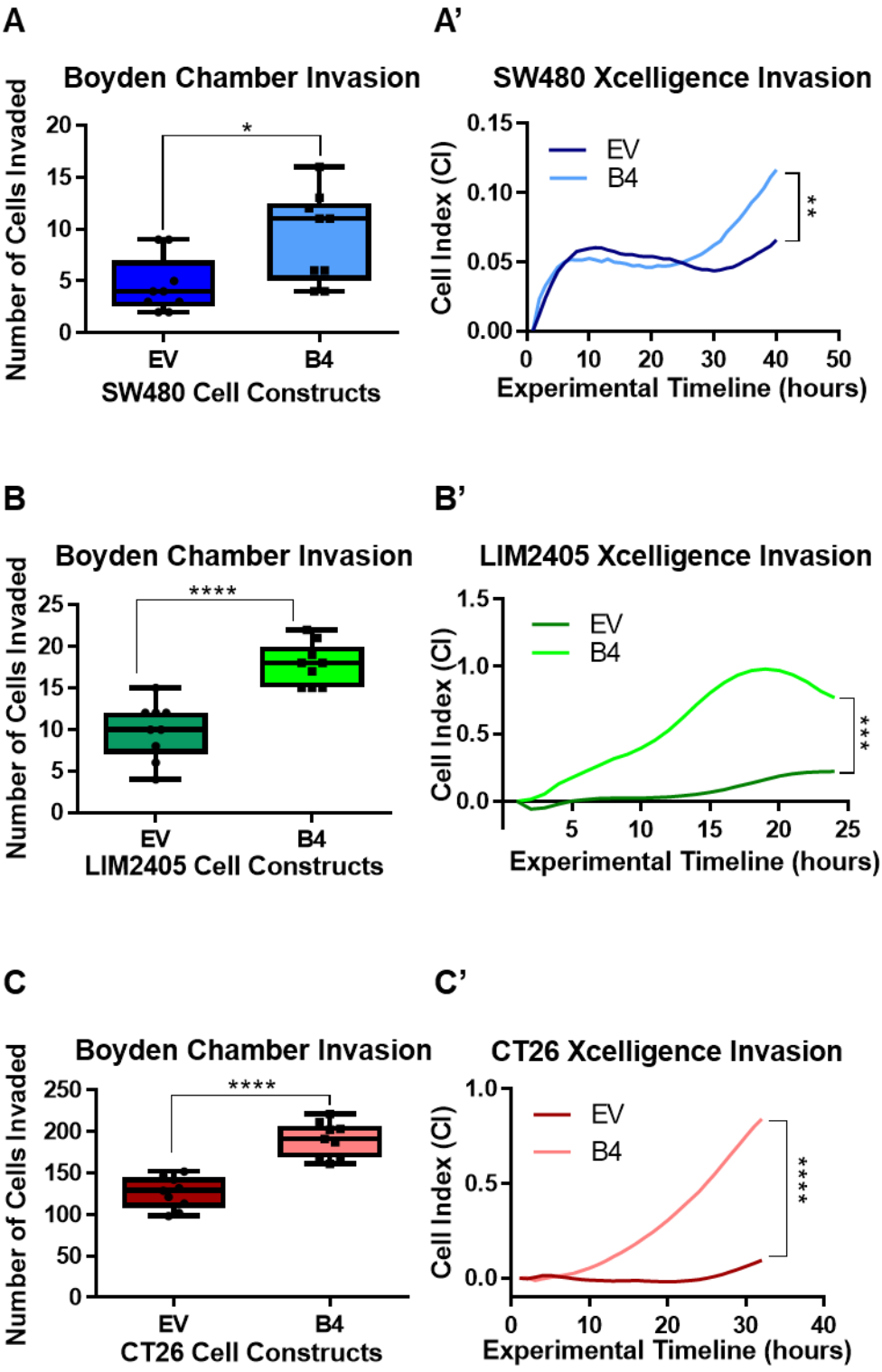
Since EphB4 overexpression enhanced the rate and number of migratory cells, its impact on cellular invasion was also investigated using the same assays. However, in these experiments the wells were coated with a layer of diluted Matrigel. This is a basement-membrane like extracellular matrix extract, which contains laminin, collagen and various other factors (Benton et al. 2011). In these experiments it mimics the *in vivo* extracellular matrix, which the cancer cells would need to degrade and invade through *in vivo*.

The number of SW480 B4 cells (9.2±4.3 cells) that invaded through the Matrigel was significantly higher than EV cells (4.6±2.7cells) (P<0.05) (**Figure 3.8A**). This cell line once again proved to be less responsive in the Xcelligence assays, since Cell Index measures of B4 and EV cells only began to significantly diverge after 24 hours (**Figure 3.8A'**). At 40 hours, B4 cells had significantly higher Cell Index values (0.12 Cell Index) than EV cells (0.07 Cell Index) (P<0.01) (**Figure 3.8A'**). Similarly, the LIM2405 B4 cells (17.8±2.6) cells also had significantly greater number of invasive cells than the EV controls (9.9±3.4) (P<0.0001) (**Figure 3.8B**). Real-time analysis showed that, the EV constructs of this cell line exhibited slower rate of invasion. The EV cells invasion becomes more prominent around 12 hours into the experiment, whereas, the invasion rate of B4 cells begin to increase around 2 hours (**Figure 3.8B'**). Overall, B4 cells have significantly higher values (0.77±0.43 Cell Index) than EV controls (0.22±0.28 Cell Index) (P<0.001) (**Figure 3.8B'**). The CT26 cells which were found to be highly migratory (**Section 3.3.5**), also appear to be the most invasive compared to SW480 and LIM2405 cell lines. The CT26 B4 constructs had on average 190.3±20.9 cells that invaded through,

which was significantly higher than the 126.2±19.3 invaded EV control cells (P<0.0001) (**Figure 3.8C**). The rate of invasion was slow and largely static for the EV constructs of this cell line even 30 hours in to the experiment. In contrast, at around 7 hours the rate of invasion for B4 cells continued on an incline and reached significance (B4: 0.46±0.08 Cell Index) compared to EV cells (-0.01±0.05 Cell Index) (P<0.0001) (**Figure 3.8C'**).

These results suggest that Matrigel provides a real physical barrier for cells, which are detected at lower numbers than in migration experiments. Cells with EphB4 overexpression can overcome this barrier at significantly greater rates and numbers compared to low expression controls.

Figure 3.8. EphB4 overexpression enhances cellular invasion. A. Quantification of the number of SW480 cells invaded through Matrigel in Boyden chamber assays at the end of 18 hours (n=3 in triplicates/cell construct). **A'.** SW480 Xcelligence assay depicting the invasion measured in real time over 40 hours (n=3/cell construct). **B.** Quantification of the number of LIM2405 cells invaded through Matrigel in Boyden chamber assays at the end of 18 hours (n=3 in triplicates/cell construct). **B'.** LIM2405 Xcelligence assay depicting the invasion measured in real time over 24 hours (n=3 in duplicates/cell construct). **C.** Quantification of the number of CT26 cells invaded through Matrigel in Boyden chamber assays at the end of 18 hours (n=3 in triplicates/cell construct). **C'.** CT26 Xcelligence assay depicting the invasion measured in real time over 32 hours (n=3 in triplicates/cell construct). *P<0.05, **P<0.01, ***P<0.001, **** P<0.0001.


A
Boyden Chamber Invasion
Number of Cells Invaded
20
15
10
5
0
*
EV
B4
SW480 Cell Constructs
A'
SW480 Xcelligence Invasion
Cell Index (CI)
0.15
0.10
0.05
0.00
EV
B4
**
0
10
20
30
40
50
Experimental Timeline (hours)
B
Boyden Chamber Invasion
Number of Cells Invaded
25
20
15
10
5
0
****
EV
B4
LIM2405 Cell Constructs
B'
LIM2405 Xcelligence Invasion
Cell Index (CI)
1.5
1.0
0.5
0.0
EV
B4
***
5
10
15
20
25
Experimental Timeline (hours)
C
Boyden Chamber Invasion
Number of Cells Invaded
250
200
150
100
50
0
****
EV
B4
CT26 Cell Constructs
C'
CT26 Xcelligence Invasion
Cell Index (CI)
1.0
0.5
0.0
EV
B4
****
0
10
20
30
40
Experimental Timeline (hours)

3.3.7 EphB4 knockout decreases migration and invasion of HT29 cells

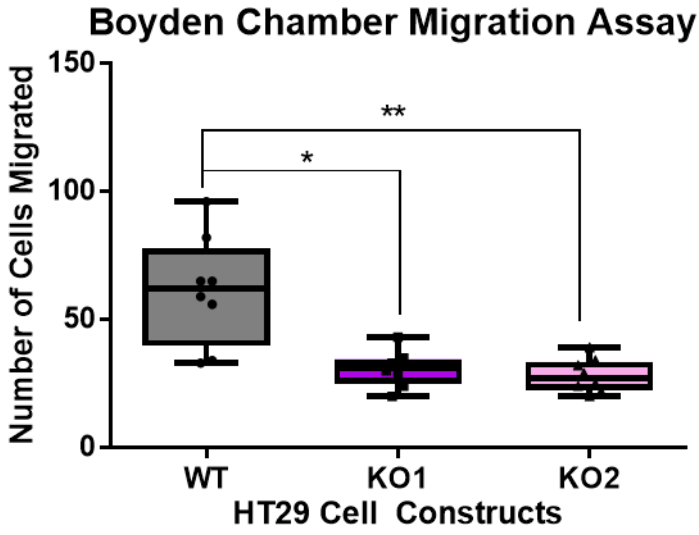
Knocking out EphB4 expression had negatively impacted proliferation rate of HT29 cells (**section 3.3.3**), in order to determine its effects on cell migration and invasion, Boyden chamber experiments were conducted as previously described. Despite multiple attempts, the HT29 cell line failed to adhere to Xcelligence plates, hence this method could not be used.

The number of migratory HT29 WT cells (61.3±21.5 cells) were significantly higher than EphB4 knockout constructs (KO1: 30.6±7 cells, KO2: 28.1±6.5 cells) (P<0.05 for WT versus KO1, P<0.01 for WT versus KO2) (**Figure 3.9A**). The number of cells counted in the invasion assays were greatly reduced for all constructs. Nonetheless, WT cells (8.0±2.8 cells) invaded through Matrigel barrier at significantly greater numbers than knockout constructs (KO1: 3.5±1.5 cells, KO2: 2.0±0.8 cells) (P<0.05 for WT versus KO1, P<0.001 for WT versus KO2) (**Figure 3.9B**).

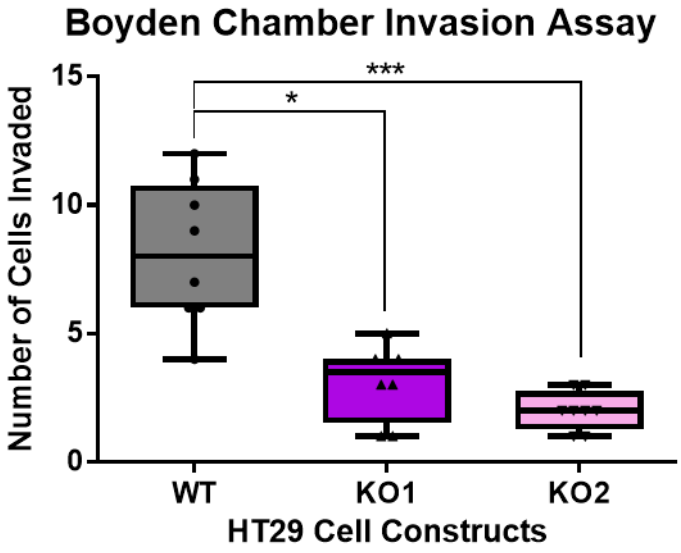
These experiments show that knocking out EphB4 expression significantly reduces migration and invasion of the HT29 cell line. The migratory numbers of EphB4 expressing WT cells are on average 45-50% higher than knockout constructs. However, the WT cell invasive potential appears to be limited. Even still, the WT cells invade around 25-44% higher than knockout constructs.

Figure 3.9. Knocking out EphB4 reduces the number of migrating and invasive cells in the HT29 cell line. A. Quantification of the number of migrated HT29 cells in Boyden chamber assays at the end of 18 hours (n=3 in triplicates/cell construct). **B.** Quantification of the number of HT29 cells invaded through Matrigel in Boyden chamber assays at the end of 18 hours (n=3 in triplicates/cell construct). **P*<0.05, ***P*<0.01, ****P*<0.001.

A


Boyden Chamber Migration Assay
Number of Cells Migrated
150
100
50
0
**
*
WT
KO1
KO2
HT29 Cell Constructs

B


Boyden Chamber Invasion Assay
Number of Cells Invaded
15
10
5
0
***
*
WT
KO1
KO2
HT29 Cell Constructs

3.4 Discussion

3.4.1 High EphB4 expression positively influences cell viability and proliferation, while EphB4 expression knockout reduces this effect

Cell growth and division is a tightly controlled process in healthy tissue, however, genetic and epigenetic changes in cancer cells lead to a loss of regulation allowing for rapid proliferation and survival (Feitelson et al. 2015). There are various ways cell division and proliferation can be measured in *in vitro*, however, due to limitations to each method, usually one or more techniques will be used to confirm results (Menyhár et al. 2016). The upregulation of EphB4 receptor expression has been reported to enhance proliferation and survival of various types of cancer cells. The results in CRC studies are contentious, as there is evidence to suggest EphB4 can supress or enhance cell proliferation and tumour growth. Several of these studies, only investigate the consequence of EphB4 expression modification in *in vivo* experiments, while, others use different methods of assessing proliferation in *in vitro*, which may all contribute to the discrepancies that are observed. For example, in Dopeso et al. (2009), EphB4 receptor influence on tumour growth and burden was investigated in transgenic mice, without *in vitro* analysis. In another study, interfering with EphB4 expression *in vivo* xenografts was shown to reduce tumour volume and *in vitro* increase sensitivity of cancer cells to tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) mediated cell death (Kumar et al. 2009). Nonetheless, its impact on cancer cell proliferation was overlooked. Similarly, in Lv et al. (2016), the SW480 cell line modified to overexpress the EphB4 receptor was found to enhance the size and growth of subcutaneous tumours, although no *in vitro* data is presented on the effect of overexpressing this receptor.

While *in vivo* experiments are better representative of human state of the disease, *in vitro* experiments are useful in determining that the mechanism or the process of gene modification itself does not interfere with or alter cell behaviour. In Davalos et al. (2006), the effect of full length and dominant negative forms of EphB4 overexpression was investigated *in vitro* using clonogenic assays. Transfected cells were subjected to antibiotic treatment then stained and counted at the end of the experimental time period. It was concluded that full length expression of the receptor reduced the number of colonies obtained, whereas truncated EphB4 increased this number. However, this was the only *in vitro* assessment conducted using these cells. Another study determined that EphB4 was an essential factor in controlling survival and proliferation of CRC cells using colourimetric alamarBlue viability, fluorescent cell-titer glo and anchorage independent growth assays (McCall et al. 2016). Using these methods, transient knockdown of EphB4 was found to reduce cell viability and proliferation in HCT116 and Caco2 CRC cell lines, whilst untransformed human colonic epithelial cells were unaffected by knockdown. In our study, we have investigated the effect of EphB4 overexpression and knockout on cell viability and proliferation using standard colourimetric assays, real-time measurements and by immunofluorescent labelling of cells with a proliferation marker.

WST-1, as described previously in **section 3.3.1**, is a tetrazolium dye that is reduced to a coloured formazan dye in metabolically active cells. WST-1 is a modified version of another commonly used tetrazolium salt, the MTT reagent. Initially, WST-1 was thought to be reduced to a formazan within the mitochondria in a similar manner to MTT, however, it has since been proven that WST-1 is cell-impermeable (Berridge et al. 1998). Due to the membrane potential of cells, the net

negative charge of sulfonated WST-1 prevents it from crossing the plasma membrane to enter the cytoplasm (Berridge et al. 1998; Ishiyama et al. 1993). It is rather reduced extracellularly in the presence of an intermediate electron acceptor (1-methoxy-5-methyl-phenazinium methyl sulfate). This molecule facilitates the transmembrane transfer of electrons from NADH and NAD(P)H oxidases to create superoxide radicals, which then reduce WST-1 molecule to a soluble formazan dye (Berridge et al. 2005). WST-1 has several advantages compared to MTT. Firstly, MTT is water insoluble and crystallises when reduced, whereas WST-1 is a soluble formazan. For this reason, the additional step needed to solubilise MTT prior to spectrophotometer absorbance measurement can be eliminated in WST-1 assays. This is advantageous since without the need for this step, the assay is not limited to endpoint assessment and can be used for real-time analysis (Berridge et al. 2005). Secondly, despite MTT having a positive charge, which allows it to penetrate the plasma membrane, it is a lipophilic molecule that can limit its internalisation and impact outcome of results. Finally, the formazan by-product of WST-1 was found to be less cytotoxic and allowed for more rapid and sensitive detection than other tetrazolium salt assays in a variety of settings (Fisichella et al. 2009; Kim et al. 2005; Ngamwongsatit et al. 2008; Yin et al. 2014). WST-1 does not directly measure cell proliferation, rather indicates the level of enzyme activity with metabolic signalling in viable cells (Berridge et al. 2005; Weir et al. 2011). This is because inhibiting or stimulating proliferation of epidermal cells was found to cause less change in absorbance readings in comparison to deoxygenation of cells, which had strong negative correlation with WST-1 absorbance (Weir et al. 2011). However, use of kinase or proliferation inhibitors were reported to have off-target effects that alter the accuracy of tetrazolium salt-based assays (Stepanenko et al.

2015). For this reason, when using chemical treatments, it is important to consider that the compound does not interfere or react with WST-1, which can limit its use in a variety of settings. Nonetheless, proliferating cells increase their metabolic activity and respiration (Mason et al. 2011). For this reason, WST-1 has been deemed a good approximation reagent for measuring cell proliferation, viability and metabolic activity under standard conditions, particularly in combination with other established methods (Berridge et al. 2005; Buttke et al. 1993; Stockert et al. 2018; Yin et al. 2013).

Using this colourimetric method, we obtained higher absorbance values for EphB4 overexpressing constructs, compared to the low expressing controls. This trend was evident across three CRC cell lines (SW480, LIM2405 and CT26). However, the range of absorbance recorded was different for each cell line, suggesting unique rate of WST-1 reduction. At low cell concentrations, the inherently slow growing SW480 cell line had a delayed incline of absorbance values. In comparison, the LIM2405 cells divide more quickly and difference in absorbance between the EV B4 constructs develops more rapidly early in the experiment. Interestingly, at the end of the experiment the absorbance values appear to plateau for these cells. The CT26 cell line has the highest overall absorbance and is the most prolific amongst the other two cell lines. One reason behind these differences could be inherent variances in cellular respiration rate and level of metabolic enzyme activity in each cell line. Another possible explanation is that, although the WST-1 reagent has a working range of 10^3-10^6 cell number, as reported by manufacturer, the outer limits of this range can affect the efficiency of the assay depending on the cell line used. For the SW480 line, it appears that low seeding concentration effects the ability of cells to reduce WST-1. On the other hand, the rate of reduction for the LIM2045

cells is most greatly impacted when the culture is near confluence, as this causes a plateau of absorbance. In comparison, CT26 does not appear to be affected by seeding density or confluence. These differences highlight the importance of using cell lines with diverse characteristics to gain insight into the influence of gene or protein modification. Nonetheless, under standard culture conditions, the reduction of WST-1 is enhanced in EphB4 overexpressing constructs of these CRC cell lines, suggesting an increase metabolically active and viable cells, which is likely to have a positive influence on rate of proliferation.

In lung cancer cells, EphB4 overexpression led to a similar increase in cell proliferation with colorimetric detection assays (Ferguson et al. 2013). In glioma cell lines, EphB4 overexpression resulted in greater number of colonies, also the growth of these cells, assessed through colourimetric detection showed a two-fold increase (Chen et al. 2013). Furthermore, knocking down EphB4 had the opposite effect and reduced proliferation. In our study, knocking out EphB4 expression in the HT29 cell line yielded similar results, negatively impacting WST-1 reduction and possibly proliferation. As previously mentioned, McCall and associates (2016), had suggested that EphB4 expression may be essential to maintain proliferation in some CRC cell lines. In our study, we observed that while EphB4 overexpression can significantly elevate proliferation in CRC cell lines, knocking out its expression in the HT29 cell line does not appear to be essential for cellular survival or proliferation. This is because despite the slowing of supposed proliferation in knockout constructs compared to unmodified WT cells that express EphB4, the knockout cells still exhibit an upward ‘growth’ trend.

3.4.1.1 Treating EphB4 overexpressing cells with EphrinB2-Fc limits their proliferative capacity

Using the same WST-1 assay, we also tested the effect of EphrinB2-Fc treatment on the EphB4 overexpressing cell lines. In **Chapter 2**, we found that EphB4 overexpressing constructs responded more to EphrinB2-Fc treatment than EV controls. For this reason, we conducted these experiments on the SW480 and LIM2405 cell lines. As discussed in **Chapter 2** (**section 2.1**), these two cell lines have morphological and mutational differences. The murine CT26 cell line resembles LIM2405 human cells morphologically, for this reason it was excluded from analysis. As previously mentioned, changing culture conditions can have off target effects if the compound used interferes directly with WST-1 or with its reduction. However, the EphrinB2-Fc should be specific to the ectodomain of Eph receptors only and is not anticipated to affect the efficiency or sensitivity of the assay. In our study we have used 1.5µg/mL of EphrinB2-Fc clustered with 0.75µg/mL anti-human IgG, based on the protocol published by Janes et al. (2011). To ensure the observed effects are a result of treatment and not growth factors or other supplements in the culture medium, cells were serum starved and then supplemented with low serum media during treatment. Low serum condition is expected to reduce metabolic rate in cells, which reflects the lower absorbance values obtained in these experiments, compared to WST-1 assays conducted under standard culture conditions. Nevertheless, in treating cells with EphrinB2-Fc, we observed a reduction in absorbance values. Similar to **Chapter 2** results, this treatment was found to have a more profound impact on EphB4 overexpressing cells, more so than EV controls. The prolonged exposure to EphrinB2-Fc had a long-lasting effect on the SW480 EphB4 overexpressing cells, significantly

reducing absorbance values compared to untreated counterparts. The values for treated B4 cells were as low as the EV constructs. Similarly, for the LIM2405 cells, EphrinB2-Fc promptly reduced absorbance values for EphB4 overexpressing cells. However, by day 7 the absorbance values of overexpressing treated cells become similar to untreated counterparts, suggesting the inhibition imposed by EphrinB2-Fc may be overcome in this cell line. Prolonged EphrinB2 treatment has been found to reduce proliferation of non-tumourigenic epithelial cell line MCF10A, which endogenously overexpresses EphrinB2, and the 22Rv1 prostate cancer cell line, which lacks EphrinB2 expression and was modified to overexpress EphB4 (Rutkowski et al. 2012). These cells were treated with 2µg/mL of clustered EphrinB2-Fc and using the MTT assay, proliferation was measured for 11 days. EphB4 overexpression did not significantly affect the growth of either cell line. However, EphrinB2 treatment significantly reduced the rate of proliferation in 22Rv1 EphB4 overexpressing cells. Like our results, the empty vector controls of the 22Rv1 cell line were unaffected by treatment. The growth of MCF10A cells was also negatively impacted by treatment (Rutkowski et al. 2012). Since these cells overexpress the EphrinB2 ligand, *cis* expression of receptor and ligand (on the same cell) can inhibit *trans* interaction (on opposing cell) and prevent forward signalling through Eph receptors (described in **Chapter 1, section 1.4.2.2**). This study demonstrated that, regardless of EphrinB2 and EphB4 expression profiles, EphrinB2 treatment can still significantly impact proliferative abilities of cells. Increased levels of apoptotic markers were detected after prolonged EphrinB2 treatment in these cell lines (Rutkowski et al. 2012). The length of EphrinB2 exposure can have varying effects. This was confirmed by a study, where a 6-day incubation with varying doses of clustered EphrinB2-Fc treatment significantly

reduced viability of a breast cancer cell line (Barneh et al. 2013). Initial treatment with EphrinB2-Fc causes EphB4 receptor activation and signalling, however, long term exposure (more than 24 hours) is suggested to cause expression downregulation of the receptor (Kumar et al. 2006). Large dose of EphrinB2-Fc (10µg/mL) treatment was shown to have more than 80% reduction in protein expression of EphB4 as early as 8 hours of treatment, occurring at a slower rate in lower concentrations (Kumar et al. 2006). Using the MTT assay, a dose dependent reduction in viability was reported to occur in a breast cancer cell line. Hence EphB4 receptor loss is suggested to impact survival and growth of cancer cells.

There are also reports on EphrinB2-Fc enhancing proliferative capacity of cells. In one study, various erythroid, lymphoid and myeloma leukaemia cell were incubated with varying concentrations of EphrinB2-Fc (0.01-1µg/mL), then WST-1 assays were used to measure proliferation (Takahashi et al. 2014). Only one erythroid leukaemia cell line was found to respond positively to this treatment, with an approximate 20% increase in proliferation. Conversely, even though it was not discussed by the authors, a myeloid leukaemia cell line showed an approximately 20% decrease in proliferation at 1µg/mL concentration of the EphrinB2-Fc, compared to untreated controls. Whilst no statistical significance is reported for this, there are also some minor reductions in proliferation for other cell lines as well, particularly at higher doses of the treatment. As such, the evidence appears to be conflicting and the authors suggest that the type of response to EphrinB2-Fc may depend on the sort of cell line used (Takahashi et al. 2014). Using WST-8 assay, which is a modified version of WST-1, 0.3-1µg/mL concentrations of EphrinB2-Fc was also found to stimulate the proliferation of human umbilical vein cells (HUVEC) in a dose dependent manner (Zheng et al. 2017). Cell cycle analysis

showed more cells in the S phase rather than G0-G1 upon treatment with this Fc. However, Eph receptor expression in these cells were not investigated, as such it is unclear which receptor is causing the effects observed. Nonetheless, these findings are supported by a study using isolated human endothelial cells from the small intestine, where EphB4 was found to be highly expressed in these populations (Steinle et al. 2002). The cells were subjected to a treatment of 50nM of the EphrinB2-Fc over two days. Once more, proliferation was assessed using WST-1. The proliferation rate of these cells increased when treated with the Fc compared to untreated controls. However, in both of these studies, it is unclear whether EphrinB2-Fc was clustered with human IgG, as we have done, prior to treatment. Treating Eph receptor expressing cells with soluble monomeric or dimeric forms of the Ephrin ligands are suggested to have different outcomes compared to Ephrin-Fc that is clustered with human IgG, as this mimics more closely the conformation of cell-bound ligand (detailed in **Chapter 1, section 1.4.2.4**). As an example, a study compared the effect of using dimeric and clustered EphrinB2-Fc on human breast cancer cells and also HUVECs (Xiao et al. 2012). Proliferation was measured using an ATP dependent fluorescent assay, after 3 or 6 days of treatment with EphrinB2-Fc alone or EphrinB2-Fc clustered human IgG. The concentrations of Fc used for these experiments are unclear. Nonetheless, EphinB2-Fc alone did not largely affect the parameters tested, however, clustered ligand caused activation of EphB4 (Xiao et al. 2012). In HUVECs this activation reduced Erk phosphorylation and slowed proliferation, while in the breast cancer cells treatment enhanced Erk activation and proliferation. These findings directly oppose those discussed previously, likely due to the different concentration of EphrinB2-Fc used, length of stimulation, the frequency of monitoring and methods used to assess proliferation.

As such, within the parameters and conditions defined in our study, the results obtained are in line with reports that clustered EphrinB2 can impose restrictions to cell viability or proliferation. Due to budget restraints we have not investigated possibility of apoptosis being induced by EphrinB2-Fc. Although, since LIM2405 exhibit a 50% recovery in absorbance values, hence appearing to regain proliferative potential, we suggest that EphrinB2-Fc treatment likely impacts proliferation or metabolism, rather than loss of viability.

3.4.1.2 Real time assessment demonstrates enhanced cell growth with EphB4 overexpressing cells

We have also used the Xcelligence system to primarily investigate influence of EphB4 expression and knockout on growth pattern of CRC cells. This method allows for real time monitoring of cell behaviour, morphology and viability (**section 3.3.1**). In this system, cellular growth and attachment to the microelectrode biosensor surfaces, impedes the current of electrons and this is reported as CI values (Kho et al. 2015; Sener et al. 2017). The CI measure of the Xcelligence system was found to be comparable to the absorbance values obtained with the MTT and WST-1 assays (Chiu et al. 2017; Martinez-Serra et al. 2014). In our experiments, the SW480 cell line produces most similar results between WST-1 and Xcelligence proliferation assays. The CI and absorbance values were within similar range and trend of growth was also comparable between the two assays. The LIM2405 and CT26 cell lines, produce slightly different results within the Xcelligence system. The upward trend in growth of EphB4 overexpressing constructs in both of these cell lines, resemble the results of the WST-1 assay. However, the EV controls have lower CI than absorbance values, as well as more stagnant growth in the

Xcelligence system than the WST-1 assay. Since cell impedance increases CI measures, factors such as cell number, viability, morphological changes affecting adherence, spread and ruffling can all affect CI values (Atienzar et al. 2011; Kho et al. 2015). Cell lines that tend to aggregate together and not grow in a monolayer when near confluence, like LIM2405 and CT26 cells, can lower CI readings (Witzel et al. 2015). Particularly, the CI of LIM2405 EV cell line falls below 0, around 40 hours of the experiment, suggesting that cells may have rounded and detached during this time and only begin to adhere again after approximately 103 hours in culture. Although the Xcelligence system provides large amount of information about cells, this in turn can make it difficult to determine exact cause of change in CI values. Coating the plates with fibronectin, collagen or Matrigel prior to seeding has been shown to improve adherence and can promote cells to grow in a consistent monolayer (Kho et al. 2015; Martinez-Serra et al. 2014). Regardless, some cells may still fail to effectively adhere to the plates. It is recommended that the cell number and the type of coating (if any) be optimised for each cell line when using this system. However, the high cost of these plates can make it difficult to do so on a low budget. We have experienced this with the HT29 cell line which, despite various attempts, failed to adhere and grow on the plates. Nonetheless, the potential difference in cellular attachment and adherence brought on by EphB4 overexpression is one that is interesting and consistent across all three cell lines. To the best of our knowledge, this was the first study to investigate the effect of EphB4 overexpression on cell proliferation, using the Xcelligence system. These results demonstrate that EphB4 overexpression can improve cell spreading, adherence and growth. These findings are in support of those observed with the WST-1 assays,

and the reports that EphB4 overexpression enhances cell proliferation in cancer cells.

3.4.1.3 Greater percentage of EphB4 overexpressing cells are positive for active mitosis at confluence

To investigate if overexpression of EphB4 and knockout could regulate CRC cells growth under confluent conditions, we used Ki67 immunofluorescence as a proliferative index when cells reached 90% confluence. When normal cells encounter each other, they are restricted by contact inhibition, which slows proliferation and drives cells into quiescence or senescence (Nelson et al. 2002). There are several important mechanisms that mediate contact inhibition, such as sensory molecules in the cell membrane and downstream signalling molecules like (p27) cyclin-dependent kinase inhibitor suppresses cell cycling by G1 phase interference (Nelson et al. 2002; Seluanov et al. 2009). Loss of sensitivity to contact inhibition in cancer cells causes them to proliferate in an unregulated manner. The degree of desensitisation toward this tumour suppressing mechanism can signify an aggressive phenotype in cancer. The Ki67 antibody is often used as a measure of proliferation and a prognostic index for various cancers, mainly because its expression is absent in G0 or resting phase of the cell cycle and is found during active mitotic stages (Li et al. 2015). Ki67 is detected at low levels during G1-S phases and protein levels build up to become most heavily expressed and phosphorylated during G2 and M phases. Ki67 is subjected to dephosphorylation and protease degradation in upon cell cycle exit. Ki67 expression can be detected in the nuclei for a relatively short time frame, lasting approximately 1-1.5 hours during mitosis. It has several epitopes and antibodies raised against Ki67 will

recognise all known types. Studies have mainly characterised Ki67 based largely on its localisation within the nucleus and its association with other proteins during mitosis (Brown et al. 2002). However, its precise functional and physiological role in cell cycling and proliferation remains unsolved. Due to sequence similarities with various other proteins, it is thought to couple and assist these molecules, as well as being able to bind directly to DNA (Bridger et al. 1998; Brown et al. 2002). During cellular division, heterochromatin undergo histone modification and compaction. Ki67 overexpression was found arrest cycling in some cells due to highly condensed chromatin, suggesting its involvement in chromatin organisation (Sobecki et al. 2016). On the other hand, knocking down this protein did not inhibit proliferation or development of mice, rather, long term silencing slowed cell division and altered gene expression. Furthermore, Ki67 was also found to be involved in nuclear segregation and reassembly. Expression depletion caused mispositioning and dissociation between several nuclear protein components during mitosis, also resulted in morphological changes and smaller nuclei in daughter cells (Booth et al. 2014). Previously, it had also been suggested to regulate ribosomal RNA synthesis (Rahmanzadeh et al. 2007). There are several limitations with using Ki67 to quantitate percentage of actively proliferating cells, based on protocols and methods used. There could be issues with false positive and background staining, which can lead to inaccurate quantification of positive cell counts. Particularly for diagnostic purposes is that areas with dense Ki67 expression hence ‘hotspots’ can introduce bias and alter results (Brown et al. 2014). Also, the range of percentage regarded as high or low Ki67 positivity can be different based on different methods of quantification. In our study, to eliminate a degree of error we have counted cells as positive when they had strong expression for Ki67. Strong expression was

defined as nucleus having more than 50% positivity for the marker. In doing so we have observed that near confluence EphB4 overexpressing constructs of SW480 and LIM2405 cell lines had strong staining, while the EV cultures tended to be largely negative for Ki67. In quantifying these results, it was established that EphB4 overexpressing cells had significantly greater percentage of Ki67 positive cells at confluence and the strong staining indicates that these cells were likely at G2-M phase. Knocking out EphB4 in the HT29 cell line negatively impacted mitosis at confluence. The knockout constructs had approximately 20% less positivity for Ki67 than WT cells. These results are in line with our colourimetric assessment of proliferation. Previously, using Ki67 immunofluorescent labelling, knocking down EphB4 in human neuronal stem cells was found to also reduce the rate of proliferation and induce rapid differentiation (Liu et al. 2017). These cells were found to cycle more slowly through G0 phase because of EphB4 knockdown. Overexpressing EphB4 had the opposite effect, resulting in enhanced self-renewal process (Liu et al. 2017).

A frequently used method to test the tumourigenic potential of modified cells is the anchorage independent colony growth assay. Although a significant change in proliferation was observed under standard assay conditions, when suspended in agar all of our CRC cell constructs failed to proliferate and form colonies under these conditions (data not shown). Conditions were changed, and this experiment was repeated several times using the cell lines, however, the largest cell clusters produced by LIM2405 and CT26 cells consisted of no more than six cells each time even after five weeks in culture. Even with small clusters, there was no notable differences between control and overexpressing cells. In order to classify as a ‘colony’ it is widely accepted that a cluster needs to have more than fifty cells. As

such, EphB4 expression or lack thereof was not found to alter the anchorage independent growth of these CRC cell lines.

Overall, our methods of measuring cell proliferation and growth suggest that when unchallenged by any additional factors, the overexpression of EphB4 can lead to increase rate of CRC cell growth. Moreover, treating overexpressing cells with clustered soluble EphrinB2 ligand and knocking out EphB4 receptor can downregulate cell growth. However, it is likely that part of the controversy surrounding EphB4 in cancer stems from the type of assay that was used to assess its effects on particularly proliferation. In colourimetric and immunofluorescent experiments, we observed significant differences between overexpressing and control cells. However, in agar experiments which would be more relevant and better representative of *in vivo* cancer cell growth, having overexpression of EphB4 or knockout of this receptor failed to yield the any marked difference. At confluence *in vitro*, culture conditions like pH increases and nutritional composition in media change can drive cells into quiescence or senescence, reduce proliferation or induce cell death (Muelas et al. 2018). Although, it is important to note that cancer cells often do not go into senescence, which is irreversible, but rather become quiescent and once replated they can begin to proliferate again (Leontieva et al. 2014; Terzi et al. 2016). Consequently, the slowing of metabolic rate and proliferation can benefit cancer cell survival. Chemotherapeutics are still the most commonly used line of treatment for most cancers, the efficiency of treatment is largely dependent on cancer cells having high rate of cell cycling and proliferation. Cells that can lower their metabolic activity and become dormant can contribute to cancer relapse and poor prognosis in patients (Lorz et al. 2017). To investigate if the observed increase in growth with EphB4 overexpression also benefits tumour growth and

survival, we have utilised animal models of cancer, which will be discussed in the upcoming chapters.

3.4.2 High EphB4 expression enhances cellular migration and invasion, knockout cells have low migratory and invasive abilities

Another well-known function of Eph receptors is their ability to regulate cytoplasmic dynamics and mediate cellular mobility (Aharon et al. 2014; Zimmer et al. 2003). The studies discussed above in relation to proliferation, also consistently report that EphB4 overexpression increases rate of migration or invasion and a knockdown or inhibition of this receptor has an opposing effect (Chen et al. 2013; Ferguson et al. 2013; Xuqing et al. 2012). In this study, we have used a Boyden chamber and the real time Xcelligence assays. Chemotaxis based assays like these, determine the ability of cancer cells to sense chemoattractive stimuli and to move through a physical barrier to reach it (Hulkower et al. 2011). Using these methods, we have found that enhanced EphB4 expression results in significant changes to cell migration in response to chemoattractants. On the other hand, knocking out of this receptor greatly reduces this effect.

As with proliferation assays, the SW480, LIM2405 and CT26 cell lines show variation in inherent migratory and invasive abilities. Of these three cell lines, the SW480 cell line yields the lowest proportion of migrating cells in these assays and CT26 cell line proves to be the most migratory and invasive in comparison. In Boyden chamber assays, EphB4 overexpression in all cell lines improves migratory and invasive cell numbers relative to the controls. In real time Xcelligence assays, the SW480 cells perform poorly as they exhibit a long plateau phase for both EV and B4 constructs, migration and invasion commences after approximately 20 hours

of the assay. For this reason, this cell line was assayed for longer. The LIM2405 EV cells perform better in the migration rather than invasion assays, nonetheless, EphB4 overexpressing constructs outperform EV cells, with an onset in migration and invasion as early as 4 hours into the experiment. Similarly, the CT26 overexpressing cells also migrate and invade more rapidly than EV controls. The HT29 cell line was only assayed using Boyden chamber methods, since it failed to adhere to Xcelligence plates. The unmodified WT cells with EphB4 expression have significantly higher number of migrated and invaded cells, than EphB4 knockout constructs. There are several factors may have influenced these outcomes. Since different cell lines migrate or invade at different velocities, it is important to run the experiment for a sufficient length of time. In Boyden chamber assays the low numbers obtained with SW480 cell line meant that the Xcelligence experiment timeline had to be extended. The issue with lengthy experiments can be that cancer cells may divide and double, leading to misinterpretation of results. Additionally, equilibration in chemotactic gradient can occur with time between the cell and chemotactic compartment of Transwell chambers slowing rate of migration or invasion (Hulkower et al. 2011). Another factor contributing to low cell mobility and invasion could be cell morphology, since cells that grow in colonies can be harder to seed in a single cell suspension. To break up colonies longer trypsinisation times may be needed, however, over-trypsinisation can sever adhesive factors that limit substrate adhesion. The SW480 cell line tends to grow in colonies in culture which may be affecting its performance in these types of assays. Cells with elongated and spindle morphology, like LIM2405 and CT26 cells, perform better in *in vitro* migration and invasion assays, while cells with an epithelial structure often perform poorly (Eccles et al. 2005). The HT29 cell line is similar to SW480

in morphology and growth, yet the HT29 WT cells on average yield greater migration and invasion numbers than overexpressing constructs of SW480. This highlights that the increase in migratory and invasive abilities is highly relative to inherent potentials. Nonetheless, it is important to consider that *in vitro* migratory and invasive abilities may not always be a true representative of metastatic potential of cells *in vivo*. There are different types of motility and invasion that can take place *in vivo,* based on environmental factors which may not be replicated successfully in *in vitro* settings (Eccles et al. 2005; Friedl et al. 2003; Kramer et al. 2013). Furthermore, not all cell lines will respond to the same type of chemoattractant. As is commonly done, we have used foetal calf serum as a chemoattractant in these experiments, however, addition of stronger chemoattractants may yield better cell numbers and more reliable comparisons (Justus et al. 2014). Finally, in our invasion assays, Matrigel was used as a barrier through which the cells need to invade. In both the Boyden chamber and Xcelligence invasions assays, cell number and CI measures are drastically reduced for all cell lines. This may be expected due to the additional physical barrier that is introduced, however, Matrigel contains growth factors which may further limit the number of cells that invade.

Nonetheless, our results are in line with various studies that report enhanced migratory and invasive abilities with EphB4 expression. Experiments conducted with pancreatic cancer cell line, showed that shRNA targeted knockdown of EphB4 significantly decreased the proliferation rate and reduced ability of cells to migrate compared to mock controls (Li et al. 2014). In a lung cancer study, cell lines were classified based on their metastatic potential (Yang et al. 2006). When cell surface expression of Eph receptors was examined with flow cytometry, EphB4 expression was found to correlate positively with migratory abilities of cells, being most highly

expressed by those that are grouped as ‘highly metastatic’ and fast migrating. When full length and truncated EphB4 was transfected into ‘non-metastatic’ slow migrating cells, the full-length receptor overexpression improved this ability. EphB4 expressing cells also performed better with scratch wound assays (Yang et al. 2006). In oesophageal cancer samples, EphB4 receptor was found to be highly expressed across the samples examined (Hu et al. 2014). Targeting high EphB4 expressing cell lines with soluble EphrinB2 was reported reduce cell proliferation, migration and colony formation. The kinase mutant EphB4 constructs were shown to have opposing effects, suggesting that in these form of cancers, EphB4 phosphorylation and activation may reduce tumourigenic potential of these cells (Hu et al. 2014). Once again, it appears that while the level of EphB4 expression can influence migratory and invasive abilities, the likelihood of them being inherited by cancer cells may depend on the setting of experimental conditions and cell lines that are being tested. Additionally, in our experiments even though there are significant differences between cells that overexpress EphB4 and controls, the migratory and invasive cell numbers are still very low considering $1x10^5$ cells are seeded into each well. The CT26 have around 350 cells counted for these assays, this number is less than 1% of the cells that were seeded. This percentage is even lower when examining some of the other cell lines. Nevertheless, the possibility of *in vivo* metastatic progression being initiated by a small amount of cancer cells cannot be overlooked. A small subset of tumour cells that are highly migratory and invasive, gaining access to vasculature and ‘seeding’ into distant organs, is a well-defined mechanism of metastasis, James Ewing in 1928 was one of the first theorisers of this process (Ewing 1928; Pienta et al. 2013). Due to the current methods of diagnosis being ineffective and not sensitive enough to detect very small

level of dissemination, up to 30% of CRC patients are reported to relapse with metastatic disease after initial treatment (Hardingham et al. 2015). Often preventative line of chemotherapy given to patients who are at risk of metastasis, also proves to be ineffective in completely eradicating these cells. Various detection methods for identifying circulating tumour cells in patient blood are being developed and tested. Patients who have less than 3 circulating tumour cells detected in 7.5mL of blood have longer survival rates than those with higher number of cells (Matsusaka et al. 2011). This shows even small numbers of migratory and invasive cells can determine the course of a patient's disease. Researchers are investigating alternative surgical methods that would reduce the presence of circulating tumour cells, such as cryosurgery, where tumours are frozen before being removed (Shi et al. 2016). This does not appear to be exclusive to colorectal cases, with similar observations being made using the blood from a breast cancer patient (King et al. 2015).

3.5 Conclusion

In conclusion, EphB4 overexpression increased proliferation across several CRC cells with mutational and morphological differences. Exposing these cells to the soluble EphrinB2 ligand greatly reduced this effect. Knocking out the EphB4 in the HT29 cell line, also had a significant negative impact on proliferation. Furthermore, the migratory and invasive abilities of CRC cells were greatly improved by overexpression of EphB4, while, knocking out the receptor significantly reduced these properties. The biological significance of these findings will be investigated in *in vivo* setting using animal models of cancer in upcoming chapters.

CHAPTER 4

Effect of EphB4 Overexpression and Knockout on Colorectal Cancer Xenografts

4.1 Introduction

A key aspect of cancer progression is the ability of a tumour to undergo vascularisation to support continuous growth. Ultimately, the cancer cells then use these blood vessels as portals into the systemic circulation and other organs in the body. The roles of EphB4 receptor and its cell-bound ligand EphrinB2 regulating of angiogenesis during embryogenesis are well characterised (Herbert et al. 2009; Wang et al. 1998). EphB4 and EphrinB2 are needed for vascular development and differentiation. Blocking EphB4 and EphrinB2 signalling results in dysregulation of adherence between pericytes and endothelia (Dimova et al. 2013). For this reason, the EphB4 receptor along with its ligand EphrinB2 have been implicated in cancer progression through enhanced vessel formation. It has been proposed that, in breast cancer tumours, even kinase dead EphB4 can stimulate formation and remodelling of tumour blood vessels (Noren et al. 2004). In addition, inhibiting EphB4 forward signalling in endothelial cells limits sprouting, migration and reduces angiogenic phenotype when tested *in vitro* and *in vivo* (Kertesz et al. 2006; You et al. 2017). Similarly, in some CRC studies, upregulation of EphB4 is thought to accelerate CRC tumour growth due to more blood vessels supplying the cancerous tissue (Martiny-Baron et al. 2004; Krasnoperov et al. 2010). Inhibition of EphB4 receptor signalling was shown to arrest vessel formation and branching, suggesting it could be used as a part of anti-angiogenic therapy (Djokovic et al. 2010; Krasnoperov et al. 2010; Kumar et al. 2009; Stephenson et al. 2001). Therefore, targeting the EphB4 receptor has been theorised as a possible way to stop the two main events in cancer progression: the vascularisation of tumours and ultimately the metastatic spread of cancers.

However, other studies have shown contradictory findings, where vasculature density and growth of EphB4 overexpressing tumours were reduced compared to EphB4 knockdown murine melanoma tumours (Huang et al. 2007). In *in vitro* experiments, EphB4 overexpressing melanoma cells were shown to induce apoptosis of EphrinB2 expressing endothelial cells. In CRC studies, EphB4 expression was found not to cause significant changes in tumour vasculature (Davalos et al. 2006; Dopeso et al. 2009). These CRC studies demonstrated that the silencing of EphB4 expression and signalling leads to an increase in mitogenic and vascular growth factors. Hence, EphB4 expression is thought to suppress the clonogenic potential of CRC cells by influencing the constituents of the tumour microenvironment (Davalos et al. 2006; Dopeso et al. 2009).

In **Chapter 3**, we found that EphB4 overexpression enhanced CRC cell viability and growth, which was maintained even at high confluence. Overexpression of this receptor improved migratory and invasiveness of CRC cells. Knocking out this receptor negatively impacted and reduced these effects, when compared to EphB4 expressing cells. To assess the biological relevance of these findings, we have used these cells in subcutaneous models of CRC. In this chapter, we aim to investigate the effects of EphB4 overexpression and knockout on subcutaneous tumour growth, vascularisation and composition.

4.2 Methods

4.2.1 Subcutaneous xenografts

All *in vivo* experiments were performed according to the Australian National Health and Medical Research Council Code of conduct on the care and use of laboratory animals for scientific purposes and approved by the Victoria University Animal Experimentation Ethics Committee (Animal Ethic Numbers 04/12 and 14/001).

Female NOD CB17-prkdc-SCID/Jasmu mice (for human cells) and Balb/c (for CT26 cells) (5-6 weeks of age, 15-20g weight) were obtained from Monash Animal Services, Victoria, Australia or Animal Resources Centre, Western Australia. Animals were kept under a 12 hour light and dark cycle with bedding, enrichment, free access to standard chow diet and water. The animals were acclimatised for a minimum of 3 days prior to experiment. The SW480, LIM2405, CT26 and HT29 cells, approximately 80% confluent, were washed with Dulbecco's Phosphate Buffered Saline (PBS), trypsinised and resuspended in Matrigel (Corning, New York, United States) at 4ºC at a concentration of $2x10^7$/mL. Mice were briefly restrained by scruffing and injected in the right flank with $1x10^6$ cells in 50 µL Matrigel. Groups of 5 animals in total were used for each construct of the cell lines. Tumour growth was monitored and measured with callipers every day until the experimental time limit of 40 days or until they reached 1cm in diameter.

4.2.2 Tissue processing

Harvested tissues were divided into three parts to be fresh frozen, paraffin embedded and snap frozen for protein extraction. Samples for protein extraction were stored at -80°C immediately after harvesting. Protein was extracted in

radioimmunoprecipitation assay buffer with PhosStop protease inhibitors (Sigma-Aldrich, Missouri, United States) using a FastPrep bead homogeniser (MP-Biomedicals, United States), cleared by centrifugation at 12,000xg at 4°C. Protein levels were quantified by Pierce BCA assay (ThermoFisher, Scorseby, Australia). Westerns blots were performed as detailed in **Chapter 2**, **section 2.2.7**. Samples for cryosectioning were frozen in moulds containing 100% Optimum Cutting Temperature compound (OCT) (Sakura FineTek, United States) slowly in 2-methyl-butane over liquid nitrogen then stored at -80°C. Tumour samples for paraffin embedding were placed in fresh 4% paraformaldehyde ~18 hours then transferred to 30% sucrose in PBS. Prior to being processed they were placed in 70% ethanol solutions. Using a spin tissue processor (Thermo Scientific), samples were put through formalin (10%, 2x), grades of ethanol (70%, 80%, 96%, 100% x3), xylene (x2) and paraffin (x2) for 1 hour. At the end of this cycle, the samples were immediately embedded in paraffin using an embedding system (TBS88) (Medite, Burgdorf, Germany) and cooled on cold block (Micro EC350) (Thermo Scientific).

4.2.3 Human cancer cell labelling

Fresh frozen sections were cut using cryostat (Zeiss, Oberkochen, Germany) at a thickness of 5µm onto saline coated slides then placed into fresh 4% paraformaldehyde solution for 5-minute fixation and rinsed in 1x PBS. Endogenous peroxidase was blocked with a 3% peroxide incubation for 30 minutes. Mouse on mouse kit (PK-2200) (Vector Laboratories, California, United States) was used according to manufacturer's instructions. Primary anti-nuclei monoclonal antibody (MAB1281) (Merck Millipore, Bayswater, Australia) at a 1:500 dilution was

applied to sections at room temperature for 2 hours. The secondary antibody containing MOM biotinylated anti-mouse IgG reagent (Vector Laboratories) was used along with Vectastain ABC reagent (Vector Laboratories) and 3-3´-diaminobenzidine chromogen (DAB) reagent (SK-4100) (Vector Laboratories) as per manufacturer's instructions. The sections were counterstained with Harris Haematoxylin (Richard-Allan Scientific, ThermoFisher). The slides were then mounted using Clearmount (Life Technologies, California, United States) which was applied directly onto the tissue and incubated for 30 minutes at 50°C. Images were taken on Olympus DP72 microscope.

4.2.4 Endothelial cell labelling

Paraffin embedded tissues were sectioned using microtome (Zeiss, Hyrax M40) at a thickness of 7µm onto saline treated slides and incubated at 60°C for 30 minutes then rehydrated through xylene (2x 10 minutes) and serial alcohol dilution washes (2x 100% for 5 minutes, 70% 5 minutes, 30% 5 minutes) then in PBS. For antigen retrieval, sections were incubated in 20µg/mL Proteinase K solution at 37°C for 20 minutes then allowed to cool at room temperature for 10 minutes before being washed twice with PBS. Endogenous peroxidase was blocked with a 3% peroxide incubation for 30 minutes. A blocking buffer (1% Bovine Serum Albumin, 0.1% cold fish skin gelatin, 0.5% Triton X-100, 0.05% sodium azide) was applied to sections for 1-hour room temperature and washed with PBS. The anti-von Willebrand factor rabbit polyclonal antibody (ab6994) (Abcam, Cambridge, United Kingdom) was then applied at a dilution of 1:100. Sections were incubated at 4°C overnight then washed using PBS. The secondary anti-rabbit antibody (ab97080) (Abcam) was applied at a dilution of 1:500 for 1 hour. Probing was visualised, and

sections were mounted as above. Four images per tumour were taken at 20x magnification on Olympus BH53 microscope. Blood vessels were counted using stereological grid count technique with the ImageJ program. Grid type was horizontal lines, area 6000 pixels^2 at a 2cm alignment and vessels intercepting the grid at any given point were marked with a dot. Results were analysed using Graphpad Prism 6 software and presented as mean ± standard deviations. Unpaired t-tests were used to analyse data.

4.2.5 Ki67 immunohistochemical staining of tumours

Fresh frozen sections were cut using cryostat (Zeiss) at a thickness of 5µm onto saline coated slides then placed into fresh 4% paraformaldehyde solution for 10-minute fixation and rinsed in 1x PBS. Endogenous peroxidase was blocked with a 3% peroxide incubation for 30 minutes. A blocking buffer (1% bovine serum albumin, 0.1% cold fish skin gelatin, 0.5% Triton X-100, and 0.05% sodium azide) was applied to sections for 1 hour at room temperature and washed with PBS. The primary anti-Ki67 antibody (Abcam) was applied at a dilution of 1:500 for 1 hour followed by a secondary anti-rabbit IgG from the Immpress reagent kit (MP-7401) (Vector Laboratories) both for 1 hour at room temperature. DAB chromogen reagent (Vector Laboratories) was then used as per manufacturer's instructions and the sections were counterstained with Harris Haematoxylin (Richard-Allan Scientific, ThermoFisher). The slides were dehydrated through alcohol (100% ethanol 2x 5 minutes) and xylene (2x 5 minutes) washes then cover-slipped using distyrene plasticizer and xylene mounting reagent. Six images/tumour were taken at 40x magnification on Olympus BH53 microscope. Cells were counted using stereological grid count technique using the ImageJ program. Grid lines were at

area per point of 157247 pixels^2 and cells within or touching the right or bottom lines of the grid were counted while cells touching the left and top sides were excluded. Positive cells were distinguished with a brown stain within the whole cell or granules, blue stained cells were counted as negative. Percentage of positive cells was analysed using Graphpad Prism 6 software and presented as mean ± standard deviations.

4.2.6 Haematoxylin & eosin staining

Paraffin embedded tissues were sectioned at a thickness of 7µm and mounted on slides which were then rehydrated as above and stained in Harris Haematoxylin (Richard-Allan Scientific, ThermoFisher) and counterstained in 1 % Eosin (Amber Scientific, Midvale, Australia). The sections were dehydrated and cleared before being mounted using distyrene plasticizer and xylene. Images were taken on Olympus BX53 microscope.

4.3 Results

To investigate the effects of EphB4 receptor expression on proliferation, growth, vascularisation and tumour composition, subcutaneous model of CRC was utilised. In this model, cancer cells were injected into the flank of immunocompromised mice (n=5/ cell construct).

4.3.1 Tumour EphB4 protein expression is maintained at similar levels to *in vitro* cell expression

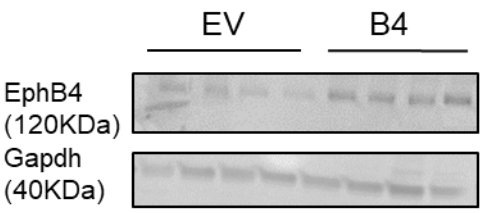
Upon harvesting, a region of the tumours was dissected and snap frozen for protein analysis. Some tumours of particularly the CT26 EV, SW480, HT29 and cell lines were fragile and contained only a small region of viable tissue, for this reason they were prioritised for immunohistochemical analysis and were not sampled for protein analysis. Furthermore, some tumour samples showed high degree of protein degradation and could not be used in western blots (data not shown). The MP-Biomedicals bead homogeniser was used to isolate protein from collected tumours; heat produced during this step and incorrect handling of samples may have contributed to protein degradation. The exclusions reflect the different sample numbers used for each cell line in **Figure 4.1**.

The protein harvested from available subcutaneous xenografts, showed that EphB4 expression levels remain similar to injected cells. SW480 and LIM2405 B4 tumours express higher levels of EphB4 than the EV control tumours (**Figure 4.1A-A', B-B'**). EphB4 protein was not detected in the HT29 KO tumours, compared to WT tumours that express moderate levels of this protein (**Figure 4.1C-C'**).

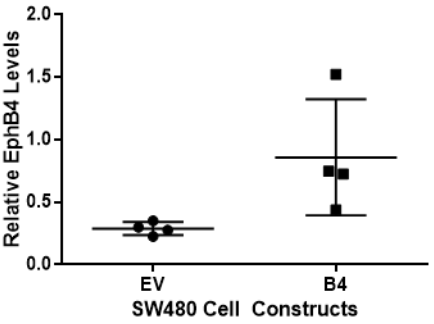
Figure 4.1. EphB4 protein expression in subcutaneous xenografts. A. Protein harvested from SW480 tumours showing the EphB4 protein band and Gapdh loading control. **A'.** Quantified EphB4 expression in SW480 EV and B4 tumours (n=4/cell construct), expression relative to EV tumours and normalised to Gapdh loading control. **B.** Protein harvested from LIM2405 tumours showing the EphB4 protein band and Gapdh loading control. **B'.** Quantified EphB4 expression in SW480 EV and B4 tumours (n=5/cell construct), expression relative to EV cells and normalised to Gapdh loading control. **C.** Protein harvested from HT29 tumours showing the EphB4 protein band and Gapdh loading control. **C'.** Quantified EphB4 expression in HT29 WT and KO tumours (n=3/cell construct), expression relative to EV cells and normalised to Gapdh loading control.

EphB4 Expression in SW480 Tumours

A

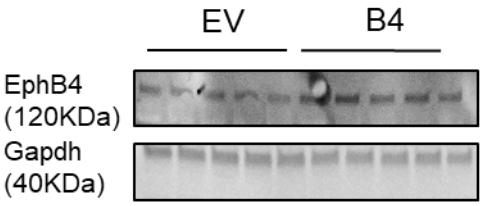


A’

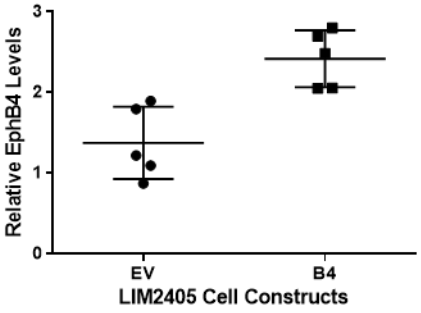


EphB4 Expression in LIM2405 Tumours

B

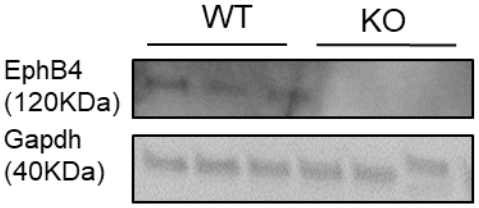


B’

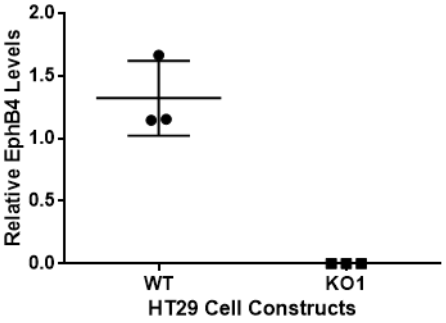


EphB4 Expression in HT29 Tumours

C



C’

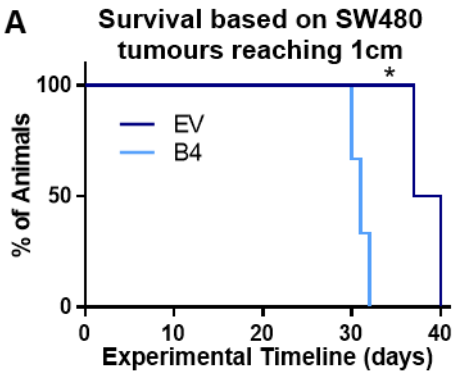


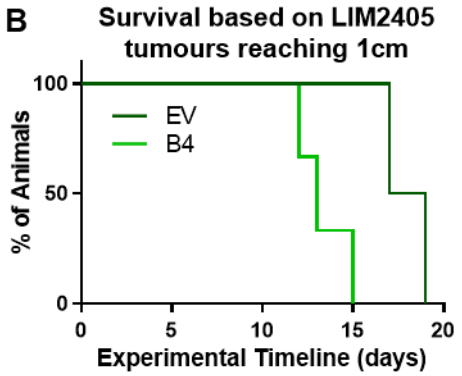
4.3.2 EphB4 overexpressing tumours have enhanced growth and viability

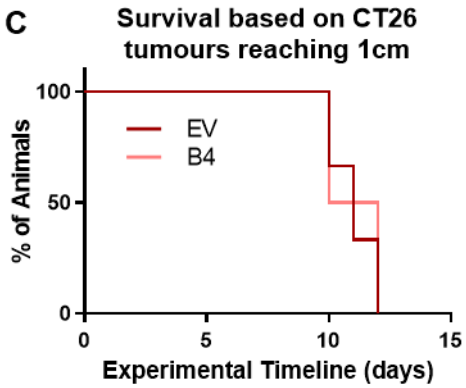
To assess the effect of EphB4 overexpression and knockout on tumour growth, subcutaneous xenografts were measured non-invasively every day. The tumours were harvested when they reached 1 cm in size or at the experimental time limit of 40 days. Survival plots were generated based on percentage of animals reaching these tumour collection times. These results suggest that, similar to the enhanced proliferation rates observed *in vitro* (**Chapter 3**), EphB4 overexpression also appears to positively influence tumour growth.

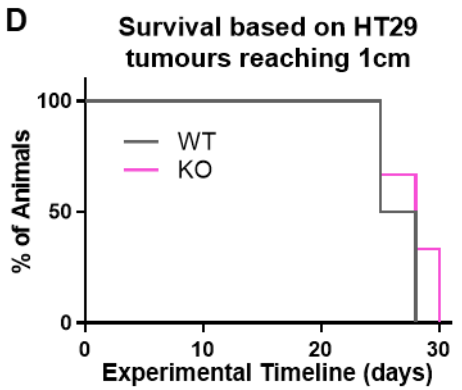
By day 32, all tumours induced by SW480 B4 cells reached the size limit of 1cm, significantly exceeding the growth rate of EV tumours ($P<0.05$) (**Figure 4.2A**). Some SW480 EV tumours were still below 1cm at the end of the experimental time limit of 40 days. Similarly, LIM2405 B4 induced tumours began reaching the size limit as early as day 12 and by day 15, 100% of these tumours were harvested. In contrast, the LIM2405 EV tumour exhibited slower growth rates, as it took 19 days for all tumours to reach the size limit (**Figure 4.2B**). The growth differences were less apparent between tumours induced by the B4 and EV constructs of the CT26 cell line (**Figure 4.2C**). The CT26 EV cell line produced highly ulcerative subcutaneous tumours. Ethically, the experiments had to be terminated and animals culled early, before the tumours reached the size limit. On the other hand, CT26 B4 tumours did not become ulcerated and grew to reach the size limit. The HT29 KO tumours also tended to grow slower than EphB4 expressing WT tumours (**Figure 4.2D**).

Figure 4.2. High EphB4 expressing tumours grow quicker than controls. A. Kaplan-Meier survival plot of animals that were culled when the SW480 EV and B4 tumours reached 1cm in diameter (n=5/group). *P<0.05 **B.** Kaplan-Meier survival plot of animals that were culled when the LIM2405 EV and B4 tumours reached 1cm in diameter (n=5/group). **C.** Kaplan-Meier survival plot of animals that were culled when the CT26 EV and B4 tumours reached 1cm in diameter (n=5/group). **D.** Kaplan-Meier survival plot of animals that were culled when the HT29 WT and KO tumours reached 1cm in diameter (n=5/group).


A
Survival based on SW480 tumours reaching 1cm
*
100
50
0
% of Animals
EV
B4
0
10
20
30
40
Experimental Timeline (days)


B
Survival based on LIM2405 tumours reaching 1cm
100
50
0
% of Animals
EV
B4
0
5
10
15
20
Experimental Timeline (days)


C
Survival based on CT26 tumours reaching 1cm
100
50
0
% of Animals
EV
B4
0
5
10
15
Experimental Timeline (days)


D
Survival based on HT29 tumours reaching 1cm
100
50
0
% of Animals
WT
KO
0
10
20
30
Experimental Timeline (days)

Macroscopic investigation of the tumours revealed that the those induced by low EphB4 expressing EV cells were found to have great extent of necrosis with hollow centres and fluid accumulation (**Figure 4.3A, B, C**). In comparison, tumours induced by high EphB4 expressing B4 cells were more solid with little to no necrosis (**Figure 4.3A', B', C'**). The histology sections of the SW480, LIM2405 and CT26 EV tumours reveal a large degree of structural tissue damage resulting from necrosis and consequent fibrosis, particularly in the central region (**Figure 4.4A, B, C**). In these EV tumours, viable tissue is only present in the outer edges of the tumour, adjacent to the capsule, although, fibrosis extends throughout these regions as well. On the other hand, the high EphB4 expressing (B4) tumours appear to have greater level of tumour viability throughout the tissue, including near the tumours centres (**Figure 4.4A', B', C'**).

In order to investigate tumour composition, the anti-human nuclei antibody was used to distinguish human CRC cells (SW480, LIM2405 and HT29) from the mouse-derived stromal cells. The results show that in EV tumours of the SW480 and LIM2405 cell lines, host-derived tissue is dispersed amongst the human cancer cells (**Figure 4.5A, B**). In the SW480 EV tumours, even though there is intermixing between the host and cancer cells, the human cancer cells cluster together forming small islets and structured colonies (**Figure 4.5A**). The LIM2405 EV human cancer cells, on the other hand, appear scattered amongst mouse derived tissue (**Figure 4.5B**). In comparison, B4 tumours of both SW480 and LIM2405 cell lines yield relatively homogenous masses, densely packed with human cancer cells and they contain minimal mouse-derived tissue (**Figure 4.5A', B'**).

Figure 4.3. Low EphB4 expressing EV tumours have largely necrotic centres, whereas high EphB4 expressing B4 tumours have better tumour viability. A-A'. SW480 subcutaneous tumours dissected in half revealing tumour centres. **B-B'.** LIM2405 subcutaneous tumours dissected in half revealing tumour centres. **C-C'.** CT26 subcutaneous tumours dissected in half revealing tumour centres (Scale bar = 2mm for A-B', 5mm for C-C').

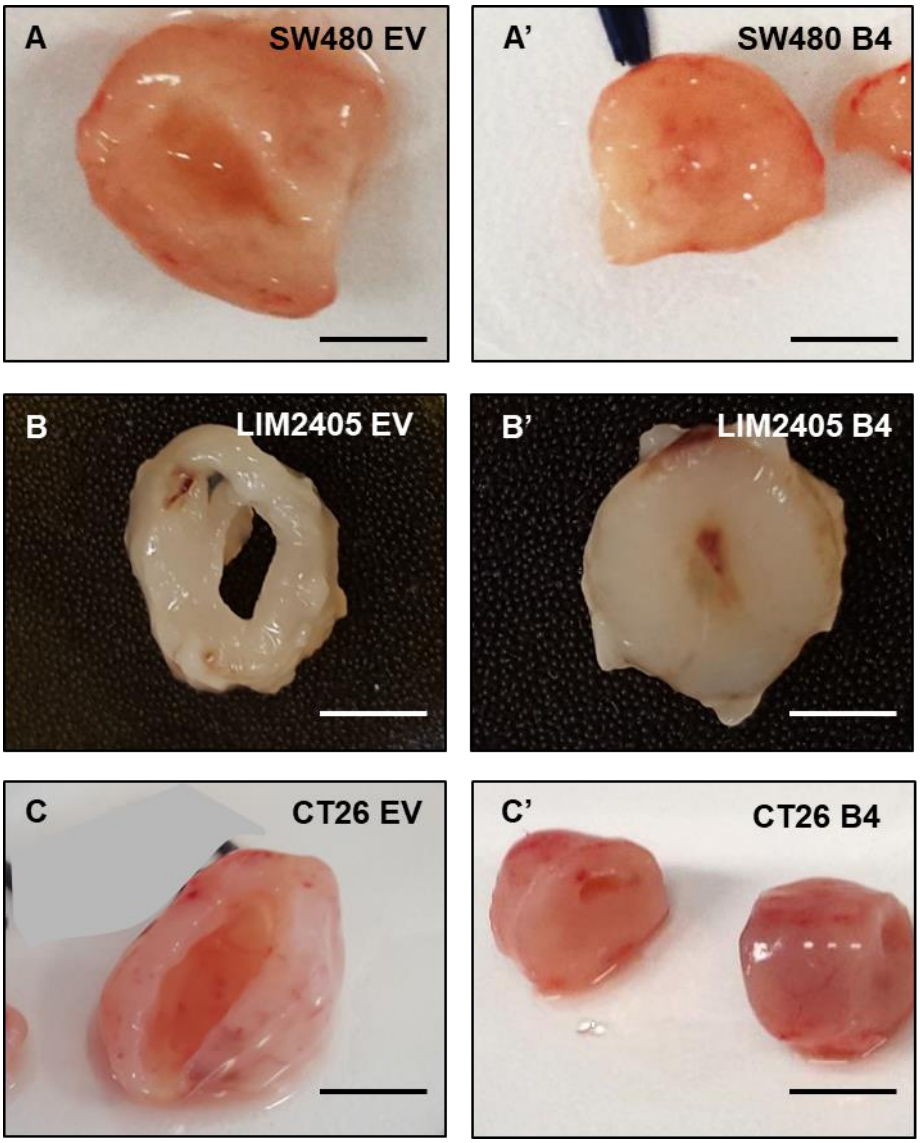

A
SW480 EV
A'
SW480 B4
B
LIM2405 EV
B'
LIM2405 B4
C
CT26 EV
C'
CT26 B4

Figure 4.4. Low EphB4 expressing EV tumours have larger degree of tissue damage and fibrosis. A-A'. SW480 subcutaneous tumours stained with Haematoxylin and Eosin. **B-B'.** LIM2405 subcutaneous tumours stained with Haematoxylin and Eosin. **C-C'.** CT26 subcutaneous tumours stained with Haematoxylin and Eosin. (Scale bar = 100µm for A-A, 200µm for B-C').

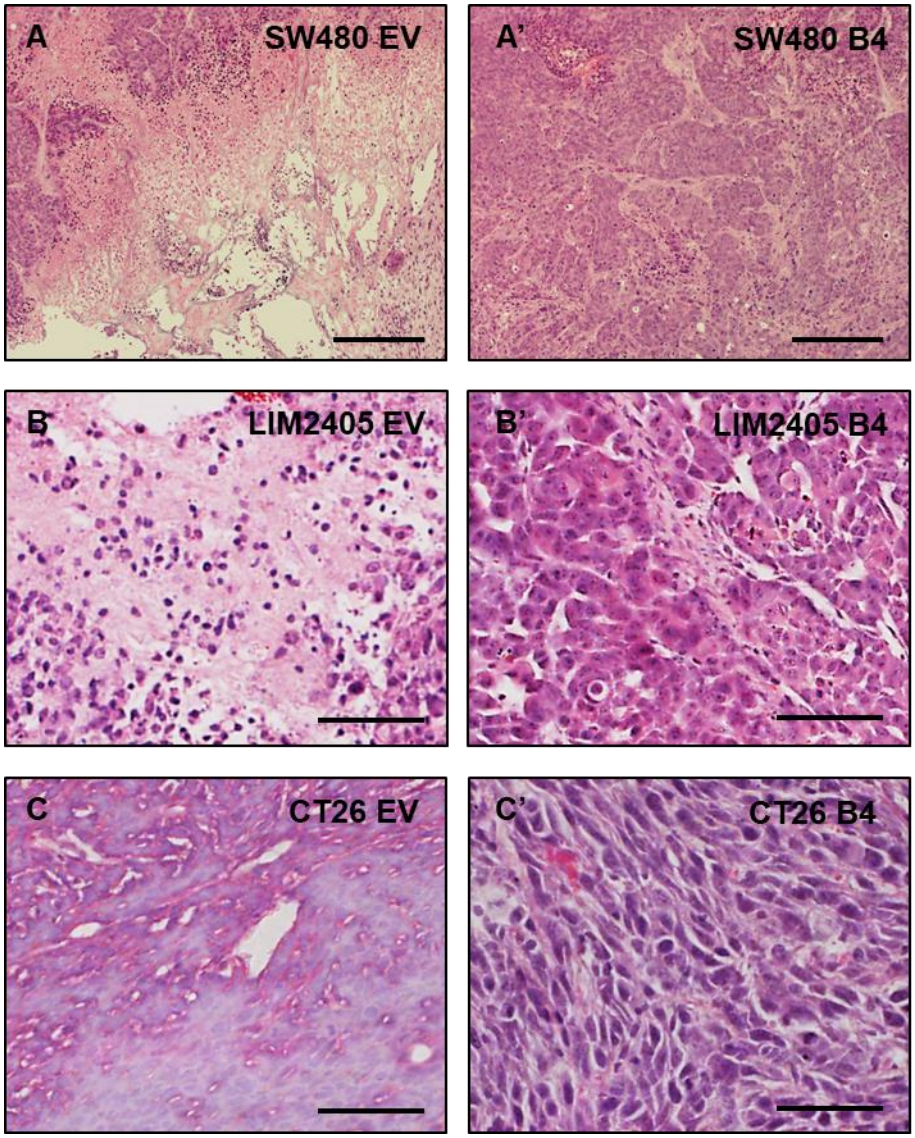
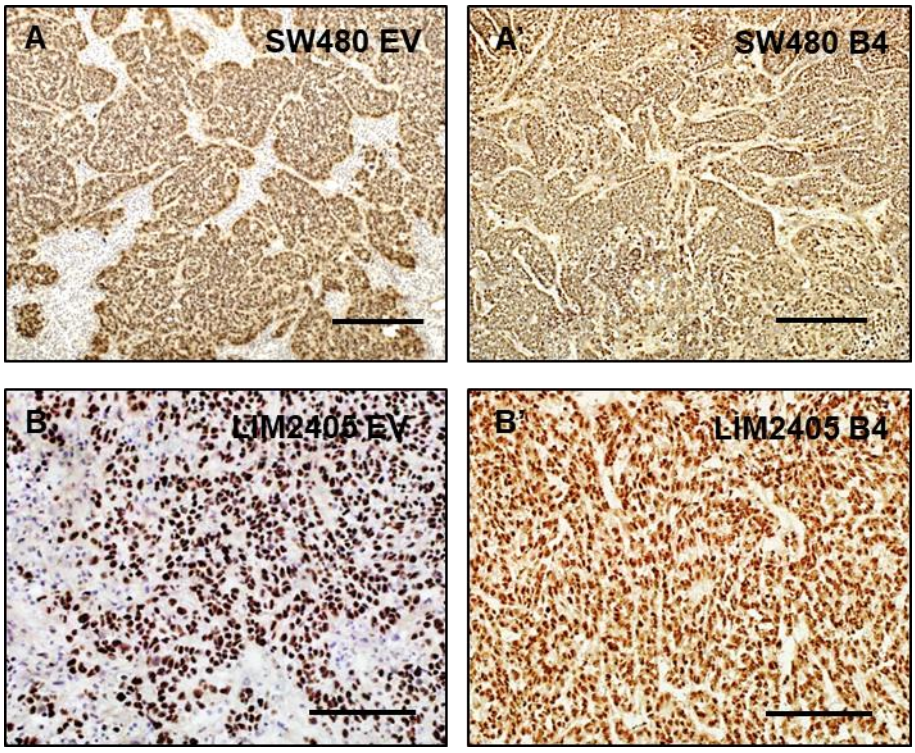

A
SW480 EV
A'
SW480 B4
B
LIM2405 EV
B'
LIM2405 B4
C
CT26 EV
C'
CT26 B4

Figure 4.5. High EphB4 expression yields tumours densely packed with human cancer cells and have less host-derived tissue. A-A'. Immunohistochemical labelling of SW480 subcutaneous tumours with anti-human antibody (brown), host tissue counter stained with haematoxylin (blue). **B-B'.** Immunohistochemical labelling of LIM2405 subcutaneous tumours with anti-human antibody (brown), host tissue counter stained with haematoxylin (blue). (Scale bar = 100μm for all).


A
SW480 EV
A'
SW480 B4
B
LIM2405 EV
B'
LIM2405 B4

4.3.3 EphB4 knockout tumours have reduced viability and significantly lower percentage of proliferating cells

Necrosis was observed in tumours induced by HT29 EphB4 expressing WT and EphB4 knockout (KO) cells. The HT29 WT induced tumours had a comparable amount of necrosis present as with the EV tumours of other cell lines (**Figure 4.6A**). This may be because, the HT29 WT tumours have EphB4 protein expression most like the EV tumours. However, HT29 KO tumours have greater degree of necrosis with white/yellow exudation in the centre of the tumour. These tumours appear fragile with small amount tumour tissue remaining (**Figure 4.6A'**). Histological images reveal high degree of structural damage to both WT and KO tumours; however, the loss of tumour viability is exacerbated within the EphB4 KO induced tumours (**Figure 4.6B-B'**). Investigation of tumour composition using anti-human nuclei antibody revealed that the WT and KO tumours have similar distributions of cancer cells and host-derived tissues (**Figure 4.6C-C'**). In the tumours, large extent of mouse stroma is present amongst the human cancer cell colonies, this level of integration appears to be only slightly higher within the KO tumours (**Figure 4.6C-C'**). Anti-Ki67 antibody was used to compare the proportion of proliferating cells within the tumours. Data were collected from the same anatomical locations within tumours, near the outer perimeter tumour capsule (**Figure 4.7A-A'**). However, EphB4 expressing HT29 WT tumours had significantly higher percentage of Ki67 positive cells in comparison to knockout tumours (WT=61±3.5% of Ki67 of positive cells, KO=45.1±8.6% Ki67 positive cells) ($P<0.01$) (**Figure 4.7B**).

Figure 4.6. Knocking out EphB4 expression reduces tumour viability and integrity. A-A'. HT29 WT and KO subcutaneous tumours dissected in half, revealing tumour centres. **B-B'.** HT29 subcutaneous tumours stained with Haematoxylin and Eosin. **C-C'.** Immunohistochemical labelling of HT29 WT and KO tumours using anti-human antibody. (Scale bar = 2mm for A-A', 100μm for B-C').

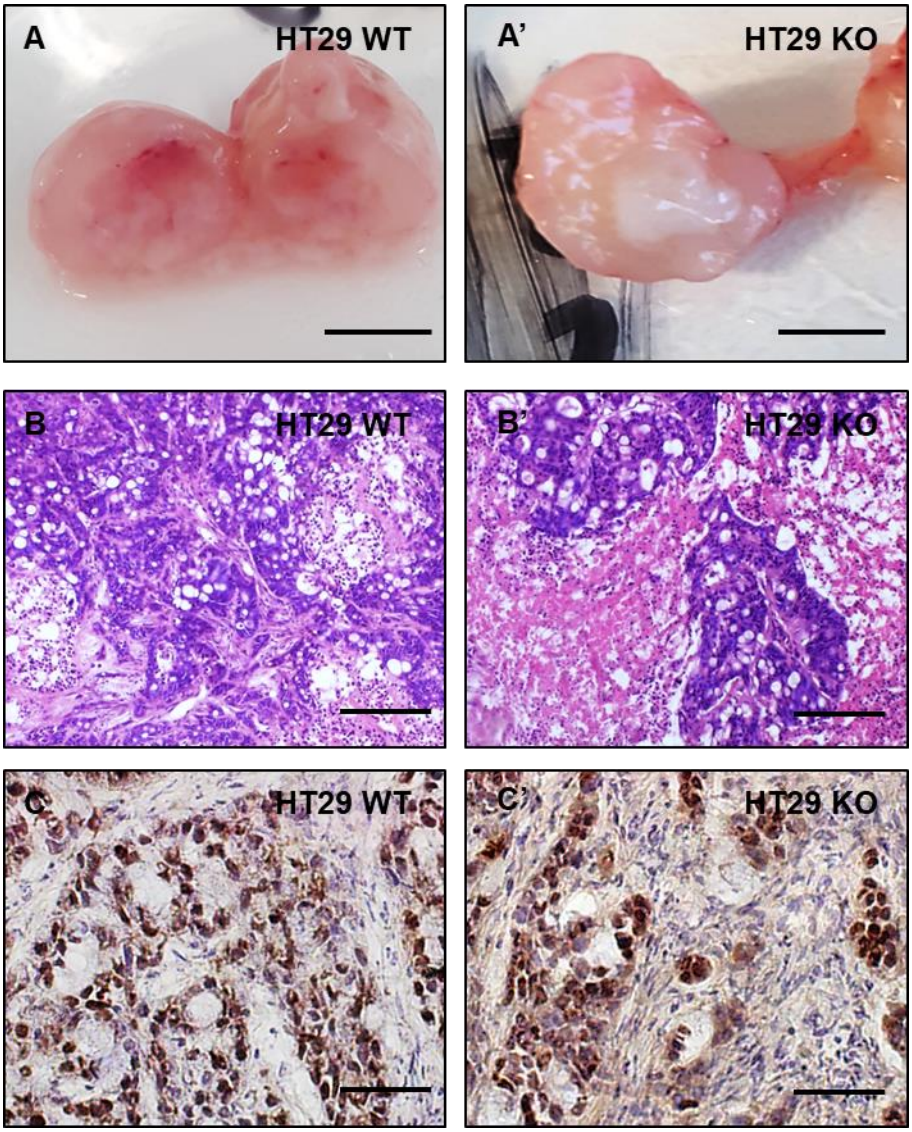
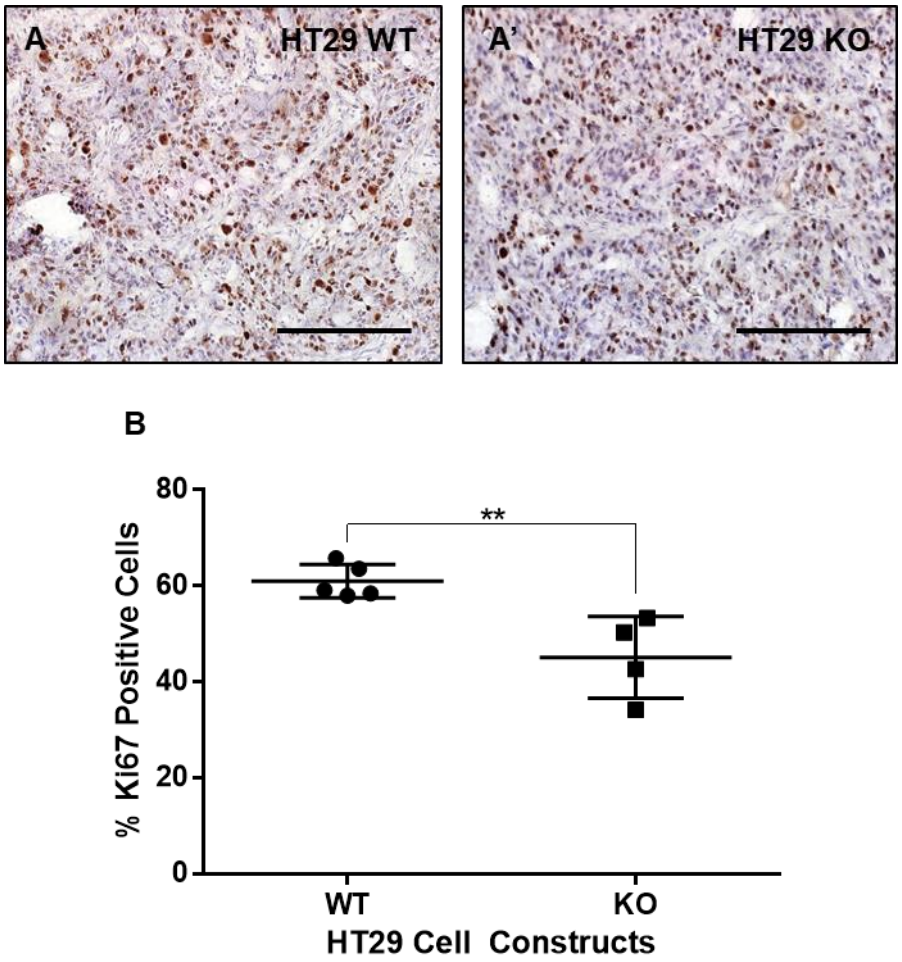

A
HT29 WT
A'
HT29 KO
B
HT29 WT
B'
HT29 KO
C
HT29 WT
C'
HT29 KO

Figure 4.7. Knocking out EphB4 expression significantly reduces proliferative marker positive cells in tumours. A-A'. Immunohistochemical labelling of HT29 WT and KO tumours with proliferation marker Ki67 (n=5 WT tumours, n=4 KO tumours) (Scale bar = 100μm). **B.** Quantification of % Ki67 positive cells. $^{**}P < 0.01$.


A
HT29 WT
A'
HT29 KO
B
80
60
40
20
0
% Ki67 Positive Cells
**
WT
KO
HT29 Cell Constructs

4.3.4 EphB4 overexpressing tumours have enhanced vascularisation and knocking out EphB4 reduces this effect

At the time of tumour collection, all the B4 tumours, induced by high EphB4 expressing SW480, LIM2405 and CT26 cells were notably supplied by thicker and more numerous blood vessels than the tumours of low EphB4 expressing EV cells (**Figure 4.8A-C'**).

We assessed the degree and quality of vascularisation in the tumours by immunohistochemistry. The EV tumour vessels appeared to be thin, fragmented and lacked orientation (**Figure 4.9A, B, C**). Whereas, vasculature was thicker and well-networked in B4 tumours (**Figure 4.9A', B', C'**). When quantified, B4 tumours had significantly higher density of blood vessels (SW480: 97.7±22.7 vessels, LIM2405: 206.7±16.7 vessels, CT26: 181±14.5 vessels) (P<0.05 for all) (**Figure 4.10A, B, C**). In comparison, the EV tumours had decreased density of vasculature (SW480: 47.6±8.8 vessels, LIM2405: 140.5±33.78 vessels, CT26: 155.8±20.8 vessels).

The HT29 WT tumours had thicker and better networking vessels, while the HT29 KO tumour vessels lacked orientation and assembly (**Figure 4.11A-A'**).The HT29 WT tumours, with EphB4 expression, also showed an enhanced blood vessel density when compared to KO tumours, which lack EphB4 expression (WT: 216.8±31 vessels, KO: 127.6±34.5 vessels) (P<0.05) (**Figure 4.11B-C**).

Figure 4.8. High EphB4 expressing B4 tumours are supplied by thicker and more numerous blood vessels than EV tumours. A-A'. SW480 EV and B4 tumour vasculature at the time of collection. **B-B'.** LIM2405 EV and B4 tumour vasculature at the time of collection. **C-C'.** CT26 tumour vasculature at the time of collection (Scale bar = 5mm for all).

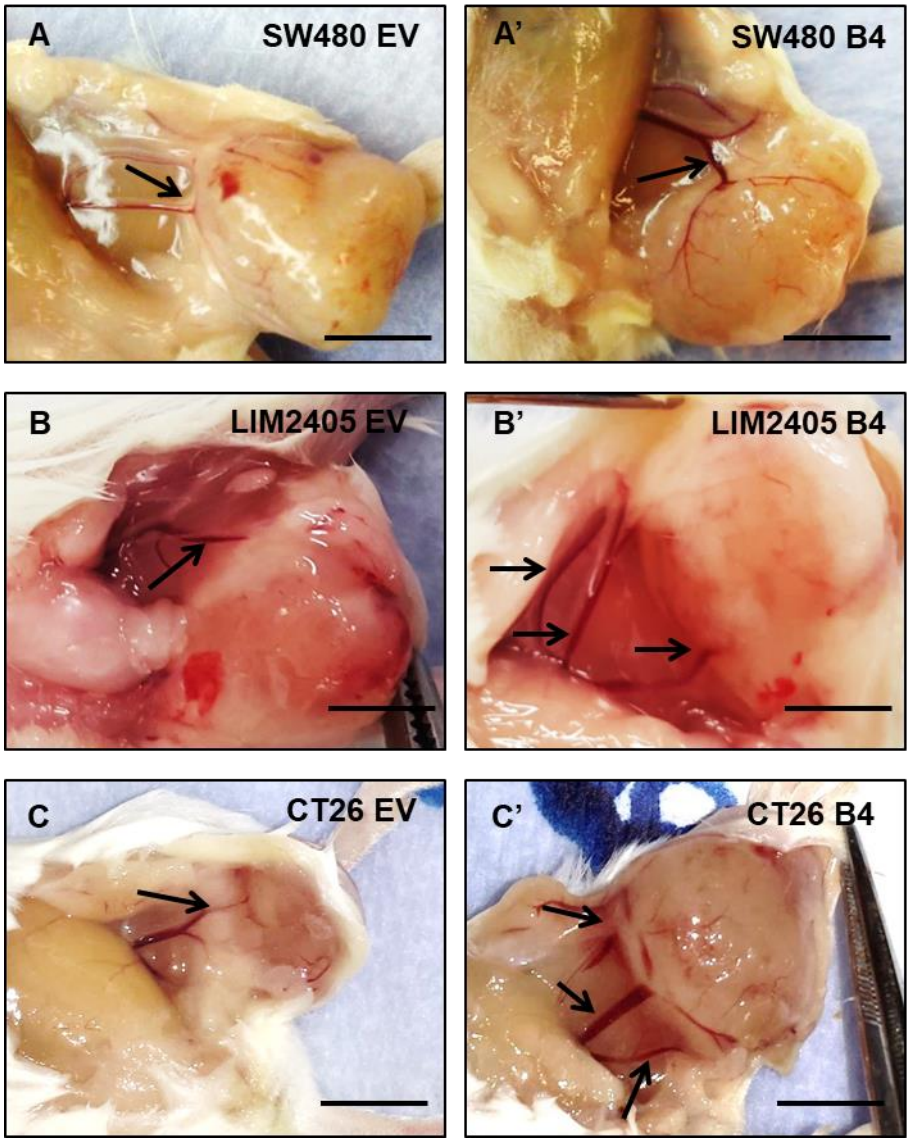

A
SW480 EV
A'
SW480 B4
B
LIM2405 EV
B'
LIM2405 B4
C
CT26 EV
C'
CT26 B4

Figure 4.9. High EphB4 expressing B4 tumours have better networking and branching vessels than EV tumours. A-A'. SW480 immunohistochemical labelling of vasculature with anti-Von Willebrand Factor in subcutaneous tumours. **B-B'.** LIM2405 immunohistochemical labelling of vasculature with anti-Von Willebrand Factor in subcutaneous tumours. **C-C'.** CT26 immunohistochemical labelling of vasculature with anti-Von Willebrand Factor in subcutaneous tumours (Scale bar = 100μm for all).

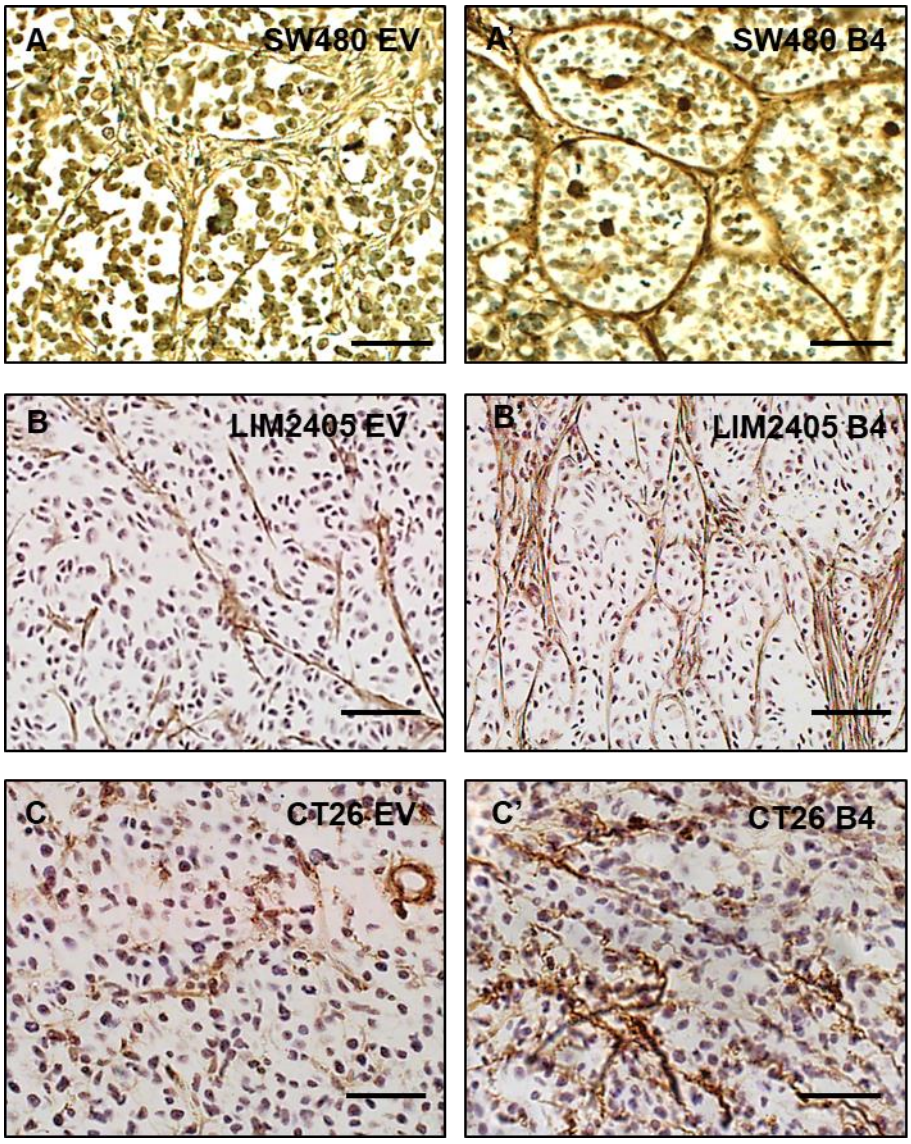

A
SW480 EV
A'
SW480 B4
B
LIM2405 EV
B'
LIM2405 B4
C
CT26 EV
C'
CT26 B4

Figure 4.10. High EphB4 expressing tumours have significantly greater number of blood vessels. A. Quantification of vessel numbers in SW480 tumours using stereological point count method (n=3 EV tumours, n=5 B4 tumours). **B.** Quantification of vessel numbers in LIM2405 tumours using stereological point count method (n=5 EV tumours, n=5 B4 tumours). **C.** Quantification of vessel numbers in CT26 tumours using stereological point count method (n=3 EV tumours, n=3 B4 tumours). *$P< 0.05$ for all.

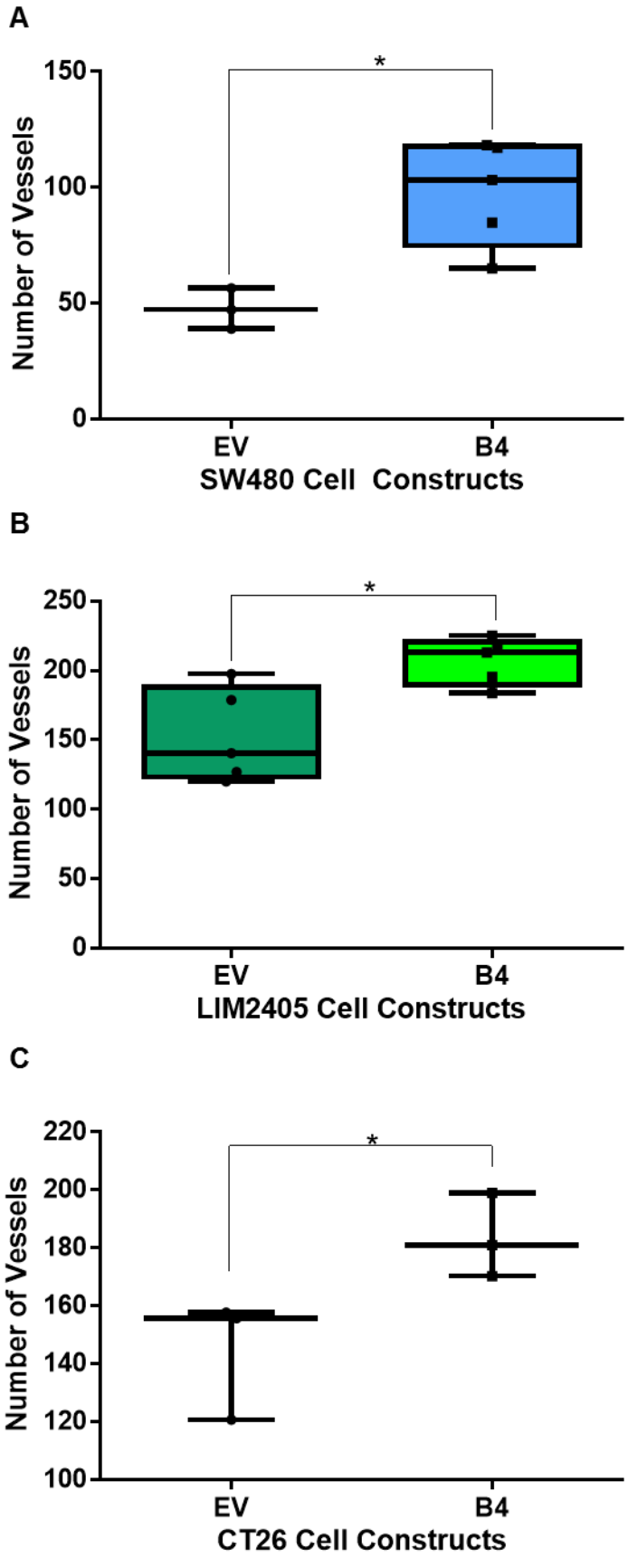
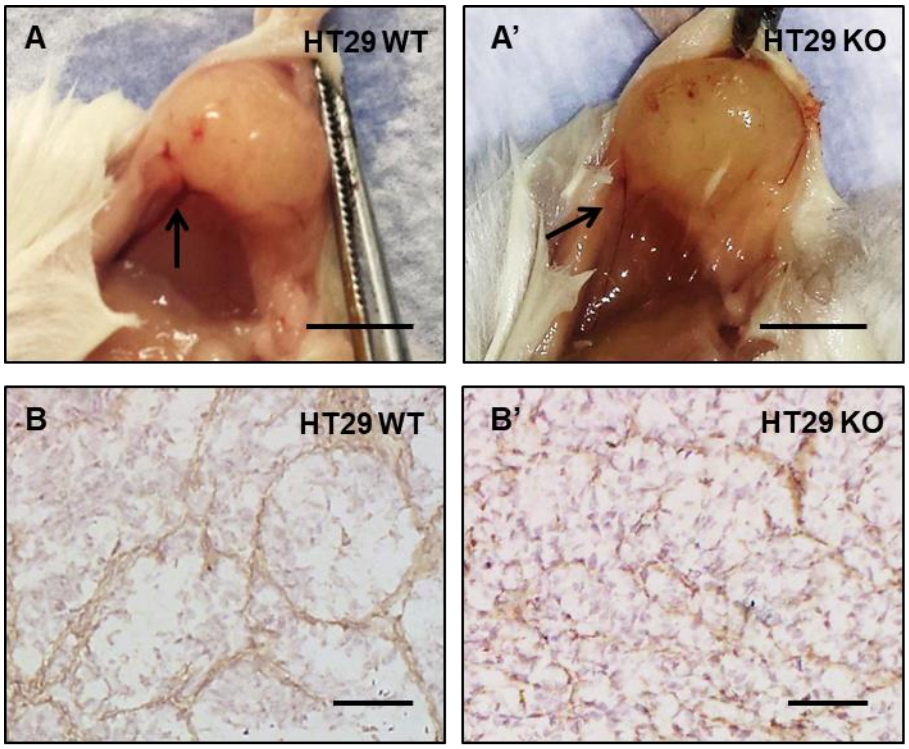

A
150
100
50
0
Number of Vessels
*
EV
B4
SW480 Cell Constructs
B
250
200
150
100
50
0
Number of Vessels
*
EV
B4
LIM2405 Cell Constructs
C
220
200
180
160
140
120
100
Number of Vessels
*
EV
B4
CT26 Cell Constructs

Figure 4.11. Knocking out EphB4 expression significantly reduces vascular density of tumours. A-A'. HT29 tumour vasculature at the time of harvesting (Scale bar = 2mm). **B-B'.** HT29 immunohistochemical labelling of vasculature with anti-Von Willebrand Factor in subcutaneous tumours (Scale bar = 100μm). **C.** Quantification of vessel numbers using stereological point count method (n=3 WT tumours, n=3 KO tumours). **P*< 0.05.


A
HT29 WT
A'
HT29 KO
B
HT29 WT
B'
HT29 KO

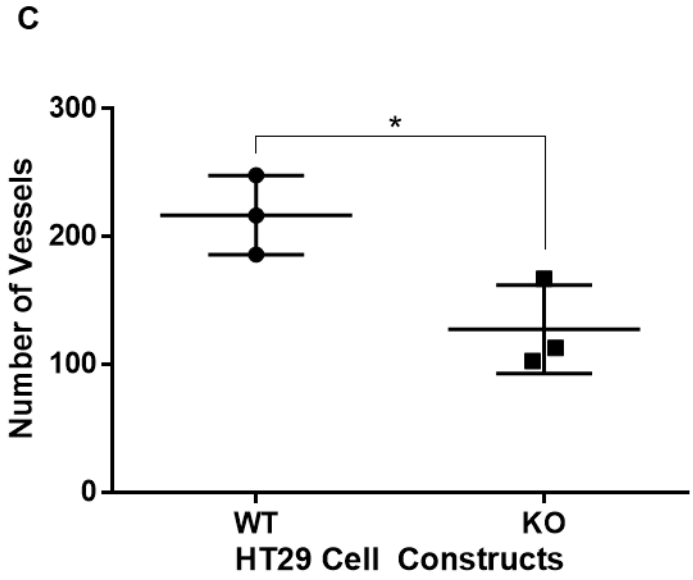

C
300
200
100
0
Number of Vessels
*
WT
KO
HT29 Cell Constructs

Table 4.1 Summary of quantitative analysis of *in vivo* findings

Cell line of tumours	Growth rate to reach 1cm (median survival days)	% of Ki67 positive cells (mean±SD)	Blood vessel density (mean±SD)
SW480 EV	38.5	42.8±14.8	47.6±8.8
SW480 B4	31.0*	45.3±2.9	97.7±22.7*
LIM2405 EV	18.0	59.6±2.9	140.5±33.8
LIM2405 B4	13.0	58.9±6.8	206.7±16.7*
CT26 EV	11.0	-	155.8±20.8
CT26 B4	11.0	-	181.0±14.5*
HT29 WT	25.0	61.0±3.5	216.8±31.0
HT29 KO	29.0	45.1±8.6$^{\#\#}$	127.6±34.5$^{\#}$

*P<0.05 compared to EV tumours

$^{\#}P$<0.05, $^{\#\#}P$<0.05 compared to WT tumours

4.4 Discussion

Validating and expanding the *in vitro* findings in animal models of cancer are of importance, as *in vitro* testing cannot properly reproduce the complex biology of animals. To examine *in vivo* effects of enhanced expression and knockout of EphB4 receptors, the SW480, LIM2405, CT26 and HT29 cell lines were used in subcutaneous xenograft models of CRC. These tumours grow as encapsulated tumour masses under the skin and above the muscle layer making this a good model for obtaining data such as rate of growth, tumour composition and vascularisation, which cannot be readily obtained from other *in vivo* models.

4.4.1 High EphB4 expression vastly improves tumour viability and incites the growth of tumours

In vitro findings may not always reflect *in vivo* tumourigenic potential of cells. One oesophageal cancer cell line showed resistance to EphB4 signalling inhibition *in vitro*, as this did not affect the rate of proliferation in cells (Hasina et al. 2013). However, EphB4 was then identified as an essential mediator in supporting the growth of tumours induced by the same cell line since *in vivo* inhibition of EphB4 signalling reduced tumour volume. Similar results were also obtained in gliomas, where EphB4 overexpression was shown to increase proliferation of cells and growth of tumours (Chen et al. 2013). Knocking down expression was shown to reduce these effects. In our study, reflecting the increased rate of proliferation observed in *in vitro* experiments (**Chapter 3**), the high EphB4 expressing tumours grew faster than the EV tumours. Also, tumours induced by EphB4 knockout cells tended have reduced the rate of tumour growth. In the colonic crypts, inhibiting

EphB receptor signalling using monomeric EphrinB-Fc is suggested to reduce number of proliferative cells and impact crypt length (Holmberg et al. 2006). For this reason, EphB receptors are suggested to be positive regulators of proliferation in the stem cell compartments. In CRC, EphB4 expression was shown to be regulated by cyclic AMP response element-binding (Creb) protein, which is suggested to allow for the rapid proliferation and dedifferentiation of cancer cells (Kumar et al. 2009). The level of EphB4 expression being a positive influence on proliferation in CRC cells and tumours have been further demonstrated by several other CRC studies (Lv et al. 2016; McCall et al. 2016). Even though our results appear to be in line with these reports, in our experiments the tumour growth trend was vastly impacted by viability. The low EphB4 expressing EV tumours were found to have large areas of central necrosis filled with pus and had increased tumour fragility. This condition had the most profound effect on the SW480 EV tumours, as some tumours grew very slowly and failed to reach the size limit of 1cm even at the end of experimental timeline of 40 days. The apparent necrotic phenotype seemed to be rescued in tumours induced by high EphB4 expressing cells, as these tissues had a greater tissue viability. In the necrotic tumours, tissue damage and brittleness were very extensive, in some instances, this made it difficult to conduct any type of analysis on the tumours. Targeting EphB4 expression in ovarian cancer xenografts was shown to reduce viability and proliferation, while increasing areas of necrosis (Kumar et al. 2007). These tumours were greatly smaller than controls and had lower number of blood vessels. These results supported *in vitro* findings were downregulation of EphB4 was also shown to decrease proliferation, migration and invasion of cells (Kumar et al. 2007). Similarly, suppression of EphB4 in lung cancer xenografts caused regression in

growth and consequently yielded smaller tumours compared to untreated controls (Ferguson et al. 2013). Long term suppression of EphB4, in combination with a chemotherapeutic agent, caused complete remission in some cases. Decreased detection in an endothelial marker also suggested reduced blood vessel formation. These results reflected *in vitro* findings of EphB4 being a positive regulator of proliferation.

In our study, evaluation of tumour cell proliferation, using the marker Ki67, revealed that there were no significant differences between the percentage of proliferating cells, in the tumours induced by high and low EphB4 expressing cells (**Table 4.1**). However, unlike the EV and B4 tumours of other cell lines, when comparing percentage of proliferative cells in the HT29 WT and KO tumours, knocking out EphB4 expression was found to significantly reduce proliferation in tumours. An explanation for these results could be that the necrotic centres in EV tumours push the proliferative compartment to the periphery to maintain growth. The B4 tumours have greater viability and proliferating cells could be seen all throughout the tumour, not just in the outer edges (data not shown). For consistency during analysis, only the tumour edges were evaluated. As such, proliferative cells in EphB4 overexpressing tumours would be significantly greater if the tumours were evaluated as a whole, because of larger area of viable tissue. A good demonstration of this theory, is the comparison of tumours induced by HT29 EphB4 knockout cells and EphB4 expressing WT cells. Since both the WT and KO tumours undergo necrosis, the proliferative compartments are confined to the periphery of both type of tumours. This allows for an equal comparison, which demonstrates significant differences in proliferating cells. Therefore, it appears that EphB4 expression can result in an increased rate of proliferation, however, this effect is

only secondary to enhanced tumour viability. The tumour growth trends observed in our study are consistent with the results of a previous study, in which low EphB4 expression, achieved by receptor knockdown, led to an 84% reduction in tumour growth (Kumar et al. 2009).

Moreover, our *in-situ* examination revealed that tumours overexpressing EphB4 were supplied by thicker and well perfused vessels, which was further investigated as a possible contributing factor to the changes observed in tumour viability.

4.4.2 High EphB4 expression leads to extensive vascularisation in subcutaneous tumours

Immunohistochemical analysis with an endothelial cell marker, to assess the level and quality of vascularisation within tumours, confirmed *in situ* observations that the high EphB4 expressing tumours have prominent and well networked vasculature throughout the tissue. In contrast, tumours of low EphB4 expression and knockout result in tumour vessels to appear malformed, fragmented and vasculature largely fail to form structured networks. Quantitation confirmed that high EphB4 expression leads to significantly greater density of tumour vessels. As we have reported, the reduced tumour viability and large areas of death observed in low expressing and knockout tumours is most likely the result of inadequate vascular development. In these tumours vessel diameter and branching was evidently insufficient. Lack of vascular development may result necrosis of tumour (Nishida et al. 2006). Angiogenesis can be affected by the site of tumour induction. Organs with high degree of pre-existing blood vessels can sufficiently support the cancer in the initial stages (Brem et al. 1976; Döme et al. 2007). However, with continuous growth of tumours, hypoxic fraction due to a lack of adequate oxygen

supply will be highest in cancer cells that are the furthest away from a blood vessel (Forster et al. 2017; Tannock et al. 1970). This can induce tumour vessel sprouting. Nonetheless, if vascular branching and density is insufficient this will trigger the onset of necrosis as a result of the tumour outgrowing its blood supply. Necrotic event in avascular tumours push the proliferative cells to the outer edges to survive (Brem et al. 1976; Folkman et al. 2003). As described above, this is observed in the subcutaneous xenografts of our study. In subcutaneous models, since cancer cells are introduced in between the skin and muscle layers, they need to attract a blood supply early during initial phase of growth. In these xenografts, tumour growth is likely supported by branching of the femoral artery (AhlstrÖM et al. 1988). Endothelial cells can then be recruited from the murine host to support the development of tumour vessels. Although, cancer derived endothelial cells have been shown in glioblastomas, where some of the endothelial cell populations had similar genetic identities to the cancer cells (Eklund et al. 2013; Ricci-Vitiani et al. 2010). Targeting these cells *in vivo* revealed a functional role as this reduced tumour size and vascular density (Ricci-Vitiani et al. 2010). Neural stem cells in the cancer cell population may have given rise to these endothelial populations, as such, cancers that are not homogenous may also have cancer stem cells that contribute to tumour vascularisation. Here using multiple colorectal cancer cell lines, we have demonstrated that regardless of the inherent morphological and mutational differences between these cell lines, the degree of tumour vessel density and networking appears to positively associate with that of EphB4 expression. The EphB4 overexpressing tumours may have earlier onset of attracting vascularisation, which continues to branch in line with tumour growth to support tissue and reduce necrosis. Evidently, the low EphB4 expressing and knockout tumours fail to attract

a sufficient blood supply and induce vascular branching inside the tumours to match the metabolic demand of growth, leading to necrosis. Recently, EphB4 overexpression in A375 melanoma cell line was found to enhance proliferation of cells and tumours, however, surprisingly it was found to impede tumour vessel formation and perfusion (Neuber et al. 2018). The lack of functional blood vessels increased hypoxia in tumours and this could negatively impact treatment of this type of cancer since drug delivery would be reduced. The proposed reason behind this observation is that tumour vessel formation may be influenced more by EphrinB2 reverse signalling, since A375 expresses low levels of the ligand its suggested to limit branching and maturation in tumour blood vessels. This is in contrast with various other reports that suggest ligand independent functions of EphB4 promote angiogenesis to support tumour growth and viability. In ovarian cancer, knocking down EphB4 expression reduced viability and growth of tumours due to decreased angiogenesis (Spannuth et al. 2010). EphB4 downregulation reduced tumour cell proliferation, to similar levels observed with chemotherapy treatment, for this reason, EphB4 was deemed as a potent target for this type of cancer therapy. These results are consistent with the well documented characteristics of EphB4 receptors in regulating angiogenesis with its corresponding EphrinB2 ligand. A knockout of the receptor or the ligand during development leads to an arrest in angiogenesis and ultimately results in embryonic lethality (Adams et al. 1999). Interaction of EphB4 receptors and EphrinB2 ligands is involved in the recruitment of pericytes to the site of angiogenesis (Salvucci et al. 2012). The pericytes in the leading edge then join forming vascular tubes. However, presumably one of the most important roles of the EphB4 receptors in this process is to recruit mural cells to the young vessels which lead to vessel

maturation (Djokovic et al. 2010). Inhibition of the Delta-like ligand 4 (DII4)/Notch pathway, known to play a role in angiogenesis, was shown to decrease tumour size and number by 50% in insulinomas (Djokovic et al. 2010). Blocking of EphB4 and EphrinB2 interaction in combination with DII4/Notch inhibition was shown to further reduce tumour size. It was found that in these tumours there was a decrease in vessel maturation with less mural cell coverage, reduced vessel diameters and perfusion compared to control animals (Djokovic et al. 2010). These findings are consistent with our results, where low EphB4 expression, therefore low signalling, results in a decrease in tumour vessel competency, poor blood delivery to tumour tissue and decrease in cancer cell viability. Fluid accumulation within the tumours induced by low EphB4 expressing cells could also be attributed to increase in vessel leakage due to lack of maturation in vessels.

Our results are similar to findings recently described by Lv and associates (2016), using only the SW480 cell line in tumour xenografts. The authors reported that EphB4 overexpressing tumours have enhanced vascularisation, in line with our findings. However, they also report that these tumours have large areas of necrosis and suggest this may be due to increased rate of tumour growth. Previously, unlike various other cancers, evaluation of CRC patient samples, revealed there was no strong association between tumour cell proliferation and level of necrosis in these types of tumours (Väyrynen et al. 2016). As described above, in our study necrotic events were mostly found in the low EphB4 expressing tumours, likely due to poor level of vascularisation, which impacted tumour viability and growth. There are several inconsistencies between our study and Lv et al. (2016) that may have led to these differences. Firstly, in our study we had both experimental and ethical endpoints. It appears that, Lv et al. (2016) collected all induced tumours at the same

time around approximately 40 days after injection. This timeline is similar to our experimental timeline. However, our ethical timeline dictated the collection of tumours at the size limit of 1cm for the welfare of the animal. For this reason, the faster growing EphB4 overexpressing tumours that reached the ethical time limit and were collected earlier (at 32 days) than EV tumours (at 40 days) for the SW480 cell line. Tumour collection times may have yielded differences in the level of tumour necrosis. Secondly, level of EphB4 overexpression in transfected cells might have significant impact on tumour behaviour and composition *in vivo*. Lv et al. (2016) did not report the level of EphB4 protein expression in their transfected cells; it may be different to the level of expression in cells used for our study. Furthermore, it is difficult to make direct comparison between our findings, as the histological images demonstrating necrosis in control tumours have not been provided in Lv et al. (2016) paper. Finally, the strains of mice used were different; in our study, we used NOD CB17-prkdc-scid/JAsmu mice, while Lv et al (2016) used athymic nude mice. The variation in immunological phenotypes as well as signalling pathways might influence the behaviour of tumour cells *in vivo*.

4.4.3 EphB4 overexpression leads to cancer cell aggregation and reduces stroma intermingling *in vivo*

The results of our *in vitro* experiments **(Chapter 2**), demonstrated that EphB4 expression leads to the segregation of 'like' expressing cells. We have also investigated the effect of EphB4 expression influencing cell composition in tumour tissues. This was visualised by an anti-human antibody to distinguish human cancer cells from background host tissue. In support of our *in vitro* findings, tumours of high EphB4 expressing cells yield homogenous masses densely packed with human

cancer cells and they contain minimal mouse-derived tissue. In comparison, in the tumours induced by low EphB4 expressing cells, host-derived tissue is widely dispersed amongst the human cancer cells. When EphB4 expression is knocked out, similar results are observed, though, these effects are less distinguished when compared to low EphB4 expressing WT control cells. It has been hypothesised that a similar interaction as between EphB2 and EphB3 receptors and EphrinB ligands contributes to tumours segregation. In *in vitro*, EphB2 and EphB3 receptor expressing CRC cells cluster together and form tight homogenous colonies upon encountering EphrinB1 ligand expressing cells (Cortina et al. 2007). Stimulation and activation of EphB2 with an EphrinB1-Fc resulted in the cells having a rounded shape and reduced adhesion to matrix proteins, suggesting onset of cellular repulsion as it has been observed in tumour models (Guo et al. 2006). We have conducted similar experiments, stimulating the cell lines used for xenografts with EphrinB2-Fc (**Chapter 2, section 2.3.3**). As a result, greater extent of cellular clustering and rounding was observed with EphB4 overexpressing cells upon encountering EphrinB2-Fc. Our *in vivo* data suggests that, similar dynamics may be at play and that EphB4 overexpression may result in tumour segregation. It has been shown that ligand stimulation with EphrinB2 in MCF-10A mammary tumour cells overexpressing EphB4 has a "tumour suppressive" effect and that EphB4 may have a tumour enhancer or suppressor role depending on the balance between ligand-independent and ligand-dependent signalling (Rutkowski et al. 2012). Furthermore, overexpression of Eph receptors in primary tumours and their epigenetic silencing in the progression of CRC have been reported (Davalos et al. 2006; Herath et al. 2009). The EphA1 receptor expression was found to be several folds higher in 53% of primary CRC tumours in comparison to matching normal

tissue (Herath et al. 2009). However, in late stages of metastasis EphA1 expression was downregulated, which associates with poorer patient survival. Similarly, inverse correlations between EphB receptor expression and stage of CRC, suggests that Eph receptors may need to be downregulated for the cancer to overcome compartmentalisation and achieve metastasis (Davalos et al. 2006). However, although local invasion may be restricted through Eph receptor mediated segregation, the high EphB4 expressing cancer cells may be under increased pressure to spread through blood vessels. EphB4 expressing cancer cells were reported to adhere to EphrinB2 expressing endothelial cells even when the receptor or the ligand was truncated (Héroult et al. 2010). These findings indicate that the enhanced vasculature in tumours and homing abilities conferred to cancer cells through high EphB4 expression could increase risk of metastatic spread. In order to investigate this, we have utilised orthotopic and intra-splenic models of metastasis, which will be discussed in the next chapter.

4.5 Conclusion

In conclusion, biological effects of EphB4 expression in tumours can support angiogenesis in cancers, supporting growth and viability. We have also found high EphB4 expression yields tumours densely packed with human cells. Whilst local invasion may be restricted due to cancer cell aggregation through EphB4 expression, high expressing cancer cells may be under increased pressure to spread through tumour blood vessels.

CHAPTER 5

Possible Implications of EphB4 and EphrinB2 Expression for Metastatic Models of Colorectal Cancer and Patient Survival

5.1 Introduction

Colorectal cancer is one of the most frequently diagnosed cancers, however, it is also one of the most treatable if diagnosed early. Nonetheless, it has been suggested that within 6 years of diagnoses, 27% of patients will progress into the metastatic stages of the disease (Luo et al. 2017). This risk increases with age at the time of diagnoses. However, CRC occurrence in young adults is reported to be on the rise in Australia (Young et al. 2015). For these reasons, being able to find predictive and prognostic biomarkers for disease progression and outcome are of importance.

EPHB gene expression appears to be low in normal colon tissue, but elevated in adenomas and carcinomas (Rönsch et al. 2011). However, a subset of carcinomas can also show a clear reduction in *EPHB* expression, which are suggested to silenced as part of disease progression in some instances (Batlle et al. 2005; Rönsch et al. 2011). In another CRC study, *EPHB4* was found to be expressed at elevated levels in primary tumours compared to normal tissue, and even more so in liver metastatic nodules (Kumar et al. 2009). When murine models of metastasis were used, *in vivo* targeting of EphB4 expression was shown to reduce the frequency of metastatic lesions. Consistent with these observations, breast cancer samples also showed high EphB4 expression in late stage disease and associated with reduced overall and recurrence-free survival (Brantley-Sieders et al. 2011). Furthermore, EphB4 expression is suggested to be an indicative factor for determining patient response to some treatments. Bevacizumab is a monoclonal antibody targeting endothelial growth factor. It has positive impact clinically, when given to metastatic CRC patients alongside chemotherapy. In search for predictive biomarkers for this kind of therapy it was found that, patients with high level of *EPHB4* mRNA were

less responsive to Bevacizumab treatment (Guijarro-Muñoz et al. 2013). Furthermore, a reduction in *EPHB4* was observed in all patients undergoing Bevacizumab and chemotherapy treatment. This reduction was more apparent in patients who displayed an objective response (Marisi et al. 2017). In another study investigating chronic myeloid leukaemia, EphB4 expression was reported to increase in patients who were deemed resistant to the tyrosine kinase inhibitor Dasatanib (Huang et al. 2014; Zhao et al. 2017). As such, having predictive and prognostic markers may help more specifically tailor cancer treatment.

On the other hand, it has been well documented that EphB4 effect on cancer cells can be dependent on the presence or absence of EphrinB2 ligand stimulation. Ligand dependent signalling in EphB4 expressing cells reduce cancer cell survival and has a tumour suppressive influence (Rutkowski et al. 2012). Conversely, it is proposed that EphrinB2 expressing tumour vasculature can cause cell rounding and detachment to promote dissemination through forward signalling of EphB4 expressing cancer cells (Noren et al. 2004). The EphrinB2 ligand can also have variable effects on cancer cells and in patients. The expression of *EPHRINB2* in neuroblastoma has been found at low levels in late stage of the disease and its expression was associated with better prognosis for patients in an age dependent manner (Tang et al. 2000). In contrary, there are reports that it can negatively impact patient survival and increase rate of disease recurrence (Castellvi et al. 2006). Additionally, elevated *EPHB4* and *EPHRINB2* expressions are also reported to negatively impact patient outcomes in uterine and cervical cancers (Alam et al. 2009).

In this study, we aimed to investigate the influence of EphB4 overexpression on the rate of CRC cell metastasis in murine models. Furthermore, clinical relevance of *EPHB4* alone and with *EPHRINB2* as potential biomarkers for CRC patient survival and disease recurrence have also been investigated in patient datasets.

5.2 Methods

5.2.1 Animal sourcing, housing and preparation of cells for surgery

All *in vivo* experiments were performed according to the Australia National Health and Medical Research Council Code of conduct on the care and use of laboratory animals for scientific purposes and approved by the Victoria University Animal Experimentation Ethics Committee (Animal Ethic Numbers 04/12 and 14/001). Details about animal sourcing, housing and strains are listed in **Chapter 4** (**section 4.2.1**). SW480, LIM2405 and CT26 cells were prepared as described in **Chapter 4** (**section 4.2.1**).

5.2.2 Orthotopic model of CRC

Animals were weighed and monitored 5 days before the treatment as well as on the day of the surgery. All equipment, surgical tools and accessories were autoclaved or pre-purchased as sterile. Anaesthetic (Ketamine (80 mg/kg) & Xylazine (10mg/kg)) and analgesic (Temgesic (0.05mg/kg)) drugs were prepared on the day of surgeries. These drugs were then adjusted as volume/body weight of animal. Surgeries were conducted on 5 mice per day. Total of 5 mice were used per group/ experiment.

Animals were briefly restrained and injected subcutaneously with the adjusted volume of anaesthetic. Pinching of the hind paws was used to determine the level of anaesthetic. When the animal was no longer responsive, it was then prepared for surgery with the close shaving of its fur around the abdominal cavity. The area was sterilized with 70% ethanol then chlorhexidine swabs. The animal was then placed on the sterile surgical drape on a heat mat, its limbs gently taped down, and sterile film was used to cover the abdominal cavity. A small incision was made in the skin then in the underlying muscle layer after which the cecum was located and externalized. Tumour cells ($1\text{-}2x10^6$) suspended in PBS or Matrigel (Corning, New York, United States) (25µL) were then injected into the wall of the cecum under a dissecting microscope. A successful injection was recorded if a bulla formed in the injection site indicating that the cell suspension was in the wall of the cecum and had not leaked into the lumen. The cecum was placed back into the abdominal cavity in the same position it was found, and muscle and skin layers were sutured. The wound was then swabbed with Betadine antiseptic solution and an adjusted volume of analgesic was administered subcutaneously and animals allowed to recover in cages placed on heat mats. Once they were fully awake, the animals were housed together with their cage mates with a supply of softened food to make it easier for consumption post-surgery. The wounds were checked; animals were weighed and monitored every day for any signs of distress, pain or change in habits. Mice were culled at 40 days post-surgery or earlier based on their condition according to ethical standards.

5.2.3 Intrasplenic model of CRC

Animals were prepared and an abdominal incision made as described above in **section 5.2.2**. The spleen was externalised, and $1x10^6$ cells were suspended in 25µL of Matrigel and injected in the splenic capsule ensuring not to penetrate too deep into the organ. As a precaution against haemorrhaging, a sterile cotton swab was used to create pressure at the site immediately after injection. The spleen was then placed back into the abdominal cavity and the abdomen was sutured as described above in **section 5.2.2**. Mice were monitored daily as above in **section 5.2.2** and culled at 40 days post-surgery or earlier based on their condition according to ethical standards. All tissue collection, processing and analyses were conducted as described in **Chapter 4, section 4.3**.

5.2.4 Data mining

Patient data were sourced from the Survexpress website: (http://bioinformatica.mty.itesm.mx:8080/Biomatec/SurvivaX.jsp). The *EPHB4* gene alone and *EPHB4+EFNB2* in combination was used to search the available colon databases. The gene *EFNB2* will be referred to as *EPHRINB2* in the text. Average expression probe was used for datasets with replicated analysis. Risk groups were set to '2' or '3' as indicated in the text. Hazard ratio was estimated by fitting a CoxPH using risk group as covariate (Aguirre-Gamboa et al. 2013). The datasets were normalised and censored for survival (in months unless otherwise indicated), disease free survival and recurrence where available. Significance was accepted as $P<0.05$. The individual databases used have been cited and referenced. There are no restrictions or limitations imposed to the data available from these sources.

5.3 Results

5.3.1 Orthotopic and intrasplenic models of CRC yield poor tumour take and metastasis

In our orthotopic and intrasplenic models of cancer, animals were operated in groups of 5 and where it was deemed necessary the experiments were repeated. Pilot experiments were run to optimise each procedure and optimal culling time points (data not shown).

Table 5.1 Orthotopic tumour incidence and number of animals with metastasis

Cell Line	No. of animals with successful cancer induction	No. animals with metastasis
SW480 EV	1 out of 15	0
SW480 B4	0 out of 15	0
CT26 EV	10 out of 10	2
CT26 B4	10 out of 10	1

Initial experiments were conducted with cells suspended in PBS, however, Matrigel was then determined to be a medium for injecting into animals. Orthotopic experiments using the SW480 cell line were repeated three times. The tumour take in each case was very low with only one animal out of the thirty showing a small tumour at the injection site (**Table 5.1**). The CT26 is a mouse CRC cell line, syngeneic with the Balb/c species. This cell line is also one of the most commonly used in the orthotopic model. In our experience, it proved to be highly aggressive. All animals injected with CT26 cells had heavy tumour burden by day 14 of the experiment, most of the animals with metastasis exhibited high degree of abdominal seeding (**Table 5.1**). For this reason, they had to be excluded from the analysis.

Moreover, three of the twenty animals showed colonic and liver invasion without abdominal spread. In each case the liver was almost completely replaced by tumour, making it difficult to draw any comparison with the rate of metastasis. This cell line proved to be too aggressive for the type of metastasis we wanted to observe. In our attempts to investigate the effect of EphB4 expression on the rate of metastasis, we have also utilised the intrasplenic model of CRC.

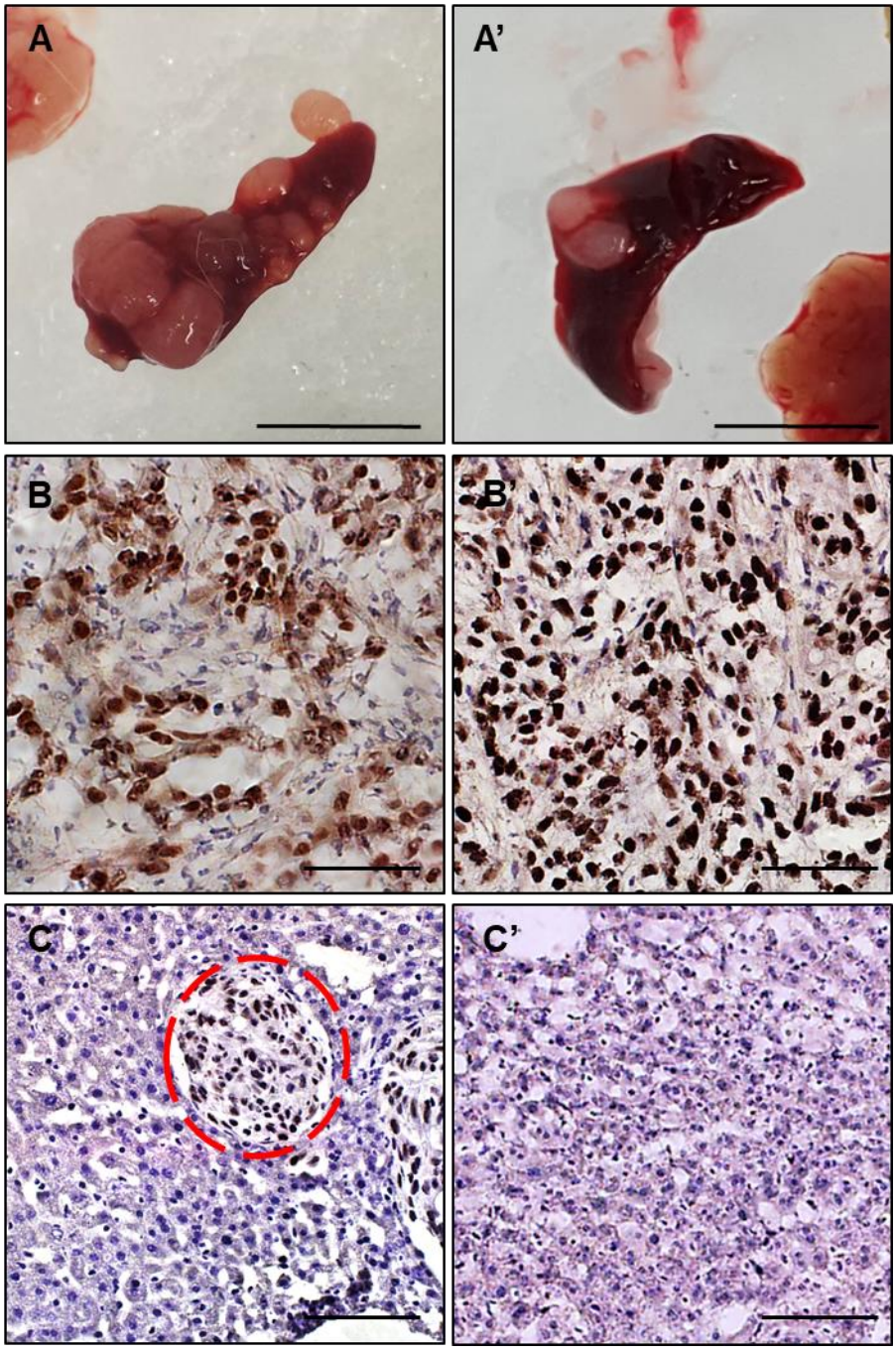
Table 5.2 Intrasplenic tumour incidence and number of animals with metastasis

Cell Line	No. of animals with successful cancer induction	No. animals with metastasis
SW480 EV	6 out of 10	0
SW480 B4	1 out of 10	0
LIM2405 EV	5 out of 10	4
LIM2405 B4	7 out of 10	0

In this model the cells were injected into the splenic capsule. Our initial experiments using SW480 cells proved to be more promising than orthotopic experiments as seven out of twenty animals had tumour growth in the spleen, although none of these animals had metastasis as the tumours were very small (**Table 5.2**). Even so, only one of these animals injected with SW480 B4 constructs developed tumours and as such no useful comparisons could be drawn in this instance (**Table 5.2**). We have tried increasing the number of cells injected ($2x10^6$), which caused several unexpected deaths soon after surgery. For this reason, all consecutive experiments were conducted with $1x10^6$ cells. The LIM2405 cell line proved to be more tumorigenic as twelve out of the twenty animals grew splenic tumours (**Table 5.2**). Unfortunately, only 4 of these tumours metastasized to the liver, all four were induced by EV construct of LIM2405. The LIM2405 B4 induced tumours tended to be much smaller in comparison to LIM2405 EV induced tumours (**Figure 5.1A,**

A'). Splenic tumours and, where present, metastatic lesions were visualised using the anti-human antibody, whereby, the human LIM2405 cells were defined in brown against the blue background of mouse spleen (**Figure 5.1B-B'**) and liver cells (**Figure 5.1 C-C'**). The LIM2405 EV cancer cells had colonised the spleen more readily and had more mouse derived tissue than LIM2405 B4 tumours (**Figure 5.1B-B'**). Metastatic spread to the liver was evident in animals injected with LIM2405 EV tumours (**Figure 5.1C**), yet no micro-lesions were found in the LIM2405 B4 animals (**Figure 5.1C'**).

Figure 5.1. LIM405 EV tumours grow more readily in the spleen **A.** LIM2405 EV induced splenic tumours. **A'.** LIM2405 B4 induced splenic tumours. **B.** LIM2405 EV splenic tumour labelled with anti-human antibody (brown), mouse tissue counter stained with hematoxylin (blue) **B'.** LIM2405 B4 splenic tumour labelled with anti-human antibody (brown), mouse tissue counter stained with hematoxylin (blue) **C.** LIM2405 EV liver lesions labelled with anti-human antibody (brown). **C'.** LIM2405 B4 liver sections not showing metastatic lesions. (Scale bar for A-A' = 1cm, B-C' = 200µm).


A
A'
B
B'
C
C'

5.3.2 Low and moderate levels of relative *EPHB4* expression correlates with better survival in CRC patients

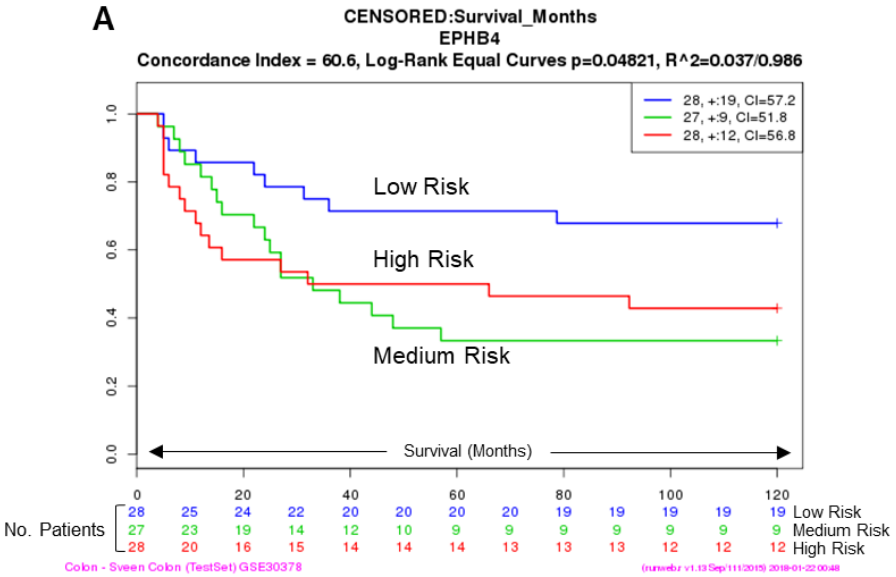
To investigate the possible effect of *EPHB4* expression on CRC patient survival and outcome, we have examined cancer gene expression databases. In seven of the thirteen CRC databases reporting on patient survival, *EPHB4* expression was higher in the tissues of patients categorised as low risk (**Table 5.3**). The remaining six databases show greater *EPHB4* expression in high risk group (**Table 5.3**). It is worth noting that, larger number of patients and samples were assayed where *EPHB4* expression was found in low risk individuals (**Table 5.3**). On the other hand, TGCA databases have categorised and evaluated tissues separately as colon, rectum or both, based on site of occurrence (**Table 5.3**). Even though, *EPHB4* gene expression was significantly different between the two risk groups in each of the datasets in **Tables 5.3**, this did not impact survival of patients. Survival rates of patients were not significantly different between high and low risk groups in any of the databases based on *EPHB4* expression as a biomarker.

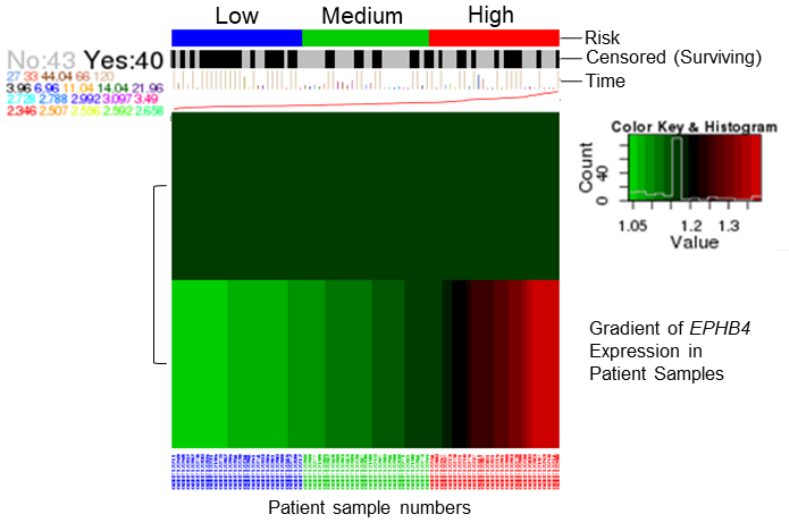
Table 5.3 Correlation of *EPHB4* expression in low and high risk categorised patients does not impact overall survival

	Database	No. of Patient Samples	Overall Impact on Survival (*P* value)	Level of *EPHB4* Expression (*P* value)
Low Risk Patients	GSE12945 (Staub et al. 2009)	62	0.48	6.26e-13
	GSE24550 (Agesen 2012; Sveen et al. 2011)	77	0.86	3.24e-08
	TCGA	151	0.39	1.71e-35
	GSE17537 (Freeman et al. 2012; Smith et al. 2010)	55	0.43	6.88e-12
	GSE28722 (Loboda et al. 2011)	125	0.75	1.01e-24
	GSE24551 (Agesen 2012; Sveen et al. 2011)	160	0.70	1.88e-60
	Colon-Metabase-Uniformized (Aguirre-Gamboa et al. 2013)	482	0.90	3.68e-93
High Risk Patients	GSE17536 (Freeman et al. 2012; Smith et al. 2010)	177	0.93	7.77e-33
	GSE30378 (Agesen 2012; Sveen et al. 2011)	83	0.10	2.03e-17
	GSE31595 (Thorsteinsson unpublished)	37	0.97	8.62e-09
	Colon adenocarcinoma (TCGA)	351	0.77	2.52e-76
	Colorectal adenocarcinoma (TCGA)	467	0.89	1.83e-99
	Rectal adenocarcinoma (TCGA)	57	0.88	8.06e-14

However, when risk group categorisation is changed to 3 to include low, medium and high risk patients, some differences can be observed in the rate of survival amongst patients with different *EPHB4* expression. In the Sveen database, the low risk individuals demonstrate the highest rate of survival over a 10 year (120 month) period (P=0.04) (**Figure 5.2A**). The low risk individuals also express the lowest level of *EPHB4* (P=9.25e-25) (**Figure 5.2B, C**). The individuals deemed medium risk also express relatively low levels of *EPHB4*, however, their survival rate was lower compared to low and high risk individuals. The number of samples designated into each group in this database is small, with about 28 samples in each category. In the TCGA database, patient numbers are greater, and each risk group has minimum of 155 individuals in it. The observation period is 11 years, similar to the previous dataset (**Figure 5.3A**). The greatest rate of survival is observed in the medium risk patients (P=0.02) (**Figure 5.3A**). The *EPHB4* gene expression trend in this database is also similar to the previous, with high level of expression correlating with increased risk score (**Figure 5.3B**). *EPHB4* expression is significantly different between the risk groups (P=6.12e-153) (**Figure 5.3C**).

Figure 5.2. Low *EPHB4* gene expression in low risk individuals correlates with better overall survival. A. Sveen database Cox survival analysis of patients categorised as high, medium and low risk groups. **B.** Categorisation of low (blue), medium (green), high risk (red) groups, heatmaps of censoring based on time/survival, gradients of *EPHB4* gene expression with respect to patient samples. **C.** *EPHB4* gene expression levels by risk group.


A
CENSORED:Survival_Months
EPHB4
Concordance Index = 60.6, Log-Rank Equal Curves p=0.04821, R^2=0.037/0.986
28, +:19, CI=57.2
27, +:9, CI=51.8
28, +:12, CI=56.8
Low Risk
High Risk
Medium Risk
Survival (Months)
No. Patients
28 25 24 22 20 20 20 20 19 19 19 19 19 Low Risk
27 23 19 14 12 10 9 9 9 9 9 9 9 Medium Risk
28 20 16 15 14 14 14 13 13 13 12 12 12 High Risk
Colon - Sveen Colon (TestSet) GSE30378


B
Low
Medium
High
Risk
Censored (Surviving)
Time
No:43 Yes:40
Color Key & Histogram
Count
Value
Gradient of EPHB4 Expression in Patient Samples
Patient sample numbers

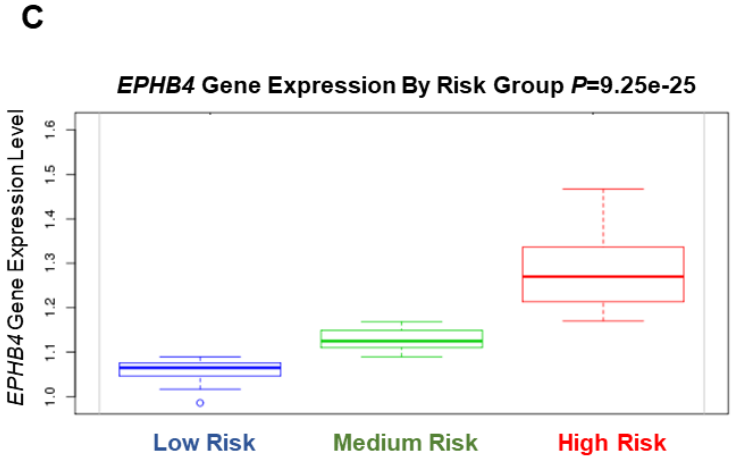
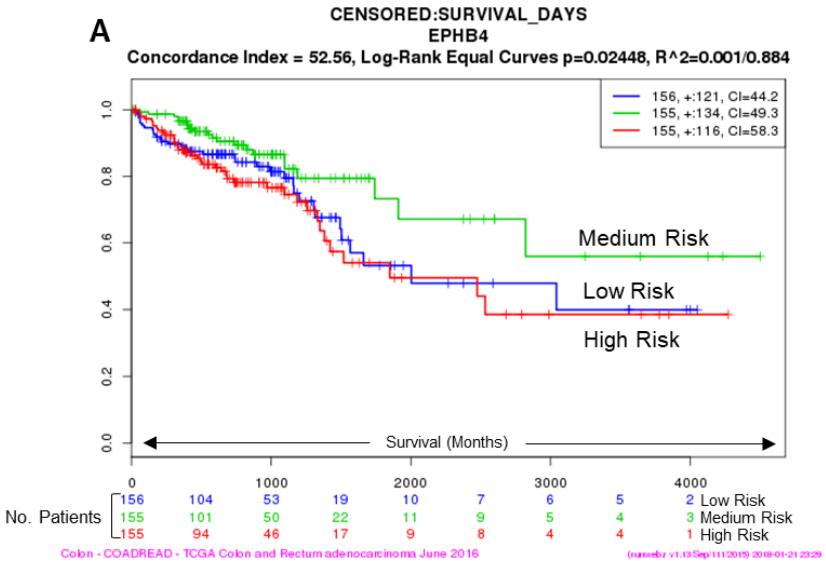
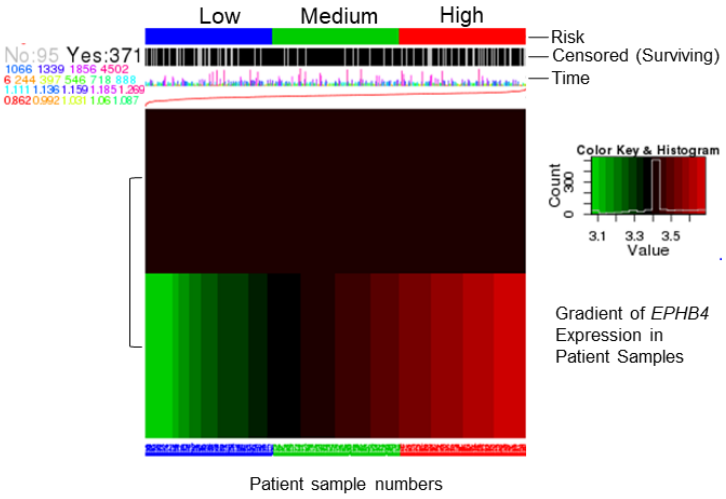
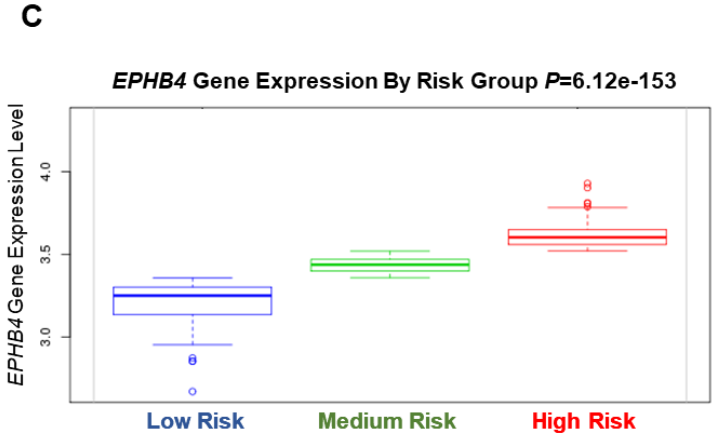

C
EPHB4 Gene Expression By Risk Group P=9.25e-25
EPHB4 Gene Expression Level
Low Risk
Medium Risk
High Risk

Figure 5.3. Patients with intermediate *EPHB4* gene expression have better overall survival. A. TCGA database Cox survival analysis of patients categorised as high, medium and low risk groups. **B.** Categorisation of low (blue), medium (green) and high risk (red) groups, heatmaps of censoring based on time/survival, gradients of gene expression with respect to patient samples. **C.** *EPHB4* gene expression levels by risk group.


A
CENSORED:SURVIVAL_DAYS
EPHB4
Concordance Index = 52.56, Log-Rank Equal Curves p=0.02448, R^2=0.001/0.884
156, +:121, CI=44.2
155, +:134, CI=49.3
155, +:116, CI=58.3
Medium Risk
Low Risk
High Risk
Survival (Months)
No. Patients
156 104 53 19 10 7 6 5 2 Low Risk
155 101 50 22 11 9 5 4 3 Medium Risk
155 94 46 17 9 8 4 4 1 High Risk
Colon - COADREAD - TCGA Colon and Rectum adenocarcinoma June 2016


B
Low
Medium
High
Risk
Censored (Surviving)
Time
Color Key & Histogram
Count
Value
Gradient of EPHB4 Expression in Patient Samples
Patient sample numbers


C
EPHB4 Gene Expression By Risk Group P=6.12e-153
EPHB4 Gene Expression Level
Low Risk
Medium Risk
High Risk

5.3.3 *EPHB4* and *EPHRINB2* expression correlates with high risk and low survival in CRC patients

Since EphB4 receptor only binds with high affinity to and is activated by the EphrinB2 ligand, the expression of their genes was analysed in combination in the datasets. From the available databases, three yielded significantly different survival rates for patients in low and high groups (**Table 5.4**). It was discovered that high expression of the *EPHRINB2* gene in high risk groups negatively affected the rate of patient survival. *EPHB4* gene expression varied between risk groups in different databases. Nonetheless, its expression was either similar in between risk groups or it is expressed in a counter gradient manner with *EPHRINB2*. In contrast, *EPHRINB2* gene expression was found to be continually more elevated in high risk groups except for two databases, where its expression was not significantly different (**Table 5.4**).

Table 5.4 *EPHB4+EPHRINB2* gene expression between low and high risk groups and its impact on patient survival

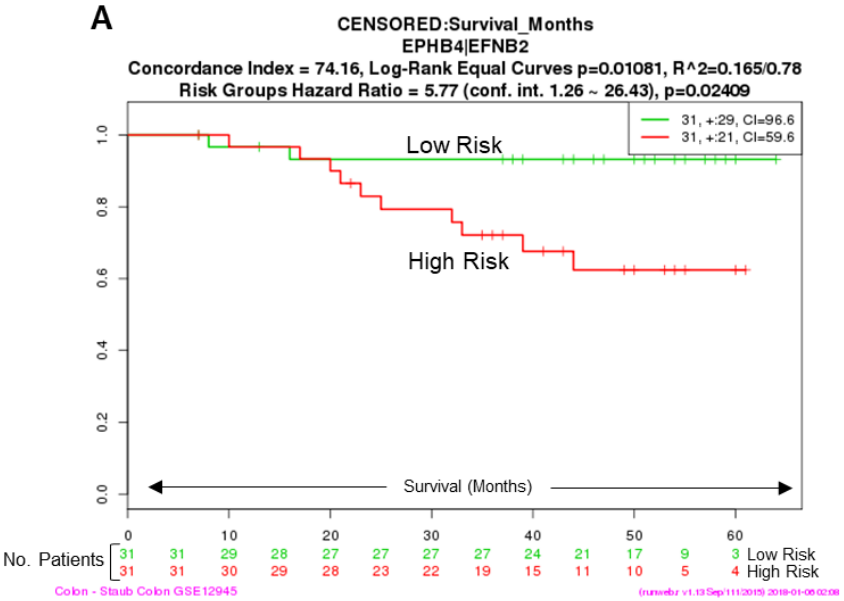
Database	**No. of Patient Samples**	**Overall Impact on Survival (*P* value)**	**Level of *EPHB4* expression between risk groups (*P* value)**	**Level of *EPHRINB2* expression between risk groups (*P* value)**
GSE12945 (Staub et al. 2009)	62	0.02*	7.21e-01	7.48e-13
GSE17536 (Freeman et al. 2012; Smith et al. 2010)	177	0.02*	4.76e-08	1.42e-33
Colon-Metabase-Uniformized (Aguirre-Gamboa et al. 2013)	482	0.02*	4.52e-02	2.97e-86
GSE24550 (Agesen 2012; Sveen et al. 2011)	77	0.91	8.54e-03	1.27e-07
GSE30378 (Agesen 2012; Sveen et al. 2011)	83	0.10	2.03e-17	1.93e-01
Colorectal (TCGA)	467	0.24	6.25e-12	1.17e-09
GSE17537 (Freeman et al. 2012; Smith et al. 2010)	55	0.49	3.29e-06	8.41e-05
GSE28722 (Loboda et al. 2011)	125	0.85	1.27e-11	7.73e-10
GSE41258 (Shefer et al. 2009)	244	0.41	2.71e-03	6.89e-45
Colon (TCGA)	351	0.24	2.90e-33	9.29e-23
Colorectal (TCGA)	467	0.19	1.59e-26	6.28e-50
Rectum (TCGA)	57	0.55	1.27e-11	9.50e-01

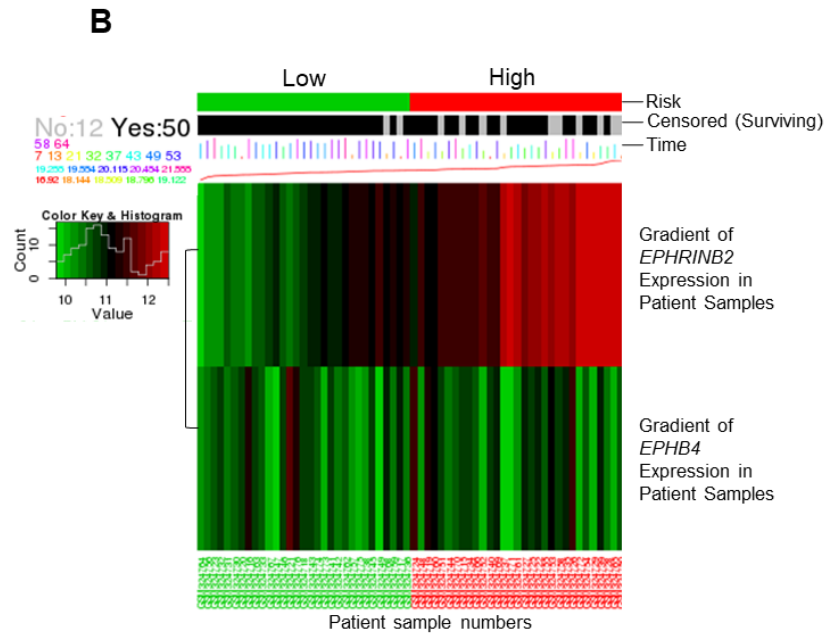
* Significant difference in the rate of overall survival between high and low risk patients

In the Staub Colon GSE12945 database, samples were collected from 62 patients undergoing surgical resectioning in Germany. In these patients, low risk group had relatively stable and better rate of survival over a 60 months period than high risk individuals (*P*=0.02) (**Figure 5.4A**). Out of the twelve individuals who reached the event (death), two were from the low risk group and the remaining ten from the high risk category. Heatmap evaluations reveal that in most of the high risk individuals who reached the event, *EPHRINB2* expression tended to be higher than that of *EPHB4* (**Figure 5.4B**). The two low risk individuals who did not survive, showed slightly higher expression of *EPHB4* and *EPHRINB2* genes, compared to other low risk individuals (**Figure 5.4B**). However, there were other low risk patient who expressed similar patterns of *EPHB4* and *EPHRINB2* who did not reach the event. Overall, there appears to be a strong gradient of *EPHRINB2* expression between the risk groups (*P*=7.48e-13), whereas, *EPHB4* expression was not significantly different (*P*=7.21e-01) (**Figure 5.4C**). For this reason, high *EPHRINB2* gene expression appears to be the more likely predictor of poor survival in this instance. A greater number of samples (177) were assayed in the Smith database. There were 73 individuals who reached the event in this dataset, 30 belonged in the low risk group (**Figure 5.5A**). The main proportion of these individuals had moderate to high levels of *EPHRINB2* expression and its expression significantly increased in the high risk group (*P*=1.42e-33) (**Figure 5.5B**). These high risk individuals reached the event in greater numbers (*P*=0.02) (**Figure 5.5A, B**). In contrast to the previous report, where no difference was observed, the level of *EPHB4* expression in this cohort was significantly higher in the high risk group (*P*=4.76e-08) (**Figure 5.5C**).

The biggest cohort containing 482 samples (Colon-Metabase) also showed a similar impact on patient survival with gene expression. Whilst, there was no significant difference in *EPHB4* expression (*P*=1.96e-01) between risk groups, high risk individuals exhibited the greatest level of *EPHRINB2* gene expression (**Figure 5.6C**). Even so, *EPHRINB2* expression gradient reaches well into the low risk group (**Figure 5.6B**). Nonetheless, *EPHRINB2* expression is significantly different between the two groups (*P*=1.06e-96) (**Figure 5.6C**). The high risk group survival was once again significantly impacted and lower than that of low risk individuals (*P*=0.02) (**Figure 5.6A**).

Figure 5.4. Patients with high *EPHRINB2* expression have reduced overall survival. A. Staub Colon GSE12945 database Cox survival analysis of patients categorized as high and low risk groups. **B.** Categorisation of low (green) and high risk (red) groups, heatmaps of censoring based on time/survival, gradients of *EPHB4* and *EPHRINB2* gene expression with respect to patient samples. **C.** Gene expression levels of *EPHB4* and *EPHRINB2* by risk group.


A
CENSORED:Survival_Months
EPHB4|EFNB2
Concordance Index = 74.16, Log-Rank Equal Curves p=0.01081, R^2=0.165/0.78
Risk Groups Hazard Ratio = 5.77 (conf. int. 1.26 ~ 26.43), p=0.02409
31, +:29, CI=96.6
31, +:21, CI=59.6
Low Risk
High Risk
Survival (Months)
No. Patients
Low Risk
High Risk
Colon - Staub Colon GSE12945


B
Low
High
No:12 Yes:50
Risk
Censored (Surviving)
Time
Color Key & Histogram
Count
Value
Gradient of EPHRINB2 Expression in Patient Samples
Gradient of EPHB4 Expression in Patient Samples
Patient sample numbers

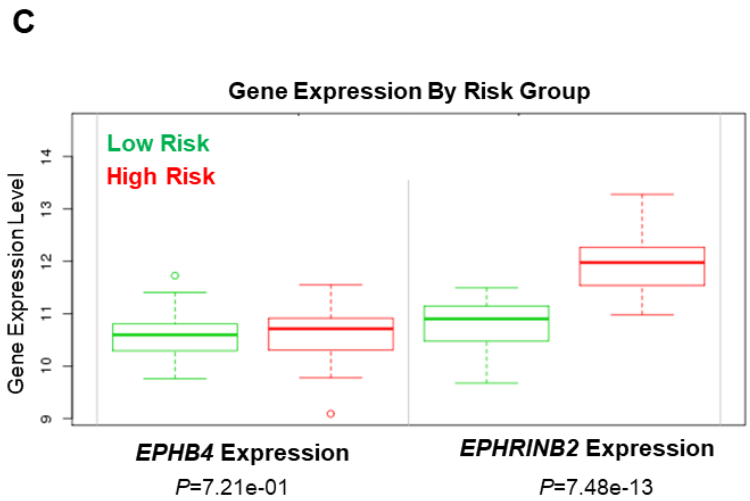
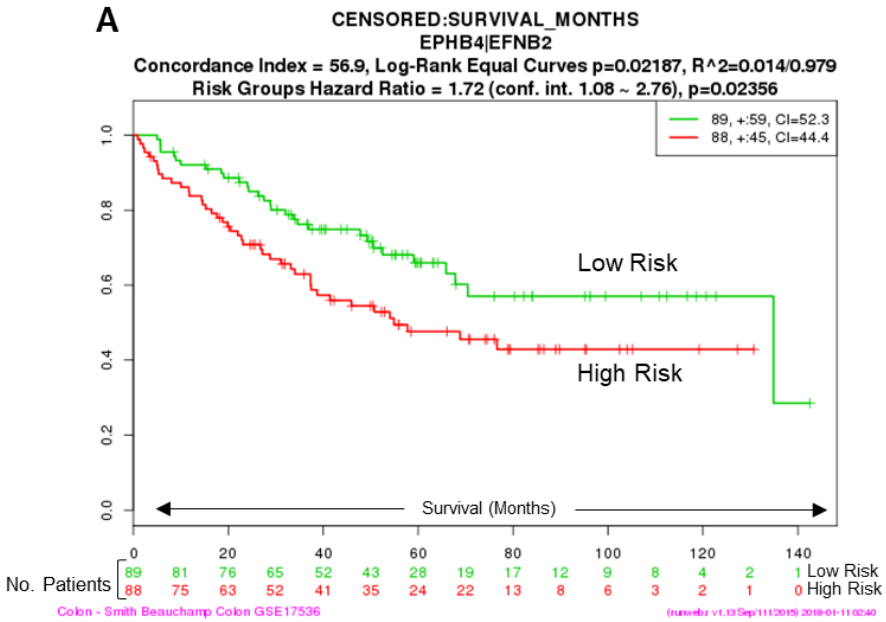
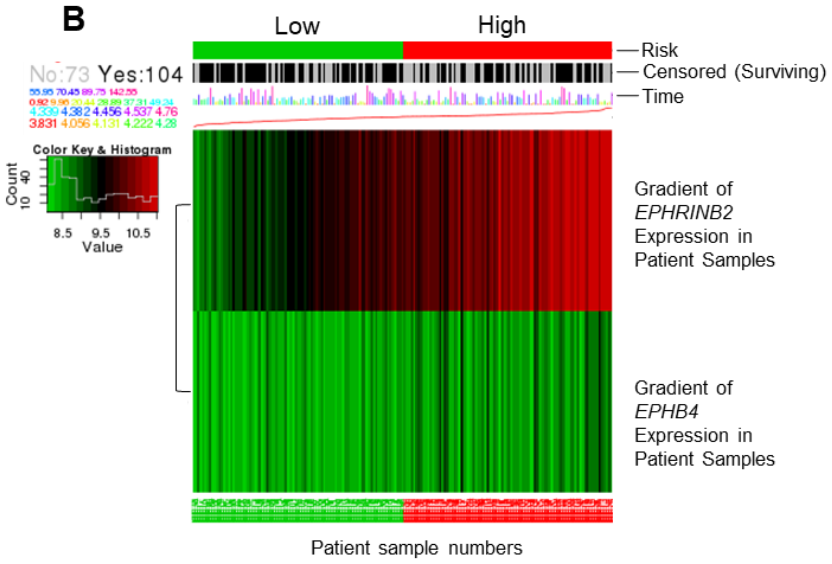

C
Gene Expression By Risk Group
Low Risk
High Risk
Gene Expression Level
EPHB4 Expression
P=7.21e-01
EPHRINB2 Expression
P=7.48e-13

Figure 5.5. High *EPHB4* and *EPHRINB2* expression negatively impacts survival of CRC patients. A. Smith database Cox survival analysis of patients categorised as high and low risk groups. **B.** Categorisation of low (green) and high risk (red) groups, heatmaps of censoring based on time/survival, gradients of *EPHB4* and *EPHRINB2* gene expression with respect to patient samples. **C.** Gene expression levels of *EPHB4* and *EPHRINB2* by risk group.


A
CENSORED:SURVIVAL_MONTHS
EPHB4|EFNB2
Concordance Index = 56.9, Log-Rank Equal Curves p=0.02187, R^2=0.014/0.979
Risk Groups Hazard Ratio = 1.72 (conf. int. 1.08 ~ 2.76), p=0.02356
89, +:59, CI=52.3
88, +:45, CI=44.4
Low Risk
High Risk
Survival (Months)
No. Patients
Low Risk
High Risk
Colon - Smith Beauchamp Colon GSE17536


B
Low
High
No:73 Yes:104
Risk
Censored (Surviving)
Time
Color Key & Histogram
Count
Value
Gradient of EPHRINB2 Expression in Patient Samples
Gradient of EPHB4 Expression in Patient Samples
Patient sample numbers

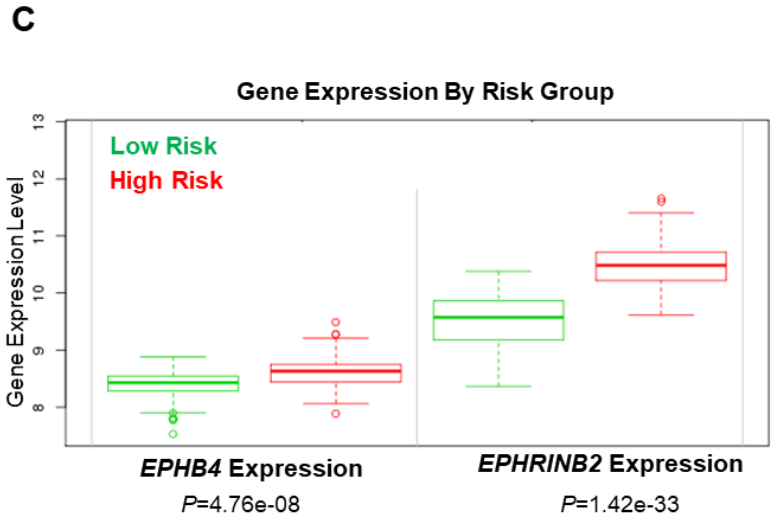
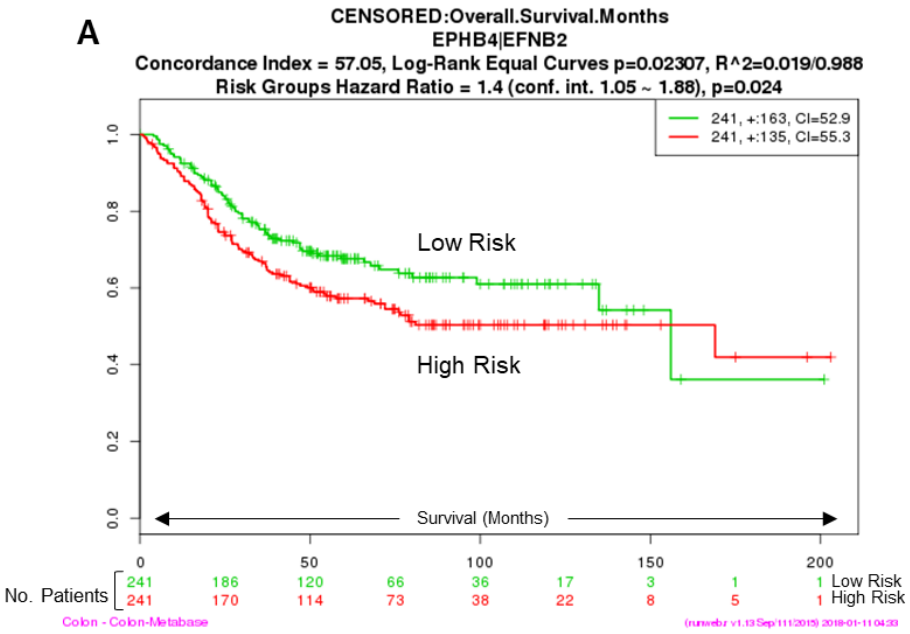
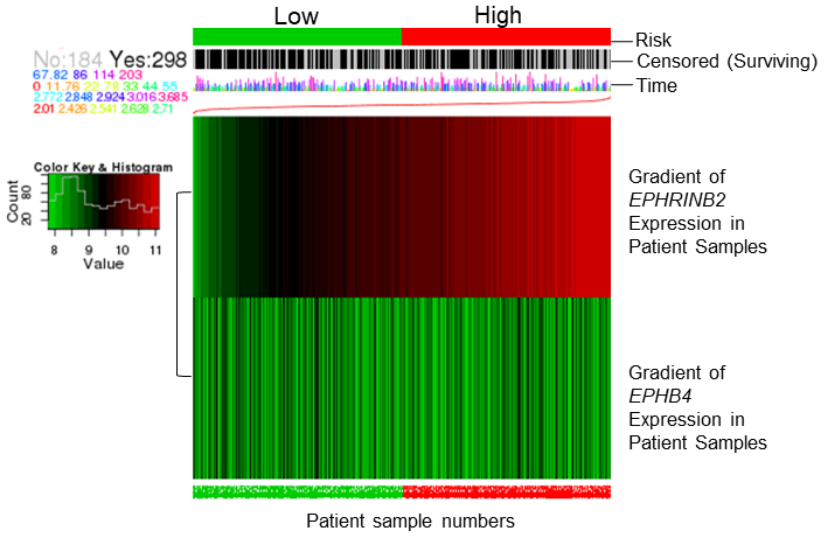
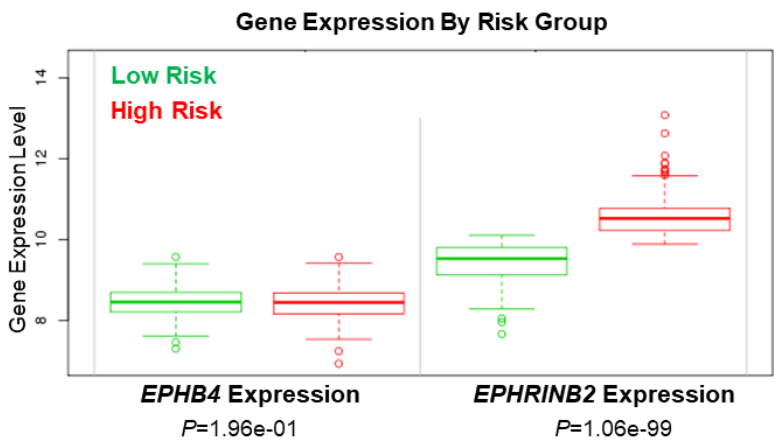

C
Gene Expression By Risk Group
Low Risk
High Risk
Gene Expression Level
EPHB4 Expression
P=4.76e-08
EPHRINB2 Expression
P=1.42e-33

Figure 5.6. Low *EPHRINB2* expression prolongs rate of survival. A. Colon-Metabase Cox survival analysis of patients categorised as high and low risk groups. **B.** Categorisation of low (green) and high risk (red) groups, heatmaps of censoring based on time/survival, gradients of *EPHB4* and *EPHRINB2* gene expression with respect to patient samples. **C.** Gene expression levels of *EPHB4* and *EPHRINB2* by risk group.


A
CENSORED:Overall.Survival.Months
EPHB4|EFNB2
Concordance Index = 57.05, Log-Rank Equal Curves p=0.02307, R^2=0.019/0.988
Risk Groups Hazard Ratio = 1.4 (conf. int. 1.05 ~ 1.88), p=0.024
241, +:163, CI=52.9
241, +:135, CI=55.3
Low Risk
High Risk
Survival (Months)
0.0
0.2
0.4
0.6
0.8
1.0
0
50
100
150
200
No. Patients
241 186 120 66 36 17 3 1 1 Low Risk
241 170 114 73 38 22 8 5 1 High Risk
Colon - Colon-Metabase


B
Low
High
No:184 Yes:298
Risk
Censored (Surviving)
Time
Color Key & Histogram
Count
Value
Gradient of
EPHRINB2
Expression in
Patient Samples
Gradient of
EPHB4
Expression in
Patient Samples
Patient sample numbers

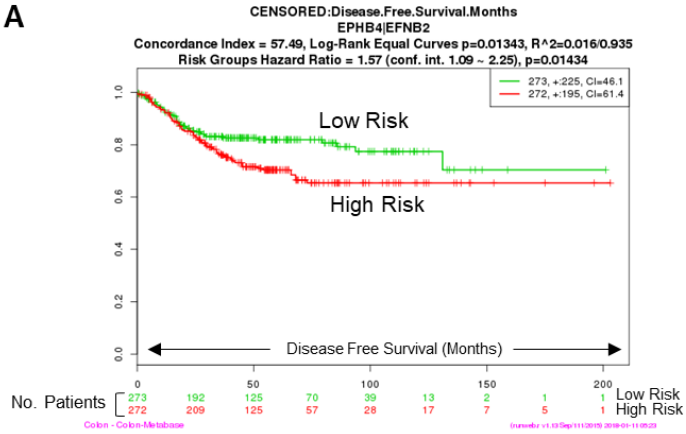

C
Gene Expression By Risk Group
Low Risk
High Risk
Gene Expression Level
8
10
12
14
EPHB4 Expression
P=1.96e-01
EPHRINB2 Expression
P=1.06e-99

5.3.4 *EPHB4* and *EPHRINB2* expression correlates with higher incidence of CRC recurrence rates in patients

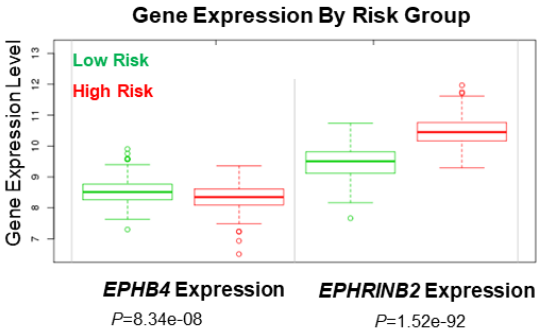
Next, the influence of *EPHB4*/*EPHRINB2* gene expression on disease free survival and recurrence was investigated. No significant difference in disease recurrence and survival was observed within most of the available databases except for two. In the first database, Colon-Metabase, the high risk group significantly overexpresses *EPHRINB2* and has higher incidence of disease recurrence (P<0.01) (**Figure 5.7A, B**). In contrast, *EPHB4* expression level is significantly higher in the low risk group (P=8.34e-08) and these individuals exhibit longer disease-free survival rates (**Figure 5.7A, B**). The other database, Sveen, assayed 160 samples and the high risk group had a significantly lower rate of disease free survival than those of low risk patients (P=0.04) (**Figure 5.7C**). The high risk group once again had greater level of *EPHRINB2* expression (P=5.15e-02) (**Figure 5.7D**). However, in contrast to previous gene expression data, this group had even higher and significant levels of *EPHB4* (P= 4.34e-22) (**Figure 5.7D**).

Figure 5.7. Impact of *EPHB4* and *EPHRINB2* gene expression on disease free survival of CRC patients. A. Colon-Metabase Cox survival analysis of patients categorised as high and low risk groups. **B.** Colon-Metabase gene expression levels of *EPHB4* and *EPHRINB2* by risk group. **C.** Sveen database Cox survival analysis of patients categorised as high and low risk groups. **D.** Sveen database gene expression levels of *EPHB4* and *EPHRINB2* by risk group.

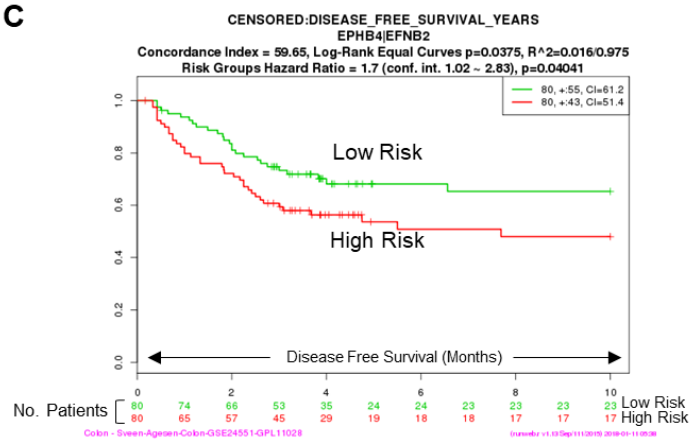
A


CENSORED:Disease.Free.Survival.Months
EPHB4|EFNB2
Concordance Index = 57.49, Log-Rank Equal Curves p=0.01343, R^2=0.016/0.935
Risk Groups Hazard Ratio = 1.57 (conf. int. 1.09 ~ 2.25), p=0.01434
273, +:225, CI=46.1
272, +:195, CI=61.4
Low Risk
High Risk
Disease Free Survival (Months)
No. Patients
273 192 125 70 39 13 2 1 1 Low Risk
272 209 125 57 28 17 7 5 1 High Risk

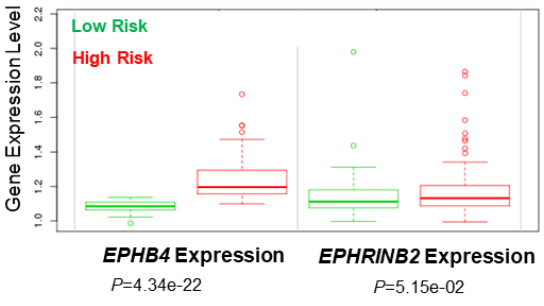
B


Gene Expression By Risk Group
Low Risk
High Risk
Gene Expression Level
EPHB4 Expression
P=8.34e-08
EPHRINB2 Expression
P=1.52e-92

C


CENSORED:DISEASE_FREE_SURVIVAL_YEARS
EPHB4|EFNB2
Concordance Index = 59.65, Log-Rank Equal Curves p=0.0375, R^2=0.016/0.975
Risk Groups Hazard Ratio = 1.7 (conf. int. 1.02 ~ 2.83), p=0.04041
80, +:55, CI=61.2
80, +:43, CI=51.4
Low Risk
High Risk
Disease Free Survival (Months)
No. Patients
80 74 66 53 35 24 24 23 23 23 23 Low Risk
80 65 57 45 29 19 18 18 17 17 17 High Risk

D


Gene Expression By Risk Group
Low Risk
High Risk
Gene Expression Level
EPHB4 Expression
P=4.34e-22
EPHRINB2 Expression
P=5.15e-02

5.4 Discussion

5.4.1 Animal models of CRC

5.4.1.1 Orthotopic model of CRC and its challenges

The step-wise progression of CRC in patients, beginning in the colon to its eventual spread to the liver, is emulated in the orthotopic murine models of CRC (Mittal et al. 2015). In this model the cells are implanted into the caecal wall of mice, successful injections are characterised by the formation of fluid filled sac termed bulla. Lack of bulla formation is indicative of the injection having leaked into the lumen or the abdomen. These cells then grow and can metastasize to the clinically relevant site of metastasis, which is the primarily to the liver. Using the CT26 mouse CRC cell line in Balb/c mice, we have obtained high tumour yield. However, despite several approaches in optimising this model using the human cancer cell line SW480 and LIM2405, the level of tumour burden and rate of metastasis was disappointing. Rate of tumour incidence and metastasis was shown to vary amongst CRC cell lines (Donigan et al 2009; Flatmark et al. 2004). It became evidentially obvious that the human cell lines used in our study may not be suitable for metastatic CRC models. There may have been several factors affecting the rate of growth of this cell line in the murine cecum including a short experimental timeline, low tumorigenicity of the cells, the medium used to inject cells and cell number.

Initially, the SW480 cell line was used for implantation into SCID mice. In early phase of pilot studies, cells were suspended in phosphate buffered saline (PBS) for injection. This medium is commonly used for this type of surgery (Liao et al. 2017; Tseng et al. 2007). However, upon injection cells dissipated making it difficult to

judge if the injection was successful and increasing the risk for leakage. The animals failed to grow any tumours, as PBS as a medium may have been inadequate to support the initial growth and colonisation phase of the injected cancer cells. For this reason, we sought to find a better alternative medium in which cells survived for several hours. We obtained reduced growth factor Matrigel (Corning), which becomes a gel at room temperature and a viscous solution at 4C°. The cells were pelleted, and all media removed, then resuspended in cold Matrigel. This suspension was kept on ice until injection. In *in vitro*, we have observed that cells suspended within this medium maintained 97% viability beyond 5 hours, which is adequate time to complete surgeries (data not shown). When injected, and exposed to body temperature, Matrigel suspension becomes a gel, keeping all the cells together making it easier to see a bulla and providing the right environment for cell growth. Despite this substitute, tumour yield using the SW480 cell line was very low. The number of injected cells ($1x10^6$) may have also influenced tumour growth. In some other studies, where orthotopic surgeries had been attempted, cell numbers greater than $1x10^6$ were used (Liao et al. 2017). However, a major limiting factor for this experiment was the experimental timeline, which was determined based on ethical regulations and housing costs. Our ethics dictates that the animal experiments need to end by day 40 of intervention. We have regularly failed to obtain adequate tumour growth and metastasis within this timeline. It may have been possible to apply for an extension from the ethics committee, however, the animal housing and maintenance costs made it increasingly difficult to fund these experiments over time.

Lack of tumour growth also raised speculation regarding the degree of immune suppression in the SCID mice. The SCID mice lack functional B and T cells,

however, they possess all the components of the innate immune system (Bankert et al. 2001; Heijstek et al. 2005). Ability to mount innate immune response is suggested to be a possible factor in human xenograft rejection and can contribute to the variable results observed between animals. Furthermore, there are reports of this strain becoming 'leaky' and acquiring a degree of functional lymphocytes particularly with age and with continual antigen exposure (Hinkley et al. 2002; Bosma et al. 1988). In housing and handling our animals, all necessary measures were taken to ensure minimal exposure to pathogens. Also, the onset of a leaky immune system in these mice typically occurs beyond 3 months of age (Hinkley et al. 2002; Bosma et al. 1988). Our mice were approximately 11 weeks (2.5 months) of age at the end of the experiments, for this reason the potential onset of a leaky immune system may not have affected the outcome of surgeries. Additionally, nude mice are also commonly used in the literature for murine models of cancer. However, the SCID strain mice withstand invasive surgery relatively better and the animal facility used in this study was not suitable for housing nude mice. Using a different strain of mouse may have altered percentage of tumour take.

Due to these difficulties, we also trialled the CT26 cell line in the orthotopic model, as these cells are syngeneic with Balb/c animals and grow more readily after implantation. Although we had high success obtaining tumours and metastasis using this cell line, it proved to be highly aggressive and it would rapidly seed all over the abdominal cavity. This is might be due to the leakage of cells from the injection site. In these experiments, since the main objective was to observe early metastasis in the liver originating from the cecum, we implemented strict criteria about abdominal spread resulting from potentially leaked cancer injection. The abdominal tumours would often attach to organs, such as the liver and kidneys,

from the surface and not invade through vessels hence making it clear that this was not a true metastatic lesion. During dissection these types of tumours would become easily detached. Furthermore, it would affect every organ in the abdominal cavity. This rapid onset of heavy tumour burden made it difficult to determine optimal culling time points for early metastatic progression. Whilst this type of heavy tumour burden may be seen in some CRC patients in very late stages of the disease, in this study we were particularly interested in the effect of EphB4 expression level on the initial stages of metastasis. For this reason, animals that did not grow tumours or animals with abdominal spread were excluded from analysis. Overall, while the orthotopic model appears to be the best representative model for imitating human CRC growth and progression, utilising it to study the rate of metastasis appears to be difficult without a well-established protocol and an *in vivo* imaging system. Imaging systems would help visualise the tumours and metastasis in live animals, rather than, having to conduct pilot experiments where animals are culled at certain intervals to determine optimal time points for metastatic progression. Often, as we have experienced, these times will vary in individual animals, requiring to increase number of animals used for consistency. The use of imaging systems that help visualise and tract the activity of luciferase tagged cancer cells are becoming more widespread, however, this was not available to us over the duration of this project.

Several studies have encountered similar hurdles and they have also tried to improve the surgical technique (Alamo et al. 2014; Zigmond et al. 2011). These studies aimed to reduce incidence of mortality amongst animals due to highly invasive surgery and to improve the accuracy of cancer cell implantation. Endoscopic imaging has been modified for its use in orthotopic surgeries (Zigmond et al. 2011). Initially, the colon is inflated and flushed, and the endoscopy tube was

inserted along with a longer hypodermic needle, which was used to inject CRC cells including CT26 and SW480 along several others. The number of cells used in this study were much lower ($1x10^5$ cells) than most other studies which could also help unwanted seeding of leaked cells. Tumour occurrence with this method is reported to be 100% in survived animals even with the SW480 cell line. Despite this the CRC cell lines used failed to produce metastatic lesions (Zigmond et al. 2011). Another CRC study also failed to produce metastatic progression in nude mice using the SW480 cell line, when injected directly into the cecum after *in vitro* culturing (Alamo et al. 2014). However, prior to orthotopic surgery, harvesting cells from subcutaneous xenografts is suggested to 'pre-condition' the cells for growth in the species, significantly improving rate of metastasis (Alamo et al. 2014). One possible limitation could be that these xenografts are introduced into different animals to the ones undergoing orthotopic implantation, which may alter cell characteristics and effect the reproducibility of molecular analysis. As such, it is suggested that the intestinal environment may not be suitable for cells to invade. In our experiments, the SW480 cell line grew tumours more frequently within the spleen compared to the colon, which could support the theory that the mouse colon microenvironment may be one of the factors hindering tumour establishment using this cell line. In breast cancer models, incidences and metastasis are observed when cancer cells are implanted in organs that are more compatible with the emerging tumour characteristics (Levy et al. 1982; White et al. 1982). An estrogen sensitive breast cancer cell line displayed highly aggressive and invasive behaviour, when it was implanted in organs that accumulated the hormone, such as uterus, brain and adipose tissue (Levy et al. 1982; White et al. 1982). Tumour growth and invasion was limited in the liver, lungs and subcutaneous grafts due to insufficient retention

of the hormone in these organs. Perhaps, the highly aggressive nature of CT26 cells and slow growing nature of SW480 cells could be explained by the differences in pro and anti-inflammatory states they induce in the colon microenvironment. The CT26 cell line has been characterised to secrete interleukin-6, which is suggested to increase the inflammatory mediators in caecal tumours, likely aiding metastasis downstream to the colon and Peyer's patches (Fisher et al. 2011; Miller et al. 2016). Inflammatory mediators and cells are observed to be higher in CRC patients and may be one of the driving forces in initiating and aiding progression of CRC (Szkaradkiewicz et al. 2009). On the other hand, the SW480 cell line was found to express interleukin-10, an anti-inflammatory cytokine *in vitro* (Miller et al 2016).

5.4.1.2 Intrasplenic model of CRC and its challenges

In a continued attempt to use a more relevant model of CRC than the subcutaneous xenografts (**Chapter 4**), we also tried to utilise an intrasplenic model of metastasis. Spleen is also a site of metastasis for colorectal cases and removing the spleen in metastatic disease is suggested to be beneficial for the longevity of patients (Fujita et al. 2000). In the intrasplenic model, the cancer cells are injected into the splenic capsule, where they grow and metastasize to the liver, through the splenic vein which connects to hepatic portal vein (Uy et al. 2017). The intrasplenic model is easier to perform than the orthotopic surgeries, although higher risk of complications due to haemorrhaging. Using this model we have obtained more tumours using the SW480 cell line, even though in less than 50% of the animals used and the tumours were very small at the experimental endpoint. These tumours may have eventually metastasised if allowed to grow well beyond the 40-day time restriction. We tried to increase number of implanted cells to $2x10^6$ to achieve

higher rate of growth and potentially metastasis. However, high number of cancer cells infiltrated the liver blocking portals within hours of surgery, which caused necrosis. This resulted in a great deal of adverse incident deaths soon after surgery. Higher number of cells may have been suitable for use in our experiments if the spleen was removed few minutes after surgery, as is done in some other studies (Burdelya et al. 2013; Oshima et al. 2016; Soares et al. 2014). Intrasplenic surgery resulting in premature deaths due to early liver infiltration has been previously reported (Lee et al. 2014). Like our study, Lee et al. (2014) did not remove the spleen after cancer cell injection. This is the likely cause for abundant liver infiltrate. Nonetheless, we then continued the study with the initial number of $1x10^6$ cells, using the LIM2405 cell line as a substitute for SW480. This cell line yielded splenic tumours in 60% of the animals used and only 33% of these animals had liver invasion. The tumours induced using EV tumours tended to be larger than B4 tumours and greater rate of metastasis was observed with EV constructs. One possible explanation behind the differential growth of tumours in the spleen may be a result of EphrinB2 and EphB4 expression in these tissues. EphrinB2 is expressed in the white and red pulps of the spleen and in some of the immune cell populations (Human Protein Atlas; Uhlén et al. 2015; Yu et al. 2003). On the other hand, EphB4 expression could not be confirmed in splenic tissues through RNA analysis, protein and immunostaining methods (Human Protein Atlas; Uhlén et al. 2015). However, it is weakly expressed in endothelial cells and some immune cells of the spleen. We have observed previously that EphrinB2-Fc stimulation, caused a large degree of cellular rounding and retraction (**Chapter 2**). Furthermore, this type of stimulation reduced cellular proliferation rates of EphB4 overexpressing cells *in vitro* (**Chapter 3**). For these reasons, when EphB4 overexpressing cells are introduced into the

spleen, they may undergo similar cellular effects of rounding and clustering resulting in smaller tumours. Using the HT29 cell line it was demonstrated that having two cell populations, expressing distinctly different levels of EphB4 and EphrinB2, can lead to separation and colonisation within the cell populations (**Chapter 2**). Immunohistochemical analysis using the anti-human antibody showed that EV tumours had greater amount of mouse cells compared to B4 tumours in the spleen. Similar observation was also made in subcutaneous experiments (**Chapter 4**). This suggests that cells with lower level of EphB4 expression may be able to intermix with EphrinB2 expressing splenic tissue, colonise and grow more readily. EphB4 overexpressing tumours may be experiencing growth restrictions imposed by EphrinB2 presence. This supports the theory that high EphB expressing tumours can be restricted and compartmentalised by EphrinB presence in surrounding tissue. In the small intestine of animals, the growing tumours were surrounded by a layer of stroma, which was reported to separate the EphB expressing tumour from EphrinB expressing villus cells (Cortina et al. 2007). One the other hand when tumours are introduced in the colon, the absence of villi was found to result in direct contact between cancer cells and normal EphrinB expressing epithelium and reduced tumour growth. To research extent of the restriction imposed on tumours by EphrinB expression, mice with reduced EphrinB expression in intestinal tract were engineered and crossed with *APC* mutant mice (Cortina et al. 2007). These tumours were not enclosed by normal mucosa suggesting the Eph/Ephrin interaction induced repulsion and compartmentalisation of the tumours had been lost. As a result, these tumours were reported to grow at an accelerated rate (Cortina et al. 2007). Another colorectal cancer study, demonstrated that EphB4 could be detected in primary tumours,

whilst its expression reduced in progressive stages of the disease (Batlle et al. 2005). The eventual silencing of EphB receptors are proposed to lift restrictive compartmentalisation occurring in the colonic crypts which also express EphrinB ligands. As such, when EphB4 overexpressing tumours are introduced in EphrinB2 expressing tissue like the spleen, this may have a limiting effect on tumour growth. These observations will need to be confirmed through more experimental repeats and perhaps targeting *in vivo* xenografts with soluble EphrinB2-Fc and observing the tumour growth trends. It is difficult conclude if the liver nodules are likely to be a result of legitimate metastatic lesions due to the low and inconsistent numbers. Overall, it was evident that due to the unrestricted drainage from splenic vein to the hepatic portal, this model appears to be a feasible representation of metastasis. In another study, cancer cell infiltration in the liver could be detected within 24 hours post-surgery (Hackl et al. 2013). This study also reported difficulty assessing metastasis using the intrasplenic model. We have also observed this with our adverse incidences of death, where within hours upon injection, perhaps due to the injected volume increasing pressure in the organ, cancer cells gain access to the liver through portal circulation. Despite our best attempts to try and investigate the influence of EphB4 expression on rate of metastasis, we have failed to obtain quantifiable results from these experiments. Nonetheless, results obtained from animal experiments can be different to what occurs in humans. For this reason, we have investigated prognostic relevance of *EPHB4* expression in patient samples using the online web tool Survexpress.

5.4.2 *EPHB4* and *EPHRINB2* gene expression as cancer biomarkers in predicting patient survival and cancer recurrence

Survexpress provides predictions about patient survival based on gene biomarker inputs (Aguirre-Gamboa et al. 2013). There are several advantages to using this tool, as it provides databases from wide variety of cancers from diverse countries and sources. Each colon database contains greater than 30 samples and besides Cox survival analysis, the grade/stage, recurrence and metastasis status for each patient are also provided in some datasets. The patients are categorised as low or high risk depending on their prognostic index, which is derived according to their clinical pathology scores.

There are very limited number of studies and databases that report on EPHB4 and its potential as a prognostic marker in CRC patients; we have analysed all CRC databases available on Survexpress. This investigation showed that, *EPHB4* expression tends to be significantly different between low and high risk groups in each dataset. However, the risk group displaying the highest level of *EPHB4* expression alternated according to the database that was examined. In some databases low risk individuals expressed greater level of *EPHB4*, while in other databases its expression was dominant in the high risk group. There could be several contributing factors to these inconsistencies, such as sample size differences, dissimilarities between tissue handling, alterations in RNA extraction and analysis as well as the protocols also level of patient detail used to determine prognosis index. Regardless, when the parameters are defined as two risk groups, being low and high, no significant differences are observed in the rate of survival amongst patients in relation to *EPHB4* expression profiles. However, when parameters are

changed to comprise of three risks groups as low, medium and high risk an interesting trend in gene expression and survival emerges in some datasets. In two of the datasets, significant differences in patient survival can be observed with three risk groups. Both datasets show lowest expression of *EPHB4* in low risk groups with an increasing trend of expression toward high risk individuals, however, survival rates vary. In the Sveen dataset, the low risk individuals with low *EPHB4* expression, exhibit better survival. There is vast amount of evidence available in literature to suggest that, increasing *EPHB4* expression associates with poor outcome for cancer patients. This is consistent with the results of our experiments in **Chapter 4** demonstrating that it enhances tumour viability, growth and vascularisation. *EPHB4* gene expression was found to be higher in gastric tumours in comparison to matched normal tissue (Li et al. 2011). Its protein level correlated positively with the size of tumour and regional lymph node spread. Consequently, high *EPHB4* levels were found to be linked with reduced disease free and overall survival rates in gastric cancer (Yin et al. 2017). *EPHB4* overexpression was found to be one of the stronger predictors of poor outcome for ovarian cancer patients (Kumar et al. 2007). Its expression was significantly greater in invasive and late stage of the disease. Similarly, in oesophageal cancer samples, *EPHB4* gene copy numbers were increased along with protein expression in patients with higher grade and aggressive tumours (Hasina et al. 2013). While for prostate cancer, *EPHB4* mRNA was detected more frequently in tumours, rather than normal match tissue (Xia et al. 2005).

Interestingly, in TCGA database the greatest rate of survival was in the medium risk group, which expresses moderate levels of *EPHB4*. According to this trend, both low and high-level expression have a similarly negative impact on survival.

Although limited in numbers, there are some studies that find *EPHB4* to be associated with prolonged survival and it is suggested to be silenced in metastatic lesions. Previously, patient samples with low *EPHB4* immunostaining were found to have reduced disease-free survival, with median recurrence rates occurring at around 1.8 years (Davalos et al. 2006). This rate was more than 9 years in patients with high level of *EPHB4* detection. Batlle and associates (2005) also suggest that *EPHB4* levels are higher in primary tumour tissues and reduced in metastatic lesions. It is suggested to be silenced like other EphB receptors in progressive disease to remove EphrinB imposed restriction on tumour growth and spread. In breast cancer, a significantly lower amount of *EPHB4* was detected in invasive carcinoma (Berclaz et al. 2002). *EPHB4* detection appeared to be exclusive to normal breast tissue and low-grade tumours. The TCGA dataset appears to represent both sides of the argument, however, several considerations must be made before a conclusion is drawn from the survival data. Firstly, in this dataset by the end of the provisional period, a data is available for 6 people out of the initial 466 individuals. Also, at 3 years (1000 days), the rate of decline in survival appear to be similar between low and medium risk groups. The strength TCGA database has over Sveen database is in the number of individuals within each risk group. However, at the 4 year (1500 days) mark the number of patients being monitored in TCGA is similar to the numbers in the Sveen database. Therefore, the advantage of large numbers used is diminished beyond this point. For these reasons, the TCGA database would be most appropriately used to interpret short-term survival. Since the Sveen database patients experience a more stable survival rate, the number of patients monitored for a longer duration of time appears to be better indicative in terms of long-term survival. Also, in these samples, the difference of *EPHB4*

expression between the low risk and high risk groups are larger than in the levels detected in the TCGA samples. Due to these limiting factors in the datasets, it is difficult to conclude if *EPHB4* expression alone will have a long lasting and important impact on CRC patient survival. However, as previously mentioned, EphB4 receptor binds to and is activated by the EphrinB2 ligand. Literature surrounding their interaction suggests that it can aid cancer progression and impact survival of patients. Since *EPHB4* gene expression alone did not appear to be an important biomarker in impacting CRC patient outcome, its expression in conjunction with *EPHRINB2* was examined using low and high risk groups.

Three databases returned significantly different survival rates amongst low and high risk groups. *EPHB4* expression was indifferent between the risk groups in all but one dataset, where it was elevated in high risk group. On the other hand, *EPHRINB2* expression was enhanced consistently in the high risk individuals and they had significantly lower rate of survival. In combination these datasets make up a decent sample size and the monitoring periods range between 5-16 years allowing for interpretation of short-term and long-term implications of *EPHB4* and *EPHRINB2* expression on patient survival. Overall, it appears that *EPHRINB2* expression could be a stronger predictive biomarker for CRC patient survival. In oesophageal squamous cell carcinoma, *EPHB4* mRNA levels were found in greater number of samples compared to *EPHRINB2* (Tachibana et al. 2007). However, *EPHRINB2* expression was found to associate with number of lymph nodes involved and also with increasing stage of the disease. Patients overexpressing *EPHRINB2* had reduced 5-year survival. Therefore, as we have found with CRC, it is suggested to be a better prognostic indicator for overall survival in this type of cancer. In glioblastomas, *EPHB4* and *EPHRINB2* were found to be independent prognostic

markers of prognosis, with both negatively affecting outcome of patients (Tu et al. 2012). Nonetheless, as is seen within the databases we have investigated, some patients with low *EPHB4* and *EPHRINB2* expression can also reach the event. Similar findings were reported for late stage head and neck squamous cell carcinoma patient samples. Where, upregulated expression of both the receptor and ligand is suggested to be a significant predictor for overall survival (Yavrouian et al. 2008). Nevertheless, some patients with low levels of *EPHB4* and *EPHRINB2* expression also did not survive. Even though *EPHB4* and *EPHRINB2* expression was more prevalent in tumours, than matched normal tissue, surrounding mucosa in these tissues also showed high levels of expression (Yavrouian et al. 2008). Hence, when expression of tumour tissue was corrected against the mucosa during analysis, *EPHB4* and *EPHRINB2* levels appeared to be lower. The authors suggest this may be tumour influencing the level of protein expression in surrounding mucosa to match its own. Thus, according to the databases examined the expression of *EPHRINB2* is more consistently elevated in high risk patients who tend to have reduced rate of overall survival. For this reason, it appears to be a stronger candidate for predicting patient survival than *EPHB4*.

5.4.3 *EPHB4* expression level varies in predicting disease free survival outcomes for CRC patients

Next, we examined how *EPHB4* and *EPHRINB2* expression impacts disease free survival. Two databases showed low risk individuals had significantly better disease free survival rates than high risk individuals. However, in Colon-Metabase, *EPHB4* expression was found to be significantly lower in the high risk group, which have greater expression of *EPHRINB2*. While in Sveen database, *EPHB4* is

significantly higher in the high risk individuals, more so than *EPHRINB2* expression, which is marginally significant in the same group. There are several notable differences between the two sources which may have led to observed discrepancies. Firstly, Colon-Metabase dataset is bigger with low risk and high risk groups having 273 and 272 patients respectively. In contrast there are only 80 patients designated for each group in the Sveen database. Secondly, the Colon-Metabase has detailed patient information including tumour localisation, lymph node spread, gender, age, grade and also comprehensive information on the gene extraction and analysis protocol. On the other hand, Sveen classifies samples only as 'colorectal cancer biopsy' without mentioning of tissue origin; other information available only states tumours stage and microsatellite instability status. The protocol listed is also much less detailed in Sveen database. Greater level of detail regarding patient information as seen in TCGA database, may lead to more accurate categorisation of individuals and better prediction for survival based on biomarkers of interest. The monitoring periods are also different, in Colon-Metabase it is approximately 16 years and in Sveen it is 10 years. However, this is unlikely to make a difference as only few patients remain in Colon-Metabase beyond the 10-year mark (125 months), which roughly equates to the period of surveying time in Sveen database. Overall, the degree of significance achieved in expression, suggests that both of these genes may be independently strong predictors for disease survival. This is also supported in the literature, where *EPHRINB2* expression was shown to associate with rate of disease recurrence and aggressiveness in ovarian tumours (Castellvi et al. 2006). High grade ovarian tumours had higher level of *EPHRINB2*, while low grade tumours and normal tissue had little positivity for it. Increase mRNA of *EPHB4* and *EPHRINB2* correlated with the stage of uterine cervical

cancers (Alam et al. 2009). Survival rates amongst patients were analysed separately for *EPHB4* and *EPHRINB2*. While reduced survival rates were observed in patients overexpressing *EPHB4*, the patients highly expressing *EPHRINB2* had poorer outcomes. Similar pattern of expression of the receptor and ligand were observed in dedifferentiated invasive tumours of endometrial cancers and ovarian cancers (Alam et al. 2007; 2008).

5.5 Conclusion

In this study, we have tried to investigate the influence of EphB4 overexpression on the rate of metastasis. For this we have conducted two types of metastatic models of CRC, the orthotopic and intrasplenic. Due to various limitations we were unable to obtain conclusive evidence from these experiments.

To explore the effect of *EPHB4* expression on patient outcome, we have conducted online analysis of various colon databases. As a result, we have found that *EPHB4* alone is unlikely to significantly impact overall patient survival, as trends in gene expression vary greatly between different datasets. On the other hand, *EPHRINB2* yield more definitive result, showing that it is greatly expressed in high risk individuals, who have reduced rate of survival. The expression between *EPHB4* and *EPHRINB2* often did not correlate in datasets that were examined in this study.

For the prediction of disease free survival, it appears that both *EPHB4* and *EPHRINB2* can have significant impact on the rate of recurrence. Individuals in the high risk groups that expressed greater levels of either one of these genes had higher incidences of tumour recurrence and therefore reduced disease-free survival rates.

CHAPTER 6

General Discussion and Conclusions

6.1 General Comments

In this study, we have engineered EphB4 overexpressing and complete EphB4 knockout clones in several CRC cell lines. In these cells, EphB4 was shown to localise in the cell membrane and upon stimulation with EphrinB2, it becomes predominantly localised in and around the nucleus. EphB4 overexpression in CRC cells and tumours enhanced the rate of proliferation and growth, while knocking out this receptor had the opposite effect. The level of EphB4 expression can affect tumour composition, as overexpression enhanced vascular density in xenografts and produced viable tumours densely packed with human cancer cells. Tumour compartmentalisation and restricted growth and spread may occur in EphB4 overexpressing cells *in vivo*. Using a metastatic model of CRC, we have observed that local EphrinB2 expression in the spleen may have affected growth of EphB4 overexpressing tumours. This is similar to *in vitro* results, where stimulation using ligand Fc caused cellular rounding, clustering and restricted proliferation. Mixing cell populations with distinct EphB4 and EphrinB2 expression also lead to further evidence of possible tumour cell compartmentalisation. Furthermore, *EPHB4* and *EPHRINB2* expression were found to be possible biomarkers in predicting patient survival and disease recurrence.

6.1.1 EphB4 receptor stimulation causes cell rounding, retraction and EphB4 localisation in the nucleus

The effect of EphB4 overexpression on different cancer types have been previously studied. Controversy has arisen concerning the expression of EphB4 receptors in CRC. Some studies suggest that EphB4 is upregulated in late and metastatic stages

of CRC (Kumar et al. 2009; Stephenson et al. 2001), while others argue that EphB4 expression is often silenced (Batlle et al. 2005; Davalos et al. 2006; Dopeso et al. 2009). It has been reported that high EphB4 expression enhances migratory abilities in CRC cells, leading to increase rate of metastasis (Kumar et al. 2009). In opposing findings, it has been stated that EphB4 expression in CRC may restrict tumour expansion and dissemination (Batlle et al. 2005; Davalos et al. 2006). One possible reason behind the controversial findings may be that EphB4 high expression or silencing may have different outcomes for cells with diverse features. As an example, in some breast cancer studies, EphB4 activation was found to inhibit cell and tumour growth (Barneh et al. 2013; Noren et al. 2006). Whereas in another study, EphB4 stimulation was proposed to benefit Akt mediated survival (Kumar et al. 2006). Amongst other factors, the set of cell lines used in these studies were different. In this study, we have chosen three cell lines that have different morphological and mutational characteristics to create EphB4 overexpressing cell lines. Previous CRC studies have employed various methods to downregulate the expression of EphB4 (Kumar et al. 2009; Lv et al. 2016). Preliminary investigation is our lab revealed that it was difficult to achieve significant level of knockdown using siRNA. As such, we have targeted the expression of EphB4 using the CRISPR-Cas9 system, to achieve complete knockout constructs at the gene level. To the best of our knowledge, this is the first time this gene editing tool has been used to target EphB4 expression in a CRC study. This system is being continually modified to improve the specific targeting of genes and reduce off-target events, at the time of application we chose the best available CRISPR-Cas9 system (Mali et al. 2013). This double strand break system has been criticized previously, as the short guide-RNA sequence makes it likely for the same sequence being found

elsewhere in the genome (Cribbs et al. 2017). The original CRISPR-Cas9 introduces a double stranded break at the recognition site (Addgene 2017). The difference between the original Cas9 and the one used in this study is that, the Cas9 molecule is modified to only introduce a nick on one strand of the DNA, instead of causing a double strand break (**Chapter 2**), hence increasing the risk of off-target effects (Addgene 2017). At the target DNA site, having two Cas9 molecules causes "nicks" that are treated as a double-strand break, whereas, single stranded nicks introduced at other sites are easily repaired, thereby reducing off-target effects. The two nicks produced by Cas9 molecules at the target site are repaired through non-homologous end joining or homology directed repair mechanism, which introduce mutations that silence the gene (Hsu et al. 2014). Using this method, we have obtained two knockout constructs that are absent for protein expression of EphB4. Morphological studies showed that altering EphB4 expression in both overexpressing and knockout constructs did not severely impact observable cell characteristics or viability. This is in contrary to reports that suggest knockdown of EphB4 negatively effects cancer cell survival (Ferguson et al. 2013; Kumar et al. 2006; Merchant et al. 2017). This may be because the electroporation method we use to transfect our cells results in a large degree of cell death, and then cells are placed under constant antibiotic selection. For this reason, any immediate effects on cell survival may not be noticeable.

We have also demonstrated the type of influence EphrinB2 stimulation has on transfected and control cells. Addition of soluble EphrinB2-Fc into cultures, caused large degree of cellular retraction and rounding (**Chapter 2**). This effect was much more pronounced in the EphB4 overexpressing cells. This type of repulsive response observed in the cells is in line with one of the most well documented

consequence of Eph and Ephrin interaction (Lisabeth et al. 2013; Park et al. 2015; Perez-White et al. 2014). Cellular repulsion is mediated through Eph receptor forward signalling upon ligand stimulation (Zimmer et al. 2003; Pasquale 2008). As an example, EphB2 receptor stimulation was found to cause cellular retraction of HeLa cells (Zimmer et al. 2003). Similarly, repulsive interaction between EphB4 and EphrinB2 is suggested to moderate arterial and venous lineage differentiation during angiogenesis (Füller et al. 2003). Additionally to these observations, we have also validated that prior to stimulation, EphB4 is largely localised on cell edges and near contact points between adjoining cells. When cells are stimulated with EphrinB2, with the collapse of cell projections, EphB4 becomes localised in and around the nucleus (**Chapter 2**). Nuclear localisation of EphB4 has also been found in prostate cancer cells (Mertens-Walker et al. 2015). This proposes that EphB4 may interact with nuclear or transcriptional factors after being internalised.

6.1.2 When unchallenged EphB4 expression positively influences proliferation rates *in vitro* and *in vivo*

Previously, downregulation of EphB4 expression was shown to reduce viable cell numbers (Kumar et al. 2009). Conversely, another study reported that reduced EphB4 receptor signalling increases the colony formation potential of CRC cell lines (Davalos et al. 2006). In our study, it was demonstrated across multiple CRC cell lines that level of EphB4 expression or knockout significantly impacts rate of cellular proliferation. In **Chapter 3**, we showed that high EphB4 expressing cells proliferate more rapidly than cells with lower expression. Furthermore, it was observed that complete silencing of EphB4 expression reduces cell division when compared to unmodified cells with endogenous EphB4 expression (**Chapter 3**). As

such, our results are in line with the finding that the level of EphB4 expression can be positively associated with the rate of proliferation in CRC cell lines. One downstream target of EphB4 mediating proliferation is likely through Erk1/2. This protein can act to regulate cell survival and proliferation through activation of transcriptional factors (Roskoski 2012). In our study, we found higher phosphorylated Erk in the overexpressing cells (**Chapter 2**). EphB4 activation enhancing cell proliferation has been reported for various cell types including human umbilical endothelial cell, melanoma, prostate, mammary epithelial cell lines and CRC cells (McCall et al. 2016; Rutkowski et al. 2012; Yang et al. 2010; Xiao et al. 2012). The trend of increased cell proliferation, was further supported by *in vivo* findings, where, subcutaneous tumours induced by cells overexpressing EphB4 also exhibited accelerated growth (**Chapter 4**). In addition to this, these tumours also had high degree of viability (**Chapter 4**). Low and knockout EphB4 expression tumours had reduced tissue integrity and growth (**Chapter 4**). These results indicate that EphB4 expression aids the survival of CRC cells and tumours.

However, in the metastatic models of cancer the opposite effect was observed; low EphB4 expressing tumours colonised the spleen and grew better than high EphB4 expressing tumours (**Chapter 5**). One possible explanation may be the presence of EphrinB2 expression in splenic tissue. Eph and Ephrin sequences are highly conserved between subtypes and across species (Bergemann et al. 1998; Flanagan et al. 1998; Himanen et al. 2010). Mouse and human EphrinB2 have high degree of sequence homology, for this reason EphrinB2 presence in the mouse spleen may be recognised and influence human cancer cells (Bossart et al. 2008). Prostate cancer cell secreting a type of protease was shown to cleave murine EphrinB2 more

efficiently than human (Lisle et al. 2015). Although in this study, functional relevance was not investigated in detail, the possibility of this cleaved ectodomain of EphrinB2 binding to and activating EphB4 in a similar manner to EphrinB2-Fc was mentioned. This is proposed to alter outcomes between mouse models of cancer in relation to human cases. We have demonstrated in **Chapter 2** that EphrinB2 stimulation results in cell rounding and clustering. This effect is more prominent in EphB4 overexpressing cells. In addition to this, in **Chapter 4**, we probed human cancer cells with an anti-human antibody to distinguish them from background host tissue. It was observed that high EphB4 expressing tumours grew in tightly packed masses of human cancer cells, with low level of mouse cells. In comparison, human tumour cells expressing low EphB4 levels are widely dispersed amongst host derived tissue. Therefore, implanting EphB4 overexpressing cells in an environment that expresses the ligand may be imposing restrictions on cancer cell growth and local spread. This phenomenon has been reported in CRC studies with other EphB receptors. Firstly, compartmentalization and segregation of cells in the colonic epithelium is achieved mostly with the counter gradient expression of EphB receptors and EphrinB ligands (Merlos-Suárez & Batlle 2008; Solanas et al. 2011). The intestinal stem cells occupy the bottom most compartment of the colonic crypts that give rise to transit amplifying cells, which, further differentiate into the mature epithelium of the colon. EphB receptor expression is largely confined in the proliferative stem cell niches in the bottom of the crypts. As cells differentiate they lose EphB receptor expression and begin to express EphrinB ligands, which is predominant in the mature epithelium. The repulsive interaction of EphB receptors and EphrinB ligands inhibit mature epithelium from migrating backward down the crypt, as such helping to maintain the integrity of the colonic cellular compartments

(Holmberg et al. 2006; Merlos-Suárez & Batlle 2008; Solanas et al. 2011). In mice colonic expression of EphB and EphrinB were found to be similar to that of human (Cortina et al. 2007). For this reason, when EphB expressing tumours were introduced in the intestines of mice, tumours were constrained, and growth was limited due to EphrinB imposed restriction. Animals engineered to have less EphrinB expression in the intestines, partially reduced this compartmentalisation effect on the EphB expressing tumours. Previous reports have shown that, when Ephs and Ephrins are highly expressed on opposing cells, cellular repulsion arising due to bidirectional signalling, does not allow for mixing between the cells (Mellitzer et al. 1999; Pasquale 2008; Zimmer et al. 2003). Since Ephs and Ephrins are expressed on the same cell surface, in different compartments, targeting the expression of either the receptor or the ligand using the CRISPR-Cas9 system, resulted in cell populations with distinct EphB4 and EphrinB2 expression (Bruckner et al. 1999; Chavent et al. 2016; Marquardt et al. 2005). When mixed together *in vitro*, cells with EphB4 expression segregate away from the EphrinB2 expressing population and colonised with cells with similar expression profiles **Chapter 2**. This mechanism of bidirectional signalling leading to cell segregation based on Eph and Ephrin expression has been most well characterised during embryonic development. As an example, EphrinB2 expressing somatic tissue is suggested to act as guidance cues for EphB4 expressing endothelial cells, which limits unnecessary intermixing between the two populations (Helbling et al. 2000). Overall, our findings suggest that EphB4 overexpression in CRC tumours can benefit growth and viability in the absence of EphrinB2. This conclusion is in line with the observations made in mammary tumour cells, where EphB4

overexpressing cell stimulation with EphrinB2 reduced proliferation, migration and invasion of cells (Rutkowski et al. 2012).

6.1.3 High EphB4 expression enhances cancer cell migration and invasion *in vitro*

One of the well-documented roles of the Eph receptors is to regulate cytoplasmic dynamics like cellular migration in physiological and pathophysiological conditions alike (Kullander et al. 2002). In developmental physiology, targeting the expression of EphB4 and EphrinB2 disrupts the migration of angioblasts, leading to impeded vessel formation (Herbert et al. 2009). In various types of cancer EphB4 expression is suggested to enhance migratory and invasive abilities of cells. In CRC studies, silencing of EphB receptors have been shown to produce more aggressive and invasive tumours (Batlle et al. 2005). It has been stated that EphB4 expression in CRC may restrict tumour expansion and dissemination (Batlle et al. 2005; Davalos et al. 2006). In contradicting evidence, high EphB4 expression has also been reported to aid the mobility of CRC cells, leading to increased rate of metastasis (Kumar et al. 2009). In these studies, methods and protocols used are slightly different, which may result in differing findings. In our study, we have used both end point and real time assays to confirm the type of impact EphB4 expression and knockout has on the migration and invasion of cancer cells. It was found that high EphB4 expression corresponds with greater responsiveness to chemotactic stimuli and, as a result, significantly improves migratory abilities of CRC cells (**Chapter 3**). We have found RhoA to be upregulated in EphB4 expressing cells, which may facilitate these outcomes (**Chapter 2**). EphB receptor prompted cell migration through Rac and Rho mediated reorganisation of the contractile cytoskeleton (Cowan et al. 2005; Gaitanos et al. 2016; Yoo et al. 2011).

Furthermore, EphB4 activation can initiate the formation of lamellipodia and filopodia in cells (Marston et al. 2003). Likewise, in **Chapter 3**, upregulated EphB4 expression also increases the number of invasive cells. In contrary, low EphB4 expressing cells perform poorly in these assays and knocking out EphB4 also negatively impacts the cells migratory and invasive capability. These results indicate that, EphB4 receptor expression could confer migratory and invasive advantage to cancer cells. However, due to a lack of adequate metastatic data in our study, it is not feasible for us to make assumptions regarding the role of this *in vitro* gain of function in altering disseminative potential *in vivo*. Nonetheless, enhanced EphB4 expression in latent stages of several cancers, such as uterine, lung and breast, have previously been observed (Alam et al. 2009; Kumar et al. 2006; Zhu et al. 2007).

6.1.4 High EphB4 expression leads to cancer cell aggregation, extensive vascularisation and reduces host derived stroma interaction in subcutaneous tumours

In **Chapter 4**, it was established that the level of EphB4 expression associated positively with vascular density of tumours. EphB4 overexpression consistently resulted in high density of blood vessels across all the cell lines used (**Chapter 4**). These tumours had prominent and well networked vasculature all through our tissue sections. The low expressing tumours had malformed, fragmented vessels which largely failed to form extensive networks. This was also evident in tumours induced by EphB4 knockout cells. The low expression and knockout tumours had poor tumour viability. Since this was consistent across all examined cell lines, a lack of vascular development and branching most likely accounts for reduced tumour

viability. EphB4 and EphrinB2 are particularly involved in angiogenesis in embryonic development. Interfering with EphB4 receptor expression during developmental stages leads to embryonic lethality due to defective heart development, inadequate branching and maturation of existing vessels (Gerety et al. 1999; Wang et al. 1998; Zhang et al. 2001). Furthermore, this receptor and its ligand are also implicated in postnatal angiogenesis of pathological conditions (Yang et al. 2016). Laying down vascular networks involves the breakdown of the extracellular matrix to enable endothelial cell branching (Kanda et al. 2003). One facilitator of vascularisation is the chemokine stromal cell-derived factor-1 (SDF-1), which induces the assembly of endothelial cells into tube formation (Kanda et al. 2003). EphB4 is proposed to enhance the migration of endothelial cell clusters in combination with this chemokine (Salvucci et al. 2006), hence prompting the establishment of a blood supply in tumours to support ongoing growth. This potentially increases the risk of metastatic spread through these portals (Heroult et al. 2010; Krasnoperov et al. 2010; Noren et al. 2007; Salvucci et al. 2006). In CRC, increase in tumour vascularization was proposed to be related to high expression of EphB4 and knockdown of this receptor resulted in inhibited tumour growth due to its anti-angiogenic effects (Kumar et al. 2009; Lv et al. 2016). Conversely, another CRC cell line showed that level of vascularisation was not affected by EphB4 expression (Dopeso et al. 2009). Our results indicate that EphB4 overexpression in multiple cell lines has a similarly significant contribution to tumour vascularisation (**Chapter 4**). Knocking out the receptor has deleterious effect on vessel formation. Previously, blocking EphB4 signalling in combination with DII4/Notch pathway was shown to reduce tumour size, due to inhibition in vessel maturation (Djokovic et al. 2010). In our experiments, low expressing cells also resulted in the generation

of smaller tumours. For these reasons, our results suggest that high EphB4 expression can mediate the rapid vascularisation in tumours, with continual branching and networking that adequately meets the demands of growing tumour tissue. Nonetheless, these observations were made only in subcutaneous xenografts, which grow with little restriction from other factors. The possibility of these results having an impact on primary tumours and metastatic potential of cells remains largely unsolved.

EphB receptor silencing with the progression of CRC to disseminative state of the disease have been demonstrated in various other studies (Clevers et al. 2006; Herath et al. 2012; Senior et al. 2010; Sheng et al. 2008). *EPHB* gene expression appears to be low in normal colon tissue and expression was elevated in adenomas and carcinomas (Rönsch et al. 2011). However, a subset of carcinomas can also show a clear reduction in *EPHB* expression, which are suggested to silenced for disease progression in some instances (Batlle et al. 2005; Rönsch et al. 2011). Previously, patients with low EphB4 expression in tumours were found to have increased disease recurrence and reduced disease-free survival than high expression patients (Davalos et al. 2006). Similarly, in breast cancer, *EPHB4* detection appeared to be exclusive to normal breast tissue and low-grade tumours (Berclaz et al. 2002). Nevertheless, there are more reports on *EPHB4* expression negatively impacting outcome of patients of various types of cancers such as gastric, ovarian and oesophageal cancers (Kumar et al. 2007; Li et al. 2011; Yin et al. 2017). From the databases we inspected (**Chapter 5**), it was established that *EPHB4* alone is likely not to be a good candidate for predicting patient survival, while high expression can possibly predict disease recurrence in some instances. When compared with *EPHB4*, *EPHRINB2* appears to be a much stronger predictor of overall patient

survival. Often EphB4 receptors influence in cancers is referred to as being 'context dependent'. Here we propose, that the 'context' may be largely dependent on the presence and level of EphrinB2 expression in the tumours and surrounding tissue.

6.2 Limitations and future directions

Initially in this project, attempts to use siRNA and other transient expression-based assays failed, due to the difficulties of transfecting the cell lines (using both electroporation and chemical methods), with efficiencies of less than 10% being typical. For this reason, we opted to use the CRISPR-Cas9 at later stages of the project. Due to the amount of time it takes to create constructs, knockouts of LIM2045, SW480 cells were not used in experiments. The CT26 cell line expresses very low levels of EphB4 inherently and knockout experiments may not be crucial for this cell line. Obtaining knockouts of all cell lines to determine the consistency of our results would add strength to the data presented in this study. Even though HT29 cell line expresses moderate levels of EphB4, obtaining overexpressing constructs would also provide a comparison for its effect in relation to knockout constructs.

There is evidence in this study for the ligand dependent and independent effects of EphB4 influence on cancer cells. Not all experiments could be repeated using EphrinB2-Fc due to budget constraints. The HT29 cell constructs, did not appear to be significant morphological changes upon EphrinB2-Fc treatment, however, long term treatment effects on cell proliferation need to be investigated. Additionally, to gain insight on the effects of EphrinB2-Fc on cellular functions other than cell proliferation, the migration and invasion assays have to be done with this EphrinB2-

Fc treatment. EphrinB2-Fc can be used *in vivo* models to observe if it has the same inhibitory effect on tumours, as it does with cells *in vitro*.

This study has provided basis for various functional implications of EphB4 receptor overexpression and knockout. We have also shown changes to some molecular targets, as determined by western blots, and changes in tumour composition and vascularisation. Further molecular studies investigating possible mechanisms underlying these changes may be useful in elucidating the role of EphB4 receptor expression.

Metastatic models will need to be optimised to fully determine the effect of EphB4 expression and knockout in disease progression. In line with this, patient samples need to be obtained to extrapolate data obtained in animal models to human state of the disease. An extensive investigation needs to be conducted into the role of EphB4 in clinical outcome of patients. The online database tool we used to investigate EphB4 and EphrinB2 as prognostic and predictive biomarkers of CRC, provides information on patient survival rate. Some databases do have grade, stage and MSI status, however, these numbers are not large or detailed enough for conclusive evidence. Surveying multiple and more informative databases will provide solid evidence of the type of influence EphB4 expressing tumours may have. As EphB4 alone is not likely to influence patient outcome, databases with more comprehensive detail about tumours may allow for more accurate predictions based on other genetic characteristics.

Despite showing poor viability in tumours, the mechanisms involved in the effects of EphB4 expression on cell and tumour survival should be further elucidated. In the future, apoptotic assays will need to be conducted *in vitro*. Markers of apoptosis,

necrosis and hypoxia can be used in xenografts to strengthen the argument that tumour viability is largely affected by lack of vascular development. Also, early harvesting of subcutaneous tumours will reveal if vascularisation has an early onset in EphB4 overexpressing tumours.

Our results revealed nuclear localisation of EphB4; the biological consequence of this needs to be determined. Screening of transcriptional factors affected by EphB4 expression might provide insight to this question.

6.3 Conclusion

This study was aimed at investigating the effects of induced upregulation in EphB4 receptors using vector systems that were transfected into the endogenously low EphB4 expressing human SW480, LIM2405 and mouse CT26 CRC cell lines. In addition to this, complete knockout constructs of the HT29 human CRC cell line were also created. Our results suggest that high EphB4 receptor expression significantly increases the rate of migration and invasion in CRC cells. Knockout of expression reduces these effects. Tumours induced by high EphB4 expressing cells grow more quickly, which is consistent with *in vitro* findings of enhanced proliferation rates. These tumours have better viability as a result of enhanced vascularisation. Tumour composition in these tumours also appear to be different, with high EphB4 expressing cells being densely packed with human cancer cells. Low and knockout tumours exhibit greater level of host tissue. When unchallenged, high EphB4 tumours can benefit tumour growth, survival and vascularisation. These effects may be reduced or inhibited with the presence of EphrinB2. Intermixing between cell populations with opposing EphB4 and EphrinB2

expression are limited and EphrinB2 stimulation reduces rate of proliferation. Evaluation of the effects of EphB4 and EphrinB2 in CRC provides evidence that EphrinB2 appears to be an important determinant of cell, tumour and patient outcome.

Chapter 7: References

Adams, R. H., Wilkinson, G. A., Weiss, C., Diella, F., Gale, N. W., Deutsch, U., . . . Klein, R. (1999). Roles of EphrinB Ligands and EphB Receptors in Cardiovascular Development: Demarcation of Arterial/Venous Domains, Vascular Morphogenesis, and Sprouting Angiogenesis. *Genes & Development, 13*, 295–306.

Agesen, T. H., Sveen, A., Merok, M. A., Lind, G. E., Nesbakken, A., Skotheim, R. I., & Lothe, R. A. (2012). ColoGuideEx: a robust gene classifier specific for stage II colorectal cancer prognosis. *Gut, 61*(11), 1560-1567. doi:10.1136/gutjnl-2011-301179

Aguirre-Gamboa, R., Gomez-Rueda, H., Martínez-Ledesma, E., Martínez-Torteya, A., Chacolla-Huaringa, R., Rodriguez-Barrientos, A., . . . Treviño, V. (2013). SurvExpress: An Online Biomarker Validation Tool and Database for Cancer Gene Expression Data Using Survival Analysis. *PLoS ONE, 8*(9), e74250. doi:10.1371/journal.pone.0074250

Aharon, R., Janes, P. W., Burgess, A. W., Hamza, K., Klebaner, F., & Lackmann, M. (2014). A Mathematical Model for Eph/Ephrin-Directed Segregation of Intermingled Cells. *PLoS ONE, 9*(12), e111803. doi:10.1371/journal.pone.0111803

Ahlstrom, H., Christofferson, R., & Lorelius, L. E. (1988). Vascularization of the continuous human colonic cancer cell line LS 174 T deposited subcutaneously in nude rats. *Apmis, 96*(8), 701-710.

Ahmed, D., Eide, P. W., Eilertsen, I. A., Danielsen, S. A., Eknaes, M., Hektoen, M., . . . Lothe, R. A. (2013). Epigenetic and genetic features of 24 colon cancer cell lines. *Oncogenesis, 2*, e71. doi:10.1038/oncsis.2013.35

Al-Ejeh, F., Offenhauser, C., Lim, Y. C., Stringer, B. W., Day, B. W., & Boyd, A. W. (2014). Eph family co-expression patterns define unique clusters predictive of cancer phenotype. *Growth Factors, 32*(6), 254-264. doi:10.3109/08977194.2014.984807

Alam, S. M., Fujimoto, J., Jahan, I., Sato, E., & Tamaya, T. (2007). Overexpression of ephrinB2 and EphB4 in tumor advancement of uterine endometrial cancers. *Annals of Oncology, 18*(3), 485-490. doi:10.1093/annonc/mdl414

Alam, S. M., Fujimoto, J., Jahan, I., Sato, E., & Tamaya, T. (2008). Coexpression of EphB4 and ephrinB2 in tumour advancement of ovarian cancers. *British Journal of Cancer, 98*(4), 845-851. doi:10.1038/sj.bjc.6604216

Alam, S. M., Fujimoto, J., Jahan, I., Sato, E., & Tamaya, T. (2009). Coexpression of EphB4 and ephrinB2 in tumor advancement of uterine cervical cancers. *Gynecologic Oncology, 114*(1), 84-88. doi:https://doi.org/10.1016/j.ygyno.2009.03.017

Alamo, P., Gallardo, A., Pavón, M. A., Casanova, I., Trias, M., Mangues, M. A., . . . Céspedes, M. V. (2014). Subcutaneous preconditioning increases invasion and metastatic dissemination in mouse colorectal cancer models. *Disease Models & Mechanisms, 7*(3), 387.

Anastas, J. N., & Moon, R. T. (2013). WNT Signalling Pathways as Therapeutic Targets in Cancer. *Nature Reviews Cancer, 13*(1), 11-26.

Arnold, M., Sierra, M. S., Laversanne, M., Soerjomataram, I., Jemal, A., & Bray, F. (2017). Global patterns and trends in colorectal cancer incidence and mortality. *Gut, 66*(4), 683-691. doi:10.1136/gutjnl-2015-310912

Arvanitis, D., & Davy, A. (2008). Eph/Ephrin Signaling: Networks. *Genes & Development, 22*, 416-429.

Astin, J. W., Batson, J., Kadir, S., Charlet, J., Persad, R. A., Gillatt, D., . . . Nobes, C. D. (2010). Competition amongst Eph receptors regulates contact inhibition of locomotion and invasiveness in prostate cancer cells. *Nat Cell Biol, 12*(12), 1194-1204. doi:10.1038/ncb2122

Atapattu, L., Saha, N., Llerena, C., Vail, M. E., Scott, A. M., Nikolov, D. B., . . . Janes, P. W. (2012). Antibodies binding the ADAM10 substrate recognition domain inhibit Eph function. *J Cell Sci, 125*(24), 6084.

Atienzar, F. A., Tilmant, K., Gerets, H. H., Toussaint, G., Speeckaert, S., Hanon, E., . . . Dhalluin, S. (2011). The use of real-time cell analyzer technology in drug discovery: defining optimal cell culture conditions and assay reproducibility with different adherent cellular models. *J Biomol Screen, 16*(6), 575-587. doi:10.1177/1087057111402825

Aune, D., Chan, D. S., Lau, R., Vieira, R., Greenwood, D. C., Kampman, E., & Norat, T. (2011). Dietary Fibre, Whole Grains, and Risk of Colorectal Cancer: Systematic Review and Dose-Response Meta- Analysis of Prospective Studies. *British Medical Journal, 343*(7833), 1082.

Australian Bureau of Statistics. (2015). *National Health Survey First Results; Australia 2014-2015*. Australia: Commonwealth of Australia.

Australian Government Department of Health and Aging. (2005). *Australia's Bowel Cancer Screening Pilot and Beyond: Final Evaluation Report*. Australia: Commonwealth of Australia.

Australian Institute of Health and Welfare. (2012a). *Cancer in Australia; An overview 2012*. Australia: Commonwealth of Australia.

Australian Institute of Health and Welfare. (2012b). *Cancer Survival and Prevalence in Australia: Period Estimates from 1982 to 2010*. Australia: Commonwealth of Australia.

Australian Institute of Health and Welfare. (2016). Australian Cancer Incidence and Mortality (ACIM) Books: Colon Cancer: Commonwealth of Australia.

Australian Institute of Health and Welfare. (2017). Australian Cancer Incidence and Mortality (ACIM) Books: Colorectal Cancer. from Australian Institute of Health and Welfare

Bagnardi, V., Rota, M., Botteri, E., Tramacere, I., Islami, F., Fedirko, V., & Scotti, L. (2015). Alcohol Consumption and Site-Specific Cancer Risk: a Comprehensive Dose-Response Meta-Analysis. *British Journal of Cancer, 112*, 580-593.

Bankert, R. B., Egilmez, N. K., & Hess, S. D. (2001). Human–SCID mouse chimeric models for the evaluation of anti-cancer therapies. *Trends in Immunology, 22*(7), 386-393. doi:https://doi.org/10.1016/S1471-4906(01)01943-3

Bardelle, C., Barlaam, B., Brooks, N., Coleman, T., Cross, D., Ducray, R., . . . Read, J. (2010). Inhibitors of the Tyrosine Kinase EphB4. Part 3: Identification of Non-Benzodioxole-Based Kinase Inhibitors. *Bioorganic & Medicinal Chemistry Letters, 20*(21), 6242-6245.

Bardelle, C., Coleman, T., Cross, D., Davenport, S., Kettle, J. G., Ko, E. J., . . . Williams, E. J. (2008). Inhibitors of the Tyrosine Kinase EphB4. Part 2: Structure-Based Discovery and Optimisation of 3,5-Bis Substituted Anilinopyrimidines. *Bioorganic & Medicinal Chemistry Letters, 18*(21), 5717-5721.

Bardelle, C., Cross, D., Davenport, S., Kettle, J. G., Ko, E. J., Leach, A. G., . . . Williams, E. J. (2008). Inhibitors of the tyrosine kinase EphB4. Part 1: Structure-based design and optimization of a series of 2,4-bis-anilinopyrimidines. *Bioorganic &*

Medicinal Chemistry Letters, 18(9), 2776-2780. doi:https://doi.org/10.1016/j.bmcl.2008.04.015

Barneh, F., Moshayedi, M., Mirmohammadsadeghi, H., Haghjooy-Javanmard, S., Sabzghabaee, A. M., & Badri, S. (2013). EphB4 Tyrosine Kinase Stimulation Inhibits Growth of MDA-MB-231 Breast Cancer Cells in a Dose and Time Dependent Manner. *Disease Markers, 35*(6), 6. doi:10.1155/2013/857895

Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., . . . Horvath, P. (2007). CRISPR provides acquired resistance against viruses in prokaryotes. *Science, 315*(5819), 1709-1712. doi:10.1126/science.1138140

Barrett, T., Wilhite, S. E., Ledoux, P., Evangelista, C., Kim, I. F., Tomashevsky, M., . . . Soboleva, A. (2013). NCBI GEO: archive for functional genomics data sets--update. *Nucleic Acids Res, 41*(Database issue), D991-995. doi:10.1093/nar/gks1193

Basu, S., Haase, G., & Ben-Ze'ev, A. (2016). Wnt Signaling in Cancer Stem Cells and Colon Cancer Metastasis. *F1000 Faculty Reviews, 5*(699), 1-10.

Batlle, E., Bacani, J., Begthe, H., Jonkeer, S., Gregorieff, A., van de Born, M., . . . Clevers, H. (2005). EphB Receptor Activity Suppresses Colorectal Cancer Progression. *Nature Letters, 435*, 1126-1130.

Batlle, E., Henderson, J. T., Beghtel, H., van den Born, M. M. W., Sancho, E., Huls, G., . . . Clevers, H. (2002). β-Catenin and TCF Mediate Cell Positioning in the Intestinal Epithelium by Controlling the Expression of EphB/EphrinB. *Cell, 111*, 251-263.

Batson, J., Astin, J. W., & Nobes, C. D. (2013). Regulation of contact inhibition of locomotion by Eph-ephrin signalling. *J Microsc, 251*(3), 232-241. doi:10.1111/jmi.12024

Becerikli, M., Merwart, B., Lam, M. C., Suppelna, P., Rittig, A., Mirmohammedsadegh, A., . . . Steinstraesser, L. (2015). EPHB4 tyrosine-kinase receptor expression and biological significance in soft tissue sarcoma. *Int J Cancer, 136*(8), 1781-1791. doi:10.1002/ijc.29244

Benton, G., Kleinman, H. K., George, J., & Arnaoutova, I. (2011). Multiple uses of basement membrane-like matrix (BME/Matrigel) in vitro and in vivo with cancer cells. *Int J Cancer, 128*(8), 1751-1757. doi:10.1002/ijc.25781

Berclaz, G., Flutsch, B., Altermatt, H. J., Rohrbach, V., Djonov, V., Ziemiecki, A., . . . Andres, A. C. (2002). Loss of EphB4 receptor tyrosine kinase protein expression during carcinogenesis of the human breast. *Oncol Rep, 9*(5), 985-989.

Bergemann, A. D., Zhang, L., Chiang, M.-K., Brambilla, R., Klein, R., & Flanagan, J. G. (1998). Ephrin-B3, a ligand for the receptor EphB3, expressed at the midline of the developing neural tube. *Oncogene, 16*, 471. doi:10.1038/sj.onc.1201557

Berridge, M. V., Herst, P. M., & Tan, A. S. (2005). Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. *Biotechnol Annu Rev, 11*, 127-152. doi:10.1016/s1387-2656(05)11004-7

Berridge, M. V., & Tan, A. S. (1998). Trans-plasma membrane electron transport: A cellular assay for NADH- and NADPH-oxidase based on extracellular, superoxide-mediated reduction of the sulfonated tetrazolium salt WST-1. *Protoplasma, 205*(1), 74-82. doi:10.1007/bf01279296

Bocharov, E. V., Mayzel, M. L., Volynsky, P. E., Mineev, K. S., Tkach, E. N., Ermolyuk, Y. S., . . . Arseniev, A. S. (2010). Left-Handed Dimer of EphA2 Transmembrane Domain: Helix Packing Diversity Among Receptor Tyrosine Kinases. *Biophysical Journal, 98*(5), 881-889.

Boland, R. C., & Goel, A. (2010). Microsatellite Instability in Colorectal Cancer. *Gastroenterology, 138*(6), 2073-2087.

Bong, Y. S., Lee, H. S., Carim-Todd, L., Mood, K., Nishanian, T. G., Tessarollo, L., & Daar, I. O. (2007). ephrinB1 signals from the cell surface to the nucleus by recruitment of STAT3. *Proc Natl Acad Sci U S A, 104*(44), 17305-17310. doi:10.1073/pnas.0702337104

Booth, D. G., Takagi, M., Sanchez-Pulido, L., Petfalski, E., Vargiu, G., Samejima, K., . . . Vagnarelli, P. (2014). Ki-67 is a PP1-interacting protein that organises the mitotic chromosome periphery. *Elife, 3*, e01641. doi:10.7554/eLife.01641

Bos, J. L., Rehmann, H., & Wittinghofer, A. (2007). GEFs and GAPs: critical elements in the control of small G proteins. *Cell, 129*(5), 865-877. doi:10.1016/j.cell.2007.05.018

Bosma, G. C., Fried, M., Custer, R. P., Carroll, A., Gibson, D. M., & Bosma, M. J. (1988). Evidence of functional lymphocytes in some (leaky) scid mice. *The Journal of Experimental Medicine, 167*(3), 1016.

Bossart, K. N., Tachedjian, M., McEachern, J. A., Crameri, G., Zhu, Z., Dimitrov, D. S., . . . Wang, L. F. (2008). Functional studies of host-specific ephrin-B ligands as Henipavirus receptors. *Virology, 372*(2), 357-371. doi:10.1016/j.virol.2007.11.011

Bowden, T. A., Aricescu, A. R., Nettleship, J. E., Siebold, C., Rahman-Huq, N., Owens, R. J., . . . Jones, E. Y. (2009). Structural Plasticity of Eph Receptor A4 Facilitates Cross-Class Ephrin Signaling. *Structure, 17*(10), 1386-1397. doi:10.1016/j.str.2009.07.018

Brantley-Sieders, D. M., Jiang, A., Sarma, K., Badu-Nkansah, A., Walter, D. L., Shyr, Y., & Chen, J. (2011). Eph/Ephrin Profiling in Human Breast Cancer Reveals Significant Associations between Expression Level and Clinical Outcome. *PLoS ONE, 6*(9), e24426. doi:10.1371/journal.pone.0024426

Brem, S., Brem, H., Folkman, J., Finkelstein, D., & Patz, A. (1976). Prolonged tumor dormancy by prevention of neovascularization in the vitreous. *Cancer Res, 36*(8), 2807-2812.

Bridger, J. M., Kill, I. R., & Lichter, P. (1998). Association of pKi-67 with satellite DNA of the human genome in early G1 cells. *Chromosome Research, 6*(1), 13-24. doi:10.1023/a:1009210206855

Brown, D. C., & Gatter, K. C. (2002). Ki67 protein: the immaculate deception? *Histopathology, 40*(1), 2-11.

Brown, J. R., DiGiovanna, M. P., Killelea, B., Lannin, D. R., & Rimm, D. L. (2014). Quantitative assessment Ki-67 score for prediction of response to neoadjuvant chemotherapy in breast cancer. *Lab Invest, 94*(1), 98-106. doi:10.1038/labinvest.2013.128

Bruckner, K., Labrador, J. P., Scheiffele, P., Herb, A., Seeburg, P. H., & Klein, R. (1999). EphrinB Ligands Recruit GRIP Family PDZ Adaptor Proteins into Raft Membrane Microdomains. *Neuron, 22*, 511-524.

Burdelya, L. G., Brackett, C. M., Kojouharov, B., Gitlin, I. I., Leonova, K. I., Gleiberman, A. S., . . . Gudkov, A. V. (2013). Central role of liver in anticancer and radioprotective activities of Toll-like receptor 5 agonist. *Proc Natl Acad Sci U S A, 110*(20), E1857-E1866. doi:10.1073/pnas.1222805110

Burgess, A. W., Faux, M. C., Layton, M. J., & Ramsay, R. G. (2011). Wnt signaling and colon tumorigenesis--a view from the periphery. *Exp Cell Res, 317*(19), 2748-2758. doi:10.1016/j.yexcr.2011.08.010

Burt, R. W., Leppert, M. F., Slattery, M. L., Samowitz, W. S., Spirio, L. N., Kerber, R. A., . . . White, R. L. (2004). Genetic Testing and Phenotype in a Large Kindred with Attenuated Familial Adenomatous Polyposis. *Gastroenterology, 127*(2), 444-451.

Bush, J. O., & Soriano, P. (2012). Eph/Ephrin Signalling: Genetic, Phosphoproteomic, and Transcriptomic Approaches. *Seminars in Cell & Developmental Biology, 23*, 26-34.

Buttke, T. M., McCubrey, J. A., & Owen, T. C. (1993). Use of an aqueous soluble tetrazolium/formazan assay to measure viability and proliferation of lymphokine-dependent cell lines. *J Immunol Methods, 157*(1-2), 233-240.

Cancer Council. (2015). *Understanding Bowel Cancer; A Guide for People with Cancer, Their Family and Friends*: Cancer Council Australia.

Carter, M., & Shieh, J. (2015). Chapter 10 - Molecular Cloning and Recombinant DNA Technology *Guide to Research Techniques in Neuroscience (Second Edition)* (pp. 219-237). San Diego: Academic Press.

Carvalho, R. F., Beutler, M., Marler, K. J., Knoll, B., Becker-Barroso, E., Heintzmann, R., . . . Drescher, U. (2006). Silencing of EphA3 through a cis interaction with ephrinA5. *Nat Neurosci, 9*(3), 322-330. doi:10.1038/nn1655

Castaño-Milla, C., Chaparro, M., & Gisbert, J. P. (2014). Systematic Review with Meta-Analysis: The Declining Risk of Colorectal Cancer in Ulcerative Colitis. *Alimentary Pharmacology & Therapeutics, 39*(7), 645-659.

Castellvi, J., Garcia, A., de la Torre, J., Hernandez, J., Gil, A., Xercavins, J., & Ramon y Cajal, S. (2006). Ephrin B expression in epithelial ovarian neoplasms correlates with tumor differentiation and angiogenesis. *Hum Pathol, 37*(7), 883-889. doi:10.1016/j.humpath.2006.02.021

Castle, J. C., Loewer, M., Boegel, S., de Graaf, J., Bender, C., Tadmor, A. D., . . . Sahin, U. (2014). Immunomic, genomic and transcriptomic characterization of CT26 colorectal carcinoma. *BMC Genomics, 15*(1), 190. doi:10.1186/1471-2164-15-190

Chan, D. S. M., Lau, R., Aune, D., Vieira, R., Greenwood, D. C., Kampman, E., & Norat, T. (2011). Red and Processed Meat and Colorectal Cancer Incidence: Meta-Analysis of Prospective Studies. *PLoS ONE, 6*(6), 20456.

Chavent, M., Seiradake, E., Jones, E. Y., & Sansom, M. S. P. (2016). Structures of the EphA2 Receptor at the Membrane: Role of Lipid Interactions. *Cell Press-Structure, 24*( ), 337-347.

Chen, T., Liu, X., Yi, S., Zhang, J., Ge, J., & Liu, Z. (2013). EphB4 is overexpressed in gliomas and promotes the growth of glioma cells. *Tumour Biol, 34*(1), 379-385. doi:10.1007/s13277-012-0560-7

Cheng, H., & Leblond, C. P. (1974). Origin, Differentiation and Renewal of the Four Main Epithelial Cell Types in the Mouse Small Intestine. *American Journal of Anatomy, 141*, 537-561.

Cheng, N., Brantley, D. N., & Chen, J. (2002). The Ephrins and Eph Receptors in Angiogenesis. *Cytokine & Growth Factor Reviews, 13*, 75-85.

Chiu, C. H., Lei, K. F., Yeh, W. L., Chen, P., Chan, Y. S., Hsu, K. Y., & Chen, A. C. (2017). Comparison between xCELLigence biosensor technology and conventional cell culture system for real-time monitoring human tenocytes proliferation and drugs cytotoxicity screening. *J Orthop Surg Res, 12*(1), 149. doi:10.1186/s13018-017-0652-6

Chrencik, J. E., Brooun, A., Kraus, M. L., Recht, M. I., Kolatkar, A. R., Han, G. W., . . . Kuhn, P. (2006). Structural and Biophysical Characterization of the EphB4EphrinB2 Protein-Protein Interaction and Receptor Specificity. *The Journal of Biological Chemistry, 281*(38), 28185–28192.

Chrencik, J. E., Brooun, A., Recht, M. I. K., Michelle, L., Koolpe, M., Kolatkar, A. R., . . . Kuhn, P. (2006). Thermodynamic Characterization of the EphB4/Ephrin-B2

Antagonist Peptide Complex Reveals the Determinants for Receptor Specificity. *Structure, 14*(2), 321-330.
Chylinski, K., Le Rhun, A., & Charpentier, E. (2013). The tracrRNA and Cas9 families of type II CRISPR-Cas immunity systems. *RNA Biol, 10*(5), 726-737. doi:10.4161/rna.24321
Clevers, H., & Batlle, E. (2006). EphB/EphrinB Receptors and Wnt Signalling in Colorectal Cancer. *American Association for Cancer Research, 66*(1), 1-5.
Colussi, D., Brandi, G., Bazzoli, F., & Ricciardiello, L. (2013). Molecular Pathways Involved in Colorectal Cancer: Implications for Disease Behavior and Prevention. *International Journal of Molecular Sciences, 14*(8), 16365-16385.
Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Habib, N., . . . Zhang, F. (2013). Multiplex genome engineering using CRISPR/Cas systems. *Science, 339*(6121), 819-823. doi:10.1126/science.1231143
Cortina, C., Palomo-Ponce, S., Iglesias, M., Fernandez-Masip, J. L., Vivancos, A., Whissell, G., . . . Batlle, E. (2007). EphB–Ephrin-B Interactions Suppress Colorectal Cancer Progression by Compartmentalizing Tumor Cells. *Nature Genetics, 39*(11), 1376-1383.
Cortina, C., Turon, G., Stork, D., Hernando-Momblona, X., Sevillano, M., Aguilera, M., . . . Batlle, E. (2017). A genome editing approach to study cancer stem cells in human tumors. *EMBO Mol Med, 9*(7), 869-879. doi:10.15252/emmm.201707550
Cowan, C. W., Shao, Y. R., Sahin, M., Shamah, S. M., Lin, M. Z., Greer, P. L., . . . Greenberg, M. E. (2005). Vav family GEFs link activated Ephs to endocytosis and axon guidance. *Neuron, 46*(2), 205-217. doi:10.1016/j.neuron.2005.03.019
Cribbs, A. P., & Perera, S. M. W. (2017). Science and Bioethics of CRISPR-Cas9 Gene Editing: An Analysis Towards Separating Facts and Fiction. *Yale J Biol Med, 90*(4), 625-634.
Dai, D., Huang, Q., Nussinov, R., & Ma, B. (2014). Promiscuous and Specific Recognition Among Ephrins and Eph Receptors. *Biochim Biophys Acta, 1844*(10), 1729-1740.
Das, A., Shergill, U., Thakur, L., Sinha, S., Urrutia, R., Mukhopadhyay, D., & Shah, V. H. (2010). Ephrin B2/EphB4 pathway in hepatic stellate cells stimulates Erk-dependent VEGF production and sinusoidal endothelial cell recruitment. *American Journal of Physiology - Gastrointestinal and Liver Physiology, 298*(6), G908-G915. doi:10.1152/ajpgi.00510.2009
Davalos, V., Dopeso, H., Castaño, J., Wilson AJ, Vilardell F, Romero-Gimenez, J., . . . Arango, D. (2006). EphB4 and Survival of Colorectal Cancer Patients. *American Association for Cancer Research, 66*, 8943-8948.
Davis, S., Gale, N. W., Aldrich, T. H., Maisonpierre, P. C., Lhotak, V., Pawson, T., . . . Yancopoulos, G. D. (1994). Ligands for EPH-related receptor tyrosine kinases that require membrane attachment or clustering for activity. *Science, 266*(5186), 816-819.
Davy, A., Bush, J. O., & Soriano, P. (2006). Inhibition of gap junction communication at ectopic Eph/ephrin boundaries underlies craniofrontonasal syndrome. *PLoS Biol, 4*(10), e315. doi:10.1371/journal.pbio.0040315
de Sousa, E. M. F., Vermeulen, L., Richel, D., & Medema, J. P. (2011). Targeting Wnt Signaling in Colon Cancer Stem Cells. *Clinical Cancer Research, 17*(4), 647.
del Solar, G., & Espinosa, M. (2000). Plasmid copy number control: an ever-growing story. *Mol Microbiol, 37*(3), 492-500.
Deltcheva, E., Chylinski, K., Sharma, C. M., Gonzales, K., Chao, Y., Pirzada, Z. A., . . . Charpentier, E. (2011). CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature, 471*(7340), 602-607. doi:10.1038/nature09886

Deveau, H., Garneau, J. E., & Moineau, S. (2010). CRISPR/Cas system and its role in phage-bacteria interactions. *Annu Rev Microbiol, 64*, 475-493. doi:10.1146/annurev.micro.112408.134123

Dimova, I., Hlushchuk, R., Makanya, A., Styp-Rekowska, B., Ceausu, A., Flueckiger, S., . . . Djonov, V. (2013). Inhibition of Notch signaling induces extensive intussusceptive neo-angiogenesis by recruitment of mononuclear cells. *Angiogenesis, 16*(4), 921-937. doi:10.1007/s10456-013-9366-5

Djokovic, D., Trindade, A., Gigante, J., Badenes, M., Silva, L., Liu, R., . . . Duarte, A. (2010). Combination of Dll4/Notch and Ephrin-B2/EphB4 targeted therapy is highly effective in disrupting tumor angiogenesis. *BMC Cancer, 10*, 641. doi:10.1186/1471-2407-10-641

Dodelet, V. C., & Pasquale, E. B. (2000). Eph Receptors and Ephrin Ligands: Embryogenesis to Tumorigenesis. *Oncogene, 19*, 5614-5619.

Doleman, J. F., Eady, J. J., Elliott, R. M., Foxall, R. J., Seers, J., Johnson, I. T., & Lund, E. K. (2010). Identification of the Eph receptor pathway as a novel target for eicosapentaenoic acid (EPA) modification of gene expression in human colon adenocarcinoma cells (HT-29). *Nutrition & Metabolism, 7*(1), 56. doi:10.1186/1743-7075-7-56

Döme, B., Hendrix, M. J. C., Paku, S., Tóvári, J., & Tímár, J. (2007). Alternative Vascularization Mechanisms in Cancer : Pathology and Therapeutic Implications. *The American Journal of Pathology, 170*(1), 1-15. doi:10.2353/ajpath.2007.060302

Donigan, M., Norcross, L. S., Aversa, J., Colon, J., Smith, J., Madero-Visbal, R., . . . Baker, C. H. (2009). Novel Murine Model for Colon Cancer: Non-Operative Trans-Anal Rectal Injection. *Journal of Surgical Research, 154*(2), 299-303. doi:https://doi.org/10.1016/j.jss.2008.05.028

Dopeso, H., Mateo-Lozano, S., Mazzolini, R., Rodrigues, P., Lagares-Tena, L., & Ceron, J. (2009). The Receptor Tyrosine Kinase EphB4 Has Tumour Suppressor Activities in Intestinal Tumorigenesis. *American Association for Cancer Research, 69*(18), 7430-7439.

Dow, L. E., O'Rourke, K. P., Simon, J., Tschaharganeh, D. F., van Es, J. H., Clevers, H., & Lowe, S. W. (2015). APC Restoration Promotes Cellular Differentiation and Reestablishes Crypt Homeostasis in Colorectal Cancer. *Cell, 161*(7), 1539-1552.

Dudanova, I., & Klein, R. (2011). The Axon's Balancing Act: cis- and trans-Interactions between Ephs and Ephrins. *Neuron, 71*(1), 1-3. doi:https://doi.org/10.1016/j.neuron.2011.06.030

Duggineni, S., Mitra, S., Noberini, R., Han, X., Lin, N., Xu, Y., . . . Huang, Z. (2013). Design, synthesis and characterization of novel small molecular inhibitors of ephrin-B2 binding to EphB4. *Biochem Pharmacol, 85*(4), 507-513. doi:10.1016/j.bcp.2012.12.005

Eccles, S. A., Box, C., & Court, W. (2005). Cell migration/invasion assays and their application in cancer drug discovery. *Biotechnol Annu Rev, 11*, 391-421. doi:10.1016/s1387-2656(05)11013-8

Edgar, R., Domrachev, M., & Lash, A. E. (2002). Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res, 30*(1), 207-210.

Egea, J., & Klein, R. (2007). Bidirectional Eph–Ephrin Signalling During Axon Guidance. *Trends in Cell Biology, 17*(5), 230-239.

Egea, J., Nissen, U. V., Dufour, A., Sahin, M., Greer, P., Kullander, K., . . . Klein, R. (2005). Regulation of EphA4 Kinase Activity is Required for a Subset of Axon Guidance

Decisions Suggesting a Key Role for Receptor Clustering in Eph Function. *Neuron, 47*, 515-528.
Eklund, L., Bry, M., & Alitalo, K. (2013). Mouse models for studying angiogenesis and lymphangiogenesis in cancer. *Mol Oncol, 7*(2), 259-282. doi:10.1016/j.molonc.2013.02.007
El-Brolosy, M. A., & Stainier, D. Y. R. (2017). Genetic compensation: A phenomenon in search of mechanisms. *PLOS Genetics, 13*(7), e1006780. doi:10.1371/journal.pgen.1006780
Emami, K. H., Nguyen, C., Ma, H., Kim, D. H., Jeong, K. W., Eguchi, M., . . . Kahn, M. (2004). A small molecule inhibitor of β-catenin/cyclic AMP response element-binding protein transcription. *Proc Natl Acad Sci U S A, 101*(34), 12682-12687. doi:10.1073/pnas.0404875101
Eph Nomenclature Committee. (1997). Unified Nomenclature for Eph Family Receptors and Their Ligands, the Ephrins. *Cell, 90*(3), 403-404.
Ewing, J. (1922). *Neoplastic diseases; a treatise on tumours*: Saunders.
Fagotto, F., Rohani, N., Touret, A. S., & Li, R. (2013). A Molecular Base for Cell Sorting at Embryonic Boundaries: Contact Inhibition of Cadherin Adhesion by Ephrin/Eph-Dependent Contractility. *Developmental Cell, 27*(1), 72-87.
Fanayan, S., Smith, J. T., Lee, L. Y., Yan, F., Snyder, M., Hancock, W. S., & Nice, E. (2013). Proteogenomic analysis of human colon carcinoma cell lines LIM1215, LIM1899, and LIM2405. *J Proteome Res, 12*(4), 1732-1742. doi:10.1021/pr3010869
Feitelson, M. A., Arzumanyan, A., Kulathinal, R. J., Blain, S. W., Holcombe, R. F., Mahajna, J., . . . Nowsheen, S. (2015). Sustained proliferation in cancer: Mechanisms and novel therapeutic targets. *Semin Cancer Biol, 35 Suppl*, S25-s54. doi:10.1016/j.semcancer.2015.02.006
Ferguson, B. D., Liu, R., Rolle, C. E., Tan, Y. H., Krasnoperov, V., Kanteti, R., . . . Salgia, R. (2013). The EphB4 receptor tyrosine kinase promotes lung cancer growth: a potential novel therapeutic target. *PLoS ONE, 8*(7), e67668. doi:10.1371/journal.pone.0067668
Ferlay, J., Soerjomataram, I., Ervik, M., Dikshit, R., Eser, S., Mathers, C., . . . Bray, F. (2013). GLOBOCAN 2013 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11. Retrieved 02-07-2016, from International Agency for Research on Cancer http://globocan.iarc.fr
Fisher, D. T., Chen, Q., Skitzki, J. J., Muhitch, J. B., Zhou, L., Appenheimer, M. M., . . . Evans, S. S. (2011). IL-6 trans-signaling licenses mouse and human tumor microvascular gateways for trafficking of cytotoxic T cells. *The Journal of Clinical Investigation, 121*(10), 3846-3859. doi:10.1172/JCI44952
Fisichella, M., Dabboue, H., Bhattacharyya, S., Saboungi, M. L., Salvetat, J. P., Hevor, T., & Guerin, M. (2009). Mesoporous silica nanoparticles enhance MTT formazan exocytosis in HeLa cells and astrocytes. *Toxicol In Vitro, 23*(4), 697-703. doi:10.1016/j.tiv.2009.02.007
Flamme, I., Frolich, T., & Risau, W. (1997). Molecular Mechanisms of Vasculogenesisand Embryonic Angiogenesis. *Journal of Cellular Physiology, 173*, 260-210.
Flanagan, J. G., & Vanderhaeghen, P. (1998). The ephrins and Eph receptors in neural development. *Annu Rev Neurosci, 21*, 309-345. doi:10.1146/annurev.neuro.21.1.309
Flatmark, K., Mælandsmo, G. M., Martinsen, M., Rasmussen, H., & Fodstad, Ø. (2004). Twelve colorectal cancer cell lines exhibit highly variable growth and metastatic capacities in an orthotopic model in nude mice. *European Journal of Cancer, 40*(10), 1593-1598. doi:https://doi.org/10.1016/j.ejca.2004.02.023

Folkman J, K. R. (2003). Beginning of angiogenesis research. In P. R. Kufe DW, Weichselbaum RR, et al., (Ed.), *Holland-Frei Cancer Medicine* (6 ed.). Hamilton: BC Decker.

Forster, J. C., Harriss-Phillips, W. M., Douglass, M. J., & Bezak, E. (2017). A review of the development of tumor vasculature and its effects on the tumor microenvironment. *Hypoxia (Auckl), 5*, 21-32. doi:10.2147/hp.s133231

Freeman, T. J., Smith, J. J., Chen, X., Washington, M. K., Roland, J. T., Means, A. L., . . . Beauchamp, R. D. (2012). Smad4-mediated signaling inhibits intestinal neoplasia by inhibiting expression of beta-catenin. *Gastroenterology, 142*(3), 562-571.e562. doi:10.1053/j.gastro.2011.11.026

Friedl, P., & Wolf, K. (2003). Tumour-cell invasion and migration: diversity and escape mechanisms. *Nat Rev Cancer, 3*(5), 362-374. doi:10.1038/nrc1075

Fu, Y., Sander, J. D., Reyon, D., Cascio, V. M., & Joung, J. K. (2014). Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. *Nature biotechnology, 32*(3), 279-284. doi:10.1038/nbt.2808

Fujita, N., Shirai, Y., Shimoda, S., Yamai, K., & Hatakeyama, K. (2000). Clinical significance of splenectomy for colorectal cancer metastases to the spleen. *International Journal of Clinical Oncology, 5*(2), 121-125. doi:10.1007/s101470050102

Fuller, T., Korff, T., Kilian, A., Dandekar, G., & Augustin, H. G. (2003). Forward EphB4 signaling in endothelial cells controls cellular repulsion and segregation from ephrinB2 positive cells. *J Cell Sci, 116*(Pt 12), 2461-2470. doi:10.1242/jcs.00426

Furukawa, K., Sato, T., Katsuno, T., Nakagawa, T., Noguchi, Y., Tokumasa, A., . . . Saito, Y. (2011). Smad3 Contributes to Positioning of Proliferating Cells in Colonic Crypts by Inducing EphB Receptor Protein Expression. *Biochemical and Biophysical Research Communications, 405*, 521-526.

Gaitanos, T. N., Koerner, J., & Klein, R. (2016). Tiam–Rac signaling mediates trans-endocytosis of ephrin receptor EphB2 and is important for cell repulsion. *The Journal of Cell Biology*.

Gale, N. W., Holland, S. J., Valenzuela, D. M., Flenniken, A., Pan, L., Ryan, T. E., . . . Yancopoulos, G. D. (1996). Eph Receptors And Ligands Comprise Two Major Specificity Subclasses And Are Reciprocally Compartmentalized During Embryogenesis. *Neuron, 17*(1), 9-19.

Gandomani, H., yousefi, S., Aghajani, M., Mohammadian-Hafshejani, A., Tarazoj, A., Pouyesh, V., & Salehiniya, H. (2017). Colorectal cancer in the world: incidence, mortality and risk factors. . *Biomedical Research and Therapy, 4*(10), 1656-1675.

Gao, Z., Guo, B., Gao, R., Zhu, Q., & Qin, H. (2015). Microbiota disbiosis is associated with colorectal cancer. *Front Microbiol, 6*, 20. doi:10.3389/fmicb.2015.00020

Gasiunas, G., Barrangou, R., Horvath, P., & Siksnys, V. (2012). Cas9–crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proceedings of the National Academy of Sciences, 109*(39), 15539.

Genander, M., & Frisen, J. (2010). Ephrins and Eph Receptors in Stem Cells and Cancer. *Current Opinion in Cell Biology, 22*, 611–616.

Georgakopoulos, A., Litterst, C., Ghersi, E., Baki, L., Xu, C., Serban, G., & Robakis, N. K. (2006). Metalloproteinase/Presenilin1 processing of ephrinB regulates EphB-induced Src phosphorylation and signaling. *Embo j, 25*(6), 1242-1252. doi:10.1038/sj.emboj.7601031

Gerety, S. S., Wang, H. U., Chen, Z. F., & Anderson, D. J. (1999). Symmetrical Mutant Phenotypes of the Receptor EphB4 and Its Specific Transmembrane Ligand Ephrin-B2 in Cardiovascular Development. *Molecular Cell, 4*, 403-414.

Goldgur, Y., Paavilainen, S., Nikolov, D., & Himanen, J. (2009). Structure of the Ligand-Binding Domain of the EphB2 Receptor of 2 Angstrom Resolution. doi:10.1107/S1744309108043078

Gong, J., Körner, R., Gaitanos, L., & Klein, R. (2016). Exosomes mediate cell contact–independent ephrin-Eph signaling during axon guidance. *The Journal of Cell Biology, 214*(1), 35.

Grivennikov, S. I. (2013). Inflammation and Colorectal Cancer: Colitis-Associated Neoplasia. *Seminars in Immunopathology, 35*(2), 229-244.

Groeger, G., & Nobes, Catherine D. (2007). Co-operative Cdc42 and Rho signalling mediates ephrinB-triggered endothelial cell retraction. *Biochemical Journal, 404*(Pt 1), 23-29. doi:10.1042/BJ20070146

Guijarro-Muñoz, I., Sánchez, A., Martínez-Martínez, E., García, J. M., Salas, C., Provencio, M., . . . Sanz, L. (2013). Gene expression profiling identifies EPHB4 as a potential predictive biomarker in colorectal cancer patients treated with bevacizumab. *Medical Oncology, 30*(2), 572. doi:10.1007/s12032-013-0572-1

Guo, D. L., Zhang, J., Yuen, S. T., Tsui, W. Y., Chan, A. S., Ho, C., . . . Chen, X. (2006). Reduced expression of EphB2 that parallels invasion and metastasis in colorectal tumours. *Carcinogenesis, 27*(3), 454-464. doi:10.1093/carcin/bgi259

Guo, F. Y., & Lesk, A. M. (2014). Sizes of interface residues account for cross-class binding affinity patterns in Eph receptor-ephrin families. *Proteins, 82*(3), 349-353. doi:10.1002/prot.24414

Hackl, C., Man, S., Francia, G., Milsom, C., Xu, P., & Kerbel, R. S. (2013). Metronomic oral topotecan prolongs survival and reduces liver metastasis in improved preclinical orthotopic and adjuvant therapy colon cancer models. *Gut, 62*(2), 259-271. doi:10.1136/gutjnl-2011-301585

Hamada, K., Oike, Y., Ito, Y., Maekawa, H., Miyata, K., Shimomura, T., & Suda, T. (2003). Distinct roles of ephrin-B2 forward and EphB4 reverse signaling in endothelial cells. *Arterioscler Thromb Vasc Biol, 23*(2), 190-197.

Hamaoka, Y., Negishi, M., & Katoh, H. (2016). EphA2 is a key effector of the MEK/ERK/RSK pathway regulating glioblastoma cell proliferation. *Cell Signal, 28*(8), 937-945. doi:10.1016/j.cellsig.2016.04.009

Hardingham, J. E., Grover, P., Winter, M., Hewett, P. J., Price, T. J., & Thierry, B. (2015). Detection and Clinical Significance of Circulating Tumor Cells in Colorectal Cancer—20 Years of Progress. *Molecular Medicine, 21*(Suppl 1), S25-S31. doi:10.2119/molmed.2015.00149

Harris, C. C., & Hollstein, M. (1993). Clinical Implications of the p53 Tumor-Suppressor Gene. *The New England Journal of Medicine, 329*(329), 1318-1327.

Hasina, R., Mollberg, N., Kawada, I., Mutreja, K., Kanade, G., Yala, S., . . . Salgia, R. (2013). Critical Role for the Receptor Tyrosine Kinase EPHB4 in Esophageal Cancers. *Cancer Research, 73*(1), 184.

Hecht, A., Vleminckx, K., Stemmler, M. P., van Roy, F., & Kemler, R. (2000). The p300/CBP acetyltransferases function as transcriptional coactivators of beta-catenin in vertebrates. *Embo j, 19*(8), 1839-1850. doi:10.1093/emboj/19.8.1839

Heijstek, M. W., Kranenburg, O., & Borel Rinkes, I. H. M. (2005). Mouse Models of Colorectal Cancer and Liver Metastases. *Digestive Surgery, 22*(1-2), 16-25.

Helbling, P. M., Saulnier, D. M. E., & Brändli, A. W. (2000). The Receptor Tyrosine Kinase EphB4 and Ephrin-B Ligands Restrict Angiogenic Growth of Embryonic Veins in Xenopus Laevis. *Development, 127*, 269-278.

Herath, N. I., & Boyd, A. W. (2010). The Role of Eph Receptors and Ephrin Ligands in Colorectal Cancer. *European Journal of Cancer, 126*(9), 2003-2011.

Herath, N. I., Spanevello, M. D., Doecke, J. D., Smith, F. M., Pouponnot, C., & Boyd, A. W. (2012). Complex Expression Patterns of Eph Receptor Tyrosine Kinases and Their Ephrin Ligands in Colorectal Carcinogenesis. *European Journal of Cancer, 48*, 753-762.

Herbert, S. P., Huisken, J., Kim, T. N., Feldman, M. E., Houseman, B. T., Wang, R. A., . . . Stainier, D. Y. (2009). Arterial-venous segregation by selective cell sprouting: an alternative mode of blood vessel formation. *Science, 326*(5950), 294-298. doi:10.1126/science.1178577

Herington, A. C., Mertens-Walker, I., Lisle, J. E., Maharaj, M., & Stephenson, S. A. (2014). Inhibiting Eph kinase activity may not be "Eph"ective for cancer treatment. *Growth Factors, 32*(6), 207-213. doi:10.3109/08977194.2014.985293

Héroult, M., Schaffner, F., Pfaff, D., Prahst, C., Kirmse, R., Kutschera, S., . . . Augustin, H. G. (2010). EphB4 Promotes Site-Specific Metastatic Tumor Cell Dissemination by Interacting with Endothelial Cell –Expressed EphrinB2. *American Association for Cancer Research, 8*(1297-1309).

Himanen, J. P. (2012). Ectodomain Structures of Eph Receptors. *Seminars in Cell & Developmental Biology, 23*, 35-42.

Himanen, J. P., Chumley, M. J., Lackmann, M., Li, C., Barton, W. A., Jeffrey, P. D., . . . Nikolov, D. B. (2004). Repelling Class Discrimination: Ephrin-A5 Binds to and Activates EphB2 Receptor Signaling. *Nature Neuroscience, 7*, 501-509.

Himanen, J. P., Henkemeyer, M., & Nikolov, D. B. (1998). Crystal Structure of The Ligand-Binding Domain of The Receptor Tyrosine Kinase EphB2. *Nature, 396*, 486-491.

Himanen, J. P., Rajashankar, K. R., Lackmann, M., Cowan, C. A., Henkemeyer, M., & Nikolov, D. B. (2001). Crystal Structure of an Eph Receptor-Ephrin Complex. *Nature, 414*, 933-938.

Himanen, J. P., Saha, N., & Nikolov, D. B. (2007). Cell-Cell Signaling via Eph Receptors and Ephrins. *Current Opinion in Cell Biology, 19*(5), 534-542.

Himanen, J. P., Yermekbayeva, L., Janes, P. W., Walker, J. R., Xu, K., Atapattuc, L., . . . Dhe-Paganon, S. (2010). Architecture of Eph Receptor Clusters. *PNAS, 107*(24), 10860-10865.

Hinkley, K. S., Chiasson, R. J., Prior, T. K., & Riggs, J. E. (2002). Age-dependent increase of peritoneal B-1b B cells in SCID mice. *Immunology, 105*(2), 196-203. doi:10.1046/j.1365-2567.2002.01360.x

Hirai, H., Maru, Y., Hagiwara, K., Nishida, J., & Takaku, F. (1987). A Novel Putative Tyrosine Kinase Receptor Encoded by the Eph Gene. *Science, 238*, 1717-1720.

Holmberg, J., Genander, M., Halford, M. M., Anneren, C., Sondell, M., Chumley, M. J., . . . Frisen, J. (2006). EphB Receptors Coordinate Migration and Proliferation in the Intestinal Stem Cell Niche. *Cell, 125*, 1151-1163.

Horvath, P., & Barrangou, R. (2010). CRISPR/Cas, the immune system of bacteria and archaea. *Science, 327*(5962), 167-170. doi:10.1126/science.1179555

Hsu, Patrick D., Lander, Eric S., & Zhang, F. (2014). Development and Applications of CRISPR-Cas9 for Genome Engineering. *Cell, 157*(6), 1262-1278. doi:10.1016/j.cell.2014.05.010

Hu, F., Tao, Z., Shen, Z., Wang, X., & Hua, F. (2014). Down-regulation of EphB4 phosphorylation is necessary for esophageal squamous cell carcinoma tumorigenecity. *Tumour Biol, 35*(7), 7225-7232. doi:10.1007/s13277-014-1955-4

Huang, B. T., Zeng, Q. C., Zhao, W. H., & Tan, Y. (2014). Homoharringtonine contributes to imatinib sensitivity by blocking the EphB4/RhoA pathway in chronic myeloid leukemia cell lines. *Med Oncol, 31*(2), 836. doi:10.1007/s12032-013-0836-9

Huang, X., Yamada, Y., Kidoya, H., Naito, H., Nagahama, Y., Kong, L., . . . Takakura, N. (2007). EphB4 Overexpression in B16 Melanoma Cells Affects Arterial-Venous Patterning in Tumor Angiogenesis. *Cancer Research, 67*(20), 9800.

Hulkower, K. I., & Herber, R. L. (2011). Cell migration and invasion assays as tools for drug discovery. *Pharmaceutics, 3*(1), 107-124. doi:10.3390/pharmaceutics3010107

Irie, F., Okuno, M., Pasquale, E. B., & Yamaguchi, Y. (2005). EphrinB-EphB signalling regulates clathrin-mediated endocytosis through tyrosine phosphorylation of synaptojanin 1. *Nat Cell Biol, 7*(5), 501-509. doi:10.1038/ncb1252

Ishiyama, M., Shiga, M., Sasamoto, K., Mizoguchi, M., & He, P. g. (1993). A New Sulfonated Tetrazolium Salt That Produces a Highly Water-Soluble Formazan Dye. *CHEMICAL & PHARMACEUTICAL BULLETIN, 41*(6), 1118-1122. doi:10.1248/cpb.41.1118

Jackson, R. N., van Erp, P. B. G., Sternberg, S. H., & Wiedenheft, B. (2017). Conformational regulation of CRISPR-associated nucleases. *Current Opinion in Microbiology, 37*, 110-119. doi:https://doi.org/10.1016/j.mib.2017.05.010

Janes, P. W., Griesshaber, B., Atapattu, L., Nievergall, E., Hii, L. L., Mensinga, A., . . . Lackmann, M. (2011). Eph receptor function is modulated by heterooligomerization of A and B type Eph receptors. *The Journal of Cell Biology, 195*(6), 1033.

Janes, P. W., Nievergall, E., & Lackmann, M. (2012). Concepts and consequences of Eph receptor clustering. *Semin Cell Dev Biol, 23*(1), 43-50. doi:10.1016/j.semcdb.2012.01.001

Janes, P. W., Saha, N., Barton, W. A., Kolev, M. V., Wimmer-Kleikamp, S. H., Nievergall, E., . . . Nikolov, D. B. (2005). Adam Meets Eph: An ADAM Substrate Recognition Module Acts as a Molecular Switch for Ephrin Cleavage in Trans. *Cell, 123*(2), 291-304.

Janes, P. W., Wimmer-Kleikamp, S. H., Frangakis, A. S., Treble, K., Griesshaber, B., Sabet, O., . . . Lackmann, M. (2009). Cytoplasmic Relaxation of Active Eph Controls Ephrin Shedding by ADAM10. *PLOS Biology, 7*(10), e1000215. doi:10.1371/journal.pbio.1000215

Jansen, R., Embden, J. D., Gaastra, W., & Schouls, L. M. (2002). Identification of genes that are associated with DNA repeats in prokaryotes. *Mol Microbiol, 43*(6), 1565-1575.

Jasperson, K. W., Tuohy, T. M., Neklason, D. W., & Burt, R. W. (2010). Hereditary and Familial Colon Cancer. *Gastroenterology, 138*(6), 2044-2058.

Ji, Y. J., Hwang, Y. S., Mood, K., Cho, H. J., Lee, H. S., Winterbottom, E., . . . Daar, I. O. (2014). EphrinB2 affects apical constriction in Xenopus embryos and is regulated by ADAM10 and flotillin-1. *Nat Commun, 5*, 3516. doi:10.1038/ncomms4516

Jiang, F., & Doudna, J. A. (2017). CRISPR-Cas9 Structures and Mechanisms. *Annu Rev Biophys, 46*, 505-529. doi:10.1146/annurev-biophys-062215-010822

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., & Charpentier, E. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science, 337*(6096), 816-821. doi:10.1126/science.1225829

Justus, C. R., Leffler, N., Ruiz-Echevarria, M., & Yang, L. V. (2014). In vitro Cell Migration and Invasion Assays. *Journal of Visualized Experiments : JoVE*(88), 51046. doi:10.3791/51046

Kanda, S., Mochizuki, Y., & Kanetake, H. (2003). Stromal Cell-derived Factor-1α Induces Tube-like Structure Formation of Endothelial Cells through Phosphoinositide 3-Kinase. *Journal of Biological Chemistry, 278*(1), 257-262. doi:10.1074/jbc.M204771200

Kang, M., Jeong, W., Bae, H., Lim, W., Bazer, F. W., & Song, G. (2018). Bifunctional role of ephrin A1-Eph system in stimulating cell proliferation and protecting cells from cell death through the attenuation of ER stress and inflammatory responses in bovine mammary epithelial cells. *J Cell Physiol, 233*(3), 2560-2571. doi:10.1002/jcp.26131

Kapuscinski, J. (1995). DAPI: a DNA-specific fluorescent probe. *Biotech Histochem, 70*(5), 220-233.

Kertesz, N., Krasnoperov, V., Reddy, R., Leshanski, L., Kumar, S. R., Zozulya, S., & Gill, P. S. (2006). The soluble extracellular domain of EphB4 (sEphB4) antagonizes EphB4-EphrinB2 interaction, modulates angiogenesis, and inhibits tumor growth. *Blood, 107*(6), 2330-2338. doi:10.1182/blood-2005-04-1655

Khan, K. H. (2013). Gene Expression in Mammalian Cells and its Applications. *Advanced Pharmaceutical Bulletin, 3*(2), 257-263. doi:10.5681/apb.2013.042

Khanna, K. K., & Jackson, S. P. (2001). DNA double-strand breaks: signaling, repair and the cancer connection. *Nat Genet, 27*(3), 247-254. doi:10.1038/85798

Kho, D., MacDonald, C., Johnson, R., Unsworth, C., #039, Carroll, S., . . . Graham, E. (2015). Application of xCELLigence RTCA Biosensor Technology for Revealing the Profile and Window of Drug Responsiveness in Real Time. *Biosensors, 5*(2), 199.

Kim, K. M., Lee, S. B., Lee, S. H., Lee, Y. K., & Kim, K. N. (2005). Comparison of Validity between WST-1 and MTT Test in Bioceramic Materials. *Key Engineering Materials, 284-286*, 585-588. doi:10.4028/www.scientific.net/KEM.284-286.585

Kim, T. K., & Eberwine, J. H. (2010). Mammalian cell transfection: the present and the future. *Analytical and Bioanalytical Chemistry, 397*(8), 3173-3178. doi:10.1007/s00216-010-3821-6

Kimura, M., Kato, Y., Sano, D., Fujita, K., Sakakibara, A., Kondo, N., . . . Tsukuda, M. (2009). Soluble form of ephrinB2 inhibits xenograft growth of squamous cell carcinoma of the head and neck. *Int J Oncol, 34*(2), 321-327.

King, M. R., Phillips, K. G., Mitrugno, A., Lee, T. R., de Guillebon, A. M., Chandrasekaran, S., . . . McCarty, O. J. (2015). A physical sciences network characterization of circulating tumor cell aggregate transport. *Am J Physiol Cell Physiol, 308*(10), C792-802. doi:10.1152/ajpcell.00346.2014

Klein, R. (2004). Eph/ephrin signaling in morphogenesis, neural development and plasticity. *Curr Opin Cell Biol, 16*(5), 580-589. doi:10.1016/j.ceb.2004.07.002

Kloor, M., Staffa, L., Ahadova, A., & von Knebel Doeberitz, M. (2013). Clinical Significance of Microsatellite Instability in Colorectal Cancer. *Langenbeck's Archives of Surgery, 399*(1), 23-31.

Knoll, B., & Drescher, U. (2004). Src family kinases are involved in EphA receptor-mediated retinal axon guidance. *J Neurosci, 24*(28), 6248-6257. doi:10.1523/jneurosci.0985-04.2004

Koolpe, M., Burgess, R., Dail, M., & Pasquale, E. B. (2005). EphB Receptor-Binding Peptides Identified by Phage Display Enable Design of an Antagonist with Ephrin-Like Affinity. *The Journal of Biological Chemistry, 280*(17), 17301-17311.

Kramer, N., Walzl, A., Unger, C., Rosner, M., Krupitza, G., Hengstschlager, M., & Dolznig, H. (2013). In vitro cell migration and invasion assays. *Mutat Res, 752*(1), 10-24. doi:10.1016/j.mrrev.2012.08.001

Krasnoperov, V., Kumar, S. R., Ley, E., Li, X., Scehnet, J., Liu, R., . . . Gill, P. S. (2010). Novel EphB4 monoclonal antibodies modulate angiogenesis and inhibit tumor growth. *Am J Pathol, 176*(4), 2029-2038. doi:10.2353/ajpath.2010.090755

Krausova, M., & Korinek, V. (2014). Wnt Signaling in Adult Intestinal Stem Cells and Cancer. *Cell Signal, 26*(3), 570-579.

Kullander, K., & Klein, R. (2002). Mechanisms and functions of Eph and ephrin signalling. *Nat Rev Mol Cell Biol, 3*(7), 475-486. doi:10.1038/nrm856

Kumar, S. R., Masood, R., Spannuth, W. A., Singh, J., Scehnet, J., Kleiber, G., . . . Gill, P. S. (2007). The Receptor Tyrosine Kinase EphB4 is Overexpressed in Ovarian Cancer, Provides Survival Signals and Predicts Poor Outcome. *British Journal of Cancer, 96*(7), 1083–1091.

Kumar, S. R., Scehnet, J. S., Ley, E. J., Singh, J., Krasnoperov, V., Liu, R., . . . Gill, P. S. (2009). Preferential induction of EphB4 over EphB2 and its implication in colorectal cancer progression. *Cancer Res, 69*(9), 3736-3745. doi:10.1158/0008-5472.can-08-3232

Kumar, S. R., Singh, J., Xia, G., Krasnoperov, V., Hassanieh, L., Ley, E. J., . . . Gill, P. S. (2006). Receptor Tyrosine Kinase EphB4 is a Survival Factor in Breast Cancer. *American Journal of Pathology, 169*(1), 278-293.

Lai, K. O., Chen, Y., Po, H. M., Lok, K. C., Gong, K., & Ip, N. Y. (2004). Identification of the Jak/Stat proteins as novel downstream targets of EphA4 signaling in muscle: implications in the regulation of acetylcholinesterase expression. *J Biol Chem, 279*(14), 13383-13392. doi:10.1074/jbc.M313356200

Lamminmaki, U., Nikolov, D., & Himanen, J. (2015). Eph Receptors as Drug Targets: Single-Chain Antibodies and Beyond. *Curr Drug Targets, 16*(10), 1021-1030.

Lau, W., Barker, N., & Clevers, H. (2007). WNT Signalling in the Normal Intestine and Colorectal Cancer. *Frontiers in Bioscience, 12*, 471-491.

Laurenti, D., & Ooi, L. (2013). Mammalian expression systems and transfection techniques. *Methods Mol Biol, 998*, 21-32. doi:10.1007/978-1-62703-351-0_2

Le Rolle, A. F., Chiu, T. K., Zeng, Z., Shia, J., Weiser, M. R., Paty, P. B., & Chiu, V. K. (2016). Oncogenic KRAS activates an embryonic stem cell-like program in human colon cancer initiation. *Oncotarget, 7*(3), 2159-2174. doi:10.18632/oncotarget.6818

Lee, W. Y., Hong, H. K., Ham, S. K., Kim, C. I., & Cho, Y. B. (2014). Comparison of colorectal cancer in differentially established liver metastasis models. *Anticancer Res, 34*(7), 3321-3328.

Lee, Y., Perren, J. R., Douglas, E. L., Raynor, M. P., Bartley, M. A., Bardy, P. G., & Stephenson, S. (2005). Investigation of the Expression of the EphB4 Receptor Tyrosine Kinase in Prostate Carcinoma. *BMC Cancer, 5*(119), 1-11.

Leontieva, O. V., Demidenko, Z. N., & Blagosklonny, M. V. (2014). Contact inhibition and high cell density deactivate the mammalian target of rapamycin pathway, thus suppressing the senescence program. *Proc Natl Acad Sci U S A, 111*(24), 8832-8837. doi:10.1073/pnas.1405723111

Levy, J. A., White, A. C., & McGrath, C. M. (1982). Growth and histology of a human mammary-carcinoma cell line at different sites in the athymic mouse. *British Journal of Cancer, 45*(3), 375-383.

Li, L. T., Jiang, G., Chen, Q., & Zheng, J. N. (2015). Ki67 is a promising molecular target in the diagnosis of cancer (review). *Mol Med Rep, 11*(3), 1566-1572. doi:10.3892/mmr.2014.2914

Li, M., Zhao, Z. W., Zhang, Y., & Xin, Y. (2010). Over-Expression of EphB4 is Associated with Carcinogenesis of Gastric Cancer. *Digestive Diseases and Sciences, 56*(3), 698-706.

Liao, H.-W., & Hung, M.-C. (2017). Intracaecal Orthotopic Colorectal Cancer Xenograft Mouse Model. *Bio-protocol, 7*(11), e2311. doi:10.21769/BioProtoc.2311

Liersch-Lohn, B., Slavova, N., Buhr, H. J., & Bennani-Baiti, I. M. (2016). Differential protein expression and oncogenic gene network link tyrosine kinase ephrin B4 receptor to aggressive gastric and gastroesophageal junction cancers. *Int J Cancer, 138*(5), 1220-1231. doi:10.1002/ijc.29865

Lisabeth, E. M., Falivelli, G., & Pasquale, E. B. (2013). Eph Receptor Signaling and Ephrins. *Cold Spring Harb Perspect Biol, 5*(9), 10.1101/cshperspect.a009159 a009159. doi:10.1101/cshperspect.a009159

Lisle, J. E., Mertens-Walker, I., Stephens, C. R., Stansfield, S. H., Clements, J. A., Herington, A. C., & Stephenson, S. A. (2015). Murine, but not human, ephrin-B2 can be efficiently cleaved by the serine protease kallikrein-4: Implications for xenograft models of human prostate cancer. *Exp Cell Res, 333*(1), 136-146. doi:https://doi.org/10.1016/j.yexcr.2015.02.014

Loboda, A., Nebozhyn, M. V., Watters, J. W., Buser, C. A., Shaw, P. M., Huang, P. S., . . . Yeatman, T. J. (2011). EMT is the dominant program in human colon cancer. *BMC Med Genomics, 4*, 9. doi:10.1186/1755-8794-4-9

Lorz, A., Botesteanu, D. A., & Levy, D. (2017). Modeling Cancer Cell Growth Dynamics In vitro in Response to Antimitotic Drug Treatment. *Front Oncol, 7*, 189. doi:10.3389/fonc.2017.00189

Lu, Z., & Xu, S. (2006). ERK1/2 MAP kinases in cell survival and apoptosis. *IUBMB Life, 58*(11), 621-631. doi:10.1080/15216540600957438

Luo, Q., O'Connell, D. L., Kahn, C., & Yu, X. Q. (2017). Colorectal cancer metastatic disease progression in Australia: A population-based analysis. *Cancer Epidemiology, 49*, 92-100. doi:https://doi.org/10.1016/j.canep.2017.05.012

Lv, J., Xia, Q., Wang, J., Shen, Q., Zhang, J., & Zhou, X. (2016). EphB4 promotes the proliferation, invasion, and angiogenesis of human colorectal cancer. *Experimental and Molecular Pathology, 100*(3), 402-408. doi:10.1016/j.yexmp.2016.03.011

Makinen, T., Adams, R. H., Bailey, J., Lu, Q., Ziemiecki, A., Alitalo, K., . . . Wilkinson, G. A. (2005). PDZ interaction site in ephrinB2 is required for the remodeling of lymphatic vasculature. *Genes Dev, 19*(3), 397-410. doi:10.1101/gad.330105

Mali, P., Aach, J., Stranges, P. B., Esvelt, K. M., Moosburner, M., Kosuri, S., . . . Church, G. M. (2013). CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. *Nature biotechnology, 31*(9), 833-838. doi:10.1038/nbt.2675

Mali, P., Yang, L., Esvelt, K. M., Aach, J., Guell, M., DiCarlo, J. E., . . . Church, G. M. (2013). RNA-guided human genome engineering via Cas9. *Science, 339*(6121), 823-826. doi:10.1126/science.1232033

Marangoni, A. G. (2003). *Enzyme Kinetics; A Modern Approach*. USA: John Wiley & Sons.

Margolis, S. S., Salogiannis, J., Lipton, D. M., Mandel-Brehm, C., Wills, Z. P., Mardinly, A. R., . . . Greenberg, M. E. (2010). EphB-mediated degradation of the RhoA GEF Ephexin5 relieves a developmental brake on excitatory synapse formation. *Cell, 143*(3), 442-455. doi:10.1016/j.cell.2010.09.038

Marisi, G., Scarpi, E., Passardi, A., Nanni, O., Ragazzini, A., Valgiusti, M., . . . Ulivi, P. (2017). Circulating VEGF and eNOS variations as predictors of outcome in metastatic colorectal cancer patients receiving bevacizumab. *Scientific Reports, 7*(1), 1293. doi:10.1038/s41598-017-01420-0

Marquardt, T., Shirasaki, R., Ghosh, S., Andrews, S. E., Carter, N., Hunter, T., & Pfaff, S. L. (2005). Coexpressed EphA receptors and ephrin-A ligands mediate opposing actions on growth cone navigation from distinct membrane domains. *Cell, 121*(1), 127-139. doi:10.1016/j.cell.2005.01.020

Marston, D., Dickinson, S., & Nobes, C. D. (2003). Rac-Dependent Trans-Endocytosis of EphrinBs regulates Eph-Ephrin Contact Repulsion. *Nature Cell Biology, 5*(10), 879-888.

Marston, D. J., Dickinson, S., & Nobes, C. D. (2003). Rac-dependent trans-endocytosis of ephrinBs regulates Eph-ephrin contact repulsion. *Nat Cell Biol, 5*(10), 879-888. doi:10.1038/ncb1044

Martínez-Maqueda, D., Miralles, B., & Recio, I. (2015). HT29 Cell Line. In K. Verhoeckx, P. Cotter, I. López-Expósito, C. Kleiveland, T. Lea, A. Mackie, T. Requena, D. Swiatecka, & H. Wichers (Eds.), *The Impact of Food Bioactives on Health: in vitro and ex vivo models* (pp. 113-124). Cham: Springer International Publishing.

Martinez-Serra, J., Gutierrez, A., Muñoz-Capó, S., Navarro-Palou, M., Ros, T., Amat, J. C., . . . Besalduch, J. (2014). xCELLigence system for real-time label-free monitoring of growth and viability of cell lines from hematological malignancies. *OncoTargets and therapy, 7*, 985-994. doi:10.2147/OTT.S62887

Martiny-Baron, G., Korff, T., Schaffner, F., Esser, N., Eggstein, S., Marmé, D., & Augustin, H. G. (2004). Inhibition of Tumor Growth and Angiogenesis by Soluble EphB4. *Neoplasia (New York, N.Y.), 6*(3), 248-257.

Mason, E. F., & Rathmell, J. C. (2011). Cell metabolism: an essential link between cell growth and apoptosis. *Biochim Biophys Acta, 1813*(4), 645-654. doi:10.1016/j.bbamcr.2010.08.011

Masood, R., Kumar, S. R., Sinha, U. K., Crowe, D. L., Krasnoperov, V., Reddy, R. K., . . . Gill, P. S. (2006). EphB4 Provides Survival Advantage to Squamous Cell Carcinoma of the Head And Neck. *International Journal of Cancer, 119*(6), 1236-1248.

Matallanas, D., Birtwistle, M., Romano, D., Zebisch, A., Rauch, J., von Kriegsheim, A., & Kolch, W. (2011). Raf Family Kinases; Old Dogs Have Learned New Tricks. *Genes & Cancer, 2*(3), 232-260.

Matano, M., Date, S., Shimokawa, M., Takano, A., Fujii, M., Ohta, Y., . . . Sato, T. (2015). Modeling colorectal cancer using CRISPR-Cas9-mediated engineering of human intestinal organoids. *Nat Med, 21*(3), 256-262. doi:10.1038/nm.3802

Matsusaka, S., Suenaga, M., Mishima, Y., Kuniyoshi, R., Takagi, K., Terui, Y., . . . Hatake, K. (2011). Circulating tumor cells as a surrogate marker for determining response to chemotherapy in Japanese patients with metastatic colorectal cancer. *Cancer Sci, 102*(6), 1188-1192. doi:10.1111/j.1349-7006.2011.01926.x

McCall, J. L., Gehring, D., Clymer, B. K., Fisher, K. W., Das, B., Kelly, D. L., . . . Lewis, R. E. (2016). KSR1 and EPHB4 Regulate Myc and PGC1β To Promote Survival of Human Colon Tumors. *Molecular and Cellular Biology, 36*(17), 2246-2261. doi:10.1128/mcb.00087-16

Mellitzer, G., Xu, Q., & Wilkinson, D. G. (1999). Eph Receptors and Ephrins Restrict Cell Intermingling and Communication. *Nature, 400*, 77-81.

Menyhart, O., Harami-Papp, H., Sukumar, S., Schafer, R., Magnani, L., de Barrios, O., & Gyorffy, B. (2016). Guidelines for the selection of functional assays to evaluate the hallmarks of cancer. *Biochim Biophys Acta, 1866*(2), 300-319. doi:10.1016/j.bbcan.2016.10.002

Merchant, A. A., Jorapur, A., McManus, A., Liu, R., Krasnoperov, V., Chaudhry, P., . . . Gill, P. S. (2017). EPHB4 is a therapeutic target in AML and promotes leukemia cell survival via AKT. *Blood Advances, 1*(20), 1635-1644. doi:10.1182/bloodadvances.2017005694

Merlos-Suárez, A., & Batlle, E. (2008). Eph–Ephrin Signalling in Adult Tissues and Cancer. *Current Opinion in Cell Biology, 20*(2), 194-200.

Mertens-Walker, I., Fernandini, B. C., Maharaj, M. S. N., Rockstroh, A., Nelson, C. C., Herington, A. C., & Stephenson, S.-A. (2015). The tumour-promoting receptor tyrosine kinase, EphB4, regulates expression of Integrin-β8 in prostate cancer cells. *BMC Cancer, 15*(1), 164. doi:10.1186/s12885-015-1164-6

Mertens-Walker, I., Lisle, J. E., Nyberg, W. A., Stephens, C. R., Burke, L., Rutkowski, R., . . . Stephenson, S. A. (2015). EphB4 localises to the nucleus of prostate cancer cells. *Exp Cell Res, 333*(1), 105-115. doi:10.1016/j.yexcr.2015.02.015

Miao, H., Li, D. Q., Mukherjee, A., Guo, H., Petty, A., Cutter, J., . . . Wang, B. (2009). EphA2 mediates ligand-dependent inhibition and ligand-independent promotion of cell migration and invasion via a reciprocal regulatory loop with Akt. *Cancer Cell, 16*(1), 9-20. doi:10.1016/j.ccr.2009.04.009

Miller, S., Senior, P. V., Prakash, M., Apostolopoulos, V., Sakkal, S., & Nurgali, K. (2016). Leukocyte populations and IL-6 in the tumor microenvironment of an orthotopic colorectal cancer model. *Acta Biochim Biophys Sin (Shanghai), 48*(4), 334-341. doi:10.1093/abbs/gmw002

Mittal, V. K., Bhullar, J. S., & Jayant, K. (2015). Animal models of human colorectal cancer: Current status, uses and limitations. *World Journal of Gastroenterology, 21*(41), 11854-11861. doi:10.3748/wjg.v21.i41.11854

Mojica, F. J., Diez-Villasenor, C., Garcia-Martinez, J., & Almendros, C. (2009). Short motif sequences determine the targets of the prokaryotic CRISPR defence system. *Microbiology, 155*(Pt 3), 733-740. doi:10.1099/mic.0.023960-0

Morkel, M., Riemer, P., Blaker, H., & Sers, C. (2015). Similar but different: distinct roles for KRAS and BRAF oncogenes in colorectal cancer development and therapy resistance. *Oncotarget, 6*(25), 20785-20800. doi:10.18632/oncotarget.4750

Muelas, M., Ortega, F., Breitling, R., Bendtsen, C., & Westerhoff, H. V. (2017). Rational cell culture optimization enhances experimental reproducibility in cancer cells. *bioRxiv*.

Munteanu, I., & Mastalier, B. (2014). Genetics of Colorectal Cancer. *Journal of Medicine and Life, 7*(4), 507-511.

National Health and Medical Research Council. (2005). *Clinical Practice Guidelines for the Prevention, Early Detection and Management of Colorectal Cancer*. Australia: Commonwealth of Australia.

Nelson, P. J., & Daniel, T. O. (2002). Emerging targets: molecular mechanisms of cell contact-mediated growth control. *Kidney Int, 61*(1 Suppl), S99-105. doi:10.1046/j.1523-1755.2002.0610s1099.x

Neuber, C., Belter, B., Meister, S., Hofheinz, F., Bergmann, R., Pietzsch, H. J., & Pietzsch, J. (2018). Overexpression of Receptor Tyrosine Kinase EphB4 Triggers Tumor Growth and Hypoxia in A375 Melanoma Xenografts: Insights from Multitracer Small Animal Imaging Experiments. *Molecules, 23*(2). doi:10.3390/molecules23020444

Ngamwongsatit, P., Banada, P. P., Panbangred, W., & Bhunia, A. K. (2008). WST-1-based cell cytotoxicity assay as a substitute for MTT-based assay for rapid detection of toxigenic Bacillus species using CHO cell line. *J Microbiol Methods, 73*(3), 211-215. doi:10.1016/j.mimet.2008.03.002

Nievergall, E., Janes, P. W., Stegmayer, C., Vail, M. E., Haj, F. G., Teng, S. W., . . . Lackmann, M. (2010). PTP1B regulates Eph receptor function and trafficking. *The Journal of Cell Biology, 191*(6), 1189.

Nievergall, E., Lackmann, M., & Janes, P. W. (2012). Eph-Dependent Cell-Cell Adhesion and Segregation in Development and Cancer. *Cellular and Molecular Life Sciences, 69*, 1813–1842.

Nikolov, D. B., Xu, K., & Himanen, J. P. (2013). Eph/ephrin recognition and the role of Eph/ephrin clusters in signaling initiation. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics, 1834*(10), 2160-2165. doi:https://doi.org/10.1016/j.bbapap.2013.04.020

Nikolov, D. B., Xu, K., & Himanen, J. P. (2014). Homotypic receptor-receptor interactions regulating Eph signaling. *Cell Adh Migr, 8*(4), 360-365. doi:10.4161/19336918.2014.971684

Nishida, N., Yano, H., Nishida, T., Kamura, T., & Kojiro, M. (2006). Angiogenesis in Cancer. *Vascular Health and Risk Management, 2*(3), 213-219.

Nishihara, R., Morikawa, T., Kuchiba, A., Lochhead, P., Yamauchi, M., Liao, X., & Imamura, Y. (2013). A Prospective Study of Duration of Smoking Cessation and Colorectal Cancer Risk by Epigenetics-Related Tumor Classification. *American Journal of Epidemiology, 178*(1), 84-100.

Nishimasu, H., Ran, F. A., Hsu, Patrick D., Konermann, S., Shehata, Soraya I., Dohmae, N., . . . Nureki, O. (2014). Crystal Structure of Cas9 in Complex with Guide RNA and Target DNA. *Cell, 156*(5), 935-949. doi:10.1016/j.cell.2014.02.001

Noberini, R., Mitra, S., Salvucci, O., Valencia, F., Duggineni, S., Prigozhina, N., . . . Pasquale, E. B. (2011). PEGylation Potentiates the Effectiveness of an Antagonistic Peptide that Targets the EphB4 Receptor with Nanomolar Affinity. *PLoS ONE, 6*(12), e28611.

Norat, T., Aune, D., Chan, D., & Romaguera, D. (2013). Fruits and Vegetables: Updating the Epidemiologic Evidence for the WCRF/AICR Lifestyle Recommendations for Cancer Prevention. *Springer-Advances in Nutrition and Cancer, 159*, 35-50.

Noren, N. K., Foos, G., Hauser, C. A., & Pasquale, E. B. (2006). The EphB4 Receptor Suppresses Breast Cancer Cell Tumorigenicity through an Abl–Crk Pathway. *Nature Cell Biology, 8*(8), 815-825.

Noren, N. K., Lu, M., Freeman, A. L., Koolpe, M., & Pasquale, E. B. (2004). Interplay between EphB4 on tumor cells and vascular ephrin-B2 regulates tumor growth. *Proc Natl Acad Sci U S A, 101*(15), 5583-5588. doi:10.1073/pnas.0401381101

Noren, N. K., & Pasquale, E. B. (2007). Paradoxes of the EphB4 Receptor in Cancer. *American Association for Cancer Research, 67*, 3994-3997.

Noren, N. K., Yang, N. Y., Silldorff, M., Mutyala, R., & Pasquale, E. B. (2009). Ephrin-independent regulation of cell substrate adhesion by the EphB4 receptor. *Biochem J, 422*(3), 433-442. doi:10.1042/bj20090014

North, H. A., Clifford, M. A., & Donoghue, M. J. (2012). Til Eph Do Us Part': Intercellular Signaling via Eph Receptors and Ephrin Ligands Guides Cerebral Cortical Development from Birth Through Maturation. *Cerebral Cortex Advance Access, 27*, 5643-5653.

Novellasdemunt, L., Antas, P., & Li, V. S. W. (2015). Targeting Wnt Signaling in Colorectal Cancer. A Review in the Theme: Cell Signaling: Proteins, Pathways and Mechanisms. *American Journal of Physiology-Cell Physiology, 309*(8), 511-521.

Oshima, G., Stack, M. E., Wightman, S. C., Bryan, D., Poli, E., Xue, L., . . . Khodarev, N. N. (2016). Advanced Animal Model of Colorectal Metastasis in Liver: Imaging Techniques and Properties of Metastatic Clones. *J Vis Exp*(117). doi:10.3791/54657

Overman, R. C., Debreczeni, J. E., Truman, C. M., Mcalister, M. S., & Attwood, T. K. (2013). Biochemical and Biophysical Characterization of Four EphB Kinase Domains Reveals Contrasting Thermodynamic, Kinetic and Inhibition Profiles. *Bioscience Reports, 33*(3), 427-437.

Overman, R. C., Debreczeni, J. E., Truman, C. M., McAlister, M. S., & Attwood, T. K. (2014). Completing the Structural Family Portrait of the Human EphB Tyrosine Kinase Domains. *Protein Science, 23*, 627-638.

Overman, R. C., Green, I., Truman, C. M., Read, J., Embrey, K. J., McAlister, M. S. B., & Attwood, T. K. (2013). Stability and Solubility Engineering of the EphbB4 Tyrosine

Kinase Catalytic Domain Using a Rationally Designed Synthetic Library. *Protein Engineering, Design & Selection, 26*(10), 695–704.
Ozgur, E., Heidenreich, A., Dagtekin, O., Engelmann, U., & Bloch, W. (2011). Distribution of EphB4 and EphrinB2 in Normal and Malignant Urogenital Tissue. *Urologic Oncology, 29*(1), 78-84.
Palmer, A., Zimmer, M., Erdmann, K. S., Eulenburg, V., Porthin, A., Heumann, R., . . . Klein, R. (2002). EphrinB phosphorylation and reverse signaling: regulation by Src kinases and PTP-BL phosphatase. *Mol Cell, 9*(4), 725-737.
Park, E. K., Warner, N., Bong, Y., Stapleton, D., Maeda, R., Pawson, T., & Daar, I. O. (2004). Ectopic EphA4 Receptor Induces Posterior Protrusions via FGF Signaling in Xenopus Embryos. *Molecular Biology of the Cell, 15*(4), 1647-1655.
Park, I., & Lee, H. S. (2015). EphB/ephrinB signaling in cell adhesion and migration. *Mol Cells, 38*(1), 14-19. doi:10.14348/molcells.2015.2116
Parker, M., Roberts, R., Enriquez, M., Zhao, X., Takahashi, T., Pat Cerretti, D., . . . Chen, J. (2004). Reverse endocytosis of transmembrane ephrin-B ligands via a clathrin-mediated pathway. *Biochemical and Biophysical Research Communications, 323*(1), 17-23. doi:https://doi.org/10.1016/j.bbrc.2004.07.209
Pasquale, E. B. (2004). Eph–Ephrin Promiscuity Is Now Crystal Clear. *Nature Neuroscience, 7*, 417-418.
Pasquale, E. B. (2008). Eph-ephrin bidirectional signaling in physiology and disease. *Cell, 133*(1), 38-52. doi:10.1016/j.cell.2008.03.011
Pasquale, E. B. (2010). Eph Receptors and Ephrins in Cancer: Bidirectional Signalling and Beyond. *Nature Reviews Cancer, 3*, 165-180.
Pawelczak, K. S., Gavande, N. S., VanderVere-Carozza, P. S., & Turchi, J. J. (2018). Modulating DNA Repair Pathways to Improve Precision Genome Engineering. *ACS Chemical Biology, 13*(2), 389-396. doi:10.1021/acschembio.7b00777
Pawlik, T. M., Raut, C. P., & Rodriguez-Biga, M. A. (2004). Colorectal Carcinogenesis: MSI-H versus MSI-L. *Disease Markers, 20*(4-5), 199-206.
Perez White, B. E., & Getsios, S. (2014). Eph receptor and ephrin function in breast, gut, and skin epithelia. *Cell Adh Migr, 8*(4), 327-338. doi:10.4161/19336918.2014.970012
Pienta, K. J., Robertson, B. A., Coffey, D. S., & Taichman, R. S. (2013). The Cancer Diaspora: Metastasis beyond the seed and soil hypothesis. *Clin Cancer Res, 19*(21), 10.1158/1078-0432.CCR-1113-2158. doi:10.1158/1078-0432.CCR-13-2158
Prelich, G. (2012). Gene Overexpression: Uses, Mechanisms, and Interpretation. *Genetics, 190*(3), 841.
Quent, V. M., Loessner, D., Friis, T., Reichert, J. C., & Hutmacher, D. W. (2010). Discrepancies between metabolic activity and DNA content as tool to assess cell proliferation in cancer research. *J Cell Mol Med, 14*(4), 1003-1013. doi:10.1111/j.1582-4934.2010.01013.x
Rahmanzadeh, R., Huttmann, G., Gerdes, J., & Scholzen, T. (2007). Chromophore-assisted light inactivation of pKi-67 leads to inhibition of ribosomal RNA synthesis. *Cell Prolif, 40*(3), 422-430. doi:10.1111/j.1365-2184.2007.00433.x
Ran, F. A., Hsu, P. D., Lin, C.-Y., Gootenberg, J. S., Konermann, S., Trevino, A., . . . Zhang, F. (2013). Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell, 154*(6), 1380-1389. doi:10.1016/j.cell.2013.08.021
Ran, X., Qin, H., Liu, J., Fan, J. S., Shi, J., & Song, J. (2008). NMR structure and dynamics of human ephrin-B2 ectodomain: the functionally critical C-D and G-H loops are highly dynamic in solution. *Proteins, 72*(3), 1019-1029. doi:10.1002/prot.21999

Raskov, H., Burcharth, J., & Pommergaard, H.-C. (2017). Linking Gut Microbiota to Colorectal Cancer. *Journal of Cancer, 8*(17), 3378-3395. doi:10.7150/jca.20497

Redding, S., Sternberg, Samuel H., Marshall, M., Gibb, B., Bhat, P., Guegler, Chantal K., . . . Greene, Eric C. (2015). Surveillance and Processing of Foreign DNA by the Escherichia coli CRISPR-Cas System. *Cell, 163*(4), 854-865. doi:10.1016/j.cell.2015.10.003

Ricci-Vitiani, L., Pallini, R., Biffoni, M., Todaro, M., Invernici, G., Cenci, T., . . . De Maria, R. (2010). Tumour vascularization via endothelial differentiation of glioblastoma stem-like cells. *Nature, 468*(7325), 824-828. doi:10.1038/nature09557

Ridley, A. J. (2015). Rho GTPase signalling in cell migration. *Current Opinion in Cell Biology, 36*, 103-112. doi:10.1016/j.ceb.2015.08.005

Riondino, S., Roselli, M., Palmirotta, R., Della-Morte, D., Ferroni, P., & Guadagni, F. (2014). Obesity and Colorectal Cancer: Role of Adipokines in Tumor Initiation and Progression. *World Journal of Gastroenterology, 20*(18), 5177-5190.

Risau, W. (1997). Mechanisms of Angiogenesis. *Nature, 386*, 671-674.

Risau, W., Sariola, H., Zerwes, H. G., Sasse, J., Ekblom, P., Kemler, R., & Doetschman, T. (1988). Vasculogenesis and Angiogenesis in Embryonic-Stem-Cell-Derived Embryoid Bodies. *Development, 102*, 471-478.

Rohani, N., Canty, L., Luu, O., Fagotto, F., & Winklbauer, R. (2011). EphrinB/EphB signaling controls embryonic germ layer separation by contact-induced cell detachment. *PLoS Biol, 9*(3), e1000597. doi:10.1371/journal.pbio.1000597

Ronsch, K., Jager, M., Schopflin, A., Danciu, M., Lassmann, S., & Hecht, A. (2011). Class I and III HDACs and loss of active chromatin features contribute to epigenetic silencing of CDX1 and EPHB tumor suppressor genes in colorectal cancer. *Epigenetics, 6*(5), 610-622.

Roovers, K., & Assoian, R. K. (2003). Effects of rho kinase and actin stress fibers on sustained extracellular signal-regulated kinase activity and activation of G(1) phase cyclin-dependent kinases. *Mol Cell Biol, 23*(12), 4283-4294.

Rosano, G. L., & Ceccarelli, E. A. (2014). Recombinant protein expression in Escherichia coli: advances and challenges. *Front Microbiol, 5*, 172. doi:10.3389/fmicb.2014.00172

Roskoski, R., Jr. (2012). ERK1/2 MAP kinases: structure, function, and regulation. *Pharmacol Res, 66*(2), 105-143. doi:10.1016/j.phrs.2012.04.005

Rutkowski, R., Mertens-Walker, I., Lisle, J. E., Herington, A. C., & Stephenson, S. A. (2012). Evidence for a dual function of EphB4 as tumor promoter and suppressor regulated by the absence or presence of the ephrin-B2 ligand. *Int J Cancer, 131*(5), E614-624. doi:10.1002/ijc.27392

Sahin, M., Greer, P. L., Lin, M. Z., Poucher, H., Eberhart, J., Schmidt, S., . . . Greenberg, M. E. (2005). Eph-dependent tyrosine phosphorylation of ephexin1 modulates growth cone collapse. *Neuron, 46*(2), 191-204. doi:10.1016/j.neuron.2005.01.030

Sakamoto, K. M., & Frank, D. A. (2009). CREB in the pathophysiology of cancer: implications for targeting transcription factors for cancer therapy. *Clin Cancer Res, 15*(8), 2583-2587. doi:10.1158/1078-0432.ccr-08-1137

Salvucci, O., Sierra, M. L., Martina, J. A., McCormick, P. J., & Tosato, G. (2006). EphB2 and EphB4 Receptors Forward Signaling Promotes SDF-1-Induced Endothelial Cell Chemotaxis and Branching Remodeling. *Blood Journal, 108*, 2914-2922.

Sander, J. D., & Joung, J. K. (2014). CRISPR-Cas systems for editing, regulating and targeting genomes. *Nature biotechnology, 32*(4), 347-355. doi:10.1038/nbt.2842

Santarius, T., Shipley, J., Brewer, D., Stratton, M. R., & Cooper, C. S. (2010). A census of amplified and overexpressed human cancer genes. *Nat Rev Cancer, 10*(1), 59-64. doi:10.1038/nrc2771

Sasaki, E., Hikono, H., Kaku, Y., Kuwana, T., Naito, M., & Sakurai, M. (2003). EphA9, A Novel Avian Receptor Tyrosine Kinase Gene. *Gene, 316*, 103-110.

Schaupp, A., Sabet, O., Dudanova, I., Ponserre, M., Bastiaens, P., & Klein, R. (2014). The Composition of EphB2 Clusters Determines the Strength in the Cellular Repulsion Response. *Journal of Cell Biology, 204*(3), 409-422.

Schmucker, D., & Zipursky, S. L. (2001). Signaling downstream of Eph receptors and ephrin ligands. *Cell, 105*(6), 701-704.

Seiradake, E., Schaupp, A., Del Toro Ruiz, D., Kaufmann, R., Mitakidis, N., Harlos, K., . . . Jones, E. Y. (2013). Structurally encoded intraclass differences in EphA clusters drive distinct cell responses. *Nature Structural & Molecular Biology, 20*(8), 958-964. doi:10.1038/nsmb.2617

Seluanov, A., Hine, C., Azpurua, J., Feigenson, M., Bozzella, M., Mao, Z., . . . Gorbunova, V. (2009). Hypersensitivity to contact inhibition provides a clue to cancer resistance of naked mole-rat. *Proc Natl Acad Sci U S A, 106*(46), 19352-19357. doi:10.1073/pnas.0905252106

Sener, L., Albeniz, G., Dinc, B., & Albeniz, I. (2017). iCELLigence real-time cell analysis system for examining the cytotoxicity of drugs to cancer cell lines. *Exp Ther Med, 14*(3), 1866-1870. doi:10.3892/etm.2017.4781

Senior, P. V., Zhang, B. X., & Chan, S. T. F. (2010). Loss of Cell-Surface Receptor EphB2 is Important for the Growth, Migration, and Invasiveness of a Colon Cancer Cell Line. *International Journal of Colorectal Disease, 25*(6), 687-694.

Shamah, S. M., Lin, M. Z., Goldberg, J. L., Estrach, S., Sahin, M., Hu, L., . . . Greenberg, M. E. (2001). EphA receptors regulate growth cone dynamics through the novel guanine nucleotide exchange factor ephexin. *Cell, 105*(2), 233-244.

Sharifpanah, F., Behr, S., Wartenberg, M., & Sauer, H. (2016). Mechanical strain stimulates vasculogenesis and expression of angiogenesis guidance molecules of embryonic stem cells through elevation of intracellular calcium, reactive oxygen species and nitric oxide generation. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 1863*(12), 3096-3105. doi:https://doi.org/10.1016/j.bbamcr.2016.10.001

Sharonov, G. V., Bocharov, E. V., Kolosov, P. M., Astapova, A. V., Arseniev, A. S., & Feofanov, A. V. (2014). Point Mutations in Dimerization Motifs of the Transmembrane Domain Stabilize Active or Inactive State of the EphA2 Receptor Tyrosine Kinase. *Journal of Biological Chemistry, 289*(21), 14955-14964.

Sheffer, M., Bacolod, M. D., Zuk, O., Giardina, S. F., Pincas, H., Barany, F., . . . Domany, E. (2009). Association of survival and disease progression with chromosomal instability: a genomic exploration of colorectal cancer. *Proc Natl Acad Sci U S A, 106*(17), 7131-7136. doi:10.1073/pnas.0902232106

Sheng, Z., Wang, J., Dong, Y., Ma, H., Zhou, H., Sugimura, H., . . . Zhou, X. (2008). EphB1 is Underexpressed in Poorly Differentiated Colorectal Cancers. *Pathobiology, 75*, 274–280.

Shi, J., Li, Y., Liang, S., Zeng, J., Liu, G., Mu, F., . . . Niu, L. (2016). Analysis of circulating tumor cells in colorectal cancer liver metastasis patients before and after cryosurgery. *Cancer Biol Ther, 17*(9), 935-942. doi:10.1080/15384047.2016.1210731

Singh, V., Braddick, D., & Dhar, P. K. (2017). Exploring the potential of genome editing CRISPR-Cas9 technology. *Gene, 599*, 1-18. doi:10.1016/j.gene.2016.11.008

Singla, N., Goldgur, Y., Xu, K., Paavilainen, S., Nikolov, D. B., & Himanen, J. P. (2010). Crystal structure of the ligand-binding domain of the promiscuous EphA4 receptor reveals two distinct conformations. *Biochem Biophys Res Commun, 399*(4), 555-559. doi:10.1016/j.bbrc.2010.07.109

Sinha, U. K., Kundra, A., Scalia, P., Smith, D. L., Parsa, B., Masood, R., & Gill, P. S. (2003). Expression of EphB4 in Head and Neck Squamous Cell Carcinoma. *Ear, Nose & Throat Journal, 82*(11), 866-887.

Smith, J. J., Deane, N. G., Wu, F., Merchant, N. B., Zhang, B., Jiang, A., . . . Beauchamp, R. D. (2010). Experimentally derived metastasis gene expression profile predicts recurrence and death in patients with colon cancer. *Gastroenterology, 138*(3), 958-968. doi:10.1053/j.gastro.2009.11.005

Soares, K. C., Foley, K., Olino, K., Leubner, A., Mayo, S. C., Jain, A., . . . Zheng, L. (2014). A Preclinical Murine Model of Hepatic Metastases. *Journal of Visualized Experiments : JoVE*(91), 51677. doi:10.3791/51677

Sobecki, M., Mrouj, K., Camasses, A., Parisis, N., Nicolas, E., Llères, D., . . . Fisher, D. (2016). The cell proliferation antigen Ki-67 organises heterochromatin. *Elife, 5*, e13722. doi:10.7554/eLife.13722

Solanas, G., Cortina, C., Sevillano, M., & Batlle, E. (2011). Cleavage of E-Cadherin by ADAM10 Mediates Epithelial Cell Sorting Downstream of EphB Signalling. *Nature Cell Biology, 13*(9), 1100-1108.

Song, J. L., Nigam, P., Tektas, S. S., & Selva, E. (2015). microRNA regulation of Wnt signaling pathways in development and disease. *Cell Signal, 27*(7), 1380-1391. doi:10.1016/j.cellsig.2015.03.018

Spannuth, W. A., Mangala, L. S., Stone, R. L., Carroll, A. R., Nishimura, M., Shahzad, M. M. K., . . . Sood, A. K. (2010). Converging Evidence for Efficacy from Parallel EphB4-Targeted Approaches in Ovarian Carcinoma. *Molecular Cancer Therapeutics, 9*(8), 2377.

Stahl, S., Branca, R. M., Efazat, G., Ruzzene, M., Zhivotovsky, B., Lewensohn, R., . . . Lehtio, J. (2011). Phosphoproteomic profiling of NSCLC cells reveals that ephrin B3 regulates pro-survival signaling through Akt1-mediated phosphorylation of the EphA2 receptor. *J Proteome Res, 10*(5), 2566-2578. doi:10.1021/pr200037u

Stapleton, D., Balan, I., Pawson, T., & Sicheri, F. (1999). The Crystal Structure of an Eph Receptor SAM Domain Reveals a Mechanism for Modular Dimerization. *Nature Structural Biology, 6*(1), 44-49.

Staub, E., Groene, J., Heinze, M., Mennerich, D., Roepcke, S., Klaman, I., . . . Rosenthal, A. (2009). An expression module of WIPF1-coexpressed genes identifies patients with favorable prognosis in three tumor types. *J Mol Med (Berl), 87*(6), 633-644. doi:10.1007/s00109-009-0467-y

Steinle, J. J., Meininger, C. J., Forough, R., Wu, G., Wu, M. H., & Granger, H. J. (2002). Eph B4 Receptor Signaling Mediates Endothelial Cell Migration and Proliferation via the Phosphatidylinositol 3-Kinase Pathway. *Journal of Biological Chemistry, 277*(46), 43830-43835. doi:10.1074/jbc.M207221200

Steinle, J. J., Meininger, C. J., Forough, R., Wu, G., Wu, M. H., & Granger, H. J. (2002). Eph B4 receptor signaling mediates endothelial cell migration and proliferation via the phosphatidylinositol 3-kinase pathway. *J Biol Chem, 277*(46), 43830-43835. doi:10.1074/jbc.M207221200

Stepanenko, A. A., & Dmitrenko, V. V. (2015). Pitfalls of the MTT assay: Direct and off-target effects of inhibitors can result in over/underestimation of cell viability. *Gene, 574*(2), 193-203. doi:10.1016/j.gene.2015.08.009

Stephenson, S., Slomka, S., Douglas, E. L., Hewett, P. J., & Hardingham, J. E. (2001). Receptor Protein Tyrosine Kinase EphB4 is Up-Regulated in Colon Cancer. *BMC Cancer, 2*(15), 1-9.

Stephenson, S. A., Douglas, E. L., Mertens-Walker, I., Lisle, J. E., Maharaj, M. S., & Herington, A. C. (2015). Anti-tumour effects of antibodies targeting the extracellular cysteine-rich region of the receptor tyrosine kinase EphB4. *Oncotarget, 6*(10), 7554-7569. doi:10.18632/oncotarget.3199

Sternberg, S. H., Redding, S., Jinek, M., Greene, E. C., & Doudna, J. A. (2014). DNA interrogation by the CRISPR RNA-guided endonuclease Cas9. *Nature, 507*(7490), 62-67. doi:10.1038/nature13011

Steven, A., & Seliger, B. (2016). Control of CREB expression in tumors: from molecular mechanisms and signal transduction pathways to therapeutic target. *Oncotarget, 7*(23), 35454-35465. doi:10.18632/oncotarget.7721

Stockert, J. C., Horobin, R. W., Colombo, L. L., & Blazquez-Castro, A. (2018). Tetrazolium salts and formazan products in Cell Biology: Viability assessment, fluorescence imaging, and labeling perspectives. *Acta Histochem*. doi:10.1016/j.acthis.2018.02.005

Stoffel, E., Mukherjee, B., Raymond, V. M., Tayob, N., Kastrinos, F., Sparr, J., . . . Gruber, S. B. (2009). Calculation of Risk of Colorectal and Endometrial Cancer Among Patients with Lynch Syndrome. *Gastroenterology, 137*(5), 1621-1627.

Surawska, H., Ma, P. C., & Salgia, R. (2004). The Role of Ephrins and Eph Receptors in Cancer. *Cytokine Growth Factor Reviews, 15*(6), 419-433.

Sveen, A., Agesen, T. H., Nesbakken, A., Rognum, T. O., Lothe, R. A., & Skotheim, R. I. (2011). Transcriptome instability in colorectal cancer identified by exon microarray analyses: Associations with splicing factor expression levels and patient survival. *Genome Med, 3*(5), 32. doi:10.1186/gm248

Szkaradkiewicz, A., Marciniak, R., Chudzicka-Strugała, I., Wasilewska, A., Drews, M., Majewski, P., . . . Zwoździak, B. (2009). Proinflammatory cytokines and IL-10 in inflammatory bowel disease and colorectal cancer patients. *Archivum Immunologiae et Therapiae Experimentalis, 57*(4), 291. doi:10.1007/s00005-009-0031-z

Tachibana, M., Tonomoto, Y., Hyakudomi, R., Hyakudomi, M., Hattori, S., Ueda, S., . . . Yoshimura, H. (2007). Expression and prognostic significance of EFNB2 and EphB4 genes in patients with oesophageal squamous cell carcinoma. *Digestive and Liver Disease, 39*(8), 725-732. doi:https://doi.org/10.1016/j.dld.2007.05.013

Takahashi, Y., Itoh, M., Nara, N., & Tohda, S. (2014). Effect of EPH-ephrin signaling on the growth of human leukemia cells. *Anticancer Res, 34*(6), 2913-2918.

Takaya, A., Hirohashi, Y., Murai, A., Morita, R., Saijo, H., Yamamoto, E., . . . Torigoe, T. (2016). Establishment and Analysis of Cancer Stem-Like and Non-Cancer Stem-Like Clone Cells from the Human Colon Cancer Cell Line SW480. *PLoS ONE, 11*(7), e0158903. doi:10.1371/journal.pone.0158903

Takemaru, K.-I., & Moon, R. T. (2000). The Transcriptional Coactivator Cbp Interacts with β-Catenin to Activate Gene Expression. *The Journal of Cell Biology, 149*(2), 249-254.

Tanaka, M., Sasaki, K., Kamata, R., & Sakai, R. (2007). The C-terminus of ephrin-B1 regulates metalloproteinase secretion and invasion of cancer cells. *J Cell Sci, 120*(Pt 13), 2179-2189. doi:10.1242/jcs.008607

Tandon, P., Showell, C., Christine, K., & Conlon, F. L. (2012). Morpholino Injection in Xenopus. *Methods in Molecular Biology, 843*, 29-46.

Tang, X. X., Zhao, H., Robinson, M. E., Cohen, B., Cnaan, A., London, W., . . . Ikegaki, N. (2000). Implications of EPHB6, EFNB2, and EFNB3 expressions in human neuroblastoma. *Proc Natl Acad Sci U S A, 97*(20), 10936-10941.

Tannock, I. F. (1970). Population Kinetics of Carcinoma Cells, Capillary Endothelial Cells, and Fibroblasts in a Transplanted Mouse Mammary Tumor. *Cancer Research, 30*(10), 2470.

Teo, J. L., Ma, H., Nguyen, C., Lam, C., & Kahn, M. (2005). Specific inhibition of CBP/beta-catenin interaction rescues defects in neuronal differentiation caused by a presenilin-1 mutation. *Proc Natl Acad Sci U S A, 102*(34), 12171-12176. doi:10.1073/pnas.0504600102

Terzi, M. Y., Izmirli, M., & Gogebakan, B. (2016). The cell fate: senescence or quiescence. *Mol Biol Rep, 43*(11), 1213-1220. doi:10.1007/s11033-016-4065-0

Thurtle-Schmidt, D. M., & Lo, T. W. (2018). Molecular biology at the cutting edge: A review on CRISPR/CAS9 gene editing for undergraduates. *Biochem Mol Biol Educ, 46*(2), 195-205. doi:10.1002/bmb.21108

Tian, T., Olson, S., Whitacre, J. M., & Harding, A. (2011). The origins of cancer robustness and evolvability. *Integrative Biology, 3*(1), 17-30. doi:10.1039/C0IB00046A

Tojkander, S., Gateva, G., & Lappalainen, P. (2012). Actin stress fibers--assembly, dynamics and biological roles. *J Cell Sci, 125*(Pt 8), 1855-1864. doi:10.1242/jcs.098087

Torres, R., Firestein, B. L., Dong, H., Staudinger, J., Olson, E. N., Huganir, R. L., . . . Yancopoulos, G. D. (1998). PDZ proteins bind, cluster, and synaptically colocalize with Eph receptors and their ephrin ligands. *Neuron, 21*(6), 1453-1463.

Tsai, J. H., Cheng, C. H., Chen, C. C., Lin, Y. L., Lin, L. I., Chen, M. L., & Liau, J. Y. (2016). Traditional serrated adenoma with BRAF mutation is associated with synchronous/metachronous BRAF-mutated serrated lesions. *Histopathology, 68*(6), 810-818. doi:10.1111/his.12814

Tseng, W., Leong, X., & Engleman, E. (2007). Orthotopic Mouse Model of Colorectal Cancer. *Journal of Visualized Experiments : JoVE*(10), 484. doi:10.3791/484

Tu, Y., He, S., Fu, J., Li, G., Xu, R., Lu, H., & Deng, J. (2012). Expression of EphrinB2 and EphB4 in glioma tissues correlated to the progression of glioma and the prognosis of glioblastoma patients. *Clinical and Translational Oncology, 14*(3), 214-220. doi:10.1007/s12094-012-0786-2

Uhlen, M., Fagerberg, L., Hallstrom, B. M., Lindskog, C., Oksvold, P., Mardinoglu, A., . . . Ponten, F. (2015). Proteomics. Tissue-based map of the human proteome. *Science, 347*(6220), 1260419. doi:10.1126/science.1260419

Uy, P. P. D., Francisco, D. M., Trivedi, A., O'Loughlin, M., & Wu, G. Y. (2017). Vascular Diseases of the Spleen: A Review. *J Clin Transl Hepatol, 5*(2), 152-164. doi:10.14218/jcth.2016.00062

Van De Wetering, M., Sancho, E., Verweij, C., De Lau, W., Oving, I., Hurlstone, A., . . . Clevers, H. (2002). The Beta-Catenin/TCF-4 Complex Imposes a Crypt Progenitor Phenotype on Colorectal Cancer Cells. *Cell, 111*(2), 241-250.

Van Raay, T., & Allen-Vercoe, E. (2017). Microbial Interactions and Interventions in Colorectal Cancer. *Microbiol Spectr, 5*(3). doi:10.1128/microbiolspec.BAD-0004-2016

Vayrynen, S. A., Vayrynen, J. P., Klintrup, K., Makela, J., Karttunen, T. J., Tuomisto, A., & Makinen, M. J. (2016). Clinical impact and network of determinants of tumour necrosis in colorectal cancer. *Br J Cancer, 114*(12), 1334-1342. doi:10.1038/bjc.2016.128

Vearing, C. J., & Lackmann, M. (2005). Eph Receptor Signalling; Dimerisation Just Isn't Enough. *Growth Factors, 23*(67-76).

Vindis, C., Cerretti, D. P., Daniel, T. O., & Huynh-Do, U. (2003). EphB1 recruits c-Src and p52(Shc) to activate MAPK/ERK and promote chemotaxis. *The Journal of Cell Biology, 162*(4), 661-671. doi:10.1083/jcb.200302073

Wang, H., La Russa, M., & Qi, L. S. (2016). CRISPR/Cas9 in Genome Editing and Beyond. *Annu Rev Biochem, 85*, 227-264. doi:10.1146/annurev-biochem-060815-014607

Wang, H. U., Chen, Z. F., & Anderson, D. J. (1998). Molecular Distinction and Angiogenic Interaction between Embryonic Arteries and Veins Revealed by Ephrin-B2 and Its Receptor Eph-B4 Angiogenesis. *Cell, 93*, 741-753.

Wang, T., Cai, G., Qiu, Y., Fei, N., Zhang, M., Pang, X., . . . Zhao, L. (2012). Structural Segregation of Gut Microbiota Between Colorectal Cancer Patients and Healthy Volunteers. *The ISME Journal, 6*, 320-329.

Wang, Y., Li, Q., Zheng, Y., Li, G., & Liu, W. (2016). *Systematic biochemical characterization of the SAM domains in Eph receptor family from Mus Musculus* (Vol. 473).

Wei, Q., Liu, J., Wang, N., Zhang, X., Jin, J., Chin-Sang, I., . . . Jia, Z. (2014). Structures of an Eph Receptor Kinase and its Potential Activation Mechanism. *Acta Crystallographica, D70*, 3135-3143.

Wei, S., Xu, G., Bridges, L. C., Williams, P., White, J. M., & DeSimone, D. W. (2010). ADAM13 induces cranial neural crest by cleaving class B Ephrins and regulating Wnt signaling. *Dev Cell, 19*(2), 345-352. doi:10.1016/j.devcel.2010.07.012

Weir, L., Robertson, D., Leigh, I. M., & Panteleyev, A. A. (2011). The reduction of water-soluble tetrazolium salt reagent on the plasma membrane of epidermal keratinocytes is oxygen dependent. *Analytical Biochemistry, 414*(1), 31-37. doi:https://doi.org/10.1016/j.ab.2011.02.030

White, A. C., Levy, J. A., & McGrath, C. M. (1982). Site-selective Growth of a Hormone-responsive Human Breast Carcinoma in Athymic Mice. *Cancer Research, 42*(3), 906.

Wiedenheft, B., Sternberg, S. H., & Doudna, J. A. (2012). RNA-guided genetic silencing systems in bacteria and archaea. *Nature, 482*(7385), 331-338. doi:10.1038/nature10886

Wiesner, S., Wybenga-Groot, L. E., Warner, N., Lin, H., Pawson, T., Forman-Kay, J. D., & Sicheri, F. (2006). A change in conformational dynamics underlies the activation of Eph receptor tyrosine kinases. *Embo j, 25*(19), 4686-4696. doi:10.1038/sj.emboj.7601315

Willis, N., Przyborski, S. A., Hutchison, C. J., & Wilson, R. G. (2008). Colonic and Colorectal Cancer Stem Cells: Progress in the Search for Putative Biomarkers. *Journal of Anatomy, 213*, 59–65.

Wimmer-Kleikamp, S. H., Janes, P. W., Squire, A., Bastiaens, P. I. H., & Lackmann, M. (2004). Recruitment of Eph Receptors into Signaling Clusters does not Require Ephrin Contact *Journal of Cell Biology, 164*, 661–666.

Witzel, F., Fritsche-Guenther, R., Lehmann, N., Sieber, A., & Bluthgen, N. (2015). Analysis of impedance-based cellular growth assays. *Bioinformatics, 31*(16), 2705-2712. doi:10.1093/bioinformatics/btv216

Wu, Q., Suo, Z., Risberg, B., Karlsson, M. G., Villman, K., & Nesland, J. M. (2004). Expression of Ephb2 and Ephb4 in breast carcinoma. *Pathology & Oncology Research, 10*(1), 26. doi:10.1007/bf02893405

Wu, W. K., Wang, X. J., Cheng, A. S., Luo, M. X., Ng, S. S., To, K. F., . . . Yu, J. (2013). Dysregulation and crosstalk of cellular signaling pathways in colon carcinogenesis. *Crit Rev Oncol Hematol, 86*(3), 251-277. doi:10.1016/j.critrevonc.2012.11.009

www.proteinatlas.org, H. P. A. a. f.

Wybenga-Groot, L. E., Baskin, B., Ong, S. H., Tong, J., Pawson, T., & Sicheri, F. (2001). Structural Basis for Autoinhibition of the EphB2 Receptor Tyrosine Kinase by the Unphosphorylated Juxtamembrane Region. *Cell, 106*(6), 745-757.

Xia, G., Kumar, S. R., Masood, R., Zhu, S., Reddy, R., Krasnoperov, V., . . . Gill, P. S. (2005). EphB4 expression and biological significance in prostate cancer. *Cancer Res, 65*(11), 4623-4632. doi:10.1158/0008-5472.can-04-2667

Xia, G., Kumar, S. R., Stein, J. P., Singh, J., Krasnoperov, V., Zhu, S., . . . Gill, P. S. (2006). EphB4 receptor tyrosine kinase is expressed in bladder cancer and provides signals for cell survival. *Oncogene, 25*(5), 769-780. doi:10.1038/sj.onc.1209108

Xiao, Z., Carrasco, R., Kinneer, K., Sabol, D., Jallal, B., Coats, S., & Tice, D. A. (2012). EphB4 promotes or suppresses Ras/MEK/ERK pathway in a context-dependent manner: Implications for EphB4 as a cancer target. *Cancer Biol Ther, 13*(8), 630-637. doi:10.4161/cbt.20080

Xiong, B., Ma, L., Hu, X., Zhang, C., & Cheng, Y. (2014). Characterization of side population cells isolated from the colon cancer cell line SW480. *Int J Oncol, 45*(3), 1175-1183. doi:10.3892/ijo.2014.2498

Xiong, C., Huang, M., Zhang, R., Song, S., Lu, W., Flores, L., . . . Li, C. (2011). In Vivo Small-Animal PET/CT of EphB4 Receptors Using 64Cu-Labeled Peptide. *Journal of Nuclear Medicine, 52*(2), 241-248.

Xu, K., Tzvetkova-Robev, D., Xu, Y., Goldgur, Y., Chan, Y. P., Himanen, J. P., & Nikolov, D. B. (2013). Insights into Eph receptor tyrosine kinase activation from crystal structures of the EphA4 ectodomain and its complex with ephrin-A5. *Proc Natl Acad Sci U S A, 110*(36), 14634-14639. doi:10.1073/pnas.1311000110

Xuqing, W., Lei, C., Zhengfa, M., Shengchun, D., Xin, F., Jianguo, Q., & Jianxin, Z. (2012). EphB4 is overexpressed in papillary thyroid carcinoma and promotes the migration of papillary thyroid cancer cells. *Tumor Biology, 33*(5), 1419-1427. doi:10.1007/s13277-012-0392-5

Yancopoulos, G. D., Klagsbrun, M., & Folkman, J. (1998). Vasculogenesis, Angiogenesis, and Growth Factors: Ephrins Enter the Fray at the Border. *Cell, 93*, 661-664.

Yang, D., Jin, C., Ma, H., Huang, M., Shi, G. P., Wang, J., & Xiang, M. (2016). EphrinB2/EphB4 pathway in postnatal angiogenesis: a potential therapeutic target for ischemic cardiovascular disease. *Angiogenesis, 19*(3), 297-309. doi:10.1007/s10456-016-9514-9

Yang, N.-Y., Pasquale, E. B., Owen, L. B., & Ethell, I. M. (2006). The EphB4 Receptor-tyrosine Kinase Promotes the Migration of Melanoma Cells through Rho-mediated Actin Cytoskeleton Reorganization. *Journal of Biological Chemistry, 281*(43), 32574-32586. doi:10.1074/jbc.M604338200

Yang, N. Y., Lopez-Bergami, P., Goydos, J. S., Yip, D., Walker, A. M., Pasquale, E. B., & Ethell, I. M. (2010). The EphB4 receptor promotes the growth of melanoma cells expressing the ephrin-B2 ligand. *Pigment Cell Melanoma Res, 23*(5), 684-687. doi:10.1111/j.1755-148X.2010.00745.x

Yang, X., Yang, Y., Tang, S., Tang, H., Yang, G., Xu, Q., & Wu, J. (2015). EphB4 inhibitor overcome the acquired resistance to cisplatin in melanomas xenograft model. *J Pharmacol Sci, 129*(1), 65-71. doi:10.1016/j.jphs.2015.08.009

Yavrouian, E. J., Sinha, U. K., Rice, D. H., Salam, M. T., Gill, P. S., & Masood, R. (2008). The Significance of EphB4 and EphrinB2 Expression and Survival in Head and Neck Squamous Cell Carcinoma. *Archives of Otolaryngology-Head & Neck Surgery, 134*, 985-991.

Yeung, T. M., Chia, L. A., Kosinski , C. M., & Kuo, C. J. (2011). Regulation of Self-Renewal and Differentiation by the Intestinal Stem Cell Niche. *Cellular and Molecular Life Sciences, 68*, 2513-2523.

Yin, J., Cui, Y., Li, L., Ji, J., & Jiang, W. G. (2017). Overexpression of EPHB4 Is Associated with Poor Survival of Patients with Gastric Cancer. *Anticancer Research, 37*(8), 4489-4497. doi:10.21873/anticanres.11845

Yin, L.-M., Wei, Y., Wang, W.-Q., Wang, Y., Xu, Y.-D., & Yang, Y.-Q. (2014). Simultaneous application of BrdU and WST-1 measurements for detection of the proliferation and viability of airway smooth muscle cells. *Biological Research, 47*(1), 75. doi:10.1186/0717-6287-47-75

Yin, L.-M., Wei, Y., Wang, Y., Xu, Y.-D., & Yang, Y.-Q. (2013). Long Term and Standard Incubations of WST-1 Reagent Reflect the Same Inhibitory Trend of Cell Viability in Rat Airway Smooth Muscle Cells. *International Journal of Medical Sciences, 10*(1), 68-72. doi:10.7150/ijms.5256

Yoo, S., Kim, Y., Noh, H., Lee, H., Park, E., & Park, S. (2011). Endocytosis of EphA receptors is essential for the proper development of the retinocollicular topographic map. *Embo j, 30*(8), 1593-1607. doi:10.1038/emboj.2011.44

You, C., Zhao, K., Dammann, P., Keyvani, K., Kreitschmann-Andermahr, I., Sure, U., & Zhu, Y. (2017). EphB4 forward signalling mediates angiogenesis caused by CCM3/PDCD10-ablation. *J Cell Mol Med, 21*(9), 1848-1858. doi:10.1111/jcmm.13105

Young, J. P., Win, A. K., Rosty, C., Flight, I., Roder, D., Young, G. P., . . . Price, T. J. (2015). Rising incidence of early-onset colorectal cancer in Australia over two decades: Report and review. *Journal of Gastroenterology and Hepatology, 30*(1), 6-13. doi:10.1111/jgh.12792

Yu, G., Luo, H., Wu, Y., & Wu, J. (2003). Ephrin B2 Induces T Cell Costimulation. *The Journal of Immunology, 171*(1), 106.

Yumoto, N., Wakatsuki, S., Kurisaki, T., Hara, Y., Osumi, N., Frisén, J., & Sehara-Fujisawa, A. (2008). Meltrin β/ADAM19 Interacting with EphA4 in Developing Neural Cells Participates in Formation of the Neuromuscular Junction. *PLoS ONE, 3*(10), e3322. doi:10.1371/journal.pone.0003322

Zenonos, K., & Kyprianou, K. (2013). RAS Signaling Pathways, Mutations and Their Role in Colorectal Cancer. *World Journal of Gastrointestinal Oncology, 5*(5), 97-101.

Zhang, R., Xiong, C., Huang, M., Zhou, M., Huang, Q., Wen, X., . . . Lia, C. (2011). Peptide-Conjugated Polymeric Micellar Nanoparticles for Dual SPECT and Optical Imaging of EphB4 Receptors in Prostate Cancer Xenografts. *Biomaterials, 32*(25), 5872–5879.

Zhang, X. Q., Takakura, N., Oike, Y., Inada, T., Gale, N. W., Yancopoulos, G. D., & Suda, T. (2001). Stromal Cells Expressing Ephrin-B2 Promote The Growth And Sprouting Of Ephrin-B2+ Endothelial Cells. *Hemostasis, Thrombosis, and Vascular Biology, 98*(4), 1028-1037.

Zhang, Z., Zhang, Y., Gao, F., Han, S., Cheah, K. S., Tse, H. F., & Lian, Q. (2017). CRISPR/Cas9 Genome-Editing System in Human Stem Cells: Current Status and Future Prospects. *Mol Ther Nucleic Acids, 9*, 230-241. doi:10.1016/j.omtn.2017.09.009

Zhao, W.-H., Huang, B.-T., Zhang, J.-Y., & Zeng, Q.-C. (2017). Distinct EphB4-mediated mechanisms of apoptotic and resistance to dasatinib in human chronic myeloid leukemia and K562 cell lines. *Leukemia Research, 63*, 28-33. doi:https://doi.org/10.1016/j.leukres.2017.10.014

Zheng, L.-C., Wang, X.-Q., Lu, K., Deng, X.-L., Zhang, C.-W., Luo, H., . . . Shi, S.-L. (2017). Ephrin-B2/Fc promotes proliferation and migration, and suppresses apoptosis in human umbilical vein endothelial cells. *Oncotarget, 8*(25), 41348-41363. doi:10.18632/oncotarget.17298

Zhu, C., Wang, X., Zheng, H., Liu, T., Li, Y., Sun, C., . . . Zhao, F. (2007). Expression of EphB4 and HIF-1α in Lung Cancer and its Clinical Significance. *The Chinese-German Journal of Clinical Oncology, 6*(1), 9-12.

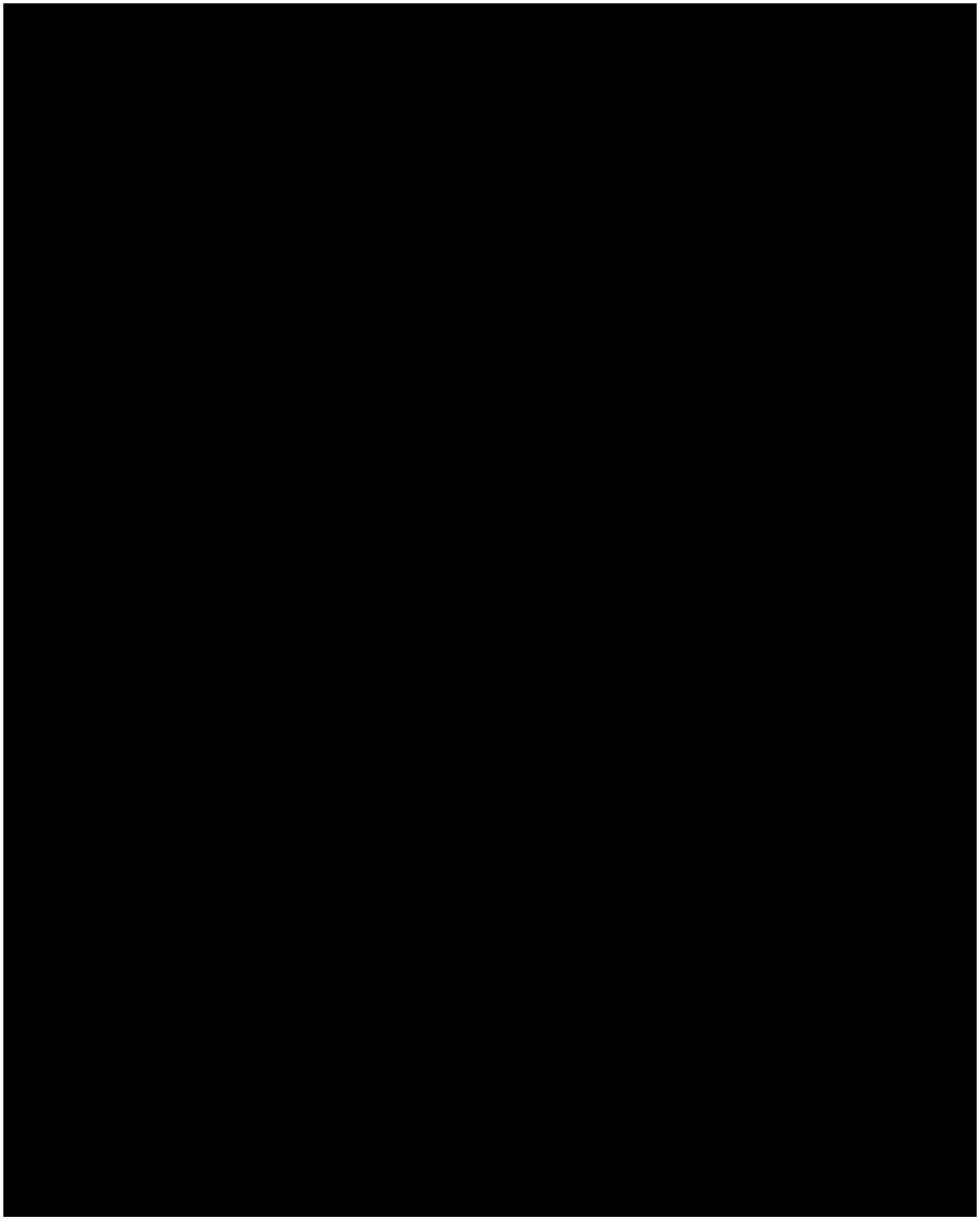
Zigmond, E., Halpern, Z., Elinav, E., Brazowski, E., Jung, S., & Varol, C. (2011). Utilization of Murine Colonoscopy for Orthotopic Implantation of Colorectal Cancer. *PLoS ONE, 6*(12), e28858. doi:10.1371/journal.pone.0028858

Zimmer, M., Palmer, A., Kohler, J., & Klein, R. (2003). EphB-EphrinB Bi-Directional Endocytosis Terminates Adhesion Allowing Contact Mediated Repulsion. *Nature Cell Biology, 5*(10), 869-878.

Zisch, A. H., Kalo, M. S., Chong, L. D., & Pasquale, E. B. (1998). Complex formation between EphB2 and Src requires phosphorylation of tyrosine 611 in the EphB2 juxtamembrane region. *Oncogene, 16*(20), 2657-2670. doi:10.1038/sj.onc.1201823

Zou, J. X., Wang, B., Kalo, M. S., Zisch, A. H., Pasquale, E. B., & Ruoslahti, E. (1999). An Eph receptor regulates integrin activity through R-Ras. *Proceedings of the National Academy of Sciences, 96*(24), 13813.

APPENDIX: Publications from this thesis



The full-text of this article is subject to copyright restrictions, and cannot be included in the online version of the thesis.

Elif Kadife, Thomas Michael Benjamin Ware, Rodney Brian Luwor, Steven Tuck Foo Chan, Kulmira Nurgali & Paul Vincent Senior (2018) Effects of EphB4 receptor expression on colorectal cancer cells, tumor growth, vascularization and composition, Acta Oncologica, 57:8, 1043-1056, DOI: 10.1080/0284186X.2018.1429650