Investigating the role of G-protein coupled receptor (GPCR) signaling as a potential target for anti-angiogenic therapy

by

Abul Azad

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Department of Medicine

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Abstract

Angiogenesis is an essential process for normal growth and development whereby new blood vessels are formed from pre-existing ones. The balance between pro-angiogenic and anti-angiogenic growth factors is critical to maintain normal physiological condition. Prolonged activation of angiogenesis leads to different pathological conditions including diabetic retinopathy, ischemic heart disease and cancer progression. In 1971, Folkman proposed that tumor could not grow beyond $2mm^3$ without the supply of nutrients and oxygen via neovascularization, suggesting that inhibition of angiogenesis would keep the tumor in a small size and dormant state. In 1990s, Ferrara's group showed that the loss of single allele of vascular endothelial growth factor (VEGF) resulted in embryonic lethality due to vessels abnormalities and inhibition of VEGF suppressed tumor angiogenesis and tumor growth *in vivo*. These works led the foundation for the development of several anti-angiogenic drugs targeting VEGF-pathway. To this end, several anti-angiogenic inhibitor (AI) drugs have been approved by FDA for the treatment of many types of cancers.

While benefits of AI therapies are clinically evident, the initial response to therapy is not durable, and in many cases, patients develop drug resistance over time due to the activation of multiple other mechanisms compensating blockade of VEGF-signaling. One of the key mechanisms that is over activated due to vessels pruning by AI therapy is intra-tumoral hypoxia, which in turn contributes to the production of plethora of alternative pro-angiogenic growth factors, thereby enabling the tumors with the continuous supply of oxygen and nutrients. Besides, patients with high level of tumor

hypoxia have higher risk of tumor progression and metastasis and reduce the tumor cells sensitivity to radiation, chemo- and immunotherapies as it becomes harder for the delivery of drugs in chronically hypoxic microenvironment. Thus, it is of utmost importance to identify broad-spectrum anti-angiogenic target(s) that can block multiple pro-angiogenic pathways, and at the same will alleviate hypoxia, for the development of better anti-angiogenic therapy.

This thesis investigates the role of alternative pro-angiogenic signaling, especially with regard to the G-protein coupled receptor (GPCR)-mediated phosphoinositide 3-kinases (PI3K) signaling activation in tumor angiogenesis. **First,** we investigated the role of endothelial cell (EC) specific p110β (PI3Kβ) isoform of PI3K signaling in regulating angiogenesis. We showed that p110β functions as a downstream target of GPCR signaling and controls angiogenesis in ex-vivo and in vivo models. We observed that patient-derived renal cell carcinoma (PD-RCC) samples release several pro-angiogenic GPCR ligands including C-X-C motif chemokine 12 (CXCL12), also known as stromal derived factor 1 (SDF-1), and apelin. We found that selective inactivation of PI3Kβ reduced PD-RCC-stimulated EC spheroid sprouting. In mice, loss of EC PI3Kβ in combination with receptor tyrosine kinase inhibitor (TKI) sunitinib treatment decreased primary tumor growth and tumor metastasis, which was accompanied by decreased tumor vessel density and angiogenesis. Further, we found that knockout of EC-specific p110β (EC-βKO) plus sunitinib resulted in decreased tumor hypoxia, but tumor vessels were normalized, suggesting that targeting EC PI3Kβ might facilitate delivery of chemo- and immunotherapeutic drugs.

Second, we studied the role of apelin, a GPCR ligand, in tumor angiogenesis. We found that loss of apelin in mice delayed the primary tumor growth in combination with sunitinib treatment, accompanied by a marked reduction in tumor vessels density, angiogenic sprouting and tip cell marker gene expression in comparison to the control group. Interestingly, in our single cell RNA (scRNA) sequencing, we observed that the loss of apelin prevented EC tip cell differentiation in comparison to the control group, suggesting that blockade of apelin captures most of the effect of PI3Kβ inhibition in EC to limit the tumor angiogenesis and the subsequent tumor growth. Together, our first two projects showed that alternative pro-angiogenic signaling operate in parallel to VEGF in tumor angiogenesis.

Third, we investigated the role of endothelial cell specific Facio-genital dysplasia 5 (FGD5) in regulating both the receptor tyrosine kinase (RTK)/PI3K and C-X-C motif chemokine receptor 4 (CXCR4)/PI3K signaling pathways. The first part of this project, i.e., FGD5 in regulating the RTK/PI3K signaling was completed in collaboration with Dr. Maikel Farhan, who was a PhD student in our laboratory, and had been published in Arteriosclerosis, Thrombosis, and Vascular Biology (ATVB) in 2017 where I was a first co-author. Therefore, we opted not to include the details work of the paper in this current thesis, rather a brief summary will be included to better facilitate the transitioning to address the role of FGD5 in CXCR4/PI3K signaling. In this later project, we showed that CXCL12 augmented EC sprouting angiogenesis and tip cell marker gene expression under conditions where VEGF was limiting, and that CXCL12-stimulated

CXCR4/PI3K signaling activation depends on PI3Kβ isoform. Knockdown of FGD5 decreases CXCL12-stimulated CXCR4/PI3K signaling. FGD5 modulates the CXCR4/PI3K signaling by acting as a Rho guanine exchange factor (RhoGEF) for Rac1 to control p110β activity. Together, our works showed that being an EC-restricted protein, FGD5 participates in regulating both RTK/PI3K and CXCR4/PI3K signaling pathways, indicating that FGD5 may serve as a potential target for anti-angiogenic therapy.

In summary, we show that GPCR/PI3K signaling participates in tumor angiogenesis along with the RTK/PI3K signaling. In endothelium, PI3Kβ is the dominant isoform of PI3K to regulate GPCR-mediated tumor angiogenesis. Inhibition of apelin, a pro-angiogenic GPCR ligand, mostly arrests the contribution of GPCR/PI3K signaling in tumor angiogenesis. Finally, we show that EC-specific FGD5 could be a potential target for the development of an effective anti-angiogenic therapy as FGD5 regulates parallel pro-angiogenic signaling coming from both RTK/PI3K and GPCR/PI3K signaling pathways.

Preface

This thesis is an original work by Abul Kalam Azad. This thesis consists of a published work, one in press for publication and an unpublished works for which manuscript has been prepared for submission to a peer reviewed journal. Chapter 3 of the thesis has been published as *Abul K. Azad, Pavel Zhabyeyev, Bart Vanhaesebroeck, Gary Eitzen, Gavin Y. Oudit, Ronald B. Moore, Allan G. Murray. Inactivation of endothelial cell phosphoinositide 3-kinase β inhibits tumor angiogenesis and tumor growth. Oncogene volume 39, pages6480–6492(2020).* The animals for this project were bred at Dr. Gavin Oudit's lab and were taken care of by Pavel Zhabyeyev. I conducted all the experiments and acquired the data, and the data analysis was performed by Dr. Murray and me.

A version of the Chapter 4 will be submitted for publication as *Abul K. Azad, Kieran Campbell, Pavel Zhabyeyev, Gavin Y. Oudit, Ronald B. Moore, Allan G. Murray. Loss of apelin blocks the emergence of sprouting angiogenesis in experimental tumors.* I conducted all the experiments and acquired the data. Single cell RNA sequencing data were analyzed by Dr. Kieran Campbell and Dr. Allan Murray. The animals for this project were bred at Dr. Gavin Oudit's lab and were taken care of by Pavel Zhabyeyev.

A version of the Chapter 5 has been accepted for publication in The FASEB Journal as *Abul K. Azad, Maikel A. Farhan, Cameron R. Murray, Kunimasa Suzuki, Nicolas Touret, Gary Eitzen, Ronald B. Moore, Allan G. Murray. FGD5 regulates endothelial cell PI3 kinase-β to promote neoangiogenesis.* The plasmids for this project were prepared by Cameron R. Murray and Kunimasa Suzuki**.** Couple of a Western blotting experiments and data analysis were done by Dr. Maikel Farhan. The data analysis for immunofluorescence confocal images was completed by Dr. Nicolas Touret**.** I conducted the rest of the experiments, data acquisition and analysis for this project.

Human renal cell carcinoma (RCC) tissue samples were obtained at surgical resection of the tumor under a protocol approved by the Human Research Ethics Board of the

University of Alberta. Patients' consents were obtained before the collection of RCC samples. All animal experiments were performed following the guidelines approved by the Canadian Council for Animal Care (CCAC), and the animal protocol was approved by the Animal Care and Use Committee at the Alberta Health Services Cross Cancer Institute (CCI).

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I am immensely grateful to my supervisor Dr. Ronald Moore. His continuous support, guidance and encouragements helped me a lot throughout my PhD program. I would like to express my gratitude to my committee members Dr. Nicolas Touret and Dr. YangXin Fu for their feedbacks and suggestions throughout my graduate study. Thank you, Dr. Nicholas, for your thoughtful comments and experimental supports. Thank you, Dr. Fu, for teaching me many of the molecular biology techniques that I have been using throughout the years.

A special thanks to Dr. Barbara Ballermann, Dr. Nadia Jahroudi and Dr. Branko Braam for their critical comments and advices during the Nephrology Research Seminar presentations. I would like to thank all the members in Dr. Barbara, Dr. Nadia and Dr. Branko's labs for their suggestions, collaborations and help I have received during this time. I must acknowledge that I was fortunate to have friends and lab mates like Maikel Farhan and Andrew Masoud who helped me a lot in preparing reagents and doing experiments.

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Akt:	Protein kinase B
Alk1:	activin receptor like kinase
Ang1:	Angiopoietin 1
APLNR:	Apelin receptor
BMDC:	Bone marrow-derived cells
BMP9:	Bone morphogenetic protein 9
BSA:	Bovine serum albumin
CAF:	Cancer-associated fibroblasts
CSC:	Cancer stem cell
Ctrl:	Control
CXCL12:	C-X-C motif chemokine 12
CXCR4:	C-X-C chemokine receptor type 4
DEG:	Differentially expressed genes
DH:	DBL homology
Dll4:	Delta-like protein 4
EC:	Endothelial cell
EC-βKO:	EC-specific p110b knockout
ECM:	Extracellular matrix
EDG1:	Endothelial differentiation sphingolipid G-protein-coupled receptor-1
EGF:	Epidermal growth factor
EGFR:	Epidermal growth factor receptor
EMT:	Epithelial-mesenchymal transition
EPC: E	Endothelial progenitor cell
ESM1:	Endothelial-specific molecule 1
FBS:	Fetal bovine serum
FGD5:	Faciogenital dysplasia 5 protein
bFGF:	Basic fibroblast growth factor
FOXO:	Forkhead transcription factor

GDP:	Guanine diphosphate
GEF:	Guanine nucleotide exchange factors
GLUT4:	Glucose transporter type 4
GPCR:	G protein-coupled receptor
GRK:	G protein-coupled receptor kinase-2
GSK3β:	Glycogen synthase kinase 3β
GTP:	Guanine triphosphate
HCC:	Hepatocellular carcinoma
HGF:	Hepatocyte growth factor
HIF:	Hypoxia-inducible factor
HUVEC:	Human umbilical vein endothelial cells
IFNa:	Interferon alpha
IHC:	Immunohistochemistry
LLC1:	Lewis lung carcinoma
LPA:	Lipopolysaccharide acid
MAPK:	Mitogen-activated protein kinase
MDSC:	Myeloid-derived suppressor cells
MEF:	Mouse embryonic fibroblasts
MMP9:	Matrix metalloproteinase 9
mRCC :	Metastatic renal cell carcinoma
mTORC1:	Mammalian target of rapamycin complex 1
nGBM:	Newly diagnosed glioblastoma
NRPs:	Neuropilin receptors
NSCLC:	Non-squamous non-small cell lung carcinoma
OS:	Overall survival
PAR1:	Protease-activated receptor
PCA:	Principal components analysis
PCR:	Polymerase chain reaction
PD-L1:	Programed death ligand 1
PD-RCC:	Patient derived renal cell carcinoma
PDGF:	Platelet-derived growth factor

PDGFRb:	Platelet-derived growth factor receptor beta
PFKFB3:	6-Phosphofructo-2-kinase/fructose-2,6 bisphosphatase 3
PFS:	Progression free survival
PH:	Pleckstrin homology
PHD:	Prolyl hydroxylase domain
PI:	Phosphoinositide
PI3K:	Phosphotidylinositide-3 kinase
PIP2:	Phosphotidylinositol (4,5) bisphosphate
PIP3:	Phosphatidylinositol (3,4,5) triphosphate
PKC:	Protein kinase C
PLGF:	Placental growth factor
PNET:	Pancreatic neuroendocrine tumor
PTEN:	Phosphatase and tensin homologue deleted on chromosome 10
qPCR:	Quantitative polymerase chain reaction
RBD:	Ras binding domain
ROS:	Reactive oxygen species
RTK:	Receptor tyrosine kinase
S1P:	Sphingosine 1-phosphate
scRNAseq:	Single cell RNA sequencing
SDF1:	Stromal cell-derived factor 1
siNS:	Short interference non-silencing
siRNA:	Small interfering RNA
sVEGFR1:	Soluble VEGFR1
TGF-β:	Transforming growth factor β
TKI:	Tyrosine kinase inhibitor
VEGF:	Vascular endothelial growth factor
VEGFR2:	Vascular endothelial growth factor receptor 2
VHL:	von-Hippel Lindau
vWF:	von Willebrand factor

Chapter 1
Introduction

1.1 Developmental angiogenesis

Formation of blood vessels is an indispensable process during the early embryonic development. Vasculogenesis and angiogenesis are the two main processes whereby vascular system and heart, the two primary functional organs, develop at the early stage of embryonic development (Betz et al. 2016, Chung and Ferrara 2011). Vasculogenesis is referred to de novo formation of blood vessels whereby endothelial precursor cells, known as angioblasts, aggregate together to form primitive vascular structures such as aorta, vein and vascular capillary plexus (Chung and Ferrara 2011, Betz et al. 2016). Vascular endothelial growth factor (VEGF) and its receptor VEGFR2/Flk1 have been identified as the two of the critical earliest markers for the generation of endothelial cell (EC) and vascular development. Mice lacking single allele of VEGF is embryonically lethal due to lack of proper vascular development (Carmeliet et al. 1996). Similarly, loss of VEGFR2 in mice results in embryonic lethality associated with the lack of development of both hematopoietic and endothelial cell lineages, highlighting the importance of VEGF/VEGFR2 in the development of blood and vascular system (Shalaby et al. 1995, Carmeliet et al. 1996). The primitive vessels thus formed then undergo remodeling, maturation and expansion to form organized vascular networks. Although primarily happened during the embryonic development, the process of vasculogenesis can be found in adults during certain pathological conditions including in

tumor angiogenesis and in ischemic heart via the incorporation of endothelial progenitor cells (EPCs) (Masuda et al. 2007, Brown 2014).

1.1.1 VEGF signaling in developmental angiogenesis

VEGF is the key pro-angiogenic growth factor that plays important role in developmental and physiological angiogenesis. The VEGF ligand family consists of VEGF-A, VEGF-B, VEGF-C and VEGF-D, as well as placental growth factor (PLGF). VEGF-A (also designated as VEGF only) plays the crucial role in vessels formation as it was shown by the works of both Ferrara and Carmeliet that inactivation of single allele of VEGF led to embryonic lethality due to abnormal vessels development (Ferrara et al. 1996, Carmeliet et al. 1996). In mouse retina, neutralization of VEGF not only prevents retinal angiogenesis but also regresses the existing blood vessels, indicating that VEGF is required for the new vessel formation as well as the survival of immature vessels during the early stage of angiogenesis (Uemura et al. 2006, Gerhardt et al. 2003). Further, VEGF also regulates the arterial and venous specification. For example, inhibition of VEGF in zebrafish by morpholino inhibits the expression of arterial marker *ephb2*, while increases the expression venous marker *flt4* (Lawson, Vogel and Weinstein 2002). While VEGF-B is highly expressed in cardiac and skeletal muscle, and regulates coronary artery system, VEGF-C and VEGF-D are the key regulators for lymph-angiogenesis (Aase et al. 2001, Rauniyar, Jha and Jeltsch 2018, Stacker and Achen 2018). VEGF-C plays crucial role in early lymphatic vessels development as mice knockout of VEGF-C die prenatally due to lack of lymphatic vessels formation (Karkkainen et al. 2004).

These VEGF ligands signal through different receptor tyrosine kinases (RTKs) such as VEGF receptor (VEGFR)1, VEGFR2 and VEGFR3, and non-RTK co-receptors neuropilin 1(NRP1) and NRP2 to regulate EC functions (Ferrara, Gerber and LeCouter 2003). Of these, VEGF/VEGFR2 signaling has been shown to play major role in developmental and tumor angiogenesis (Waldner et al. 2010, Kowanetz and Ferrara 2006, Shalaby et al. 1995). Mice deficient in VEGFR2 or mutation in VEGFR2 tyrosine residue (Tyr-1173) die in utero between days 8.5 and 9.5 associated with vascular abnormalities (Shalaby et al. 1995, Sakurai et al. 2005). Further study in mouse retina shows that neutralization of VEGFR2 suppresses the formation of tip cell filopodia and vascular plexus, suggesting that VEGF/VEGFR2 signaling is indispensable in vascular development during normal physiological conditions (Gerhardt et al. 2003). VEGFR1 has high affinity for VEGF, but has weak mitogenic potential in EC, indicating that VEGFR1 might work as a decoy receptor for VEGF and could negatively regulate angiogenesis in certain condition especially during early embryonic development. Indeed, knockout of VEGFR1 has been shown to result in embryonic lethality associated with severe vessel overgrowth, possibly due to the overactivation of VEGF/VEGFR2 signaling in the absence of VEGFR1 (Fong et al. 1995). VEGFR3 is mainly expressed in lymphatic vessels, and mice knockout of VEGFR3 is embryonically lethal due to cardiovascular failure (Dumont et al. 1998).

1.2 Angiogenesis

Angiogenesis is referred to a process whereby new vessels are formed from the pre-exiting vessels. The process of angiogenesis can be initiated by the sprouting of new vessels from the pre-exiting vessels or by the splitting of the existing vessel into two new vessels (intussusception).

1.2.1 Sprouting angiogenesis

In the presence of pro-angiogenic growth factors, EC becomes activated and initiates sprouting angiogenesis. The activated EC at the distal end of each sprout is designated as tip cell, and the finger like projections coming out of the tip cells are known as filopodia (Gerhardt et al. 2003). Tip cell filopodia secrete matrix degrading enzymes to break down the extracellular matrix (ECM), becomes motile and invasive, and guides the ECs migration towards the pro-angiogenic growth factor cues (De Smet et al. 2009, Eelen et al. 2020). These migratory tip cells are followed by stalk cells, which continue to proliferate and extend to maintain the stability of the growing vessels. The developing sprout then connects to the neighbouring sprout to form a functional vessel by the subsequent recruitment of pericytes to ensure the stability and maturity of the nascent vessels.

One of the important aspects during the sprouting angiogenesis is the selection of tip cells and stalk cells. The distinct gene profiles that are expressed by tip cells include delta-like protein 4 (*Dll4*), endothelial cell specific molecule 1 (*Esm1*), *Vegfr2*, platelet derived growth factor beta (*Pdgfb*) and *Cxcr4* (del Toro et al. 2010), whereas, the stalk

cells are enriched with *Vegfr1* and von Willebrand (*vWF*) (Hellström et al. 2007, Chen et al. 2019). The tip cell-stalk cell specification is dynamically regulated by VEGF and Notch signaling pathways (Fig. 1.1) (Kangsamaksin, Tattersall and Kitajewski 2014, Jakobsson, Bentley and Gerhardt 2009). VEGF binds to the VEGFR2 and up-regulates Dll4 expression in the leading tip cells, which in turn activates Notch signaling in the adjacent cells. The Notch signaling then down regulates the expression of VEGFR2 within the adjacent cells, while up-regulates the expression of VEGFR1, which further decreases the VEGFR2 activation by acting as a weak receptor for VEGF (Kangsamaksin et al. 2014, Jakobsson et al. 2009, Hellström et al. 2007). Thus, the Notch activated neighbouring cells adopt the stalk cell behaviour, which proliferate to establish lumen formation and support the sprouting elongation. Indeed, accumulating evidence shows that inactivation of Notch signaling in EC increases the number of tip cells formed in mouse retina, whereas activation of Notch decreases tip cell formation (Jakobsson et al. 2009, Hellström et al. 2007). Importantly, global or EC specific knockout of Notch1 is embryonically lethal due sever defects in angiogenic vascular remodeling (Krebs et al. 2000, Limbourg et al. 2005). Similarly, inactivation of one allele of *Dll4* in EC increases number of tip cell in mouse retina, and mouse knockout of *Dll4* has similar phenotype as of Notch1 knockout mice, indicating that Dll4 is the cognate Notch1 ligand and participate in maintaining appropriate number of tip cells during vascular development (Hellström et al. 2007, Suchting et al. 2007, Krebs et al. 2004).

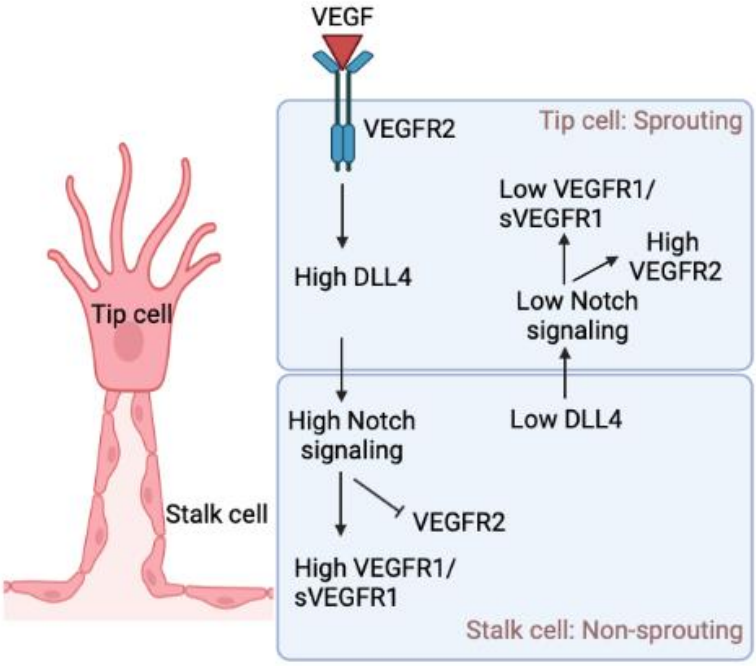

Fig. 1.1: A schematic representation VEGF/Notch signaling in tip and stalk cell specification during sprouting angiogenesis.

1.2.2 Intussusceptive angiogenesis

Intussusceptive angiogenesis, also known as splitting angiogenesis, is the process whereby interstitial tissue pillars get inserted into the lumen of pre-existing vessels, thereby splitting the vessels into halves. It's a faster process than sprouting angiogenesis and can be found during embryogenesis, but it is not very common in adults (Djonov, Kurz and Burri 2002, De Spiegelaere et al. 2012). This process was first identified in developing lung and subsequently in several other developing tissues including in retina, kidney and mammary gland (Caduff, Fischer and Burri 1986, Burri and Djonov 2002, Makanya et al. 2005). While sprouting participates in early stage of embryonic development, the studies have shown that intussusceptive angiogenesis is

mainly involved in the later stage of vascular growth and remodeling of the vascular beds (Makanya et al. 2005, Djonov, Baum and Burri 2003, Makanya, Hlushchuk and Djonov 2009). The involvement of intussusceptive angiogenesis has been reported in tumor growth and in several rodent disease models such as in glomerular nephritis and in inflammation induced murine colitis (Konerding et al. 2010, Notoya et al. 2003, Hillen and Griffioen 2007). The detail of intussusceptive angiogenesis is yet to be understood, however, in some high flow regions of the circulation, hemodynamic forces can play an important role in inducing intussusceptive angiogenesis (Djonov et al. 2002, le Noble et al. 2005).

1.3 Blood vessels maturation

In normal physiological conditions, pericytes and vascular smooth muscle cells are recruited covering around the newly formed blood vessels to confer vessels stability and maturation (Conway, Collen and Carmeliet 2001, Udan, Culver and Dickinson 2013). PDGFB is secreted by sprouting EC and has been shown to play critical role in vessels maturation by recruiting platelet PDGF receptor beta (PDGFRβ) expressing mural cells (Jain 2003, Hellberg, Ostman and Heldin 2010). Indeed, mice lacking either PDGFB or PDGFRβ have been shown to embryonically lethal associated with hyperactive EC sprouts, dilated and leaky vessels due to lack of proper pericytes coverage (Levéen et al. 1994, Soriano 1994, Hellström et al. 2001). Further, antibody-mediated neutralization of PDGFRβ in neonatal pups prevents retinal vascular maturation due to the lack of mural cell coverage (Uemura et al. 2002). The presence of high level VEGF itself contributes to vessels abnormalities by suppressing the PDGFRβ signaling in mural

cells, thereby preventing pericytes recruitment to the newly developed vessels (Greenberg et al. 2008). In addition, knockout of endothelial differentiation sphingolipid G-protein-coupled receptor-1 (EDG1, S1P1), receptor for spingosine-1-phosphate (S1P), has similar phenotype like the PDGFB or PDGFRβ knockout mice, suggesting the involvement of S1P/EDG1 signaling in vascular maturation (Liu et al. 2000). Indeed, it has been shown that S1P/EDG1 signaling is required for the recruitment and activation cell-cell adhesion molecule N-cadherin and the subsequent formation of tight junction between EC and mural cells (Paik et al. 2004). Angiopoietin 1 (Ang1)/Tie2 signaling another critical signaling for vessels maturation. Ang1 is predominately expressed in mural cells and stabilizes the nascent vessels by bridging to Tie2 receptor on EC (Augustin et al. 2009). Consequently, genetic deletion of either of Ang1 and Tie2 in mice is embryonically lethal due to severe vascular defects (Suri et al. 1996). Furthermore, the crosstalk of EC and mural cells through Ang1/Tie2 signaling has also been shown to be involved in the formation of the basement membrane surrounding nascent blood vessels by inducing the transforming growth factor beta (TGFβ) signaling pathway (Stratman et al. 2009, Holderfield and Hughes 2008).

1.4 Tumor angiogenesis

Growth of primary tumor and distant metastases are accompanied by the formation of new blood vessels to ensure the adequate supply of nutrients and oxygens. Tumor cells are highly proliferative, and as tumor continues to grow, tumor becomes hypoxic due to the consumption of nutrients form the surrounding blood vessels. Hypoxic tumors in turn upregulate multiple pro-angiogenic growth factors including VEGF, PLGF, CXCL12 and

FGF to facilitate the recruitment of new blood vessels (Fig. 1.2) (Lampert and Hardman 1984, Rankin and Giaccia 2008).

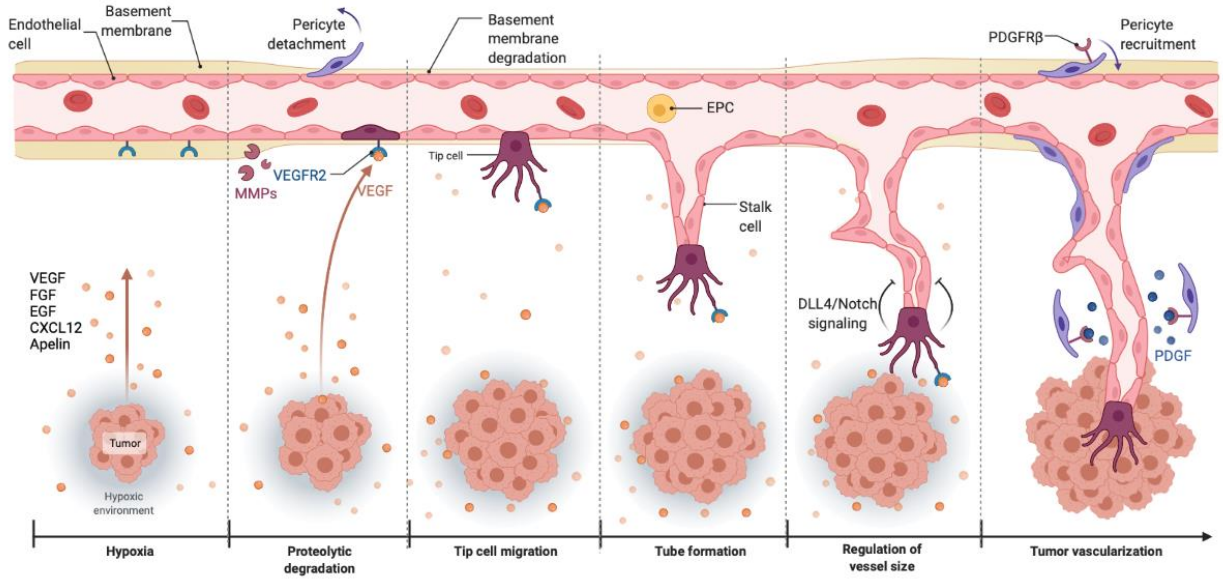

Fig. 1.2: A schematic representation of different steps of tumor angiogenesis (picture created on Biorender).

However, the structural and functional features of tumor vessels are distinct from the normal blood vessels. Tumor blood vessels are structurally abnormal as such characterized by tortuous, irregular shape and size, chaotic branching overlapping with adjacent vessels (Baluk, Hashizume and McDonald 2005). They are unstable and immature due to the lack of proper pericytes, smooth muscle cells and basement membrane coverages (Fig 1.3) (Hellberg et al. 2010, Raza, Franklin and Dudek 2010). The structural stability to the blood vessel is provided by the basement membrane, however, the tumor vessel is loosely associated with basement membrane, thus leaving

the immature nascent vessels for chaotic organizations (Baluk et al. 2003, Baluk et al. 2005). The stability and integrity of the growing vessels is further provided by the recruitment of pericytes which position around EC junctions, thereby tightening the gaps between ECs (Baluk et al. 2005, Martin, Seano and Jain 2019). However, tumor vessels often have fewer pericytes and in many cases are not well covered (Martin et al. 2019, Carmeliet and Jain 2011). Being not properly covered by basement membrane and pericytes, EC can detach from vessels wall and can pile up on each other, and might not be aligned to form a uniform and intact monolayer for optimal blood perfusion (Viallard and Larrivée 2017, Lugano, Ramachandran and Dimberg 2020). On the other hand, without having proper junctions between ECs, tumor vessels become leaky (Weis et al. 2004). The abnormal tumor vessels in turn create a hostile microenvironment where malignant cells adapt to survive and achieve more aggressiveness and metastatic potential (Rapisarda and Melillo 2012, Jain 2014). Indeed, the tortuous, disorganized and high permeability of tumor vessels leads to increase the interstitial fluid pressure and acidosis, which in turn facilitate tumor cells dissemination, invasion and favours the establishment of secondary tumor at distant sites (Finger and Giaccia 2010, Petrova et al. 2018).

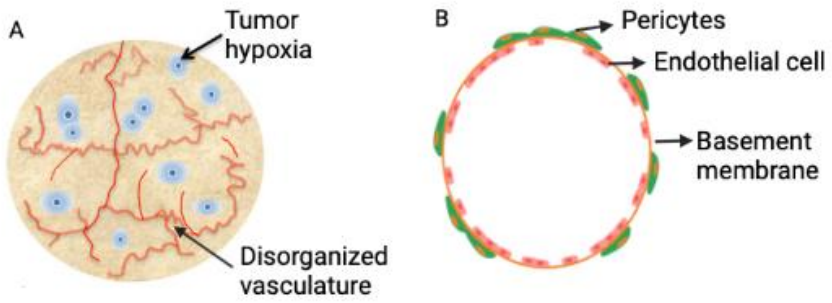

Fig. 1.3: A schematic representation of abnormal tumor vasculatures. A, Chaotic and disorganized tumor vessels promote generation of tumor hypoxia. **B,** Tumor vessels are lacking proper pericytes coverage and endothelial cells are not in close contact with each other.

Moreover, the abnormal structure and leakiness of tumor vessels impedes the efficacy of chemo-, immune- and radiotherapy (Viallard and Larrivée 2017, Chang and Lai 2020, Moeller, Richardson and Dewhirst 2007). The heterogenous blood perfusion within the tumor micro-environment creates an uneven pressure gradient, thereby limiting the delivery and effectiveness of chemotherapeutics drugs (Martin et al. 2019, Jain 2014). In addition, the disorganized and non-functional tumor vessels create an immunosuppressive micro-environment that prevent the infiltration of host immune cells, thus making the tumor resistance immunotherapy as well (Huang et al. 2013). On the other hand, radiation therapy relies on oxygen availability to generate reactive oxygen species (ROS) as a mean to kill cancer cells by inducing DNA damage (Moeller et al. 2007). However, lack of proper oxygenation due to aberrant tumor vessels and the concomitant intra-tumoral hypoxia render the tumor resistance to radiation therapy (Moeller et al. 2007).

1.5 Anti-angiogenic inhibitor (AI)

Among the many pro-angiogenic growth factors that are upregulated in a rapidly growing and hypoxic tumor, VEGF is the most studied and is recognized as the pre-dominant angiogenic cue for tumor neovascularization as it regulates EC proliferation, growth and survival, and vessels permeability and lumen formation (Nagy, Dvorak and

Dvorak 2007, Gerhardt 2008). It has been shown that elevated levels of VEGF expression are associated with tumor progression and poor prognosis, and inhibition of tumor vascularization by blocking VEGF-signaling reduces tumor growth, thus establishing the indispensable function of VEGF-mediated angiogenesis in tumor growth (Kim et al. 1993, Niu and Chen 2010).

Given the importance of VEGF signalling output in vascular development and angiogenesis, drugs targeting VEGF/VEGFR pathway have been approved to treat many cancer types (Fig. 1.4) (Veeravagu et al. 2007, Niu and Chen 2010). The humanized monoclonal antibody bevacizumab against VEGF-A was the first anti-angiogenic drug approved by the FDA to treat metastatic colon cancer, advanced NSCLC and advanced cervical cancer (Hurwitz et al. 2004, Sandler et al. 2006, Tewari et al. 2014). Soon after the approval of bevacizumab, several other anti-angiogenic drugs targeting VEGF/VEGFRs pathway were approved to treat different types of cancers (Jain 2014, Maj, Papiernik and Wietrzyk 2016). Among these, ramucirumab is another monoclonal antibody against VEGFR2, aflibercept is a chimeric protein consisting of the VEGFA-binding domains from VEGFR1 and 2 and is fused to the fragment-crystallizable region (Fc) of a human IgG. Aflibercept works as a trap against VEGF isoforms A and B, and PLGF. Several small molecule inhibitors such as sunitinib, sorafenib and pazopanib work as a receptor tyrosine kinase inhibitors (TKIs).

The main goal of anti-angiogenic drugs is to reduce the tumor growth by blocking the growth of new tumor vessels. Preclinical studies showed that tumor bearing mice

treated with anti-angiogenic drugs reduced tumor growth by decreasing the number of tumor vessel densities (Korn and Augustin 2015, Baffert et al. 2006). AI drugs also have been shown to target the tumor and stromal cells, especially by inhibiting the VEGF-dependent growth and survival of tumor cells, and by preventing the recruitment bone marrow derived progenitors (BMDCs) and macrophages (Reguera-Nuñez et al. 2019). For example, the anti-tumor effect of TKI sunitinib has been reported in multiple mouse xenograft models and found that sunitinib exerts its anti-tumor effect by blocking tumor angiogenesis mediated through VEGF and PDGFB signaling pathways (Marzola et al. 2005, Mendel et al. 2003). In addition, sunitinib inhibits tumor growth by blocking the VEGF, PDGFB and KIT signaling required for tumor cells survival and proliferation (Mendel et al. 2003, Abrams et al. 2003).

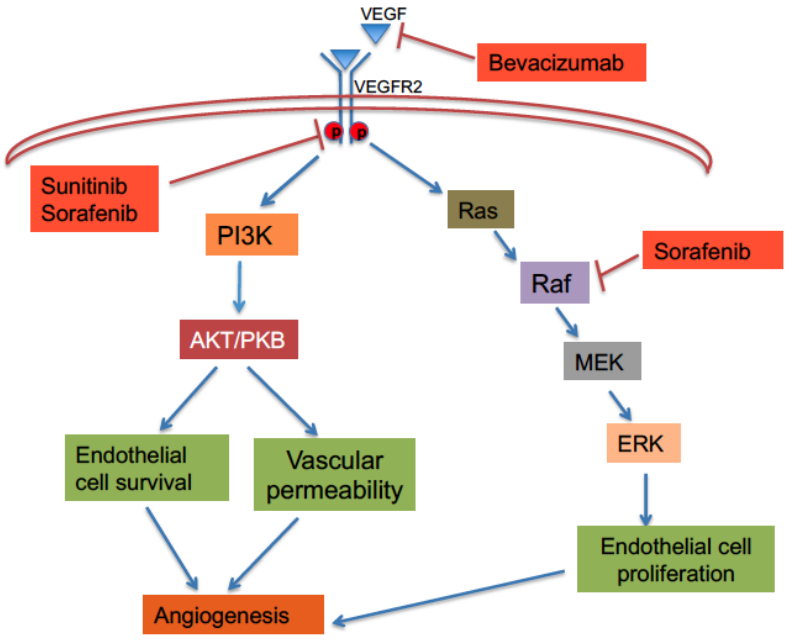

Fig. 1.4: A simplified schematic depiction of anti-angiogenic drugs targeting VEGF/VERF2 signaling

Moreover, AI agents have been shown to actively remodel the tumor vasculatures by improving the vessels normalization by integrating pericytes, thereby facilitating the delivery of chemo-, immune- and radiotherapy (Viallard and Larrivée 2017, Martin et al. 2019). For example, it has been reported that a single dose bevacizumab in patients with rectal carcinoma reduced the vascular densities, however, it promoted the vessels function by increasing the mural cells coverage and lowering the interstitial fluid pressure (Willett et al. 2004, Willett et al. 2009). This improvement in vessels function further facilitated the effectiveness of radiation therapy (Willett et al. 2004, Willett et al. 2009). Similarly, TKI cediranib improved the tumor vascular functionality and overall survival in patients with newly diagnosed glioblastoma (nGBM) when combined with chemoradiation than the chemoradiation alone (Batchelor et al. 2013). However, the window of tumor vessels normalization is relatively short and vary from patient to patient, and dose and scheduling of AI drugs used (Ellis and Hicklin 2008).

1.5.1 Clinical outcome of AI therapy

The clinical benefits of anti-VEGF therapy are reflected by the improvement in progression free survival (PFS) for several months and overall survival (OS) for weeks to few months. Bevacizumab is a monoclonal antibody and first anti-angiogenic drug approved to treat several cancer types, including colorectal cancer and glioblastoma (Hurwitz et al. 2004, Escudier et al. 2010). Clinical observations showed that when combined with chemotherapy, bevacizumab resulted in improved PFS (10.6 vs. 6.2 months) and OS (23 vs. 15.3 months) in patients with metastatic colorectal cancer than

the chemotherapy treatment alone (Berretta et al. 2008). The combination treatment strategy has also been found to be effective in improving PFS and/or OS in patients with non-squamous non-small cell lung carcinoma (NSCLC) and advanced cervical cancer (Sandler et al. 2006, Perren et al. 2011). Bevacizumab in combination with interferon alpha (IFNα) is used as the standard care for the treatment of metastatic renal cell carcinoma (mRCC) (Escudier et al. 2010). However, bevacizumab as monotherapy failed to show any clinical benefits except in case of glioblastoma (Kreisl et al. 2009). Another monoclonal antibody ramucirumab against VEGFR2 in combination with chemotherapy showed significant clinical benefits in patients with metastatic gastric cancer and NSCLC (Wilke et al. 2014, Nakagawa et al. 2019).

Sorafenib (targets VEGFRs, PDGFRs, RAF, KIT) is the first TKI which demonstrated significant anti-tumor efficacy in mRCC by improving the PFS and OS in comparison to the placebo treated group (Llovet et al. 2008). Sunitinib is a multitargeted TKI that targets VEGFRs, PDGFRs, c-Kit and FLT3, and have been approved for the treatment of mRCC and gastrointestinal stromal tumors (Motzer et al. 2007, Raymond et al. 2011). As a single agent, sunitinib has been shown to be clinically effective for mRCC by improving both the PFS and OS (Motzer et al. 2007). Pazopanib, another multitargeted TKI, showed similar efficacy as of sunitinib, both of which are now used as a standard first line of treatment for the patients with mRCC (Motzer et al. 2013). Interestingly, unlike the bevacizumab or aflibercept which works effectively in combination with chemotherapy, it has been shown that while TKIs are effective as monotherapy in certain tumors, nevertheless fail in others when used in combination with chemotherapy

(Carrato et al. 2013, Van Cutsem et al. 2011). However, recent clinical studies show that TKIs are effective when combined with immunotherapy (Motzer et al. 2019, Bergerot et al. 2019). For example, combination of avelumab, an antibody against programed death ligand 1 (PD-L1), with TKI axitinib has been proven to increase PFS and OS in patients with mRCC (Motzer et al. 2019). Interestingly, combining axitinib with avelumab for the treatment of PD-L1 positive patients has longer PFS than avelumab and sunitinib combination therapy (Motzer et al. 2019).

1.6 Tumor resistance to AI therapy

While benefits of anti-VEGF therapy, either monotherapy or in combination, are clinically evident, the initial response to therapy is not durable, and in many cases, tumors escape therapy (Ebos and Kerbel 2011, Pinto et al. 2016). Although, AI drugs in combination with chemotherapy increases PFS for months in certain cancers, but in many cases the improvement in OS cannot be achieved. Moreover, when the AI treatment is stopped, the so-called drug holidays, tumor vascularization rapidly re-emerges with the subsequent regrowth of the tumors (Mancuso et al. 2006, Vasudev et al. 2013). This limited clinical benefit might be due to some tumors are intrinsically refractory to AI therapy or the involvement of multiple acquired molecular resistance mechanisms. To address the intrinsically resistant tumors, it is important to identify molecular biomarkers in tumor tissue or in circulation of patients who will be enrolled to AI therapy. For example, it has been shown that patients with hepatocellular carcinoma (HCC) and metastatic colorectal cancer have elevated level of circulating soluble VEGFR1 (sVEGFR1), which functions as a decoy receptor for endogenous VEGF, and

did not respond to bevacizumab in combination with chemotherapy (Raut et al. 2012, Zhu et al. 2013). In such a case, expression of alternative pro-angiogenic growth factors such as basic fibroblast growth factor (bFGF) and PDGFB and others might be utilized for tumor vessels growth (Nissen et al. 2007). Indeed, preclinical studies showed that overexpression of bFGF and PDGFB in mouse fibrosarcoma resulted in tumor hyper-vascularization, thereby accelerating the tumor growth (Nissen et al. 2007). Therefore, selection of cancer patients based on circulating biomarkers such as sVEGFR1 and bFGF, and the knowledge of mechanisms of vascularization would improve the efficacy of AI therapy.

1.6.1 Alternative modes of tumor vascularization

Understating the modes of angiogenesis is another important factor for AI therapy to be effective. Anti-VEGF drugs inhibit vessels branching and reduce the number of pre-existing vessels (Nissen et al. 2007). However, AI might not be effective if tumors rely on alternative modes of vascularization such as vessel co-option, vascular mimicry and vessels splitting.

1.6.1.1 Intussusceptive angiogenesis

It is a faster process than the sprouting angiogenesis since it requires vascular rearrangement only from the pre-existing vessels, and as such could be deployed by tumor as the easiest means to get nutrients supply. Several tumor types such as melanoma and glioblastoma have been shown to use intussusceptive angiogenesis (Nico et al. 2010, Ribatti et al. 2005). The molecular mechanisms of intussusceptive

angiogenesis are not properly understood yet, however, studies have shown that VEGF and erythropoietin can initiate the process (Baum et al. 2010, Crivellato et al. 2004).

1.6.1.2 Vascular mimicry

In vascular mimicry, the aggressively growing tumor cells act like ECs and get aligned to form blood vessels like structures. The involvement of vascular mimicry has been reported in many cancer types including melanoma, glioblastoma and lung cancer (Maniotis et al. 1999, Wang et al. 2010, Williamson et al. 2016). The tumor cells participating in vascular mimicry simultaneously could express both the tumor cell and endothelial cell markers. For instance, Wang *et. al*. showed that glioblastoma stem-like cells ($CD133^{+}$) express vascular endothelial cadherin (VE-cadherin, CD144), enabling the glioblastoma stem-like cells to form tumor vasculatures (Wang et al. 2010). This alternative form of vascularization helps tumors to get sufficient oxygen and nutrients supply in one hand, and at the same time could evade the anti-angiogenic therapy.

1.6.1.3 Vessels co-option

Vessels co-option refers to the non-angiogenic process where tumor cells use the pre-existing non-malignant blood vessels to ensure the supply of oxygen and nutrients. The evidence of vessel co-option has been demonstrated in histopathological specimens of many cancer types including glioblastoma, lung and liver cancer (Sardari Nia et al. 2008, Nakashima et al. 1999, Baker et al. 2014). Especially in the context of NSCLC, studies have shown that cancer can enlarge by using the alveolar capillaries without inducing neoangiogenesis (Pezzella et al. 1997, Sardari Nia et al. 2008). Clinically,

about 10-25% patients with advanced NSCLC show patterns of vessel co-option (Offersen et al. 2001, Yousem 2009). Since this type of tumor growth bypasses neoangiogenesis, emerging evidence suggests that vessel co-option make the tumors intrinsically resistance to anti-angiogenic therapy (Baker et al. 2014, Kuczynski et al. 2019).

1.6.2 Acquired resistance to AI therapy

On the other hand, several possible mechanisms of acquired resistance compensating antiangiogenic therapies might include exaggeration of tumor hypoxia, activation of alternative pro-angiogenic signaling pathways, activation of tumor epithelial-mesenchymal transition (EMT) and promotion of tumor invasiveness and metastasis, all of these processes will be discussed briefly in the following sections.

1.6.2.1 Hypoxia promotes tumor progression

One of the critical acquired mechanisms that is over activated due to vessels pruning by anti-angiogenic therapy is exacerbation of intra-tumoral hypoxia (Jain 2014). Hypoxia is considered as the main driver of angiogenesis in solid tumors and has been implicated in anti-cancer therapy resistance by modulating tumor microenvironment. For instance, hypoxia induces the expression of vessels permeability factors such as Ang-2 and VEGF, leading to dissemination of tumor cells from primary site to distant sites (Semenza 2014). Hypoxia also promotes and maintains cancer stem cell (CSC) phenotypes within the tumor microenvironment, thereby facilitates aggressive tumor growth and metastasis (Wilson and Hay 2011, Semenza 2016). In addition, hypoxia

causes the immunosuppression by driving the elevated expression of PDL-1 from the cells of the tumor microenvironment including macrophages and myeloid derived suppressor cells (Palazón et al. 2012, Noman et al. 2014).

Tumor adaptation to the hypoxic micro-environment is mainly mediated by the stabilization of hypoxia inducible factor (HIFs), consisting of oxygen sensing HIFα and oxygen independent HIFβ subunits (Semenza 2003, Schito and Semenza 2016). Higher expression of HIFs are associated with poor prognosis and metastasis in multiple cancer types including breast, metastatic renal cell carcinoma (mRCC) and colon cancer (Zhong et al. 1999, Semenza 2003). Under normal physiological conditions, HIFs are regulated by oxygen-sensing prolyl hydroxylase domain proteins (PHD1-3) whereby HIFs are hydroxylated, which are then recognized by von-Hippel Lindau (VHL) tumor suppressor protein, ubiqutinylated and targeted for proteasomal degradation (Huang et al. 1998, Pagé et al. 2002). The loss of function or mutation in *VHL* gene has been reported in many highly vascularized tumors including in mRCC and pancreatic neuroendocrine tumor (PNET) (Kim and Zschiedrich 2018, Tirosh et al. 2018). In the absence of VHL or in hypoxic condition where HIFs are not hydroxylated by oxygen sensing PHD, HIFs are stabilized and regulate the transcription of many genes involving in EC cell survival, proliferation and angiogenesis (Krock, Skuli and Simon 2011, Tarade and Ohh 2018).

1.6.2.2 VEGF independent angiogenic signaling pathways

The inhibition of VEGF-pathway by AI therapies has been shown to upregulate the production of different alternative pro-angiogenic factors and chemokines, which in turn participate in vascular regrowth and tumor progression. Several clinical trials as well as pre-clinical studies showed that anti-VEGF treatment significantly upregulated the plasma level of PLGF, hepatocyte growth factor (HGF), FGF and other angiogenic growth factors (Willett et al. 2005, Kopetz et al. 2010). In glioblastoma patients, increased plasma level of bFGF and stromal CXCL12 were observed after treatment with a pan-VEGF RTK inhibitor AZD2171 (Batchelor et al. 2007). Elevated levels of CXCL12 were also found in patients with HCC after sunitinib treatment (Zagzag et al. 2006). The increased expression of CXCL12 and its receptor CXCR4 in ECs during tumor progression under anti-VEGF treatment indicates the participation of alternative angiogenic pathway to compensate VEGF blockade (Li, Gomez and Zhang 2007, Zhu et al. 2009). Similar results have been validated in pre-clinical studies as well. AI therapy-induced hypoxia promotes the recruitment of bone marrow-derived cells (BMDCs) including monocytes/macrophages, endothelial progenitor cells (EPCs), myeloid-derived suppressor cells (MDSCs) and cancer-associated fibroblasts (CAFs) into the tumor microenvironment (Quail and Joyce 2013, Lewis and Murdoch 2005, Shojaei et al. 2007a). These stromal cells in turn contribute for the production of alternative angiogenic factors such as FGF, BV8 and CXCL12, and contribute to tumor regrowth (Shojaei et al. 2007b, Cascone et al. 2011). For instance, CXCL12 can act as a chemoattractant to recruit EPC, macrophages and $CXCR4^{+}$ hemangiocytes, the hematopoietic progenitor cells, to the tumor micro-environment to induce tumor

vascularization (Jin et al. 2006, Orimo et al. 2005, Tseng, Vasquez-Medrano and Brown 2011). In addition, CXCL12 at higher concentration can induce angiogenesis *in vivo*, and can augment the angiogenic effect of low VEGF (Kryczek et al. 2005).

The involvement VEGF-independent angiogenesis has further been reported in pre-clinical model of RIP-Tag model of islet cell carcinogenesis by Casanova *et. al.* where they showed that cancer initially responded to anti-VEGFR2 treatment as measured by decreased tumor size accompanied by the reduction in tumor vascular densities. However, cancer eventually developed resistance to therapy with concomitant re-emergence of vascular densities and regrowth of tumor (Casanovas et al. 2005). They found that the reactivation of tumor angiogenesis was independent of VEGF, but involved another pro-angiogenic FGF. It was next found that combining the anti-VEGFR2 along with FGF inhibition increased the effectiveness of the treatment. Therefore, identifying other pro-angiogenic factors that are upregulated upon VEGF inhibition and their attenuation in combination would improve the efficacy of AI therapy.

1.6.2.3 Metastasis

In 2000, Hanahan and Weinberg reported invasion and metastasis as hallmarks of cancer (Hanahan and Weinberg 2000). About 90% of cancer-related deaths are associated with metastatic spread of primary tumours (Christofori 2006). Metastasis is a multistep process involving local invasion, intravasation into blood or lymphatic vessels, circulation of cancer cells throughout the blood or lymph vessels, extravasation to distant sites and finally colonization to form secondary tumours (Tsai et al. 2012). Ebos *et. al.* showed that short term AI therapy resulted in accelerated metastasis and

decreased survival (Ebos et al. 2009). They injected human metastatic breast cancer 231/LM2-4^{LUC+} cells through tail vein (experimental metastasis model) in SCID mice or surgically removed the 231/LM2-4^{LUC+} primary tumor (spontaneous metastasis model) when the tumor size reached to 400mm^3. They treated the mice with AI inhibitors such as sunitinib or sorafenib for 7 days, either before or after tumor injection, and found that metastatic tumor burden was significantly increased leading to shorter survival. Similar results were also reported by Paez-Ribes *et. al.* where they showed that inhibition of VEGF signaling by DC101 (a monoclonal antibody against VEGFR2) or sunitinib in RIP1-Tag2 mouse model supressed the primary tumor growth accompanied by a reduction in tumor vasculatures. However, they observed in an increased cancer invasive front extending to the surrounding acinar tissue (Pàez-Ribes et al. 2009). Interestingly, they further showed that tumor-specific deletion *Vegf-A* reduced the angiogenesis, but led to increased invasiveness, indicating the consequence of VEGF/VEGFR2 signaling blockage in tumor invasiveness (Pàez-Ribes et al. 2009). To gain insight into the possible molecular mechanism of tumor invasiveness and metastasis, they found that the tumor hypoxia was significantly higher in mice treated with either DC101 or sunitinib than the vehicle treated mice.

However, other groups observed that sunitinib administration increased the metastases in mice only when used at a high dose, but not when administered at lower dose (Singh et al. 2012). Using different mouse models of metastasis, Chung *et. al.* showed that antibody-mediated inhibition of VEGF pathway did not accelerate metastasis, while the administration of RTKs, sunitinib in particular, promoted metastasis at higher dosages,

indicating the dose and drug dependent side effects on tumor vasculatures (Chung et al. 2012).

1.6.2.4 Epithelial-mesenchymal transition (EMT)

EMT is considered as an essential step to spread cancer cells from the primary tumor to distant organs. In EMT, epithelial cells lose their cell-cell adhesion and convert to migratory and invasive mesenchymal cells, which is characterized by the expression of mesenchymal markers such as N-cadherin, vimentin, and fibronectin, and the down-regulation of epithelial markers such as cell adhesion proteins including E-cadherin and claudins (Voulgari and Pintzas 2009, Ouyang et al. 2010). Several transcription factors such as Snail, Zeb, and Twist are also involved in triggering EMT by inhibiting the expression E-cadherin (Peinado, Olmeda and Cano 2007). Anti-angiogenic treatments have been reported to induce EMT. Federico *et. al.* demonstrated that inhibition of angiogenesis in RIP-Tag2 mice by sunitinib markedly increased the level of Snail1 and Vimentin, while supressing the expression of E-cadherin (Maione et al. 2012). In addition, therapy induced HIFα-mediated activation of TGFβ pathway has been suggested as the main inducer of EMT (Katsuno, Lamouille and Derynck 2013), since it is involved in the induction of core EMT-transcription factors such as Snail1/2, Zeb1/2, and Twist1 (Thiery et al. 2009, Eckert et al. 2011). These transcription factors induce EMT by inhibiting E-cadherin expression, as well as through the degradation of the basement membrane and extracellular matrix, thus facilitating tumor metastasis (Nieto 2013).

Moreover, cancer hypoxic microenvironment and the concomitant induction of EMT enable the selection of drug-resistant CSC, leading to more invasive and metastatic cancer (Singh and Settleman 2010). It has been shown that the expression of Twist1 or Snail1 in human mammary epithelial cells promotes a CSC phenotype as measured by their tumor initiating properties, mammosphere formation and expression of cell surface markers ($CD44^{high}/CD24^{low}$) (Mani et al. 2008). In addition, CSCs isolated from mouse and human normal and neoplastic mammary glands exhibit EMT markers, such as low levels of E-cadherin and increased expression of Snail1 and Twist1 (Mani et al. 2008).

1.7 Tumor vessels normalization

The theory of 'vascular normalization' has been proposed in order to mitigate the problems of AI therapy induced hypoxia and tumor vessels abnormality. It is hypothesized that normalization of abnormal tumor vessels would decrease vessels leakiness and improve vessels perfusion, thus would decrease tumor invasiveness and metastasis (Fig. 1.5) (Martin et al. 2019, Jain 2014). Moreover, this will also increase the efficiency of chemo-, radio- and immunotherapies by facilitating the drug's delivery to the malignant cells. Studies show that the inhibition of VEGF-signaling leads to tumor vessel normalization, and in turn facilitate chemotherapeutics drugs delivery (Li, Zhang and Hong 2020, Helfrich et al. 2010, Winkler et al. 2004). For instance, Huang *et. al.* showed that the lower dose of anti-VEGFR2 antibody in a breast cancer model improved the effectiveness of immunotherapy by creating a more homogeneous distribution of functional tumor vessels (Huang et al. 2012). However, it should be noted the window for vessels normalization with AI therapy is relatively transient, and depend

on the types, doses and the scheduling of the drugs used, and as well as the tumor types being treated (Batchelor et al. 2007, Martin et al. 2019).

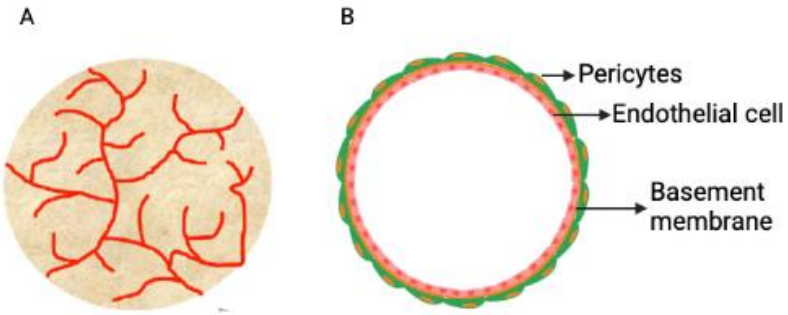

Fig. 1.5: A schematic representation of normalized tumor vessels. A, More homogenous tumor vessels with improved perfusion and blood supply. **B,** Mature tumor vessels with intact endothelial monolayer and proper pericytes coverage.

Several recent preclinical studies also explore the targets for vascular normalization beyond the anti-VEGF pathway. Park *et. al.* showed that activation of Tie2 by blocking Ang2 in mice model of glioma and Lewis lung carcinoma reduced tumor growth and metastasis by improving tumor vessels normalization, increasing blood perfusion, and favoring chemotherapeutic drug delivery (Park et al. 2016). Endothelial cell-specific deletion or pharmacological inhibition of adhesion molecule L1 decreases angiogenesis and normalizes tumor vessels in mouse model of pancreatic carcinoma, leading to decreased tumor growth and metastasis (Magrini et al. 2014). Moreover, activation of endothelial cell-specific activin receptor like kinase (Alk1)/bone morphogenetic protein 9 (BMP9) axis by overexpression of BMP9 in LLC1 promotes tumor vessel normalization

accompanied by decreased hypoxia and vessels leakage, while increasing the vessel's perfusion and trafficking of immune cell infiltration (Viallard et al. 2020). Interestingly, Carmeliet's group found that blocking EC metabolism itself could improve tumor vessel normalization (Cantelmo et al. 2016). They inhibited the EC glycolytic activator 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3) by using a low dose of the small molecule 3PO [3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one). They found that the blocking of PFKFB3 did not inhibit cancer cell proliferation, instead normalized the tumor vessels by increasing pericytes coverage and tightening the EC barrier by reducing VE-cadherin endocytosis, leading to decreased metastasis and increased chemotherapeutic drug delivery. In this context, recent studies show that EC metabolism actively participates in angiogenesis by ensuring the sustained energy demand of highly proliferative and motile EC tip cells (De Bock et al. 2013, Eelen et al. 2013). For example, EC-specific inactivation of PFKFB3 has been shown to prevent sprouting angiogenesis by impeding the formation of tip cell filopodia and vessels branching in mouse retina (De Bock et al. 2013).

1.8 VEGF/PI3K signaling

After VEGF binding, VEGFR2 undergoes receptor dimerization and autophospho-rylation leading to the activation of downstream signaling such as the PI3K/protein kinase B (Akt) pathway to exert numerous cellular processes including protein synthesis, cell growth and proliferation, metabolism, migration and survival (Fruman et al. 2017, Thorpe, Yuzugullu and Zhao 2015). In the endothelium, PI3K/Akt is the major signaling pathway for most angiogenic cues and is critically required for the growth,

survival and proliferation ECs and the formation of tip cell filopodia (Gerber et al. 1998, Takahashi, Ueno and Shibuya 1999, Shaik et al. 2020). There are 3 classes of PI3K (Class I, II and III) based on their structure and substrate specificities. Class I PI3K consists of four catalytic subunits (p110-α, β, γ and δ) that are bound to one of the 5 types of p85 regulatory subunits. In mammals, p110α and -β are ubiquitously expressed, whereas p110γ and -δ are highly enriched in leukocytes (Vanhaesebroeck et al. 2010). The subunits p110α and p110δ are preferentially activated by RTKs, whilst p110β and p110γ by GPCRs (Soler, Angulo-Urarte and Graupera 2015, Guillermet-Guibert et al. 2008). In addition, all the Class I PI3K isoforms are also activated by Ras through interactions with their Ras binding domain (RBD) (Rodriguez-Viciana et al. 1996, Rodriguez-Viciana et al. 1994), with only exception for p110β which instead is activated by small Rho-GTPases such as Rac1 and CDC42 (Guillermet-Guibert et al. 2008, Fritsch et al. 2013). Upon binding to the ligands, class I PI3Ks convert phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3, 4, 5-trisphosphate (PIP3), which acts as a second messenger and interacts with diverse protein effectors to activate multiple downstream signaling pathways. Pleckstrin homology (PH) containing effector proteins such as Ser/Thr protein kinase B (PKB/AKT) and its activating kinase, phosphoinositide dependent kinase 1 (PDK1), binds to PIP3 and are recruited to the plasma membrane. In an inactive state, AKT activity is negatively regulated by its PH domain. However, the interaction of AKT with PIP3 releases its kinase domain and facilitates PDK1-mediated AKT phosphorylation at Thr 308 site. Although the phosphorylation at Thr 308 sufficiently regulates many downstream events, the full activation of AKT depends on further phosphorylation at Ser

473 site by mammalian target of rapamycin complex 2 (mTORC2) (Manning and Toker 2017). Activated AKT in turns regulates diverse cellular functions including cell proliferation, metabolism, survival and motility by the phosphorylation of a number of downstream targets including mTORC1, glycogen synthase kinase 3 (GSK3) and forkhead box subgroup O (FOXO) (Manning and Toker 2017). The PI3K/Akt signaling is counteracted by the phosphatase and tensin homologue (PTEN), a critical tumor suppressor gene which dephosphorylates PIP3 to PIP2 (Fig.1.6) (Manning and Toker 2017, Fruman et al. 2017).

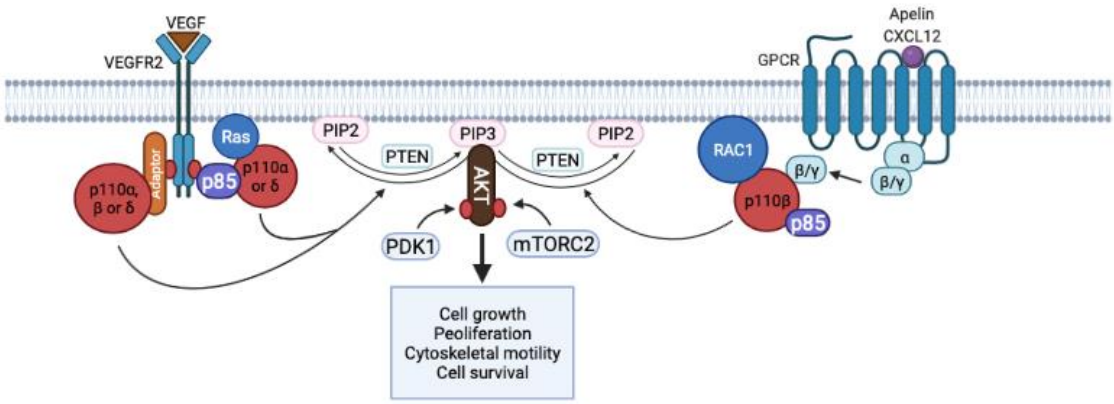

Fig. 1.6: PI3K/AKT signaling. PI3K is a convergence node among pro-angiogenic cell signaling pathways in endothelial cells to regulate cell growth, proliferation, migration and cell survival.

1.8.1 PI3K in developmental angiogenesis

The PI3Ks in the endothelium paly an essential role in normal vascular growth and development. P110α being the predominant isoform of PI3K in EC, it plays the most crucial function in regulating EC migration, angiogenesis, as well as EC's barrier function and junctional morphology (Graupera et al. 2008). Mouse with global or EC-

specific knockout of p110α is embryonically lethal due to severe vascular defects associated with impaired angiogenic sprouting and vascular remodeling (Graupera et al. 2008). In contrast, global and EC-specific p110β knockout mice develop normally without any obvious vascular abnormalities, suggesting more tolerability for p110β blockade (Graupera et al. 2008, Kulkarni et al. 2011). PTEN restricts the over activation of PI3K downstream signaling and has been shown that EC-specific PTEN deficiency resulted in embryonic lethality due to excessive bleeding and cardiac dysfunction associated with the lack of vascular maturity (Hamada et al. 2005). Furthermore, mutations in PTEN are associated with the development of Cowden syndrome, which predisposes the patients for the development of several cancer types including breast and thyroid cancers (Dahia et al. 1997, Hollander, Blumenthal and Dennis 2011). Among the three isoforms of AKT, AKT1 plays the most predominant role in EC proliferation, migration and survival (Chen et al. 2005). AKT1 null mice are viable, however, results in defective angiogenesis and vascular maturation, and an increased in vascular permeability (Chen et al. 2005, Ackah et al. 2005). On the other hand, embryonic deletion of AKT1 and AKT2 at the same time led to lethality shortly after birth (Peng et al. 2003), indicating that one AKT isoform may compensate the loss of function of another isoform. However, the pre-dominant role of AKT1 in angiogenesis has been further validated in the mouse model of retinal angiogenesis where it has been shown that EC-specific inactivation of AKT1 alone, regardless of the presence of other isoforms of AKT, resulted in defective angiogenesis in mouse retina (Lee et al. 2014). Furthermore, Foxo1, a downstream target of PI3K/AKT, knockout mice are embryonically lethal due to lack of angiogenesis (Furuyama et al. 2004). In line with this

study, higher expression of Foxo1 in EC-tip cells has been reported and found that EC-specific deletion of Foxo1 led to defective mouse retinal angiogenesis (Fukumoto et al. 2018).

1.8.2 PI3K in tumor angiogenesis

In endothelium, PI3K/Akt is the major signaling pathway for most angiogenic cues. VEGF-mediated p110α activation is the major signaling event that drives angiogenesis via PI3K/Akt pathway. Soler *et. al.* showed that inhibition of p110α, genetically or pharmacologically, in syngeneic mouse model with B16F1 cells resulted in decreased tumor growth (Soler et al. 2013). Interestingly, they found that tumor vessels density was not reduced after p110α inactivation, rather tumor growth reduction was associated with non-functional tumor angiogenesis that failed to provide growth supplements for exponential growth (Soler et al. 2013). Yet in another study, EC-specific knockout mice with four Class 1A PI3K regulatory subunits (p85α, p55α, p50α, and p85β) with the retention of single allele of p85a were viable and developed normally (Yuan et al. 2008). They implanted B16F1 cells in these heterozygous mice and found significant growth delayed in comparison to the wild type (WT) mice. They found no significant differences in tumor vessel densities between the groups, however, the vessels in WT mice were larger and chaotic in distribution, thus fueling the tumor growth (Yuan et al. 2008). Soler *et al.* further showed that selective pharma-cological inhibition of p110α or mice with heterozygous p110α kinase dead domain in RIP-Tag2 model of pancreatic cancer resulted in decreased tumor size as well as accompanied by decreased vascular densities, indicating the effect of PI3K inhibition in tumor vascularization depends on the

tumor models used (Soler et al. 2016). As mentioned before, patients with Cowden syndrome due to PTEN mutations are susceptible to develop several cancer types. Interestingly, it has been shown that EC-specific heterozygous deletion of PTEN increases tumor growth in vivo due to increased number and larger size of the tumor vessels, indicating the involvement of PTEN/PI3K in tumor angiogenesis (Hamada et al. 2005). Although VEGF-stimulated angiogenesis is predominantly mediated by p110α, the compensatory functions of other isoforms could also play crucial roles in tumor vascularization. For example, we previously showed that inhibition of p110β in EC decreases sprouting angiogenesis *in vitro* and *in vivo* angiogenesis in a Matrigel plug implanted in EC-specific p110β knockout mice (Haddad et al. 2014). Therefore, it could be expected that inactivation of p110β would also play an important role in tumor vascularization as well.

Inhibitors of PI3K/Akt pathway have been shown to exhibit anti-angiogenic effects in multiple preclinical models depending on the types of the inhibitors and the doses used (Pons-Tostivint, Thibault and Guillermet-Guibert 2017, Thorpe et al. 2015), albeit with weaker anti-angiogenic potential than the anti-VEGF therapies. In pre-clinical models, pan-PI3K inhibitors such as LY294002 at higher doses lead to tumor vascular pruning, but the resulting vessels were leaky and disorganized (Hu, Hofmann and Jaffe 2005). Therefore, toxicity and off target effects of pan-inhibitors are major obstacles to be effective in such a setting for clinical use. However, in a lower dose, pan-PI3K inhibitor GDC-0941 have been shown to facilitate doxorubicin delivery and a substantial reduction in tumor growth (Qayum et al. 2012). This may be due to the improved

vascular function after the administration of PI3K inhibitors. Indeed, several studies have shown that low dose Class IA PI3K specific inhibitors, such as GDC-0941 and PI-103, resulted in vascular normalization, even if it was given for a short term (3 days treatment), which in turn facilitated delivery of chemotherapy (Qayum et al. 2009, Qayum et al. 2012).

1.9 GPCR signaling in regulating PI3K signaling

Although VEGF/RTK is the main angiogenic pathway, emerging evidence suggests that GPCRs also play crucial roles in developmental and tumor angiogenesis. GPCRs are seven-transmembrane receptors, which are involved in numerous cellular processes including vascular remodeling, ECs migration, invasion and angiogenesis ((Allende, Yamashita and Proia 2003, Cui et al. 2014). Aberrant activation of GPCRs signaling has been reported to cause diverse pathological conditions such as inflammatory and cardiovascular diseases, and many aspects of cancer development including proliferation, angiogenesis, invasion, and metastasis (Khalil et al. 2016, Dbouk et al. 2012, Kuba et al. 2019). Therefore, understanding the roles of GPCRs with respect to tumor growth, metastasis and angiogenesis could lead to the development of promising anti-cancer therapy.

In an inactive state, GPCRs are coupled to G proteins consisting of α, β, and γ subunits. Gα is further subdivided into four isoforms: Gαq/11, Gαi/o, Gαs and Gα12/13 (Rosenbaum, Rasmussen and Kobilka 2009, Weis and Kobilka 2018). The function of Gα is regulated by the bound inactive state of guanine diphosphate (GDP). Upon

binding to ligands, GPCRs undergo conformational changes, Gα-GDP is converted to active Gα-guanine triphosphate (GTP), then Gα and Gβγ are separated, both of which in turn can activate multiple downstream signaling pathways including PI3K/AKT, MAPK and Rho- and Ras-GTPases (Rosenbaum et al. 2009, Weis and Kobilka 2018). Gβγ can regulate PI3K/AKT pathway by directly interacting and activating p110β and p110γ isoforms of PI3K (Fig. 1.2) (Schwindinger and Robishaw 2001). On the other hand, Gα subunits can activate PI3K by transactivating RTKs and through second messenger molecules (Weis and Kobilka 2018). For example, GPCR agonists, lysophosphatidic acid (LPA) and thrombin, have been shown to activate RTK epidermal growth factor receptor (EGFR) in Rat1 fibroblasts cells (Daub et al. 1996). LPA can further act through Gαq/11 and Gαi/o to activate protein kinase C (PKC)/Ca2+ and PI3K/AKT pathways (Choi et al. 2010, van Meeteren and Moolenaar 2007). Sphingosine 1-phosphate (S1P) activates PI3K signaling by transactivating EGFR and PDGFRβ in vascular smooth muscle cells (Tanimoto, Lungu and Berk 2004). As indicated before, activated GPCR convert GDP bound inactive Gα to active GTP-Gα, implying that GPCR can function as a GEF to activate small GTPases including Rho and Ras family of GTPases. Indeed, studies show that GPCR can activate Rho-GTPases directly by interacting with Rho-GEFs or indirectly by activating PKC and PKA (Aittaleb, Boguth and Tesmer 2010, Bos, Rehmann and Wittinghofer 2007). For instance, once activated, Gα12/13 in turn interacts and activates Rho-GEF family of p115-RhoGEF, postsynaptic density 95, disk large, zona occludens (PDZ)-RhoGEF, leukemia-associated RhoGEF (LARG) and lymphoid blast crisis (Lbc)-RhoGEF (Aittaleb et al. 2010, Siehler 2009).

Uncontrolled activation of GPCRs signaling contributes to tumor cell proliferation and survival by regulating PI3K/AKT, mitogen-activated protein kinase (MAPK), small GTPases and Wnt/β catenin pathways, as well as by transactivating RTKs (Nogués et al. 2018, Dorsam and Gutkind 2007). The aberrant activation of GPCR signaling is associated with many pathological conditions including in cardiovascular diseases and cancer (Heng, Aubel and Fussenegger 2013). The hyperactivation of GPCRs is regulated by G-protein-coupled receptor kinases (GRKs). GRKs phosphorylate the intracellular domain of GPCRs, which are then recognized by β-arrestins to initiate the receptors endocytosis process, followed by proteasomal degradation (Nogués et al. 2018, Magalhaes, Dunn and Ferguson 2012). Interestingly, both GRKs and β-arrestin function beyond their known role as GPCRs signaling terminators to signal transducers by affecting cancer cell proliferation, migration, invasion and metastasis (Nogués et al. 2018, Dorsam and Gutkind 2007).

1.9.1 GPCR in developmental angiogenesis

In addition to VEGF/RTKs signaling axis, GPCRs signaling also regulates different aspect developmental angiogenesis. The expression of GPCRs have been reported in EC, EPC, as well as in tip cells and vascular smooth muscle cells (De Francesco et al. 2017). GPCRs such as CXCR4, S1P1/EDG1, and protease-activated receptor 1 (PAR-1) mediated signaling have been implicated in vascular development and homeostasis, self-renewal, lineage specification and homing processes (Zou et al. 1998, Lu et al. 2015, Gong et al. 2016). S1P1 receptor has been shown to regulate maturation of blood vessels during vascular development as demonstrated by EDG1 knockout mice, where

mice die between E12.5 and E14.5 without any apparent defect in vasculogenesis and angiogenesis, but vessels' maturity was incomplete due to lack of smooth muscle cells and pericytes coverage (Liu et al. 2000). Furthermore, EC-specific deletion of S1P1 receptor led to embryonic lethality similar to global S1P1 receptor mice associated with lack of vascular maturation, implying that the S1P1 receptor in EC regulates vascular maturation by facilitating the mural cell coverage (Allende et al. 2003). The Wnt/β-catenin signaling through its GPCR receptors, Frizzled-4 and Frizzled-7, is important in vascular developmental processes as it regulates EC differentiation and vessels sprouting, leading to vascularization of the neural retina and formation of blood-brain barrier (BBB) (Peghaire et al. 2016, Paes et al. 2011, Liebner et al. 2008). Furthermore, the thrombin receptors, PARs, regulate vessel tone and permeability, as well as early stages of vasculo-genesis. Indeed, mice embryos lacking PAR-1 die with varying frequency at mid-gestation due to hemorrhage and cardiovascular failure, while this was reversed by the overexpression of PAR-1 in EC (Connolly et al. 1996).

1.9.1.1 CXCL12/CXCR4 in developmental angiogenesis

In particular, higher level of CXCR4 has been reported in developing blood vessels. CXCR4 knockout mice die at E18.5 or within an hour after birth due to defect in vascular development in gastrointestinal tract (Tachibana et al. 1998). Interestingly, deletion of CXCL12, ligand for CXCR4, in mice is also lethal as mice die shortly after birth associated with vascular abnormalities in the gut, indicating the essential role of CXCL12/CXCR4 in normal vascular development (Nagasawa et al. 1996). The importance of the CXCL12/CXCR4 axis in vascular development has been further

demonstrated in kidney glomeruli. Takabatake *et. al.* showed that CXCL12 is secreted from podocytes and remains in close proximity to CXCR4 expressing glomeruli ECs (Takabatake et al. 2009). They showed that EC-specific deletion of CXCR4 led to defective blood vessels formation in kidney glomeruli characterized by disorganized renal vasculatures and ballooning of the glomerular tufts, which were similar to global CXCR4 knockout mice (Takabatake et al. 2009). In addition, it has been shown that antibody-mediated inhibition of CXCL12 or pharmacological inhibition of CXCR4 in neonatal mice prevents retinal vascular development accompanied by a reduction in filopodia extension and tip cell marker gene expression (Unoki et al. 2010).

1.9.1.2 Apelin and apelin receptor in developmental angiogenesis

Apelin and its receptor APJ play an important role in vascular development and contribute to heart development and in maintaining cardiovascular homeostasis (Cox et al. 2006, Zeng et al. 2007, Kang et al. 2013, Masoud et al. 2020). Apelin is a potent angiogenic-secreted peptide mainly expressed in EC during embryonic vascular development, however, the expression of apelin gets reduced in adults except in certain pathological conditions including in ischemic heart and in tumor growth (Liu et al. 2015, Sheikh et al. 2008). Loss of function of apelin or APJ in the frog embryo shows aberrant vascular development specially the intersegmental vessel formation was markedly reduced or completely lost (Cox et al. 2006). Furthermore, the gain of function study in frog embryo and chorioallantoic-membrane (CAM) model demonstrates the angiogenic potential of apelin that acts independent of VEGF-signaling (Cox et al. 2006). In a zebrafish model of caudal fin regeneration, knockout of apelin or APJ has been shown

to prevent hypoxia-induced vessel regeneration, indicating the role of apelin/APJ signaling developmental angiogenesis (Eyries et al. 2008). Interestingly, apelin expression has been found to be upregulated in the EC tip cell during retinal vascular development (Saint-Geniez et al. 2002). Study reveals that although apelin knockout mice are viable but have defects in retinal vascularization, and fail to elicit proper angiogenesis even in the presence of VEGF (Kasai et al. 2008, Zeng et al. 2007). While apelin knockout mice are viable, it has been shown that almost 50% of APJ knockout mice die at E10 due to defect in cardiac development, indicating that in the absence of apelin, other ligand maybe working through APJ receptor (Charo et al. 2009, Ishida et al. 2004). Indeed, Elabela/Toddler has been identified as a new ligand for APJ receptor (Chng et al. 2013, Perjés et al. 2016). Like apelin, Elabela/Toddler is also highly expressed during the early embryonic stage and participates in fetal vascular and heart development (Liu et al. 2020, Kuba et al. 2019). For example, embryonic inactivation of Elabela in zebrafish leads to development of rudimentary hearts and in some cases total absence of heart altogether (Chng et al. 2013).

1.9.2 GPCR in tumor angiogenesis

Apart from the roles in normal blood vessels formation, maturation and maintenance, GPCRs and their respective ligands are important in regulating multiple aspects of tumor angiogenesis. Several GPCRs ligands such LPA, CXCL12, S1P, angiotensin, other cytokines and chemokines have been shown to regulate tumor growth, metastasis and neovascularization (Song et al. 2009, Xue et al. 2017). Some of these chemokines exert their effect on angiogenesis directly acting on ECs, while others function in

paracrine manner by recruiting monocytes, neutrophils and macrophages to the tumor microenvironment, which then induce the release of different angiogenic factors including HIF-1α, VEGF and matrix metalloproteinases 9 (MMP9) (Lappano et al. 2016, De Francesco et al. 2017). For instance, the signal transduction through angiotensin II/ angiotensin II receptor type 1 (AGTR1) has been shown to induce VEGF production by tumor-associated macrophages (Oh et al. 2016, Egami et al. 2003). Overexpression of AGTR1 in breast cancer cells promoted tumor angiogenesis *in vivo*, while ablation of AGTR1 reduced tumor vascularization in mice implanted with melanoma cells (Oh et al. 2016, Egami et al. 2003). LPA receptor 1 (LPAR1) has been shown to increase VEGF production and vascularization in multiple myeloma *in vivo* (Kanehira et al. 2017). Chae *et. al.* showed that during tumor angiogenesis *in vivo*, intratumoral capillaries and juxtatumoral large vessels highly expressed S1P1, and the inhibition of S1P1 suppressed tumor angiogenesis and delayed tumor growth (Chae et al. 2004).

1.9.2.1 CXCL12/CXCR4 in tumor angiogenesis

Among the chemokines that are expressed in the cancer microenvironment, CXCL12/ CXCR4 signaling plays the most critical role in tumor angiogenesis and distant metastasis (Jin et al. 2012, Li et al. 2007, Zagzag et al. 2006). Activation of chemokines receptors such as CXCR2 and CXCR4 are important modulators of tumor cell survival, growth and metastatic homing upon the release of several chemokines from the tumor microenvironment (Liu et al. 2016). In particular, CXCR4 overexpression is found to be the most crucial GPCR responsible for increased migration and metastasis of tumor

cells (Jin et al. 2012, Müller et al. 2001). As mentioned earlier, both CXCL12 and CXCR4 are upregulated upon the activation of tumor hypoxia after VEGF blockade, and CXCL12/CXCR4 axis functions as an alternative angiogenic signaling (Jin et al. 2012, Zagzag et al. 2006). Indeed, clinical and pre-clinical studies have found that CXCL12/CXCR4 signaling is involved in tumor vascularization in multiple cancer types including breast, ovarian, prostate, colorectal cancer, and glioma (Balkwill 2004, Müller et al. 2001). In an interesting study, Manish *et. el.* showed that the elevated level of tumor CXCL12 was associated with tumor vasculogenesis, the homing and incorporation of bone marrow derived EPCs into tumor vessels, whereas inhibition of CXCR4 by AMD3100 reversed the process (Aghi et al. 2006). Further, they showed that overexpression of CXCL12, but not VEGF, in a mouse glioma model induced vasculogenesis, suggesting that tumor CXCL12 secretion by tumor cells alone was sufficient for the recruitment and incorporation of EPCs into blood vessels (Aghi et al. 2006). In fact, studies have shown that VEGF primed the EC by inducing the expression of CXCR4 and the subsequent EC migration and tube formation were directed by tumor CXCL12 (Stratman, Davis and Davis 2011, Salcedo et al. 1999).

1.9.2.2 Apelin/APLNR axis in tumor angiogenesis

Recent studies have identified apelin and its receptor APJ as an another potent signaling pathway for tumor vascularization (Uribesalgo et al. 2019, Zhao et al. 2018). Studies have revealed that apelin/APLNR pathway is upregulated in tumors and in the tumor microenvironment, especially in endothelial cells, and elevated expression of apelin is linked to tumor progression and poor clinical outcome (Berta et al. 2010,

Tolkach et al. 2019). In pre-clinal studies, the role of apelin in tumor growth and angiogenesis depends on the use of the particular tumor models. For instance, Uribesalgo *et. al.* showed that apelin inactivation increases the survival of mice implanted with breast and lung cancer (Uribesalgo et al. 2019). To get insight into the molecular mechanisms of apelin in tumor growth, they found that loss apelin reduced tumor vascular density, and at the same time improved the vessel's function by enhancing normalization as was measured by an increased in mural cell coverage and decreased in tumor hypoxia. Similarly, Mastrella *et. al*. showed that inhibition of apelin in orthotopic glioblastoma model reduced tumor vascular density, however rendering the APLNR-positive glioblastoma cells more invasive (Mastrella et al. 2019). When they treated the mice with apelin-F13A, a mutant apelin receptor ligand, they were able to block both the tumor angiogenesis and tumor cell invasion.

1.9.3 GPCR/p110β axis

In general, as it has been mentioned before, EC specific RTK signaling is mainly mediated through p110α, while GPCR is through p110β. P110β is unique among the isoforms of PI3K as it incorporates signaling coming from both RTKs and GPCRs, albeit it has weak effect on PI3K signaling after RTK activation (Guillermet-Guibert et al. 2008, Murga, Fukuhara and Gutkind 2000). Upon ligands binding to the GPCRs, the activated Gβγ directly interacts with p110β and activates it (Schwindinger and Robishaw 2001, Vadas et al. 2013). It has been shown that a point mutation in p110β for Gβγ binding site blocked AKT activation, suggesting site-specific targeting of PI3K signal inhibition

(Dbouk et al. 2012). The site-specific targeting of p110β has further been validated by disrupting p110β

interaction with Rho-GTPases, suggesting the involvement of both Gβγ and Rho-GTPases for p110β activation. For example, the intact Ras binding domain of p110β is required for its activation by small Rho-GTPases such Rac1 and CDC42 (Rodriguez-Viciana et al. 1996, Fritsch et al. 2013). Ralph *et. al.* showed that active Rac1 or CDC42 failed to stimulate RBD-mutated p110β *in vitro*, and PI3K signaling was significantly reduced in mice with germline mutation in RBD domain of p110β (Fritsch et al. 2013). They further showed that knockdown or pharmacological inhibition of Rac1 in mouse embryonic fibroblasts (MEFs) strongly reduced LPA or S1P-induced AKT phosphorylation, while blocking CDC42 had only minor changes in AKT phosphorylation (Fritsch et al. 2013). Rac1 dependent p110β activation was further solidified by the work of Thomas Roberts' group where they showed that Rac1 was required for p110β membrane raft localization and subsequent activation by Gβγ (Cizmecioglu et al. 2016). Together, these studies suggest a complex mechanism of p110β activation by GTPases and heterotrimeric G-proteins.

1.10 FGD5 as a potential target for anti-angiogenic therapy

FGD5 is a member of FYVE, Rho-GEF, and pleckstrin homology (PH) domain-containing family proteins, which is robustly expressed in highly vascularized organs and especially in ECs (Kurogane et al. 2012). All the six FGD family members (FGD1-6) encoded by human genome contain, chronologically, a DBL homology (DH) domain, first PH1 domain, a FYVE domain and a second PH2 domain (Kurogane et al. 2012,

Cheng et al. 2012) . The DH domain of FGD1-4 have been shown to function as Rho-GEF for CDC42 by exchanging GDP for GTP, whereas Rho-GEF activities of FGF6 are so far uncharacterized (Miyamoto, Yamauchi and Itoh 2003, Huber et al. 2008). Recently, Park *et. al.* showed that FGD5 preferentially interacts and activates Rac1 *in vitro* (Park et al. 2021). Like most of the PH domain containing proteins, the PH domains of FGD family interact with phosphoinositides (PIs) and play roles in recruiting proteins to the cell membrane (Lenoir et al. 2015). Proteins with FYVE domains recognize the phosphatidylinositol- 3´-phosphate (PI3P) preferentially in an acidic microenvironment along the endocytic route, suggesting FYVE domain plays role in endosomal trafficking (Heldin et al. 2017). However, it is not still unclear about the function FGD FYVE domain in endosomal trafficking events. Among the FGDs, FGD5 is drawing more attention due its selective expression on EC and its role in VEGF-signaling pathway (Farhan et al. 2017, Kurogane et al. 2012). Several studies have shown that FGD5 is involved in EC survival, migration and angiogenesis by modulating VEGF/PI3K pathway (Farhan et al. 2017, Kurogane et al. 2012, Nakhaei-Nejad et al. 2012). Indeed, deletion of FGD5 in mice is embryonically lethal due to vascular defect, indicating its critical role in vascular development (Gazit et al. 2014).

Our recently published data showed that loss of FGD5 in EC has the following effects: 1) reduced VEGF/PI3K signalling (particularly mediated by p110α) output, 2) decreased cancer cell-stimulated growth factors-mediated angiogenic sprouting, and 3) reduced VEGF-stimulated expression of tip cells marker genes (Farhan et al. 2017). Mechanistically, FGD5 regulates VEGFR2 retention in recycling endosomes, and

coupling to PI3K/mTORC2-dependent Akt and cortactin activation. Furthermore, our unpublished data (which will be presented as a manuscript in Chapter 6) show that FGD5 in EC: 1) decreases Akt activation in response to SDF-1-stimulation through GPCR, particularly mediated by p110β, and 2) functions as a Rho-GEF for Rac1 to control p110β activity. Thus, our studies have identified a novel role of FGD5 in regulating PI3K/Akt activity mediated by both VEGF/RTK and GPCR pathways, making it as a potential target for anti-angiogenic therapy**.**

1.11 Hypothesis and objectives

VEGF is the most critical growth factor for tumor neoangiogenesis. AI drugs targeting VEGF signalling to the EC are used to treat various cancers. However, tumors become resistant to this therapy due to the recruitment of alternative growth factors, acting via cognate endothelial cell RTK or g-protein coupled GPCR, to cue neo-angiogenesis. This thesis investigates the role of alternative pro-angiogenic signaling along with the canonical VEGF/RTK signaling. Our hypothesis is that GPCR signaling acts as an alternative pro-angiogenic pathway in EC for tumor vascularisation.

Objective 1: To examine the role of EC p110β isoform of PI3K in tumor growth and angiogenesis. **Aims:** We will evaluate the expression of pro-angiogenic GPCR ligands in PD-RCC samples and examine whether inhibition of p110β as a downstream GPCR target can block *ex-vivo* angiogenesis. We will further extend our study in EC-specific p110β knockout mice to study tumor angiogenesis *in vivo*.

Objective 2: To determine the role of apelin as a GPCR ligand in tumor growth and angiogenesis. **Aims:** We will use apelin knockout mice to study tumor angiogenesis. We will examine the changes in tumor EC transcriptome in apelin knockout mice in comparison to the control mice.

Objective 3: To define the role of EC specific FGD5 in angiogenesis mediated through GPCR signaling. **Aims:** We will study whether FGD5 regulates CXCL12-stimulated CXCR4/PI3K signaling and controls angiogenesis. We will further evaluate if FGD5 functions as a GEF in EC to control Rac1-mediated p110β activation.

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Chapter 2

Extended Materials and Methods

2.1 Reagents

M199 media and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific. Endothelial cell growth supplement (ECGS) was purchased from BD Biosciences. VEGF-A was obtained from Peprotech, CXCL12 from R&D, AS252424, Cdc42 and Rac1 inhibitors from Tocris, and TGX-221 and BYL719 from Cayman Chemical. Hiperfect, non-silencing short interfering RNA (siRNA) and all the gene specific siRNAs were purchased from Qiagen Inc. Cytofect^{TM} HUVEC transfection kit was purchased from Cell Applications. Anti-phospho-Akt^{S473}, anti-Akt, anti-p110β and anti-Actin were from Cell Signaling Technology. Anti-FGD5 was obtained from Protein Tech. Details of all the antibodies used are listed in Table 2.1.

For tumor cell isolation, 70 µm cell strainer was purchased from BD Biosciences, ultrapure BSA from Ambicon, 10X MACS red blood cell (RBC) lysis buffer from Miltenyi Biotec, BSA fraction V and DMEM medium from Sigma, and DMEM 12 from Gibco, Dynabeads® Sheep Anti-Rat antibody ThermoFisher Scientific and EasySep™ PE Positive Selection Kit II StemCell Technologies.

2.2 Cell culture

Human umbilical vein endothelial cells (HUVECs) were cultured in M199 (Life Technologies) medium containing 20% fetal bovine serum (FBS) supplemented with 1% endothelial cells growth supplement (ECGS; VWR) and 100 U/ml penicillin and

100 µg/ml streptomycin (Life Technologies). B16F10 mouse melanoma and mouse Lewis lung carcinoma (LLC1) cell lines (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) with 10% FBS and 100 U/ml penicillin and 100 µg/ml streptomycin (Life Technologies). Cells were tested negative for mycoplasma contamination. All the cells were cultured in a humidified incubator at 37^0C containing 5% CO_2.

Before any drug treatment or growth factor stimulation for Western blot analysis, confluent HUVECs were at first washed twice with plain M199 medium, then incubated with the plain M199 for 4h, a step we mentioned as serum starvation to reduce the background interference from the unknown growth factors that might be present in the serum itself. Drugs at desired concentrations were prepared in plain M199 and pretreated the cells for another 1 hour. Growth factors stimulation were done in the presence of the drug where we wanted to block a specific pathway. To study PI3K isoforms specificity, cells were treated with PI3K-α specific inhibitor BYL-719 (30nM), PI3K-β specific inhibitor TGX-221 (100nM) and PI3K-γ specific inhibitor AS252424 (100nM) for 1h. Cells were then stimulated with either 30ng/mL VEGF or 50ng/mL CXCL12 for the specific time as indicated in the experiment.

2.3 Human tissue material (PD-RCC samples)

Human RCC tissue samples were obtained at surgical resection of the tumor with the patient's consent under a protocol approved by the Human Research Ethics Board of the University of Alberta. Freshly harvested samples from individual patients were

placed in iced saline, weighed, minced, and aliquoted for immediate use, or placed in RNALater for RNA isolation, or snap frozen for protein isolation, or Zn fixative for histology. HUVEC were isolated as described earlier under a protocol approved by the Human Research Ethics Board of the University of Alberta (Chakrabarti and Davidge 2012).

2.4 Western blotting

Tissue lysate from PD-RCC samples were prepared in lysis buffer consisting of 100mg Tris-HCl, 8% glycerol, 4.8% SDS, 100mM β-mercaptoethanol, 1mM phenylmethylsulfonyl fluoride, 10ng/mL leupeptin and 1X protease inhibitor cocktail. Tissue lysate from mice tissues and HUVECs were collected in RIPA lysate buffer (Thermofisher). Samples were subjected to mechanical homogenization followed by brief sonication and cleared by centrifugation at 13000rpm at for 15min. Protein concentrations for all the cell lysates were quantified using the DC protein assay (Bio-Rad) and an equal amount of protein was boiled at 95^0C in SDS sample buffer for 5 min. Protein samples were separated by electrophoresis on 10% SDS polyacrylamide gel, and then transferred to nitrocellulose membranes (Bio-Rad). Membranes were incubated with primary antibodies overnight at 4^0C. Bound proteins were detected by IRDye secondary antibodies (LiCor Biosciences) and the images were analyzed and quantified by using an Odyssey® IR scanner and Odyssey® imaging software 3.0 (LiCor Biosciences).

2.5 RNA isolation and cDNA synthesis and qPCR

Total cell RNA was extracted from HUVECs in 3D culture using Trizol (Life Technologies) and RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Briefly, 500µl of Trizol was added to dissolve the cells in 3D fibrin gel. 150µl chloroform was used for phase separation. The upper aqueous phase was transferred to a fresh tube and mixed with equal volume of 70% ethanol. RNeasy Mini Kit (Qiagen) was used for column separation of RNA and finally eluted with 20µl of RNase free water. Reverse transcription was performed to synthesize cDNA with a total 500ng of RNA by using QuantiTect Reverse Transcription Kit (Qiagen) following the manufacturer's instructions.

Tissue samples obtained from mice tumors were preserved in RNAlater (Life Technologies) and stored in -80^{0}C until the RNA isolation. Less than 20mg sample was homogenized in RLT lysis buffer to isolate tissue RNA using RNeasy Mini Kit (Qiagen) and 1µg of total tissue RNA was used for reverse transcription. qRT-PCR was carried out using the Mastercycler®ep realplex real-time PCR system (Eppendorf). The reaction mixture consisted of 1µl of cDNA, 1µl of 10µM primers, and 10µl of SYBR Select Master Mix (Applied Biosystems) in a total volume of 20µl reaction mixture. Experimental samples were first normalized to internal control HPRT, then to the control samples, and the fold change was calculated based on $2^{\Delta DCT}$ method. The PCR primers used are listed in Table 2.2.

2.6 RNA interference and plasmid overexpression

HUVECs were transfected twice with either 50nM non-silencing (siNS) or specific siRNA using Hiperfect transfection reagent according to the manufacturer's instructions. Briefly, cells were seeded in 6-well plates. Next day when the cells were around 60% confluent, medium was aspirated and washed twice with plain M199 and then covered with 500µl of 2% FBS containing M199 medium without the antibiotics. 50nM siRNA was prepared in 100µl of plain M199 along with transfection reagent and waited for 5-10min, and then added dropwise to the plate and was kept in the 37^0C incubator. After 4-6h, 1ml of complete HUVEC medium was topped up. This process was repeated for a second time next day. The following day, cells were transiently transfected with 1.5µg of empty vector or GFP-FGD5 or GFP-FGD5$^{\Delta DBL}$ or GFP-FGD5$^{\Delta FYVE}$ or RBD-mutated p110β or by wild type (WT)-p110β plasmids by using *Cytofect™ HUVEC* transfection kit following the manufacturer's instructions. The knockdown of specific gene or the transgenes overexpression was evaluated by Western blotting.

2.7 3D angiogenesis assay

In vitro 3D angiogenesis assay was performed as described previously (Nakatsu and Hughes 2008). Briefly, Cytodex-3 micro-beads (Sigma) were coated with HUVECs in medium containing 10% FBS and CellTracker Green (Life Technologies), then pre-treated for 2h in the presence of 100nM TGX-221 or vehicle control. Beads coated with cells were washed twice and resuspended in fibrinogen (2mg/mL) matrix containing aprotinin (0.15 U/mL) and thrombin (0.625U/mL) and then cultured in 24 well plates. Once the gel was formed, 1ml of angiogenesis growth media (M199, 10% FBS, 15ng/ml

VEGF and/or 100ng/ml CXCL12) was added on top. For PD-RCC stimulated angiogenesis, 1mL of 10% FBS medium with vehicle or minced 150mg fragments of a patient derived renal cell carcinoma (PD-RCC) or PD-RCC+TGX-221 was added on top of the gel. After 18h incubation, images of at least 30 beads from each group were taken using a DM-IRB fluorescent microscope (20X; Leica). The number and length of sprouts were analyzed by OpenLab software. Beads plus PD-RCC were collected in Trizol (Life Technologies) for RNA analysis.

2.8 Wound healing

HUVEC cells were transiently transfected with empty vector or FGD5$^{\Delta DBL}$ or FGD5$^{\Delta FYVE}$. The following day, a scratch was made across a confluent HUVEC monolayer using a sterile pipette tip. CXCL12-driven cellular migration to cover the scratch area was monitored at the indicated time points. Images were taken by 5X objective lens and a CCD camera-equipped inverted microscope (Leica, Concord, ON). Data represent the distance of cellular migration (the difference in size between the time zero scratch area and the scratch area at each time point).

2.9 FGD5 FYVE Immunofluorescence

Cells were fixed with 3% formaldehyde for 10min. Cells were blocked and permeabilized with 3%BSA and 0.1% TritonX for 30 minutes and then incubated with anti-EEA1 (BD Bioscience) for overnight at 4^0C, processed for immunofluorescence and visualized using confocal microscope with a 63X objective. Enrichment of GFP-FGD5 on the EEA1$^+$ endosomes was determined using custom Matlab scripts (The

Mathworks, Natick, MA). Images were background-subtracted and both the $EEA1^+$ endosomes and the outline of the transfected cells were segmented using the Otsu thresholding method. FGD5 enrichment on the endosomes was defined as the ratio of its mean intensity in the $EEA1^+$ mask divided by the mean intensity in the cell mask outside the endosomes. Data represents the cumulative of 30 cells from three independent experiments.

2.10 Animal models

Animal experiments were performed following the guidelines approved by the Canadian Council for Animal Care, and the animal protocol was approved by the Animal Care and Use Committee at the Alberta Health Services Cross Cancer Institute (CCI).

The generation of EC-specific $p110\beta^{flx/flx}$ mice was described previously [26]. $p110\beta^{flx/flx}$ mice were bred with Tie2-$Cre^{ERT2+/-}$/$p110\beta^{flx/flx}$ to generate Tie2-$Cre^{ERT2+/-}$/$p110\beta^{flox/flox}$ mice (EC-βKO) or Tie2-$Cre^{ERT2-/-}$/ $p110\beta^{flx/flx}$ littermate control mice. Tamoxifen (Sigma-Aldrich, 80 mg/kg/day) was administrated intraperitoneally to 10-week old mice for 5 days to activate Cre-recombinase in EC. This resulted in EC-specific truncated p110β (exon 21 and 22 deleted from the Pik3cb gene), lacking catalytic activity. Expression of truncated *Pik3cb*$^{\Delta 21,22}$ mRNA was determined by endpoint PCR. Littermate mice were used as controls for the experiments.

The generation of apelin knockout mice (*Apln*$^{-/y}$) in the background of C57BL/6 mice was described previously (Kuba et al. 2019). Apelin is coded on X-chromosome, therefore the apelin knockout male mice are designated as *Apln*$^{-/y}$. The *Apln* knockout

was confirmed by genotyping by Gavin Oudit, laboratory, University of Alberta. 10 to 12 weeks of old male mice were used for the tumor study. Littermate mice were used as controls for the experiments.

2.11 Primary tumor models

LLC1 or B16F10 mouse melanoma cell lines were cultured in DMEM medium supplemented with 10% FBS. Fresh medium was added day before the cells were prepared for the mice injection. 80% to 90% confluent cells were washed with 1XPBS and trypsinized and collected in 10% FBS containing DMEM. The cell pellets were washed twice again in 1XPBS and were resuspended in 1XPBS (without Ca^{2+} and Mg^{2+}). For the primary tumor growth, 1 X 10^6 cells were implanted into the subcutaneous of 10-12 weeks old EC-βKO or apelin KO or their respective littermate control mice. Tumor volume was measured every 3 days using slide calipers by a blinded investigator using the formula: π x width2 x length/6. The groups were designed to detect differences in tumor growth between sunitinib-treated-wild type versus EC-βKO or *Apln*$^{-/y}$ mice. A power calculation was not performed. Administration of vehicle or sunitinib (40mg/kg i.p. daily) was decided by coin toss, and was initiated when the tumor volume reached an average size of 200mm^3, and mice were euthanized at day 16 post injection or when tumor volume reached an average size >1500mm^3. 60mg/kg pimonidazole (Hypoxyprobe™-1 kit; NPI Inc) was administrated through the tail vein 1h before euthanization. Primary tumors were harvested in RNAlater for PCR analysis and in Zinc fixative for immunohistochemical analyses. Primary tumors preserved in RNAlater were also used for the preparation of protein lysate in RIPA lysate buffer.

Each data point represents an individual mouse. All mice were included in the analyses. Investigators were blinded to group allocation during tumor growth assessment and analysis of the tumor vasculature.

2.12 Experimental metastasis models

In the experimental metastasis model, B16F10 mouse melanoma cells (2 X 10^5) were injected into the tail vein of 12 weeks old EC-βKO or control mice. After tumor cell implantation, mice were treated with either vehicle or sunitinib (40mg/kg/day) for 20 days and were euthanized at day 21. 60mg/kg pimonidazole (Hypoxyprobe™-1 kit; NPI Inc) was administrated through the tail vein 1h before euthanization. Surface tumor nodules were scored visually on the left lung of each mouse. Rest of the lung samples were preserved in RNAlater for PCR analyses and in zinc fixative for immunohisto-chemical analyses. Each data point represents an individual mouse. All mice were included in the analyses. Investigators were blinded to group allocation during tumor growth assessment and analysis of the tumor vasculature.

2.13 Immunohistochemical (IHC) analysis

Tissue samples were collected in IHC-zinc fixative (BD Bioscience) and kept at room temperature for 48h, then the samples were paraffin-embedded and 5µm sections were prepared for immunofluorescent analysis. Sections were immunostained for tumor hypoxia (pimonidazole) and tumor vascular density (αCD31; Dianova), pericytes coverage (αNG2; Millipore Sigma; and αPDGFRβ; ThermoFisher Scientific), basement membrane (αCollagen type IV; Millipore Sigma), apoptosis marker (αCaspase3;

Novusbio). Briefly, after deparaffinization and rehydration, samples were subjected for antigen retrieval in citrate buffer solution (pH 6.0) for 15min by heating at microwave. Samples were blocked by 10% normal goat serum or by 10% rabbit serum for 1h depending on the secondary antibodies used. Primary antibodies are listed in Table 2.1. For CD31 (1:40), pimonidazole (1:100), NG2 (1:100), PDGFRβ (1:100), Col IV (1:100) and active Caspase 3 (1:200) were prepared in Dako diluent buffer and incubated overnight at 4^0C. For immunofluorescence imaging, slides were incubated for 1h at room temperature with secondary antibodies: FITC-conjugated goat anti-rat (1:200; ThermoFisher), red fluorescent conjugated goat anti-rabbit antibody (1:200; Thermo-Fisher) and red fluorescent conjugated donkey anti-goat antibody (1:200; Thermo-Fisher). After washing, slides were counterstained with DAPI and then mounted with prolong gold mounting medium.

2.14 Tumor hypoxia detection

For pimonidazole, endogenous peroxidase activity was quenched using 6% H_2O_2 for 30min, blocked with 10% rabbit serum, incubated with FITC-mouse monoclonal anti-pimonidazole antibody (Mab, clone 4.3.11.3) overnight at 4^0C. Next day, after washing 3X, sections were incubated with rabbit anti-fluorescein isothiocyanate-horseradish peroxidase antibody (1:150) for 1h at room temperature. Immune complexes were visualized with 3,3′-Diaminobenzidine (DAB), washed and counterstained with hematoxylin, dehydrated and mounted with prolong gold mounting medium.

2.15 Tumor vessel perfusion assay

For lectin perfusion assay, 0.2mg/100µl DyLight® 649 Lycopersicon Esculentum Tomato lectin (Vectorlabs) was injected via tail vein of the mice. After 30 mins, mice were euthanized, and tumor tissues were collected in zinc fixative. Paraffin embedded sections were deparaffinized, rehydrated and incubated overnight at 4^0C with CD31 for tumor vessels staining. Next day, after washing, slides were counterstained with DAPI and then mounted with prolong gold mounting medium.

2.16 Drugs preparations

Sunitinib (Pfizer) was dissolved in 1X PBS (without Ca^{2+} and Mg^{2+}) containing 0.1% DMSO at a concentration 5mg/ml. This stock solution of sunitinib was kept at 4^0C and used within a week. Hypoxyprobe™-1 (NPI Inc) was prepared at a concentration of 100mg/ml in 0.9% saline and kept at 4^0C.

2.17 Single cells isolation from tumor for scRNAseq

Before euthanizing the mice, tumor dissociation solution was prepared by adding 4mL of 10X collagenase A in 26 mL of DMEM F12, 10mL of BSA fraction V and 0.5mg/mL DNase and was kept on ice. Primary tumor was harvested and washed with cold 1XPBS. Tumor section (~3 cm^3) was placed in a 10cm dish containing 5mL of dissociation solution. Tumor tissue was minced with a scalpel into small pieces (~1mm^3) and transferred to 10mL tubes. Another 5mL of dissociation solution was added and the tubes were placed in 37^0C incubator for 20 mins rotating slowly in a Mini Tube Rotator (Fisher Scientific). The tissue suspension was passed through 70µm cell strainer for

twice. The left-over unfiltered tissue was grinded with a 3ml syringe plunger. Cell pellet was collected by spinning at 300g for 5mins at 4^0C, resuspended and incubated in 1mL RBC solution for 10min at room temperature. After centrifugation, cell pellet was resuspended in 2mL of PBS-based washing buffer containing 0.5% BSA. Cell suspension was incubated with 7.5μg/mL E-cadherin (CD34, eBioscience) antibody for 10 min to deplete tumor cells by using Dynabeads® Sheep Anti-Rat antibody (ThermoFisher Scientific). The remaining cells were used to enrich CD31-positive endothelial cells by using 7.5μg/mL anti-CD31 antibody (eBioscience) and the EasySep™ PE Positive Selection Kit II (Stemcell Technologies). The obtained cells were resuspended in 1mL PBS + 0.04% BSA and were used for single cell sequencing by 10X genomics (CA, USA).

2.18 Image analysis

Immunofluorescence images were taken by using a DM-IRB fluorescent inverted microscope (Leica, magnification 63X oil). At least 3 random images from each slide were acquired for data analysis and were analyzed by ImageJ software. Pimonidazole and H&E-stained lung sections were photographed with Olympus BX53 with Infinity Capture (magnification 1.25X), analyzed by ImageJ software, and presented as percentage of hypoxic areas and lung metastases per lung section. Pimonidazole-positive areas in LLC1 was measured by ImageJ program and presented as percentage of total tumor area. Images for lectin perfusion were captured by Olympus WaveFX spinning disk confocal microscope (magnification, 40X oil) and then analyzed by ImageJ software.

2.19 Statistical analysis

Statistical analyses were performed by using GraphPad Prism 8 software. Where ANOVA was used, the data were first tested for normality using the D'Agostino test, and found to have similar variance. Primary tumor growth curves of the repeated measure data were analyzed by two-way ANOVA. The difference between two groups were evaluated by Mann-Whitney *U*-test or the pairwise comparisons were done by paired Student *t*-test. Error bars represent as mean ± SEM. *P* value < 0.05 was considered significant.

Table 2.1 Names and sources of antibodies

Primary antibodies	Supplier	Cat#	Dilution
CD31	Dianova	DIA310	1:40 (IHC)
Ki67	Abcam	AB15580	1:100 (IHC)
NG2	Millipore Sigma	AB5320	1:100 (IHC)
Collagen type IV	Millipore Sigma	AB769	1:100 (IHC)
PDGFRb	ThermoFisher	MA5-15143	1:100 (IHC)
VHL	Abcam	AB140989	1:100 (IHC)
ESM1	MyBiosource.com	MBS2006250	1:100 (IHC)
FITC-Mab1	Hypoxyprobe, Inc	HP2-200KIT	1: 100 (IHC)
VEGF	Millipore Sigma	ABS82	1: 1000 (WB)
SDF1/CXCL12	Cell Signaling	3740	1: 1000 (WB)
NDRG1	Cell Signaling	5196S	1: 1000 (WB)
Zeb1	Cell Signaling	3396T	1: 1000 (WB)
p-AKT^{S473}	Cell Signaling	4060S	1: 1000 (WB)
t-Akt	Cell Signaling	40D4	1: 1000 (WB)
Actin	Cell Signaling	4967	1: 1000 (WB)
Ang-2	Abcam	AB8451	1: 1000 (WB)
Slug	Cell Signaling	9585T	1: 1000 (WB)
FGD5	Proteintech	20910-1-AP	1: 1000 (WB)
p110β	Cell Signaling	3011	1: 1000 (WB)
EEA1	BD Biosciences	610456	IF- 1:100
GFP	Cell Signaling	2555	1: 1000 (WB)

Secondary antibodies	Supplier	Cat#	Dilution
Goat anti Rat (Alexa flour 488)	ThermoFisher	A-11036	1:200
Goat anti Rabbit Alexa flour 568	ThermoFisher	A-11036	1:200
Donkey anti Goat Alexa flour 568	ThermoFisher	A-11057	1:200
Rabbit anti-FITC-HRP	NPI, Inc	HP2-200KIT	1:150

Table 2.2 PCR primers

Targets	Sequences
hVEGFA	F: 5' CCGAAACCATGAACTTTCTGC 3' R: 5' GACTTCTGCTCTCCTTCTGTC 3'
hESM1	F: 5' GGTGGACTGCCCTACACT 3' R: 5' GTCGTCGAGCACTGTCCTCTT 3'
hCXCL12/*SDF1*	F: 5' CATGAACGCCAAGGTCGT 3' R: 5' CATCTGTAGCTCAGGCTGAC 3'
hCXCR4	F: 5' GTCATGGGTTACCAGAAGAAACTGA 3' R: 5' AGAGGAGGTCGGCCACTGA 3'
hDLL4	F: 5' TGGGTCAGAACTGGTTATTGGA 3' R: 5' CTGCAGATGACCCGGTAAGAG 3'
hCXCL7	F: 5' GAGCCTCAGACTTGATACCAC 3' R: 5' AGAGCAGTCAGCAGCAATG 3'
hAPLN	F: 5' CCCATGCCCACATATTGCA 3' R: 5' TCAGTTTGAGGCCACTTGACCTA 3'
mEsm1	F: 5' AGCGAGGAGGATGATTTTGGT 3' R: 5' TGCATTCCATCCCGAAGGT 3'
mCxcr4	F' ATGGATTGGTGATCCTGGTCA 3' R' GACAGGTGCAGCCGGTACTT 3'
mDll4	F' CAGCTCAAAAACACAAACCAGAA 3' R' GCAGTTTGCCACAATTGGACTT 3'
mVegfa	F: 5'GCAGGCTGCTGTAACGATGA 3' R: 3' TCCGCATGATCTGCATGGT 3'
mCxcl12/*Sdf1*	F' CCAGAGCCAACGTCAAGCAT 3' R' TTCTTCAGCCGTGCAACAATC 3'
mApln	F: 5' TAGCCCCTGACACTGGTTGTC 3' R: 5' TTCTCCATCCCCCAAAAGC 3'
mPdgfb	F' CCCTCGGCCTGTGACTAGAA 3' R' AATGGTCACCCGAGCTTGAG 3'
mGlut1/*Slc2a1*	F: 5' GTGGTGAGTGTGGTGGATG 3' R: 5' AGTTCGGCTATAACACTGGTG 3'
mNDRG1	F: 5' GTCCTGTCATCCTCACGTATC 3' R: 5' GTGTGATCTCCTGCATGTCC 3'
mTwist1	F: 5' AGTTATCCAGCTCCAGAGTCT 3' R: 5' ATGTCCGCGTCCCACTA 3'
mZeb1	F: 5' ATGTGAGCTATAGGAGCCAGA 3' R: 5' GTACAAACACCACCTGAAAGAG 3'
mSnail2	F: 5' GTGAGGATCTCTGGTTTTGGT 3' R: 5' ACATTTCAACGCCTCCAAGA 3'
mP110β (exon 16-24)	F: 5' CACTCCTGCTGTGTCCGTACA 3' R: 5' TCAGTGCTTCCTCCTCGCTCT 3'

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Chapter 3

Inactivation of endothelial cell phosphoinositide 3-kinase β inhibits tumor angiogenesis and tumor growth

Abul K. Azad[1], Pavel Zhabyeyev[1,2], Bart Vanhaesebroeck[3], Gary Eitzen[4], Gavin Y. Oudit[1,2], Ronald B. Moore[5], Allan G. Murray[1]

[1]Department of Medicine, [2]Department of Physiology, [4]Department of Cell Biology, [5] Department of Oncology and Department of Surgery, University of Alberta, Edmonton, Canada; [3] University College London Cancer Institute, University College London, London, UK.

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3.1 Abstract

Angiogenesis inhibitors, such as the receptor tyrosine kinase (RTK) inhibitor sunitinib, target vascular endothelial growth factor (VEGF) signaling in cancers. However, only a fraction of patients respond, and most ultimately develop resistance to current angiogenesis inhibitor therapies. Activity of alternative pro-angiogenic growth factors, acting via RTK or G-protein coupled receptors (GPCR), may mediate VEGF inhibitor resistance. The phosphoinositide 3-kinase β (PI3Kβ) isoform is uniquely coupled to both RTK and GPCRs. We investigated the role of endothelial cell (EC) PI3Kβ in tumor angiogenesis. Pro-angiogenic GPCR ligands were expressed by patient-derived renal cell carcinomas (PD-RCC), and selective inactivation of PI3Kβ reduced PD-RCC-stimulated EC spheroid sprouting. EC-specific PI3Kβ knockout (EC-βKO) in mice potentiated the sunitinib-induced reduction in subcutaneous growth of LLC1 and B16F10, and lung metastasis of B16F10 tumors. Compared to single-agent sunitinib treatment, tumors in sunitinib-treated EC-βKO mice showed a marked decrease in microvessel density, and reduced new vessel formation. The fraction of perfused mature tumor microvessels was increased in EC-βKO mice suggesting immature microvessels were most sensitive to combined sunitinib and PI3Kβ inactivation. Taken together, EC PI3Kβ inactivation with sunitinib inhibition reduces microvessel turnover and decreases heterogeneity of the tumor microenvironment, hence PI3Kβ inhibition may be a useful adjuvant anti-angiogenesis therapy with sunitinib.

3.2 Introduction

Kidney cancer is among the most common cancers, pre- dominantly affecting those over 45 years, with a lifetime risk of about 2.1% among men and 1.2% among women (Siegel, Miller and Jemal 2019). A high fraction of these tumors carry a poor prognosis due to metastatic spread at the time of diagnosis (Choueiri and Motzer 2017). The opportunity for curative surgery is therefore limited, and first-line treatment for these poor-prognosis cancers is directed at inhibition of tumor neoangiogenesis to indirectly limit tumor growth (Choueiri and Motzer 2017).

Vascular endothelial growth factor (VEGF) is recognized as the dominant growth factor for embryonic vascularization (Ferrara et al. 1996). Similarly, in the adult, malignant cells and tumor stromal cells exploit VEGF to drive vascular endothelial cell (EC) sprouting and expansion from the existing mature vasculature (Yuan et al. 1996, Grunstein et al. 1999, Stockmann et al. 2008, De Palma, Biziato and Petrova 2017). This pathway is targeted by angiogenesis inhibitors, for example neutralizing antibodies to VEGF, or small molecule inhibitors of the VEGF receptor tyrosine kinase (RTK) activity (Gougis et al. 2017). Treatment with angiogenesis inhibitors results in arrested tumor progression or tumor regression in a fraction of cancer patients (Choueiri and Motzer 2017, Thomas and Kabbinavar 2015). However, this antitumor effect is not sustained, and tumor neoangiogenesis and growth eventually escape drug inhibition (Casanovas et al. 2005).

Other pro-angiogenic pathways are thought to be upregulated by the tumor when the VEGF pathway is drug-inhibited (Jiménez-Valerio and Casanovas 2016). These include hepatocyte growth factor via the c-met RTK among others, which can be targeted by extended-spectrum RTK inhibitors (Zhou et al. 2016). In contrast, pro- angiogenic ligands for endothelial G-protein coupled receptors (GPCRs), also expressed by the cancer and tumor stromal cells, are not blocked. These include pro-angiogenic inflammatory chemokines, such as interleukin-8 (Grepin et al. 2012) or chemokine (C-X-C motif) ligand 7 (CXCL-7) (Grépin et al. 2014), and developmental angiogenic cues, such as stromal cell derived factor-1/CXCL12 (CXCL12) and apelin (Orimo et al. 2005, Seaman et al. 2007). These pro-angiogenic RTK and GPCR pathways converge to efficiently activate mammalian target of rapamycin (mTOR) kinase signaling. Temsirolimus, a salvage anti-angiogenesis agent that inhibits mTOR activity, is approved to treat advanced kidney cancer, but is limited by systemic toxicity (Hudes et al. 2007).

Endothelial phosphoinositide 3-kinase (PI3K) activity couples pro-angiogenic cell surface receptors to mTOR and other effectors such as Akt (Ackah et al. 2005, Phung et al. 2006). PI3K activity in ECs has been shown to be both required and rate-limiting for angiogenesis (Graupera et al. 2008, Huang and Kontos 2002, Hamada et al. 2005). Among the three classes of PI3K, the class I group is the most extensively studied. Aberrant signaling via these enzymes downstream of RTKs, GPCRs, and small GTPases promote many human cancers (Zhao and Vogt 2008, Bilanges, Posor and Vanhaesebroeck 2019). The class I PI3Ks comprise four catalytic subunits (p110α, β, γ,

and δ) that are bound to p85 regulatory subunits (Vanhaesebroeck, Whitehead and Piñeiro 2016). Whereas p110α and p110β show a broad tissue distribution, p110γ and p110δ are mainly found in leukocytes (Bilanges et al. 2019). The p110α isoform is the dominant form coupled to RTKs such as the VEGF receptor-2 (VEGFR2) in EC (Graupera et al. 2008), whereas p110β and p110γ are coupled to pro-angiogenic endothelial GPCRs (Graupera et al. 2008).

In this report we tested the hypothesis that renal cell carcinomas (RCCs) express pro-angiogenic GPCR ligands as alternative cues to VEGF to support tumor neoangiogenesis. We further examined if selective inhibition of GPCR-stimulated PI3Kβ activity alters neoangiogenesis and tumor growth under chronic sunitinib-mediated VEGFR2 inhibition.

3.1 Results

3.1.1 Inhibition of PI3Kβ decreases patient-derived RCC- stimulated sprouting angiogenesis

High-risk RCCs are currently managed with antiangiogenic therapies mainly targeting VEGF-dependent neoangiogenesis. The role of alternative pro-angiogenic GPCR ligands produced by RCCs or tumor stromal cells to stimulate vascularization of the tumor is poorly defined. We evaluated eight patient-derived RCCs (PD-RCC) obtained by surgical excision, and characterized the ability of these tumors to elicit an angiogenic response from EC spheroids in 3D culture *in vitro* (Fig. 3.1A, B). Four of the eight PD-RCC samples stimulated angiogenic sprouting. Von Hippel-Lindau was lost in all tumors

that stimulated EC sprouts, but not in the four non-angiogenic PD-RCCs (Supplementary Table 3.1).

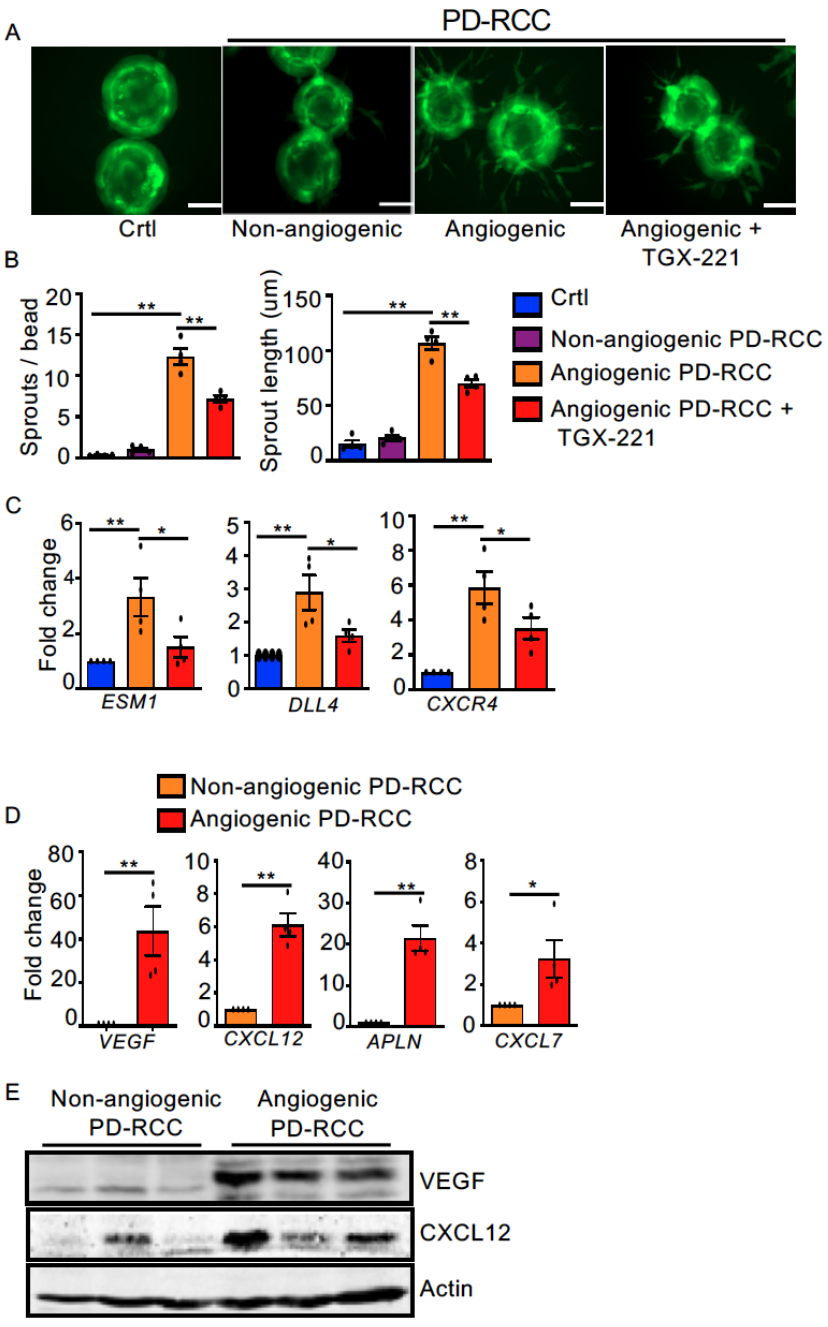


Fig. 3.1: Inhibition of PI3Kβ decreases sprouting angiogenesis in HUVECs co-cultured with patient-derived renal cancer (PD-RCC) samples. A, Freshly harvested

PD-RCC samples were minced, then co-cultured with HUVEC spheroids with vehicle or 100nM TGX-221 as described in Methods. Mock-treated EC cultures were used as the control (Ctrl). Scale bars 95 μm. **B,** Quantification of the number and length of angiogenic sprouts (mean ± SEM; $n = 8$ independent PD-RCC samples; $^{**}P < 0.01$; two-way ANOVA). **C,** Endothelial tip cell marker-gene expression in PD-RCC/ EC spheroid 3D co-culture (mean ± SEM; $n = 8$ independent samples; $^{*}P < 0.05$; two-way ANOVA). Mock-stimulated EC spheroids were used as the reference. **D,** Quantification of pro-angiogenic growth factor expression by PD-RCC. PD-RCC samples that did not stimulate angiogenesis were used as the reference (mean ± SEM; $n = 8$ independent samples; $^{*}P < 0.05$, $^{**}P < 0.01$; Mann–Whitney *U* - test). **E,** Expression of VEGF and CXCL12 in PD-RCC samples by western blot.

We previously observed that optimal *in vitro* angiogenic sprouting occurs in the context of dual receptor tyrosine kinase and GPCR pro-angiogenic ligands (Haddad et al. 2014). VEGF- stimulated PI3K-dependent activation of Akt in cultured EC was found to critically depend on PI3Kα, whereas pro- angiogenic CXCL12 signals required PI3Kβ (Supplementary Fig. 3.1; (Graupera et al. 2008)). We next tested the effect of TGX-221, a highly specific inhibitor of PI3Kβ (Jackson et al. 2005), on PD-RCC-stimulated *in vitro* sprouting. Treatment with TGX-221 decreased the number and length of endothelial sprouts in the co-cultures (Fig. 3.1A, B), correlating with reduced expression of *ESM1*, *DLL4*, and *CXCR4*, genes that mark the lead, or ‘tip cell’ of an angiogenic sprout (del Toro et al. 2010, Strasser, Kaminker and Tessier-Lavigne 2010) (Fig. 3.1C).

Angiogenic PD-RCCs were found to express more VEGF as compared to non-angiogenic PD-RCCs, as analysed by qPCR and western blot (Fig. 3.1D, E). We next probed PD-RCCs for specific pro-angiogenic GPCR ligands such as CXCL12 and apelin, loss-of-function of which during embryogenesis is associated with defects in vascular development (Tachibana et al. 1998, Kasai et al. 2008). CXCL12 is predominantly produced by the tumor, whereas apelin is produced by EC. We also examined expression of CXCL7, a pro-angiogenic chemokine produced by tumor stromal cells in RCC (Grépin et al. 2014). Expression of each of these pro-angiogenic growth factors was found to be higher in freshly isolated angiogenic versus non-angiogenic PD-RCC samples, as assessed by qPCR (Fig. 3.1D) or immunoblotting (for VEGF and CXCL12; Fig. 3.1E). TGX-221 treatment did not affect VEGF, CXCL12, or APLN transcript abundance in the co-cultures compared to paired vehicle controls (Supplementary Fig. 3.2). These data show the angiogenic PD-RCCs express both VEGF and pro-angiogenic GPCR ligands, the production of which is independent of PI3Kβ.

3.1.2 EC PI3Kβ KO decreases primary tumor growth

Since endothelial PI3Kβ activity is a convergence node for CXCL12, apelin, and CXCL7 GPCR signaling, we sought to determine if EC-selective PI3Kβ loss would alter tumor growth and neoangiogenesis. Twelve-week-old C57Bl/6 Pik3cb$^{fl/fl}$/Tie2-Cre$^{ERT2^{+/-}}$ (EC-βKO) and littermate control C57Bl/6 Pik3cb$^{fl/fl}$/Tie2-Cre$^{ERT2^{-/-}}$ (wild-type) mice were

treated with tamoxifen as described (Haddad et al. 2014), leading to effective Cre-recombinase-mediated disruption of Pik3cb (Supplementary Fig. 3.3).

We next implanted Lewis lung carcinoma (LLC1) or B16F10 mouse melanoma cells subcutaneously into the flank of EC-βKO or littermate wild-type mice, followed by treatment with vehicle or the VEGFR2 inhibitor sunitinib when the implanted tumor volume reached an average size of 200 mm^3. This design of the sunitinib treatment has been shown to optimally reduce tumor growth and invasiveness (Ebos et al. 2007). In agreement with earlier publications, we observed a delay in LLC1 and B16F10 tumor growth in wild-type mice treated with sunitinib (Fig. 3.2A, Supplementary Fig. 3.4). We observed a trend to delayed tumor growth in untreated EC-βKO mice, and further delayed upon sunitinib treatment. These data indicate that inactivation of endothelial PI3Kβ potentiates the reduction in tumor growth obtained with VEGF-pathway inhibition.

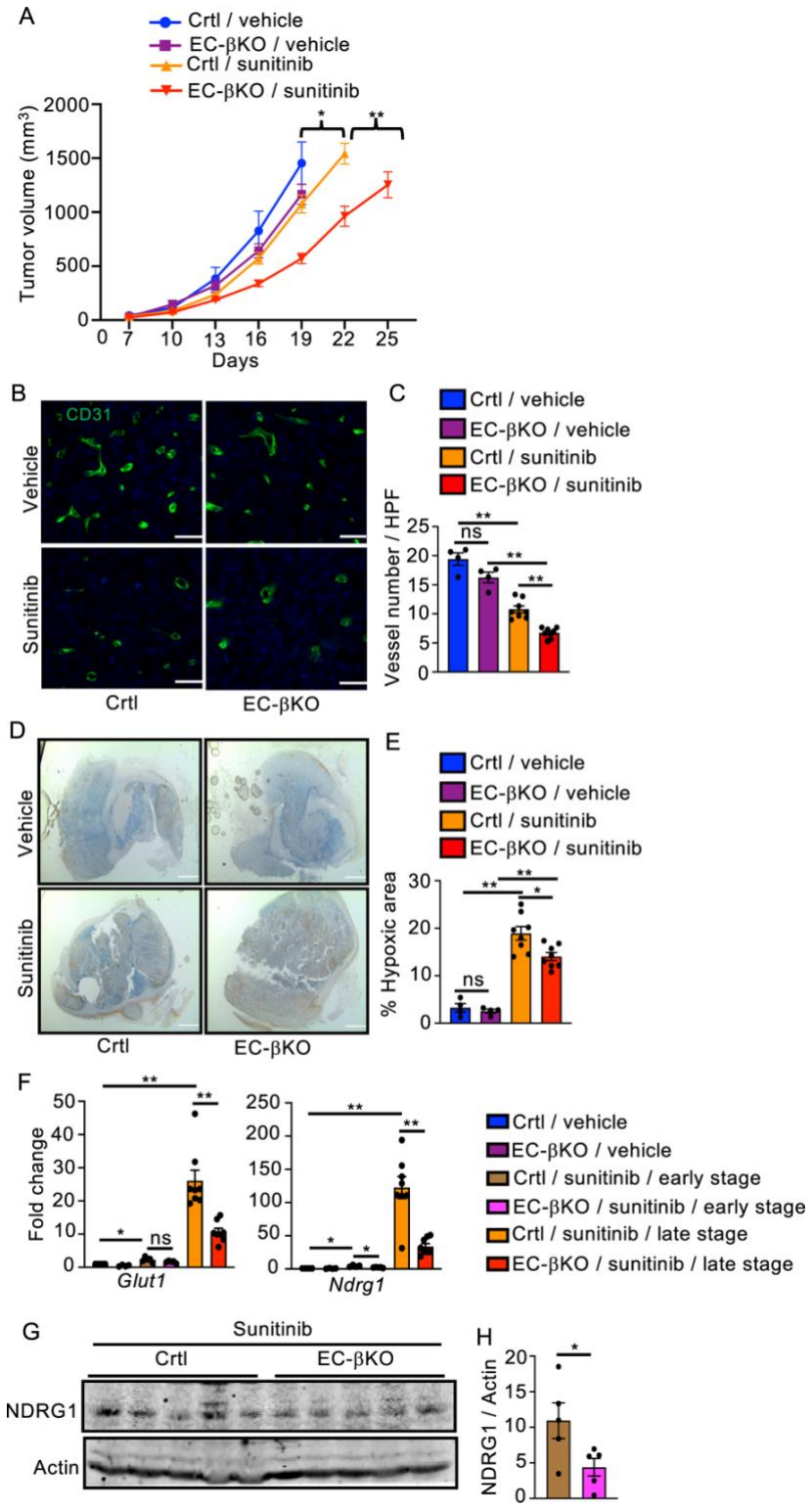


Fig. 3.2: Inactivation of endothelial PI3Kβ potentiates sunitinib treatment to delay tumor growth and reduce tumor vessel density.

A, 1 × 106 mouse Lewis lung carcinoma (LLC1) cells were implanted subcutaneously into wild-type (Ctrl) or EC-βKO mice (n = 4-8 mice/group). When the tumor volume reached 200mm^3, mice were treated with sunitinib (40 mg/kg/day) or vehicle. Tumor volume was measured every 3 days using slide calipers as described in Methods. Results are presented as the mean ± SEM; *$P < 0.05$, **$P < 0.01$ by two-way ANOVA, repeated measure. **B,** Representative immunohistochemical (IHC) images of CD31$^+$ vessels. Scale bar, 50μm. **C,** Quantification of CD31$^+$ vessels per high power field (HPF) from (**B**) (mean ± SEM; n = 4-8 mice/group; **$P < 0.01$; Mann–Whitney *U* -test). **D,** Representative images of pimonidazole$^+$ hypoxic areas (scale bar, 1 mm) and **E**, quantification of tumor pimonidazole$^+$ area (mean ± SEM; n = 4-8 mice/group; *$P < 0.05$, **$P < 0.01$; Mann-Whitney *U* test). **F,** Hypoxia-responsive marker-gene expression in vehicle or sunitinib-treated control vs EC-βKO mice (mean ± SEM; mice n = 4-8 mice/group; *$P < 0.05$, **$P < 0.01$; Mann-Whitney *U* -test). **G,** NDRG1 protein level was probed by western blot in early-stage tumors collected from sunitinib-treated wild-type vs EC-βKO mice. **H,** Quantification of western blot images among the groups (mean ± SEM; n = 5 mice/group; *$P < 0.05$; Mann-Whitney *U* test).

3.1.3 EC PI3Kβ KO decreases tumor vascular density

To understand the mechanism of the endothelial PI3Kβ inactivation-mediated delay of primary tumor growth, we examined the tumor microvasculature in sunitinib-treated EC-βKO and wild-type mice. In agreement with previous reports, we found that the density of CD31$^+$ tumor micro-vessels was decreased in sunitinib-treated wild-type mice (Fig. 3.2B, C). Endothelial PI3Kβ inactivation combined with sunitinib treatment further reduced tumor vascularization versus sunitinib treatment or endothelial PI3Kβ inactivation alone. This was associated with increased tumor cell apoptosis, quantified by active caspase-3 immunostaining (Supplementary Fig. 3.5).

Next, we evaluated the impact of EC PI3Kβ KO on tumor hypoxia in late tumors at the time of maximum tumor growth (1500mm^3). As anticipated, the reduced tumor vascularization in sunitinib-treated mice was associated with reduced nutrient delivery and increased tumor hypoxia compared to vehicle- treated wild-type mice (Fig. 3.2D, E). We observed a reduction in the pimonidazole-positive hypoxic tumor area in sunitinib-treated EC-βKO versus wild-type mice (Fig. 3.2D, E). We further evaluated the expression of the hypoxia-dependent marker genes, glucose transporter 1 (Glut1) and N-myc downstream- regulated gene (Ndrg1), in the late stage tumors. The abundance of both *Glut1* and *Ndrg1* transcripts was increased in tumors from sunitinib-treated mice (Fig. 3.2F), consistent with the data from the pimonidazole staining. Expression of *Glut1* and *Ndrg1* was reduced in sunitinib-treated-EC-βKO mice versus -wild-type mice.

We investigated day 16 (early) post-implantation sunitinib-treated tumors, a timepoint that corresponds to maximal tumor growth in untreated littermate wild-type mice, to better define the effect of endothelial PI3Kβ loss during tumor growth. In wild-type mice, tumor expression of the hypoxia reporter genes was higher in sunitinib-treated than untreated tumors at the similar timepoint, despite the smaller volume of the sunitinib-treated tumors. Endothelial PI3Kβ inactivation mitigated this effect of sunitinib treatment. We found decreased *Ndrg1* and a trend for decreased *Glut1* expression in EC-βKO versus wild-type mice (Fig. 3.2F). We confirmed this finding by western blot of early tumors recovered from sunitinib-treated mice (Fig. 3.2G, H). Moreover, since hypoxia drives expression of *Vegfa*, *Cxcl12*, and *Apln*, we examined transcript abundance of these pro-angiogenic genes. We found that expression of *Vegfa*, *Cxcl12*, and *Apln* were

each reduced in tumors in sunitinib-treated EC-βKO mice versus -wild-type mice at the early and late stage of tumor growth (Supplementary Fig. 3.6). Together, these data show that while combined endothelial PI3Kβ inactivation and sunitinib treatment maximally slowed tumor growth and decreased microvascular density, endothelial PI3Kβ inactivation reduced tumor hypoxia versus sunitinib treatment alone at early and late timepoints in tumor growth.

3.1.4 EC PI3Kβ KO decreases tumor vessel remodeling

The observation that EC PI3Kβ inactivation decreased tumor microvascular density and tumor hypoxia, indicated that endothelial PI3Kβ inactivation may favor tumor vessel normalization, which is characterized by pericyte coverage that stabilizes blood vessels (De Palma et al. 2017). To gain insight into this, we evaluated pericyte association with $CD31^+$ microvessels using the pericyte markers, NG2 and PDGFRβ, to identify mature, stabilized vessels in late-growth tumors. Our analyses showed that the fraction of both NG2-positive (Fig. 3.3A, B) and PDGFRβ-positive (Supplementary Fig. 3.7A, B) tumor microvessels were higher in EC-βKO mice compared to wild-type controls. Furthermore, larger tumor 'mother' vessels were also well-covered by pericytes in EC-βKO mice compared to wild-type mice (Supplementary Fig. 3.7C, D). Consistent with these findings, we observed that tumor *Pdgfb* expression, a major pericyte growth factor and chemo-attractant (Abramsson, Lindblom and Betsholtz 2003), was elevated in tumors from EC-βKO mice (Supplementary Fig. 3.7E).

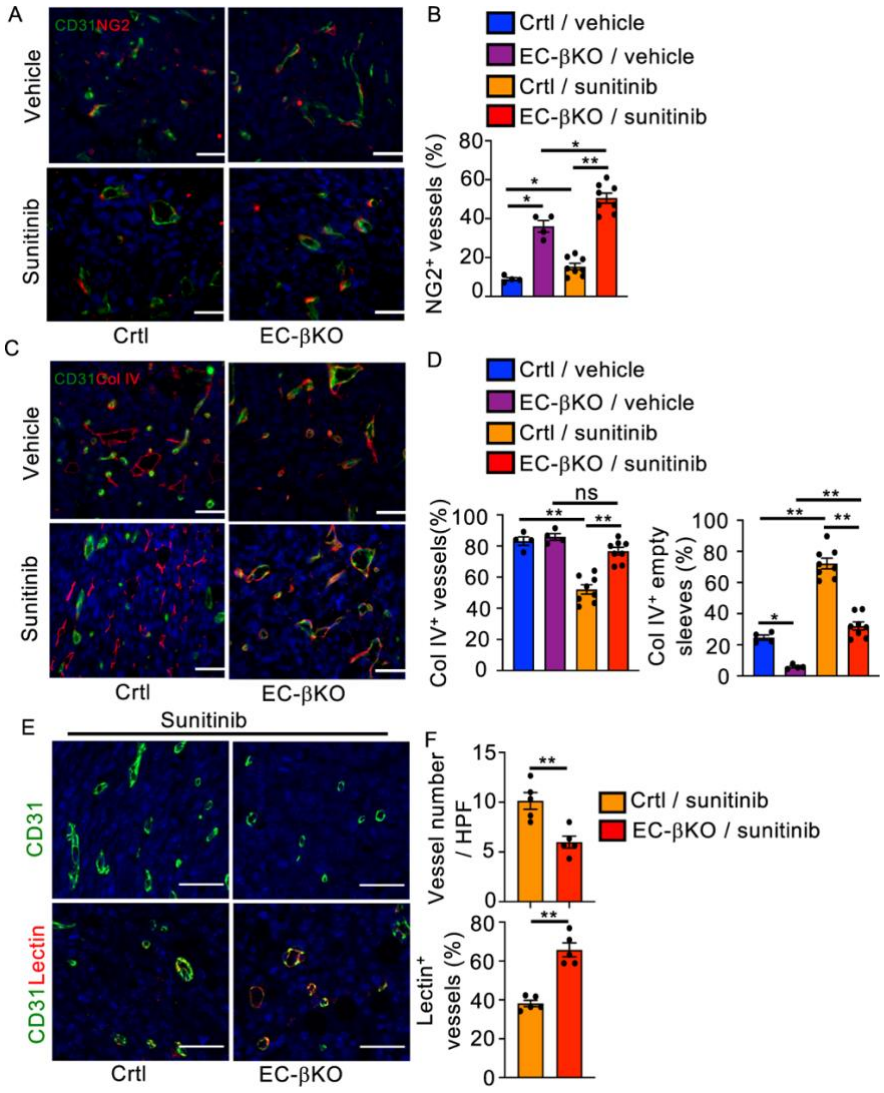


Fig. 3.3: Inactivation of endothelial PI3Kβ promotes tumor vessel normalization in sunitinib-treated mice. Vessel maturity was evaluated by using NG2$^+$ pericyte and Collagen IV$^+$ (Col IV) basement membrane staining. **A,** Representative images of NG2 (red) coverage of CD31$^+$ (green) tumor vessels in vehicle or sunitinib-treated wild-type (Ctrl) vs EC-βKO mice. Scale bar, 50μm. **B,** Quantification of the fraction of NG2-associated CD31$^+$ vessels (mean ± SEM; n = 4-8 mice/group; **P* < 0.05, ***P* < 0.01; Mann-Whitney *U* - test). **C,** Representative images of CD31$^+$ (green) vessels and Col IV$^+$ (red) capillary basement membranes. Scale bar, 50μm. **D,** Quantification of Col IV$^+$ coverage of CD31$^+$ vessel profiles (left panel), and Col IV$^+$ empty sleeves (right) in vehicle or sunitinib-treated wild-type vs EC-βKO mice (mean ± SEM; n = 4-8 mice/group;

$P < 0.01$; Mann-Whitney U - test). **E, Representative images of lectin$^+$ (red) perfused tumor vessels in sunitinib-treated wild-type vs EC-βKO mice. Scale bar, 20μm. **F,** Quantification of the fraction of lectin-positive CD31$^+$ vessels (mean ± SEM; $n = 5$ mice/group; **$P < 0.005$; Mann–Whitney U test).

We further assessed if endothelial PI3Kβ inactivation altered tumor microvessel remodeling. First, we quantified the fraction of CD31$^+$ EC microvessels associated with collagen IV staining, marking maturing microvessels. We found sunitinib treatment reduced the fraction of mature EC CD31$^+$ microvessels covered by collagen IV$^+$ basement membranes in wild-type mice (Fig. 3.3C, D left panel). However, loss of endothelial PI3Kβ was associated with a normalization of the fraction of mature tumor microvessels in sunitinib-treated mice (Fig. 3.3C, D left panel).

Regression of established microvessels is associated with remnant basement membrane sleeves from which ECs have been lost (Mancuso et al. 2006). Sunitinib treatment markedly increased the fraction of collagen IV$^+$ remnant vessel profiles lacking an endothelium in wild-type mice (Fig. 3.3C, D right panel). However, we observed a reduction in the number of remnant micro-vessel basement membrane sleeves in the tumors of sunitinib-treated EC-βKO mice (Fig. 3.3C, D right panel). Moreover, remnant vessel profiles were also reduced in the tumors from carrier-treated EC-βKO mice. Together, these data indicate that VEGFR2 inhibition is associated with a high turnover of immature tumor micro-vessels. Endothelial PI3Kβ loss blunts both immature vessel initiation or stability, and regression of mature micro-vessels.

We next assessed the effect of endothelial PI3Kβ inactivation on vessel perfusion under sunitinib treatment. We studied early postimplant tumors from sunitinib-treated mice. Consistent with our evaluation of the late-growth tumors, endothelial loss of PI3Kβ was associated with a reduction in microvessel density (Fig. 3.3E, F upper panel). However, the fraction of perfused microvessels, labeled by antemortem intravenous lectin staining, was increased in the EC-βKO versus wild-type control mice (Fig. 3.3E, F lower panel). These data indicate that inactivation of endothelial PI3Kβ further reduces sunitinib-induced microvascular remodeling, promotes tumor vessel normalization, and increases the net fraction of perfused vessels.

3.1.5 EC PI3Kβ KO suppresses sprout formation

We observed that TGX-221-mediated inhibition of PI3Kβ reduced *in vitro* endothelial spheroid sprouting in PD-RCC co-cultures (Fig. 3.1). We therefore examined the effect of endothelial PI3Kβ inactivation on tumor microvessel sprouting *in vivo*. As shown in Fig. 3.4A, B, sunitinib treatment was associated with a reduction in sprouts from tumor mother vessels. Combined endothelial PI3Kβ inactivation and sunitinib treatment was associated with a further reduction in sprout formation (Fig. 3.4A, B). We characterized angiogenic endothelial tip cell marker-gene expression among the tumors from sunitinib-treated mice in both early- and late-growth postimplant tumors. We found EC PI3Kβ inactivation co-ordinately reduced the expression of *Esm1*, *Dll4*, and *Cxcr4* versus wild-type hosts, in tumors from sunitinib-treated mice (Fig. 3.4C). Tip cell gene expression in the tumors was reduced in the EC-βKO versus the wild-type mice at both the early- and late-growth sunitinib-treated mice. This suggests that delayed tumor

growth and reduced microvessel density in sunitinib-treated EC-βKO mice is associated with a sustained decrease in tumor sprouting neoangiogenesis.

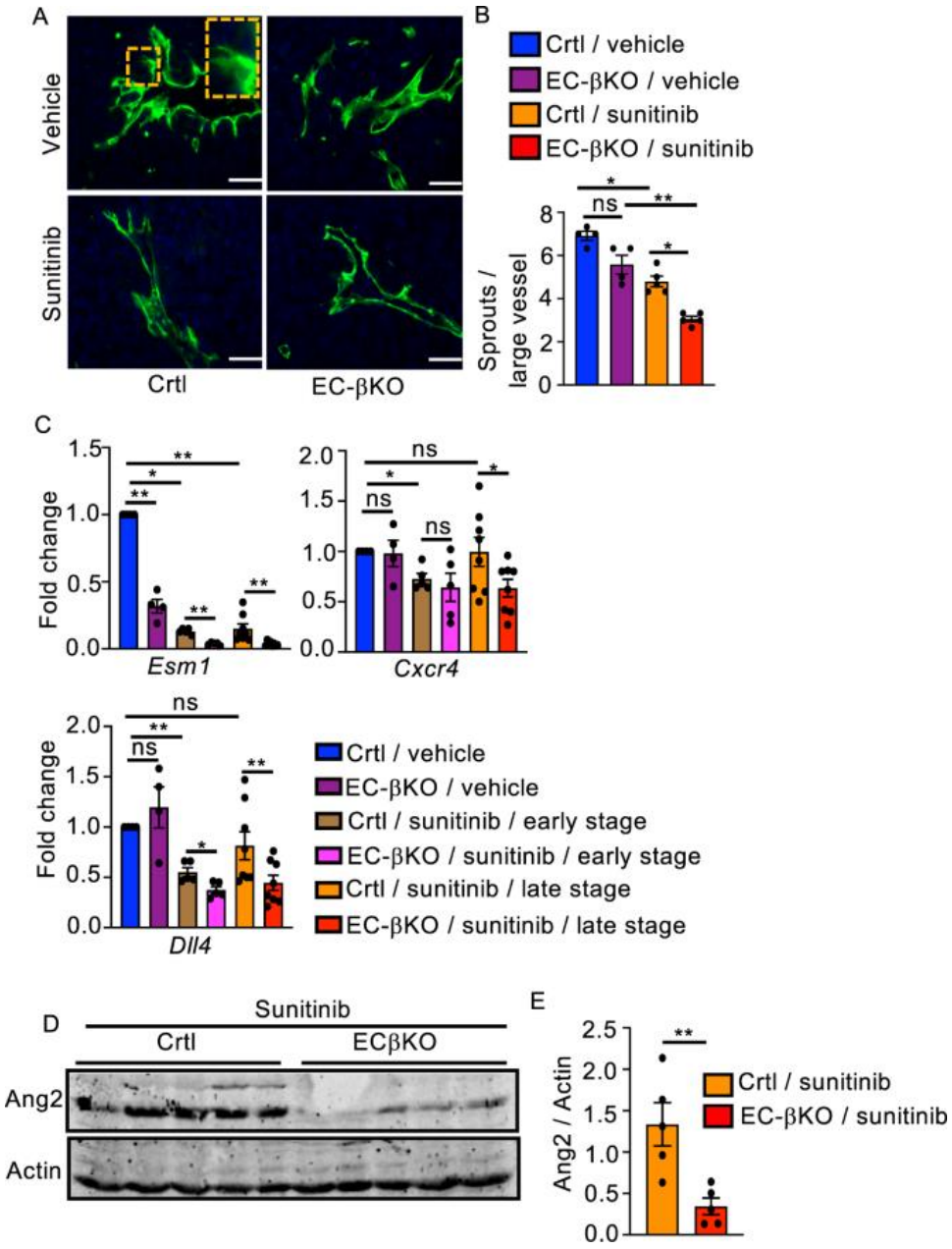


Fig. 3.4: Inactivation of endothelial PI3Kβ reduces tumor angiogenesis.

A, Representative images of sprouting from large vessels in sunitinib-treated wild-type

(Ctrl) vs EC-βKO mice. The inset shows a sprout with tip cell filopodia. Scale bars, 50 μm. **B,** Quantification of angiogenic sprouts in LLC1 tumors from sunitinib-treated wild-type vs EC-βKO mice (mean ± SEM; n = 5 mice/group; **P* < 0.05, ***P* < 0.01; Mann-Whitney *U* test). **C,** Quantification of endothelial tip cell marker-gene expression in vehicle or sunitinib-treated wild-type vs EC-βKO mice (mean ± SEM; *n* = 4-8 mice/group; **P* < 0.05, ***P* < 0.01; Mann-Whitney *U* - test). **D, E,** Western blot analysis of angiopoietin-2 (Ang2) protein level in early-stage tumor collected from sunitinib-treated wild-type vs EC-βKO mice (mean ± SEM; n = 5 mice/group; ***P* < 0.01; Mann–Whitney *U* - test).

Further, we evaluated tumor angiopoietin-2 expression, another characteristic tip cell marker, by western blot in early tumors from sunitinib-treated mice. We observed that angiopoietin-2 was reduced in tumors from ECβ-KO versus wild-type sunitinib-treated mice (Fig. 3.4D). Similarly, ESM1 matrix protein deposits were reduced in tumors from the EC-βKO versus wild-type tumors (Supplementary Fig. 3.8). These data indicate that inactivation of endothelial PI3Kβ activity in combination with sunitinib-mediated VEGFR2 inhibition, markedly reduces neovessel sprouting and microvessel density, while sparing pericyte-covered established vessels.

3.1.6 EC PI3Kβ KO dampens sunitinib-associated tumor cell epithelial-to-mesenchymal transition

Our data show that endothelial PI3Kβ loss combined with sunitinib treatment optimally reduces microvessel density, tumor growth, and promotes tumor apoptosis. Next, we evaluated the effect of sunitinib treatment with and without endothelial PI3Kβ deficiency on the tumor cells. We observed a decreased frequency of Ki-67^{+} proliferating cells in

tumor cortex from ECβ-KO versus wild-type sunitinib-treated mice (Supplementary Fig. 3.9). We further evaluated if vascular normalization in EC-βKO mice reduced tumor cell epithelial-to-mesenchymal transition (EMT), a process that is involved in tumor progression and metastatic spread to distant sites (Brabletz et al. 2018). To test this, we determined the expression of the EMT-driving transcription factors, *Twist1*, *Zeb1*, *Snail1*, and *Slug* (Brabletz et al. 2018). Sunitinib treatment markedly upregulated expression of these transcription factors in late-growth tumors in wild-type mice (Fig. 3.5A). Expression of each of these transcription factors was lower in tumors from EC-βKO versus wild-type control sunitinib-treated mice, in both early- and late-growth tumors (Fig.3.5A). Further, we evaluated Zeb1 and Slug expression by western blot in early growth tumors. We confirmed reduced expression in tumors from EC-βKO versus wild- type sunitinib-treated mice (Fig.3.5B). Together, these data indicate that EC PI3Kβ inactivation with sunitinib treatment optimally reduces tumor cell proliferation, and blunts sunitinib treatment-associated EMT.

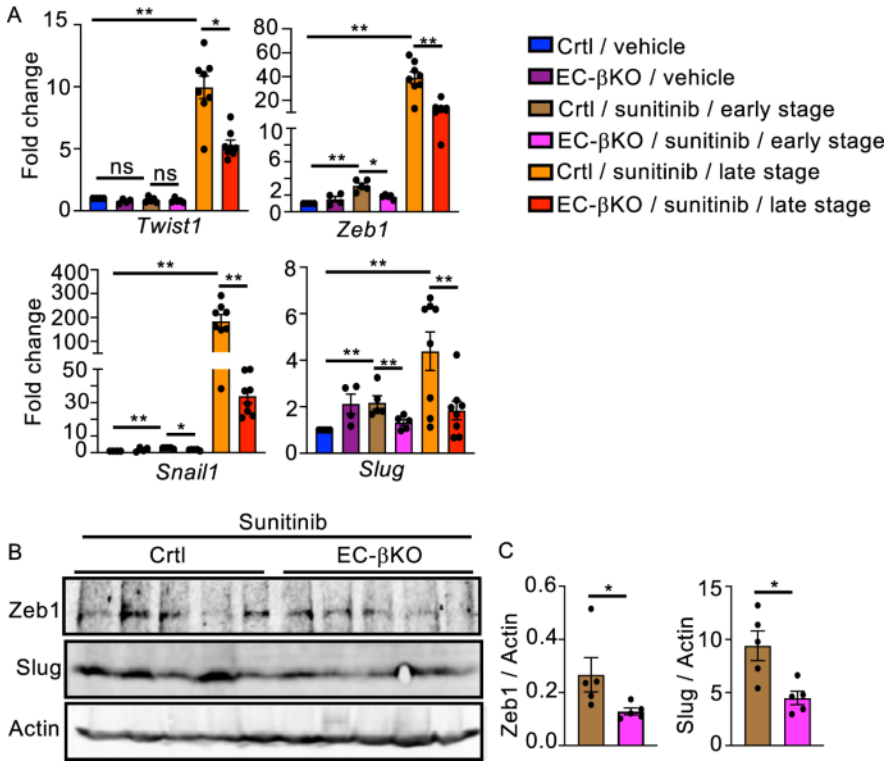


Fig. 3.5: Cancer cell epithelial-to-mesenchymal transition (EMT) is reduced in EC-βKO mice. A, EMT marker-gene expression in vehicle or sunitinib-treated wild-type (Ctrl) vs EC-βKO (mean ± SEM; mice $n = 4$-8 mice/group; $^{*}P < 0.05$, $^{**}P < 0.01$; Mann-Whitney *U* - test). **B,** The expression of Zeb1 and Slug proteins were probed by western blot in early-stage tumors collected from sunitinib-treated wild-type vs EC-βKO mice. **C,** Quantification of western blot images among the groups (mean ± SEM; n = 5 mice/group; $^{*}P < 0.05$; Mann-Whitney *U* - test).

3.1.7 EC PI3Kβ KO reduces tumor metastasis

Tumor growth at metastatic sites requires tumor cell seeding in the naive vascular bed, with subsequent growth dependent on neoangiogenesis or co-option of the native microvasculature (Bridgeman et al. 2017). VEGF inhibitor treatment has been implicated to sensitize the lung vasculature to support tumor cell extravasation (Ebos et al. 2009). We evaluated the effect of endothelial PI3Kβ inactivation in the meta- static

seeding potential of B16F10 mouse melanoma cells under sunitinib treatment. B16F10 cells were injected into the tail vein of 12–15 weeks EC-βKO or littermate wild-type mice. Sunitinib alone did not affect tumor metastasis (Fig. 3.6A). However, endothelial PI3Kβ inactivation resulted in reduced tumor foci establishment in the lung, and decreased tumor area per lung in EC-βKO mice versus wild-type mice (Fig. 3.6A, B; Supplementary Fig 3.10A, B). Similar to the LLC1 primary tumor model, B16F10 metastases showed a decrease in $CD31^+$ micro- vessels in sunitinib-treated EC-βKO versus -wild-type mice (Fig. 3.6C, D). This was accompanied by a reduction in endothelial tip cell marker-gene expression, consistent with a reduction in sprouting neoangiogenesis (Supplementary Fig. 3.11). Furthermore, the pimonidazole-positive hypoxic area in the metastatic tumors was substantially reduced in sunitinib-treated tumors from ECβ-KO versus wild-type mice (Fig. 3.6E, F). Consistent with the subcutaneous primary LLC tumor model, a greater fraction of microvessels were found to be covered with pericytes in sunitinib-treated tumors from the ECβ-KO versus wild-type mice (Fig. 3.6G, H). These data indicate that metastatic B16F10 tumor growth and angiogenesis is optimally reduced by combined endothelial PI3Kβ inactivation with sunitinib-mediated VEGF receptor inhibition.

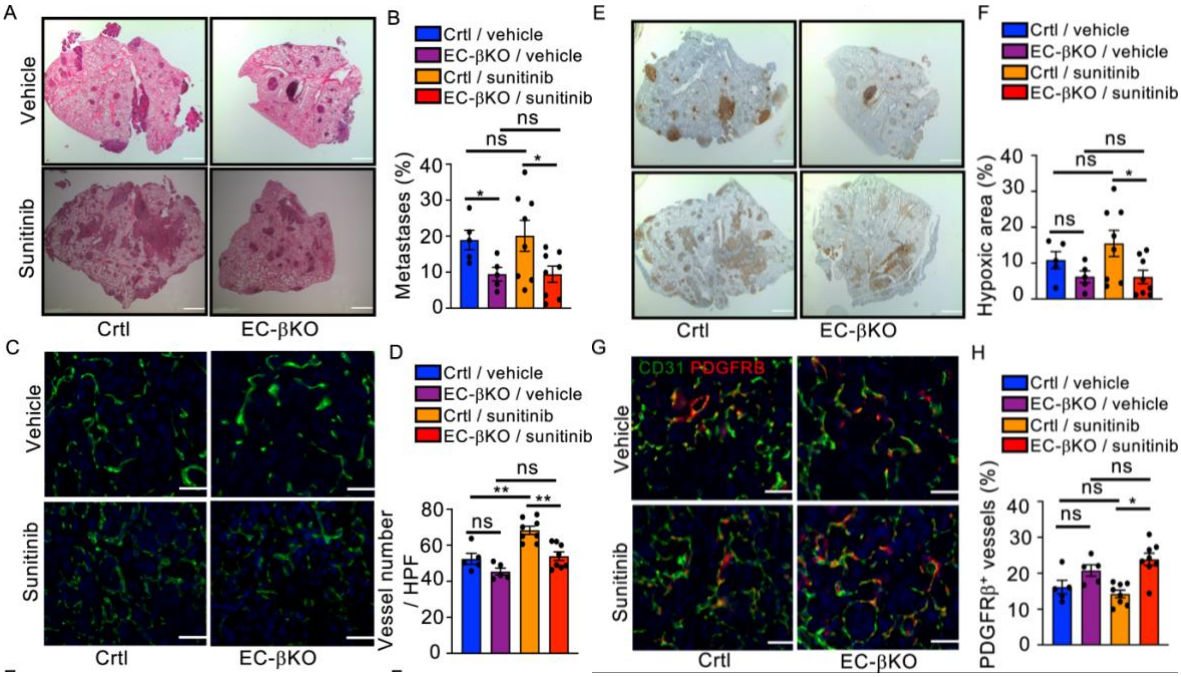


Fig. 3.6: Inactivation of endothelial PI3Kβ decreases lung metastases, tumor vessel density, and tumor hypoxia in sunitinib-treated mice. Mouse B16F10 melanoma cells (2×10^5) were injected into the tail vein of wild-type (Ctrl) or EC-βKO mice. Immediately after tumor cell inoculation, mice were treated with vehicle or sunitinib (40 mg/kg/day) for 20 days, then were euthanized at day 21. **A,** Representative images of H&E stained sections showing tumor foci among vehicle or sunitinib-treated wild-type vs EC-βKO mice. Scale bars, 1mm. **B,** Quantification of tumor area in H&E stained sections (mean ± SEM; n = 5-8 mice/group; **P* < 0.05; Mann Whitney *U* - test). **C,** Representative images of CD31⁺ tumor vessels and **D,** quantification (mean ± SEM; n = 5-8 mice/group; **P* < 0.05, ***P* < 0.01; Mann-Whitney *U* - test). **E,** Representative images and **F,** quantification of pimonidazole⁺ tumor hypoxic area among vehicle or sunitinib-treated wild-type vs EC-βKO mice (mean ± SEM; n = 5-8 mice/group; **P* < 0.05; Mann-Whitney *U* - test). Scale bars, 1mm. **G,** Representative images of PDGFRβ⁺ pericyte (red) coverage of CD31⁺ (green) tumor vessels among vehicle or sunitinib-treated wild-type vs EC-βKO mice. Scale bars, 50μm. **H,** Quantification of the fraction of CD31⁺ vessels covered by PDGFRβ⁺ pericytes (mean ± SEM; n = 5–8 mice/group; **P* < 0.05; Mann-Whitney *U* - test).

3.4 Discussion

The anticipated benefit of angiogenesis inhibition therapies to control advanced or chemotherapy-resistant cancer is not fully realized, since not all tumors respond to anti-VEGF treatment, and those that do initially respond eventually become resistant (Sennino and McDonald 2012). Further, the unstable immature vessels associated with anti-VEGF treatment may compromise the delivery and effect of chemotherapy or immune- modulating antitumor drugs. This is particularly important, since emerging clinical trial data suggest that a combination of angiogenesis inhibitors and immune-modulating agents optimizes outcomes versus either approach alone (McDermott et al. 2018, Bergerot et al. 2019).

In this study, we sought to understand the role of pro- angiogenic ligands for endothelial GPCRs in tumor neoangiogenesis. We show that a subset of PD-RCC samples are able to elicit angiogenic sprouting from endothelial spheroids *in vitro*. These PD-RCCs express VEGF as well as pro-angiogenic ligands (CXCL12, CXCL7, and APLN) for endothelial GPCRs. Inhibition of PI3Kβ, a common signal integration node for GPCRs, in these co-cultures did not reduce pro-angiogenic growth factor gene expression by the cancer and stromal cells, but inhibited tumor-driven angiogenic sprouting. These data suggest these mediators participate to cue tumor EC neoangiogenesis in humans, acting through endothelial PI3Kβ.

We directly tested this idea in mice by inactivating PI3Kβ selectively in the host endothelium, then evaluated tumor growth under anti-VEGFR2-treatment. PI3Kβ

inactivation alone reduced subcutaneous growth of LLC1 and B16F10 tumors. Further, endothelial PI3Kβ KO markedly potentiated sunitinib-driven growth inhibition of both LLC1 and B16F10 tumors in mice. This finding is supported by an increase in cleaved caspase-3^+ apoptotic tumor cells, and reduced fraction of Ki-67^+ proliferating tumor cells, in tumors with combined endothelial PI3Kβ inactivation and VEGFR2 inhibition. Sunitinib treatment alone reduced tumor microvessel density and markedly increased tumor hypoxia, expression of hypoxia reporter genes, and tumor EMT in late-growth tumors. EC PI3Kβ inactivation combined with sunitinib treatment mitigated tumor hypoxia, hypoxia-responsive gene expression, and expression of the EMT markers versus sunitinib treatment alone in early and late stage tumors. Earlier work has shown the effects of VEGFR2 inhibition to reduce tumor neoangiogenesis and vascular density and yet increase tumor oxygenation are transient (Winkler et al. 2004, Matsumoto et al. 2011). Stabilization of the tumor micro-vasculature, mediated by combined endothelial PI3Kβ and VEGFR2 inactivation, reduces vascular heterogeneity in the early and late tumor microenvironment consistent with a sustained decrease in cyclic tumor hypoxia. Hypoxic niches in the tumor have been linked to a cancer cell EMT program that may facilitate metastasis or resistance to chemotherapy (Terry et al. 2018, Nieto 2017). Our data suggest cyclic tumor hypoxia associated with immature microvessel turnover is an important driver of LLC cancer cell EMT that can be mitigated by dual inactivation of pro-angiogenic RTK and GPCR signaling.

Tumor microvessels in the late-growth tumors from ECβ-KO hosts were found to be more mature, covered by both basement membranes and pericytes, with tumors

featuring fewer empty basement membrane sleeves arising from regressed microvessels (Mancuso et al. 2006). The combination of endothelial PI3Kβ inactivation and sunitinib-mediated VEGFR2 inhibition suppressed sprouting from tumor mother vessels better than VEGFR2 inhibition alone, consistent with the effect of pharmacological PI3Kβ inhibition in the *in vitro* model of PD- RCC angiogenesis. Expression of endothelial tip cell genes was consistently lower in early- and late-growth tumors from ECβ-KO hosts. The finding was confirmed by the observation that angiopoietin-2 was decreased in the early growth sunitinib-treated tumors in ECβ-KO hosts. These data suggest that both VEGF and non-VEGF angiogenic cues drive angiogenesis in these tumors. Moreover, these four lines of evidence support the interpretation that compared to mature microvessels, tip cell differentiation and sprout formation are particularly sensitive to the combination blockade of VEGFR2 and pro-angiogenic GPCR cues dependent on PI3Kβ. The net result is a higher fraction of the tumor vessels are perfused if endothelial PI3Kβ is inactivated.

Under VEGF pathway inhibition, cancer or tumor stromal cell (Ma et al. 2018, Lyons et al. 2017) recruitment of neovascularization using alternative pro-angiogenic RTK ligands contributes to acquired drug resistance (Shojaei et al. 2010, Jahangiri et al. 2013), and is partially mitigated by the broader receptor inhibition profile of new-generation antiangiogenic agents such as cabozantinib (Zhou et al. 2016). However, RTK antagonists do not inhibit pro-angiogenic GPCR ligands generated by cancer or stromal cells. Earlier work has identified upregulation of ligands such as CXCL12, IL- 8, and CXCL-7 that might contribute to neovascularization under VEGF/VEGF receptor

blockade (Grépin et al. 2014, Huang et al. 2010, Kryczek et al. 2005). Moreover, autocrine apelin receptor signaling in angiogenic ECs participates in developmental and tumor angiogenesis (Charo et al. 2009, Mastrella et al. 2019). Since tumors are heterogeneous for these and other mediators, our data suggest that targeting an important common GPCR signal integration node in the EC, such as PI3Kβ, may complement RTK inhibitors of angiogenesis.

Neoangiogenesis and dysfunctional vascular remodeling are associated with progression and metastasis of several cancer types (Hanahan and Weinberg 2011). The effects of anti-angiogenesis drugs, such as sunitinib, on the tumor microvasculature promote non- homogeneous oxygen and nutrient delivery that results in microenvironmental niches favoring cancer cell transition to more aggressive forms (Ebos et al. 2009, Pàez-Ribes et al. 2009). In addition, these experi- mental findings suggest anti-VEGF pathway inhibitors may condition the systemic vasculature to facilitate metastatic cancer cell spread (Ebos et al. 2009). Combined inhibition of VEGF RTK and PI3Kβ normalized tumor hypoxia-responsive, and EMT gene expression versus sunitinib alone. In addition, we observed reduced expression of Vegf and Cxcl12 in these tumors, suggesting reinforced angiogenesis inhibition upon combined sunitinib and PI3Kβ inactivation. Future experiments will be needed to determine if such combination treatment reduces metastatic behavior of the primary tumor. However, we find that this regimen reduced tumor seeding, growth and hypoxia, and promoted tumor microvessel maturation in the B16 lung metastasis model. These data suggest combined inhibition favorably promotes host micro- vessel resistance to metastasis.

In the endothelium, PI3Kα is coupled to the VEGF RTK, and is indirectly inhibited by VEGF pathway inhibitors. In contrast to endothelial PI3Kβ inhibition, however, PI3Kα inactivation is associated with chaotic tumor neoangiogenesis (Soler et al. 2013), in part mediated by impaired expression of DLL4 NOTCH ligand, in turn associated with uncontrolled endothelial tip cell differentiation (del Toro et al. 2010). A reduction in gene expression markers for endothelial tip cells, combined with a reduction in remnant, non-vascularized microvessels in tumors in the sunitinib-treated EC-βKO mice suggests that pro-angiogenic GPCR ligands functionally drive tumor neoangiogenesis in these models, and contribute to unbalanced PI3K isoform signaling.

In summary, our findings in preclinical models reveal the potential benefit of combined inhibition of VEGF RTK and PI3Kβ to inhibit tumor growth. Freshly isolated human RCCs were found to express several pro-angiogenic GPCR ligands that can couple to endothelial PI3Kβ, and PD-RCC- stimulated angiogenesis was sensitive to pharmacological PI3Kβ inhibition. This suggests that PI3Kβ inhibition to target the subset of such human tumors merits further investigation. Systemic PI3Kβ inhibition in human cancer has been found to be well-tolerated (Mateo et al. 2017). Our data suggest that clinical PI3Kβ inhibitors could be useful as an adjuvant to VEGF-based antitumor therapies.

3.5 Materials and methods

The details of the Material and methods section for this chapter are included in **Chapter 2.**

3.5.1 Human tissue material

Human renal cell carcinoma tissue samples were obtained at surgical resection of the tumor with the patient's consent under a protocol approved by the Human Research Ethics Board of the University of Alberta. The characteristics of the tumors is summarized in Table 3.1.

3.5.2 Cell culture

HUVEC, B16F10 mouse melanoma, and mouse LLC1 cell lines were cultured as described in Extended Methods.

To investigate the involvement of specific PI3K isoforms, cells were treated for 1 h with the PI3Kα-specific inhibitor BYL-719 (30nM) or the PI3Kβ-specific inhibitor TGX-221 (100nM), followed by stimulation of cells with 30 ng/ml VEGF and 50 ng/ml CXCL12 as indicated in the experiment. The data are representative of three independent experiments.

3.5.3 Drugs

Sunitinib (Pfizer) was dissolved in 1× PBS (without Ca^{2+} and Mg^{2+}) containing 0.1% DMSO at a concentration 5mg/ml. This stock solution of sunitinib was kept at 4 °C and used within a week. Hypoxyprobe^{TM}-1 (NPI Inc) was prepared at a concentration of 100

mg/ml in 0.9% saline and kept at 4^0C. TGX-221 (Cayman chemical) was reconstituted in alcohol and further diluted to 100μM in PBS.

3.5.4 3D angiogenesis assay

In vitro 3D angiogenesis assay was performed as described previously (Haddad et al. 2014, Nowak-Sliwinska et al. 2018). HUVECs were mounted on Cytodex-3 micro-beads (Sigma) in medium containing 10% FBS and CellTracker Green (Life Technologies), then pre-treated for 2 h in the presence of 100 nM TGX-221 or vehicle control. Beads coated with cells were washed twice and resuspended in fibrinogen (2 mg/ml) matrix containing aprotinin (0.15 U/ml) and thrombin (0.625 U/ml) and then cultured in 24-well plates. Once the gel was formed, 1 ml of 10% FBS medium with vehicle or minced 150 mg fragments of a patient-derived renal cell carcinoma (PD-RCC) or PD-RCC+TGX-221 was added on top of the gel. After 18 h incubation, images of at least 30 beads from each group were taken using a DM-IRB fluorescent microscope (20X; Leica). The number and length of sprouts were analyzed by OpenLab software. Beads plus PD-RCC were collected in Trizol (Life Technologies) for RNA analysis. Each data point reflects one independent PD-RCC/HUVEC co-culture.

3.5.5 Animal model

Animal experiments were performed following the guidelines approved by the Canadian Council for Animal Care, and the animal protocol was approved by the Animal Care and Use Committee at the Alberta Health Services Cross Cancer Institute.

Mouse LLC1 (ATCC) or B16F10 mouse melanoma (ATCC; 1 × 106) cells were subcutaneously injected in 12-week-old mixed sex EC-βKO or control mice. Tumor volume was measured every 3 days. Vehicle or sunitinib (40 mg/kg i.p. daily) was started when the tumor volume reached an average size of 200 mm^3. Mice were euthanized at day 16 post injection or when tumor volume reached an average size >1500 mm^3. In the experimental metastasis model, B16F10 mouse melanoma cells (2 × 105) were injected into the tail vein of 12 weeks old mice.

3.5.6 Western blot

Tissue lysate from PD-RCC samples or mouse tissues and HUVECs were collected and processed as described in Extended Methods. Each data point reflects one independent PD-RCC/ HUVEC co-culture. A list of the antibodies is in Table 2.2.

3.5.7 RNA isolation and quantitative PCR

Total cell RNA was extracted from HUVECs in 3D culture or from mouse tumors were processed as described in Extended Methods. Each data point represents an individual mouse. The PCR primers used are listed in Table 2.3.

3.5.8 Immunohistochemistry

Tissue samples were collected in ICH-zinc fixative (BD Bioscience) and kept at room temperature for 48 h, then the samples were paraffin-embedded and 5 μm sections prepared for immunohistochemical analysis. Sections were immunostained for tumor hypoxia (pimonidazole) and tumor vascular density (αCD31; Dianova), pericyte

coverage (αNG2; Millipore Sigma; and αPDGFRβ; ThermoFisher Scientific), basement membrane (αCollagen type IV; Millipore Sigma), proliferation marker (αKi-67; Abcam), and an apoptosis marker (αCaspase-3; Novusbio). Technical details of the processing are described in Extended Methods. Each data point represents an individual mouse.

3.5.9 Image analysis

Immunofluorescence images were taken by using a DM-IRB fluorescent inverted microscope (Leica, magnification 63X oil). At least 3 random images from each slide were acquired for data analysis and were analyzed by ImageJ software. Pimonidazole and H&E-stained lung sections sections were photographed with Olympus BX53 with Infinity Capture (magnification 1.25X), analyzed by ImageJ software, and presented as percentage of hypoxic areas and lung metastases per lung section. Pimonidazole-positive areas in LLC1 was measured by ImageJ program and presented as percentage of total tumor area. Images for lectin perfusion were captured by Olympus WaveFX spinning disk confocal microscope (magnification, 40X oil) and then analyzed by ImageJ software.

3.5.10 Statistical analysis

Statistical analyses were performed using GraphPad Prism software. Differences between two groups were analyzed by Mann-Whitney *U* -test. Where ANOVA is used, the data were first tested for normality using the D'Agostino test, and found to have similar variance. Primary tumor growth curves of the repeated measure data were

analyzed by two- way ANOVA. Error bars represent the mean ± SEM. P value < 0.05 was considered significant.

Supplementary Table 3.1 Patient-derived renal cell carcinoma (PD-RCC) characteristics

Samples ID	Histology	Grade	Stage at presenta-tion	Pathologic Stage	VHL status	Angiogenic response
1	Chromophobe type (predominantly classic type with pale cells with some eosinophilic cells).	n/a	T1aN0M0	pT3a	Positive	No
2	Clear cell renal cell carcinoma	3	T1aN0M0	pT1aNXM0	Positive	No
3	Clear cell renal cell carcinoma	4	T3NxM0	pT3aN0M0	Negative	Yes
4	Clear cell renal cell carcinoma	4	T3aN0M0	pT3aN0M0	Negative	Yes
5	Papillary renal cell carcinoma	3	T1aN0M0	pT1aNxM0	Positive	No
6	Clear cell renal cell carcinoma	2	T1aN0M0	pT1aNXM0	Negative	Yes
7	Clear cell renal cell carcinoma	2	T1bN0M0	T1bN0M0	Negative	Yes
8	Right complex renal cyst	No high grade features	T1bNXM0	pT1bNXM0	Positive	No

Supplementary Table 3.2 Antibodies

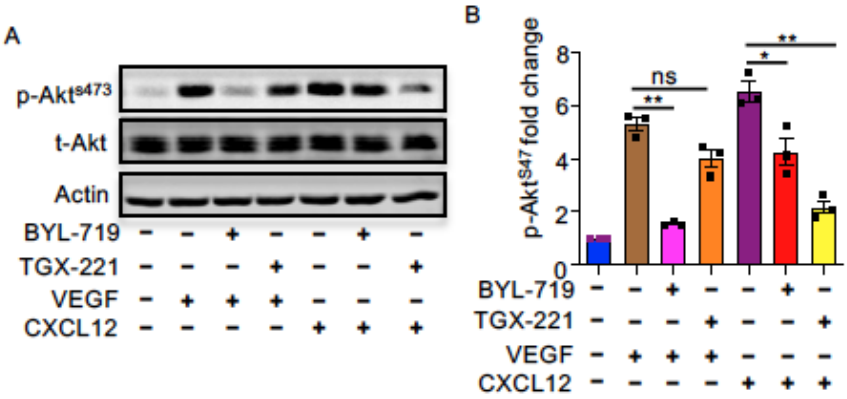
Primary	Supplier	Cat#	Dilution
CD31	Dianova	DIA310	1:40 (IHC)
Ki67	Abcam	AB15580	1:100 (IHC)
NG2	Millipore Sigma	AB5320	1:100 (IHC)
Collagen type IV	Millipore Sigma	AB769	1:100 (IHC)
PDGFRb	ThermoFisher	MA5-15143	1:100 (IHC)
VHL	Abcam	AB140989	1:100 (IHC)
ESM1	MyBiosource.com	MBS2006250	1:100 (IHC)
FITC-Mab1	Hypoxyprobe, Inc	HP2-200KIT	1: 100 (IHC)
VEGF	Millipore Sigma	ABS82	1: 1000 (WB)
SDF1/CXCL12	Cell Signaling	3740	1: 1000 (WB)
NDRG1	Cell Signaling	5196S	1: 1000 (WB)
Zeb1	Cell Signaling	3396T	1: 1000 (WB)
p-AKT^{S473}	Cell Signaling	4060S	1: 1000 (WB)
t-Akt	Cell Signaling	40D4	1: 1000 (WB)
Actin	Cell Signaling	4967	1: 1000 (WB)
Ang-2	Abcam	AB8451	1: 1000 (WB)
Slug	Cell Signaling	9585T	1: 1000 (WB)

Secondary	Supplier	Cat#	Dilution
Goat anti Rat (Alexa flour 488)	ThermoFisher	A-11036	1:200
Goat anti Rabbit Alexa flour 568	ThermoFisher	A-11036	1:200
Donkey anti Goat Alexa flour 568	ThermoFisher	A-11057	1:200
Rabbit anti-FITC-HRP	NPI, Inc	HP2-200KIT	1:150

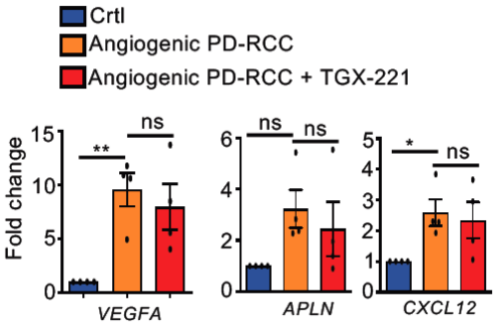
Supplementary table 3.3 PCR primers

Targets	Sequences
hVEGFA	
hESM1	F: 5' GGTGGACTGCCCTACACT 3' R: 5' GTCGTCGAGCACTGTCCTCTT 3'
hCXCL12/*SDF1*	F: 5' CATGAACGCCAAGGTCGT 3' R: 5' CATCTGTAGCTCAGGCTGAC 3'
hCXCR4	F: 5' GTCATGGGTTACCAGAAGAAACTGA 3' R: 5' AGAGGAGGTCGGCCACTGA 3'
hDLL4	F: 5' TGGGTCAGAACTGGTTATTGGA 3' R: 5' CTGCAGATGACCCGGTAAGAG 3'
hCXCL7	F: 5' GAGCCTCAGACTTGATACCAC 3' R: 5' AGAGCAGTCAGCAGCAATG 3'
hAPLN	F: 5' CCCATGCCCACATATTGCA 3' R: 5' TCAGTTTGAGGCCACTTGACCTA 3'
mEsm1	F: 5' AGCGAGGAGGATGATTTTGGT 3' R: 5' TGCATTCCATCCCGAAGGT 3'
mCxcr4	F' ATGGATTGGTGATCCTGGTCA 3' R' GACAGGTGCAGCCGGTACTT 3'
mDll4	F' CAGCTCAAAAACACAAACCAGAA 3' R' GCAGTTTGCCACAATTGGACTT 3'
mVegfa	F: 5'GCAGGCTGCTGTAACGATGA 3' R: 3' TCCGCATGATCTGCATGGT 3'
mCxcl12/*Sdf1*	F' CCAGAGCCAACGTCAAGCAT 3' R' TTCTTCAGCCGTGCAACAATC 3'
mApln	F: 5' TAGCCCCTGACACTGGTTGTC 3' R: 5' TTCTCCATCCCCCAAAAGC 3'
mPdgfb	F' CCCTCGGCCTGTGACTAGAA 3' R' AATGGTCACCCGAGCTTGAG 3'
mGlut1/*Slc2a1*	F: 5' GTGGTGAGTGTGGTGGATG 3' R: 5' AGTTCGGCTATAACACTGGTG 3'
mNDRG1	F: 5' GTCCTGTCATCCTCACGTATC 3' R: 5' GTGTGATCTCCTGCATGTCC 3'
mTwist1	F: 5' AGTTATCCAGCTCCAGAGTCT 3' R: 5' ATGTCCGCGTCCCACTA 3'
mZeb1	F: 5' ATGTGAGCTATAGGAGCCAGA 3' R: 5' GTACAAACACCACCTGAAAGAG 3'
mSnail2	F: 5' GTGAGGATCTCTGGTTTTGGT 3' R: 5' ACATTTCAACGCCTCCAAGA 3'
mP110β (exon 16-24)	F: 5' CACTCCTGCTGTGTCCGTACA 3' R: 5' TCAGTGCTTCCTCCTCGCTCT 3'

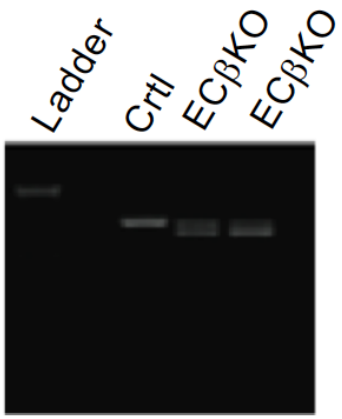
Supplementary Figs:



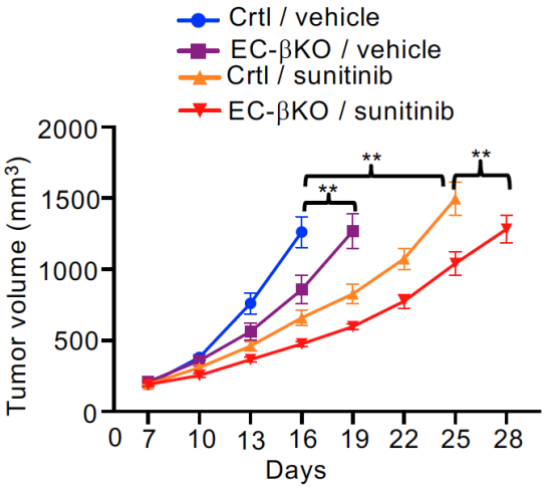
Supplementary Fig. 3.1: Pharmacological Inhibition of PI3Kβ by TGX-221 decreases PI3K/AKT signaling. A, HUVECs were pretreated with 30nM PI3Ka specific inhibitor BYL719 or 100nM PI3Kβ specific inhibitor TGX221 for 1h. Serum starved cells were treated with 50ng/mL VEGF or CXCL12 for 10min. Active p-Akt (p-Akt^{S473}) was determined by Western blot. **B,** Quantification of pAkt (n = 3 independent experiments, *P < 0.05, **P < 0.01 by two-way ANOVA).



Supplementary Fig. 3.2: Treatment with TGX-221 does not block growth factor expression in PD-RCC / HUVEC spheroid 3D co-culture. RNA was isolated from PD-RCC / HUVEC spheroid 3D co-culture and the expression of growth factors were measured by qPCR analysis (mean ± SEM; n = 8 independent samples; *P < 0.05; two-way ANOVA).

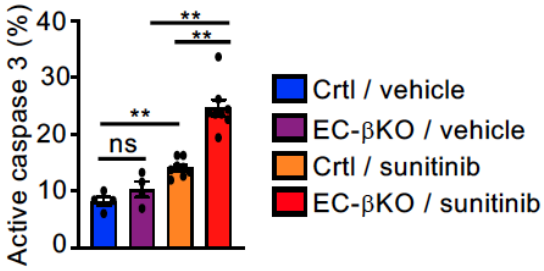


Supplementary Fig. 3.3: PCR analysis of expression of truncated p110β in lung samples harvested from EC-βKO or control mice. The amplicon covers exon 16 to exon 24 and indicates successful p110β inactivation.

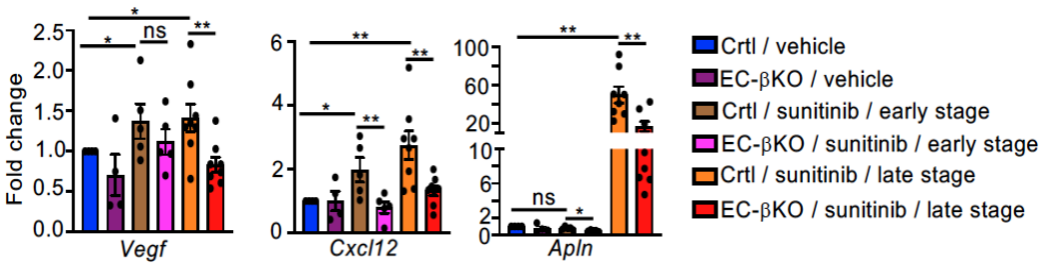


Supplementary Fig. 3.4: Inactivation of endothelial PI3Kβ decreases primary tumor growth. 1 X 106 B16F10 mouse melanoma cells were implanted subcutaneously into EC-βKO or control mice (n = 7 mice/group). When the tumor volume reached 200mm^3, mice were treated with vehicle or sunitinib (40mg/ kg/ day). Tumor volume was measured every three days using slide calipers as described in

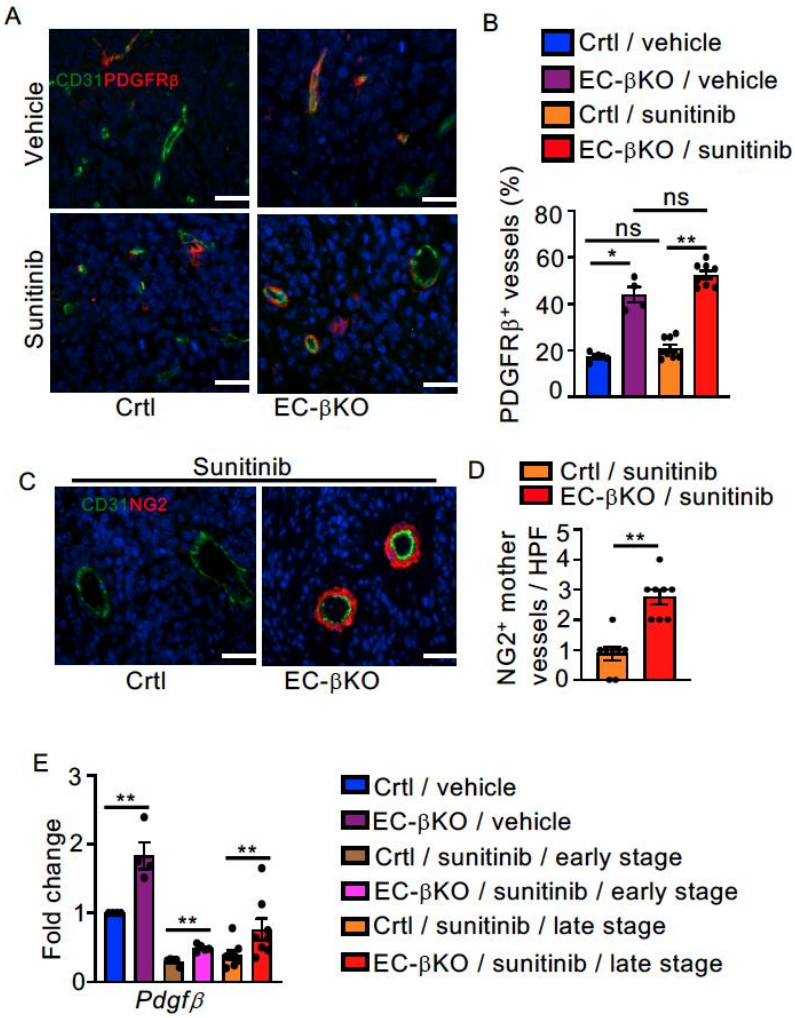
Methods. Results are presented as the mean ± SEM; *P < 0.05, **P < 0.01 by two-way ANOVA, repeated measure.



Supplementary Fig. 3.5: Endothelial PI3Kβ inactivation increases tumor cells apoptosis in sunitinib-treated mice. Quantification of active caspase 3^{+} cells in vehicle or sunitinib-treated EC-βKO vs control mice (mean ± SEM; n = 4-8 mice/group; **P < 0.01; Mann-Whitney U - test).

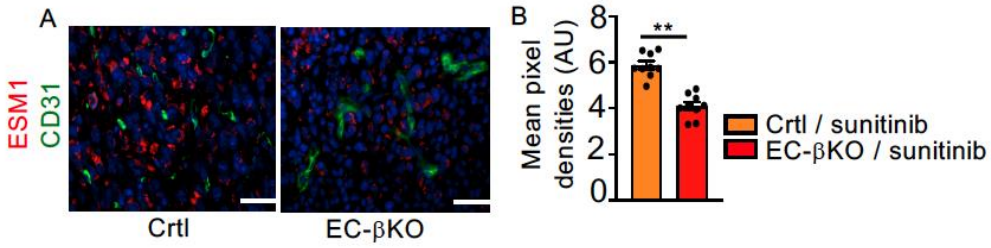


Supplementary Fig. 3.6: Inactivation of endothelial PI3Kβ reduces growth factor expression in the LLC1 primary tumor model. qPCR analysis of pro-angiogenic growth factors expression among the groups (mean ± SEM; n = 4-8 mice/ group; *P < 0.05, **P < 0.01; Mann-Whitney U - test).

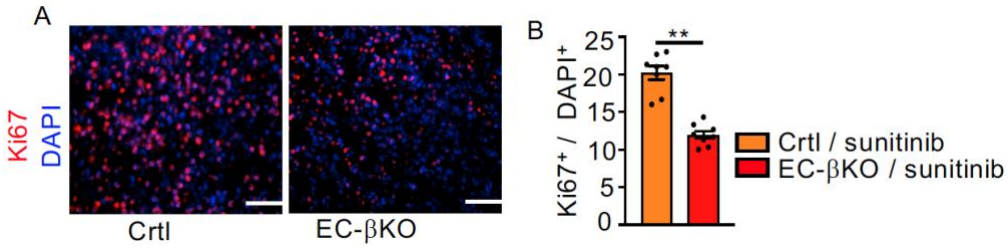


Supplementary Fig. 3.7: Inactivation of endothelial PI3Kβ promotes tumor vessel normalization among sunitinib-treated mice. A, Representative IHC images of PDGFRB (red) coverage of CD31+ (green) tumor microvessels in vehicle or sunitinib-treated EC-βKO and control mice. Scale bars, 50μm. **B,** Quantification of the fraction of PDGFRB-associated CD31+ vessels (mean ± SEM; n = 4-8 mice/group; $^{**}P < 0.01$; Mann-Whitney *U* - test). **C,** Representative IHC images of $NG2^+$ pericyte (red) coverage

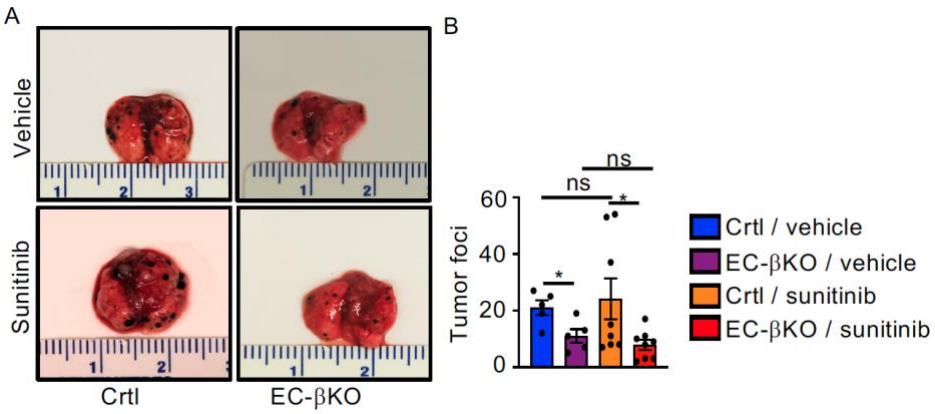
of CD31+ (green) tumor mother vessels among sunitinib-treated EC-βKO and control mice. Scale bars, 50μm. **D,** Quantification of CD31+ large vessels covered by NG2+ pericytes (mean ± SEM; n = 8 mice/group; ***P* < 0.01; Mann-Whitney *U* - test). **E,** *Pdgfb* growth factor expression among the groups (mean ± SEM; n = 4 - 8 mice/ group; ***P* < 0.01; Mann-Whitney *U* - test).



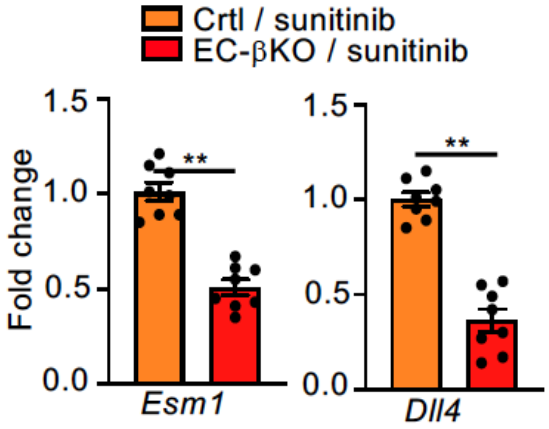
Supplementary Fig. 3.8: Inactivation of endothelial PI3Kβ reduces the expression of the endothelial tip cell marker ESM1 in sunitinib-treated LLC1 tumors. A, Representative images of ESM1 (red) and CD31+ (green) tumor vessels in sunitinib treated EC-βKO or control mice. Scale bars, 50μm. **B,** Quantification of ESM1^{+} pixels among EC-βKO and control mice (mean ± SEM; n = 8 mice/ group; ***P* < 0.01; Mann-Whitney *U* - test).



Supplementary Fig. 3.9: Endothelial PI3Kβ inactivation reduces tumor cell proliferation of LLC1 tumor in sunitinib-treated mice. A, Representative IHC images of Ki67+ (red) proliferating tumor cells among sunitinib-treated EC-βKO and control mice. Scale bars, 50μm. B, Quantitation of Ki67 co-localization to DAPI (mean ± SEM; n = 8 mice/group; ***P* < 0.01; Mann-Whitney *U* - test).



Supplementary Fig.10. Inactivation of endothelial PI3Kβ decreases the tumor metastasis burden. 2 X 10^5 B16F10 mouse melanoma cells were injected into the tail vein of EC-βKO or control mice. Immediately after tumor cell inoculation, mice were treated with sunitinib (40mg/ kg/ day) or vehicle for 20 days, then the mice were euthanized at day 21. **A,** Representative images of lung metastases and **B,** the quantification (mean ± SEM; n = 5 - 8 mice/ group; **P* < 0.05; Mann-Whitney *U* - test).



Supplementary Fig. 3.11: Inactivation of endothelial PI3Kβ decreases EC tip cell gene expression in B16F10 lung metastasis model. Quantification of tip cell marker

genes among the groups (mean ± SEM; n = 8 mice/ group; $^{**}P < 0.01$; Mann-Whitney *U* - test).

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Chapter 4

Loss of apelin blocks the emergence of sprouting angiogenesis in experimental tumors*

Abul K. Azad[1], Kieran Campbell[2], Pavel Zhabyeyev[1,3], Gavin Y. Oudit[1,3], Ronald B. Moore[4], Allan G. Murray[1]

[1]Department of Medicine, University of Alberta, Edmonton
[2]Lunenfeld-Tanenbaum Research Institute, University of Toronto, Toronto
[3]Department of Physiology, University of Alberta, Edmonton
[4]Department of Oncology and Department of Surgery, University of Alberta, Edmonton

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4.1 Abstract

Angiogenesis-inhibitor (AI) drugs targeting vascular endothelial growth factor (VEGF) signalling to the endothelial cell (EC) are used to treat various cancers types. However, initial response to therapy is not durable, and often times tumors become resistance to therapy. Clinical and pre-clinical studies suggest that other alternative pro-angiogenic factors are up-regulated after VEGF-pathway inhibition. Therefore, identifying alternative pro-angiogenic pathway(s) is critical for the development of more effective anti-angiogenic therapy. Here we study the role of Apelin as a por-angiogenic G-protein coupled receptor (GPCR) ligand in tumor growth and angiogenesis. We found that loss of Apelin in mice delayed the primary tumor growth of Lewis lung carcinoma (LLC1) and B16F10 melanoma when combined with a receptor tyrosine kinase inhibitor (RTK) Sunitinib. Targeting Apelin in combination with Sunitinib treatment markedly reduced the tumor vessels density, which was accompanied by a significant reduction in angiogenic sprouting and tip cell marker genes expression in comparison to the control group. Interestingly, in our single cell RNA (scRNA) sequencing, we observed that the loss of Apelin markedly prevented endothelial cell (EC) tip cell differentiation in comparison to the control mice. Together, our data suggest that targeting Apelin may be useful as adjuvant therapy in combination with VEGF-signaling inhibition to prevent the growth of advanced tumors.

4.2 Introduction

Angiogenesis in embryonic development is a process in which new blood vessels are formed from pre-existing vessels. In the adult, maladaptive responses to tissue ischemia, such as proliferative retinopathies, invoke similar events to stimulate new microvessel growth (Folkman 1995). Similarly, neoangiogenesis is essential to supply oxygen and nutrients for tumor growth and to support tumor metastasis (Lugano, Ramachandran and Dimberg 2020). The chaotic tumor neovasculature is characterized by immature, leaky, and unstable blood vessels, associated with dynamic cycles of vessel extension, perfusion, and regression, resulting in an inhomogeneous microenvironment thought to provoke tumor aggressiveness and metastasis (Bennewith and Durand 2004, Fukumura et al. 2010).

Vascular endothelial growth factor (VEGF) is the dominant pro-angiogenic cue in development and is upregulated in rapidly-growing, hypoxic cancers (Kerbel 2008). Therapy directed to decrease VEGF-dependent neovascularization has been deployed clinically, but is limited by variable initial responses, and ultimately escape from current angiogenesis inhibitor drugs (Kerbel 2015, Hawkins et al. 2020). Recruitment of diverse alternative pro-angiogenic growth factors, in part, accounts for drug resistance (De Palma, Biziato and Petrova 2017, Apte, Chen and Ferrara 2019). Development of better

anti-angiogenic strategies targeting these alternative pro-angiogenic cues or common signalling pathways are needed.

In development, additional cues collaborate with VEGF to promote angiogenic sprouting, marked by differentiation of the leading or tip endothelial cell (EC) that guides sprout extension from an existing vessel (Gerhardt et al. 2003). Among these, apelin is a secreted endogenous peptide ligand for the G-protein coupled apelin receptor, APLNR, that is preferentially expressed in endothelial tip cells in the embryo and tumor neovasculature (del Toro et al. 2010, Goveia et al. 2020a). Loss of apelin in zebrafish causes defects in endothelial tip cell morphology and sprouting, and apelin knockout mice, while viable, have defects in retinal vascularization (Eyries et al. 2008, Kasai et al. 2008). Hence, an approach to interrupt apelin/ apelin receptor signalling, in combination with anti-VEGF therapy, is a candidate to increase the depth of inhibition of pathological angiogenesis.

In experimental tumors, over-expression of apelin has inconsistent effects on tumor growth and neovascularization (Sorli et al. 2007, Kidoya et al. 2012, Mastrella et al. 2019, Frisch et al. 2020, Berta et al. 2014), that may be confounded by variable cancer cell expression of the apelin receptor and cancer cell response to apelin stimulation (Harford-Wright et al. 2017, Mastrella et al. 2019, Tolkach et al. 2019, Podgorska et al. 2021, Picault et al. 2014). Host loss of apelin decreases tumor neoangiogenesis, and additively increases the effect of anti-VEGF-dependent angiogenesis inhibition (Mastrella et al. 2019, Uribesalgo et al. 2019). However, despite reducing tumor

vascular density, apelin inactivation renders apelin receptor-positive glioblastoma cells more invasive in experimental tumors (Mastrella et al. 2019).

How apelin participates in angiogenesis is not yet fully understood. Here, we study the role of apelin, alone and in combination with VEGFR2 inhibition, in tumor neoangiogenesis, and characterize the effect of apelin loss on tumor EC. We show that inactivation of host apelin delays primary tumor angiogenesis and growth. Further, loss of apelin prevents angiogenic sprouting and endothelial tip cell-enriched gene expression in tumor EC.

4.3 Results

4.3.1 Apelin loss delays tumor growth

To analyze the role of apelin in tumor growth, we implanted Lewis lung carcinoma (LLC1) or B16F10 melanoma cells into the flank of *Apln*$^{-/y}$ or wild-type control mice. In addition, since VEGF is the primary pro-angiogenic cue inhibited in the clinic, we used sunitinib to inhibit VEGF receptor-2 tyrosine kinase signaling to test the effect of combined blockade of VEGFR-2 and apelin receptor signaling. Sunitinib (40mg/ kg/ day) or vehicle control was administered by intra-peritoneal injection when the tumor volume reached an average size of 200mm^3 as described (Ebos and Kerbel 2011). As expected, sunitinib slowed but did not stop tumor growth in the wild-type control mice (Fig. 4.1A, B). We found there was little delay in tumor growth in the vehicle-treated *Apln*$^{-/y}$ versus wild-type control mice (Fig 4.1A, B). However, tumor growth was markedly delayed in *Apln*$^{-/y}$ versus the wild-type mice when combined with sunitinib treatment

(Fig. 4.1A, B). A similar effect on B16F10 tumor growth was evident in sunitinib-treated *Apln*$^{-/y}$ compared to wild-type mice. Together, these data indicate that loss of apelin production by the tumor stroma reduces tumor growth when combined with VEGF-pathway inhibition more than VEGF pathway inhibition alone.

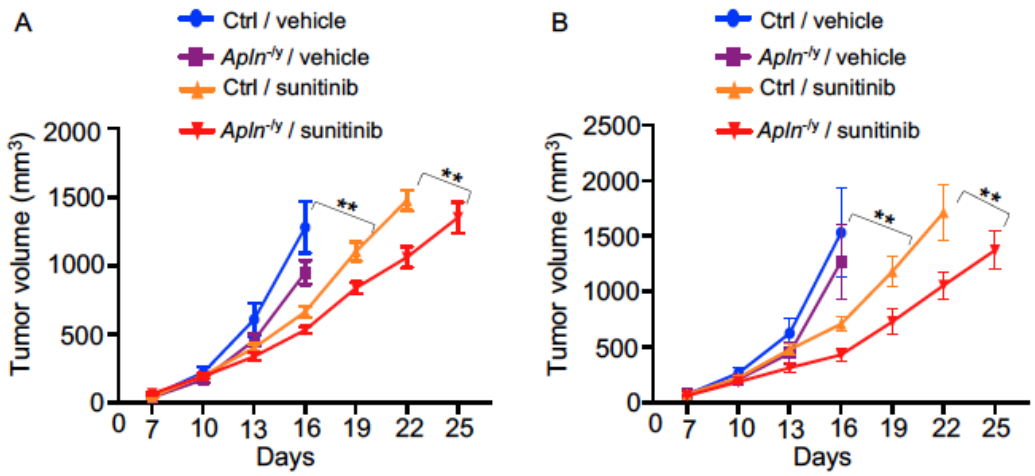


Fig. 4.1: Loss of host apelin delays subcutaneous tumor growth. A, 1X106 mouse Lewis lung carcinoma (LLC1) cells were implanted subcutaneously into wild-type (Ctrl) or Apelin knockout (*Apln*$^{-/y}$) mice (n = 7-8 mice/group). When the tumor volume reached 200 mm3, mice were treated with sunitinib (40mg/kg/day) or vehicle. Tumor volume was measured every 3 days using slide calipers as described in Methods. Results are presented as the mean ± SEM; $^{**}P < 0.01$ by two-way ANOVA, repeated measure. **B,** A second tumor cell line, B16F10, was used for similar experiment as in Fig. 1A. Results are presented as the mean ± SEM; $^{**}P < 0.01$ by two-way ANOVA, repeated measure; n = 6-8.

4.3.2 Loss of apelin reduces tumor angiogenesis and promotes vessels normalization

To understand how apelin loss delays primary tumor growth, we evaluated LLC1 tumor neovascularization among the groups. When wild-type mice were treated with sunitinib, the density of $CD31^+$ tumor microvessels was reduced compared to the vehicle-treated mice, confirming the effect of sunitinib on the tumor vasculature (Fig 4.2A, B). We observed that the tumor vascular density was somewhat reduced in vehicle-treated *Apln*$^{-/y}$ versus wild-type mice (Fig 4.2A, B). However, the $CD31^+$ tumor vessel density was further decreased in *Apln*$^{-/y}$ mice when combined with sunitinib, and was reduced by ~50% in sunitinib-treated *Apln*$^{-/y}$ versus the sunitinib-treated wild-type littermate mice (Fig 4.2A, B).

Tumor vascular density reflects the net of a dynamic process of new microvessel growth and regression of more mature microvessels (Mancuso et al. 2006, Inai et al. 2004). We next examined the effect of VEGF and apelin inactivation on established tumor microvessels. Mature CD31+ endothelial cell-lined tumor microvessels are surrounded by type IV collagen basement membranes and enveloped by pericytes. We found that sunitinib treatment decreased the fraction of CD31+ tumor microvessels with established basement membranes (Fig. 4.2C, D left panel). In contrast, apelin loss alone did not alter the fraction of mature vessels, but normalized the fraction of mature tumor vessels in sunitinib-treated mice. Further, regression of endothelial cells from mature vessels was quantified. Sunitinib treatment of wild-type mice increased the fraction of empty collagen IV^+ basement membrane sleeves, lacking $CD31^+$ EC (Fig. 4.2C, D right panel). Apelin loss alone did not change the fraction of empty basement membrane sleeves, and normalized the fraction of sleeves when combined with

sunitinib. Moreover, we observed that apelin loss increased the fraction of mature microvessels covered by $NG2^+$ pericytes, after either vehicle or sunitinib treatment (Fig. 4.2E, F). Together these data indicate that sunitinib inhibition of VEGF signalling is associated with regression of mature vessels, and impaired maturation of new vessels. Apelin loss alone or in combination with perturbed VEGF signalling does not appear to promote regression of established vessels or impair the maturation of new vessels.

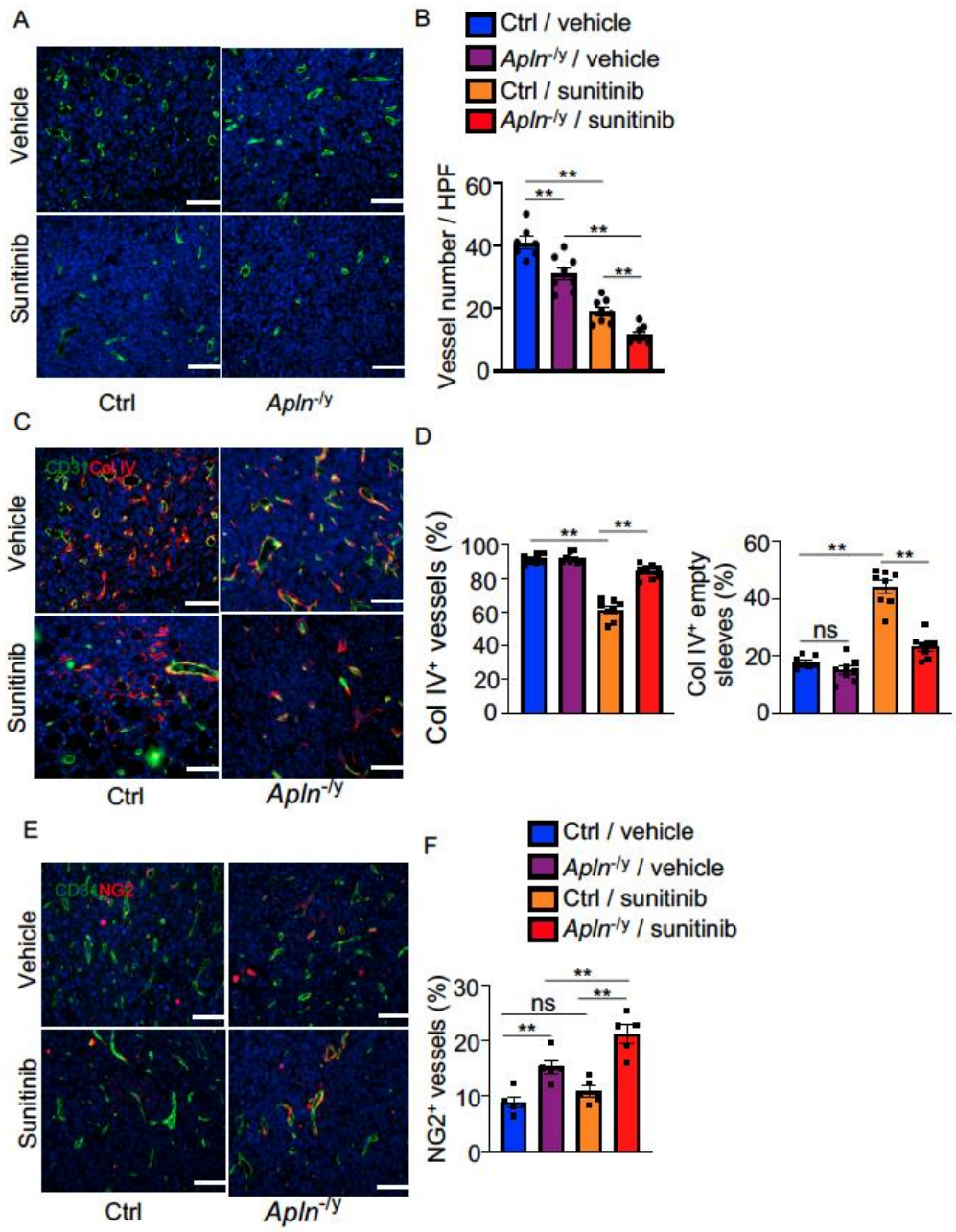


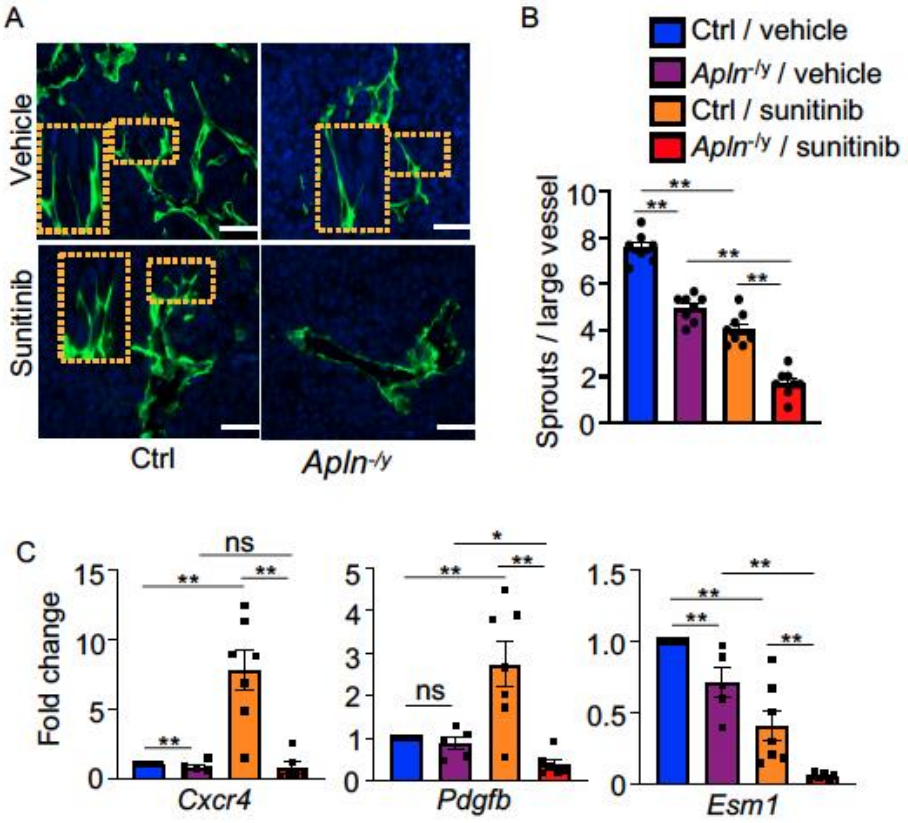
Fig. 4.2: Fig. Loss of apelin reduces tumor vessels density and promotes vessels maturity. A, Representative images of CD31-positive tumor vessels in vehicle or sunitinib-treated Ctrl vs *Apln*$^{-/y}$ mice.Scale bar, 70µm. **B,** Quantification of CD31+ vessels per high power field (HPF) (mean ± SEM; n = 7-8 mice/group; ***P* < 0.01 by one-way ANOVA). **B,** Representative images of CD31+ (green) vessels and Col IV (red) capillary basement membranes. Scale bar, 70µm. **C,** Quantification of Col IV+ basement membrane coverage of CD31+ vessel profiles (left panel), and Col IV empty

sleeves (right) in vehicle or sunitinib-treated wild-type vs *Apln*$^{-/y}$ mice (mean ± SEM; n = 7-8 mice/group; $^{**}P < 0.01$ by one-way ANOVA). Vessel maturity was evaluated by using NG2+ pericyte staining. **E,** Representative images of NG2 (red) coverage of CD31+ (green) tumor vessels in vehicle or sunitinib treated wild-type Ctrl vs *Apln*$^{-/y}$ mice. Scale bar, 70μm. **F,** Quantification of the fraction of NG2-associated CD31+ vessels (mean ± SEM; n = 5 mice/group; $^{**}P < 0.01$ by one-way ANOVA).

We examined the effect of apelin loss on new microvessel sprouting. Since the quantitative assessment of tumor vascular densities does not necessarily represent ongoing neovascularization, we sought to determine the effect of apelin deficiency in tip cell development. We evaluated outgrowth of new microvessels, marked by characteristic tip cell filopodia, from tumor mother vessels. We found sunitinib treatment of wild-type mice decreased the number of tip cell sprouts (Fig 4.3A, B). Apelin loss alone similarly decreased tip cell sprouts, and additively decreased tip cell sprouting events observed when combined with sunitinib.

Next, we studied expression of tip cell marker genes as a surrogate of new tumor vessel sprouting. Previous work has identified tip cell gene expression gene profiles from bulk transcriptome sequencing, and more recently from single-cell RNAseq (del Toro et al. 2010, Zhao et al. 2018, Goveia et al. 2020b)**.** We examined the expression of the tip cell markers *Pdgfb*, *Cxcr4*, *Esm1* (Zhao et al. 2018). Sunitinib treatment alone had a variable effect on tip gene induction compared to vehicle treatment in wild-type mice. However, we found that loss of apelin in combination with sunitinib treatment consistently inhibited the expression of the tip cell marker genes versus sunitinib

treatment alone (Fig 4.3C). Together, these data suggest that loss of tumor stromal apelin complements VEGF receptor inhibitor to reduce the emergence of differentiated tip cell sprouts.



4.3. Loss of apelin reduces tumor angiogenesis. A, Representative images of sprouting from large vessels in sunitinib-treated Ctrl vs *Apln*$^{-/y}$ mice. The inset shows a sprout with tip cell filopodia. Scale bars, 50μm. **B,** Quantification of angiogenic sprouts in LLC1 tumors from sunitinib-treated wild-type vs *Apln*$^{-/y}$ mice (mean ± SEM; n = 7-8 mice/group; $^{**}P < 0.01$ by one-way ANOVA). **C,** Quantification of endothelial tip cell marker gene expression by qPCR in vehicle or sunitinib-treated Ctrl vs *Apln*$^{-/y}$ mice (mean ± SEM; n = 5-7 mice/group; $^{*}P < 0.05$, $^{**}P < 0.01$ by one-way ANOVA).

4.3.3 Loss of apelin reduces endothelial tip cell gene expression

Loss of apelin in the tumor microenvironment decreases tumor vascular density and sprouting angiogenesis. To better understand the role of apelin in tumor angiogenesis, we analyzed the effect of apelin loss on the tumor EC transcriptome. We used positive immunomagnetic bead enrichment on CD31 to obtain tumor EC from 4 groups of mice: wild-type and *Apln*$^{-/y}$ mice treated with either vehicle or sunitinib, each pooled from two replicate mice. Single-cell transcriptome libraries were generated, pooled, and analyzed by Seurat clustering. *Pecam*1 and *Cdh*5, used as EC markers, identified 401 EC as shown in Fig 4.4A.

The EC population was examined further. Unsupervised Seurat clustering identified 2 EC subpopulations within the tumor EC (Fig 4.4B). Principal component analysis was applied to describe the relatedness of the two EC populations, displayed as pseudotime in PC1 (Fig 4.4C). EC in cluster 1 were represented in each tumor group (Fig 4.4D). However, EC in cluster 0 were strikingly absent from tumors grown in *Apln*$^{-/y}$ mice.

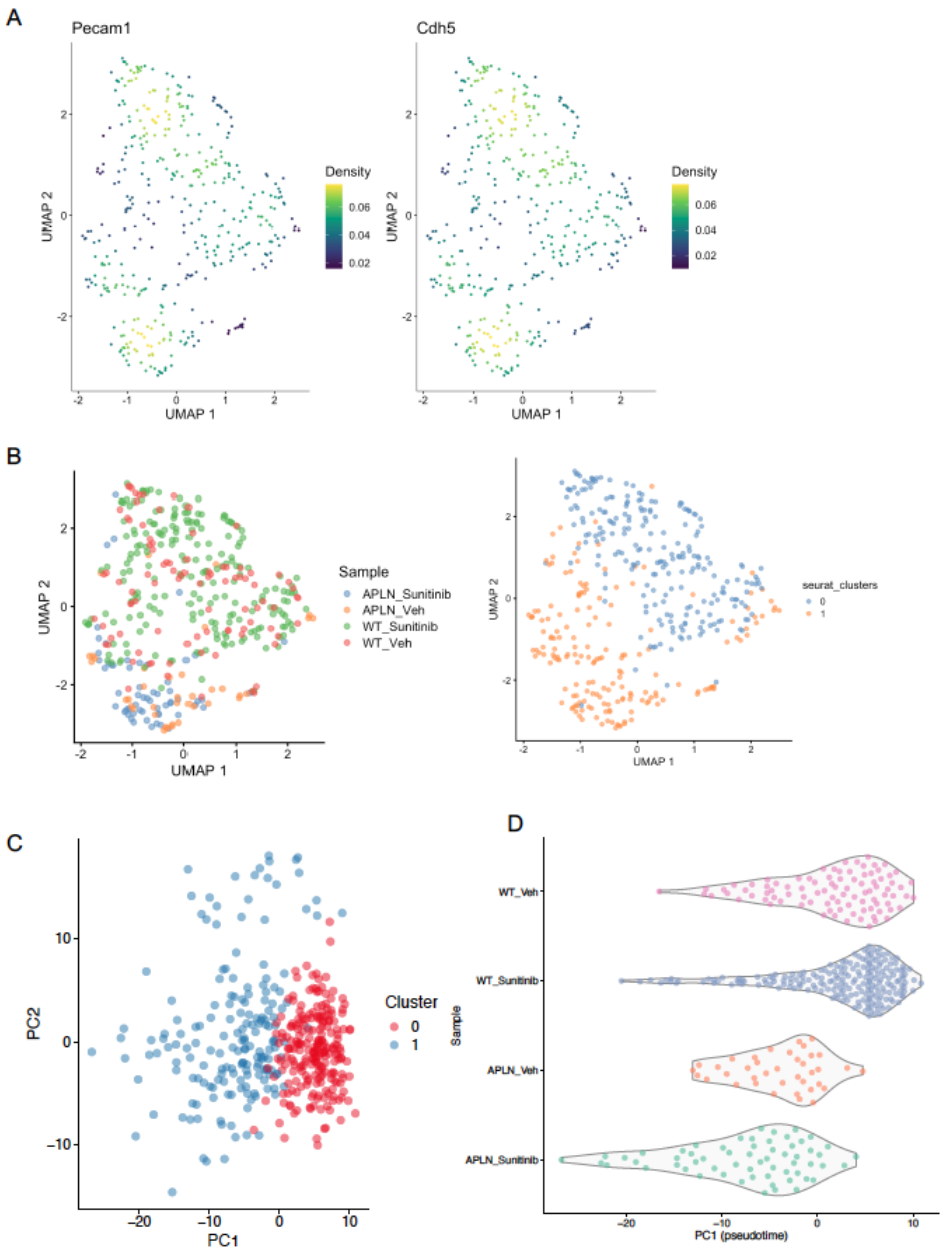


Fig. 4.4: Loss of apelin alters tumor EC transcriptome. **A,** Identification of EC by using EC-specific marker genes Pecam-1 and Cdh5. **B,** Seurat clustering of EC transcriptome among the groups (left panel), and re-clustering the cells into 2 EC sub-populations (right panel). **C,** Representation of EC sub-populations in principal component analysis (PCA) showing similar genes clustering together. **D,** Representation of EC transcriptome showing ECs in cluster 0 are almost absent in *Apln*$^{-/y}$ mice.

The genes with expression that most discriminate EC between the two clusters are shown in Fig 4.5A. Differentially-expressed genes between cluster 0 and 1 are shown in Fig 6.5B. Among the most highly differentially-expressed genes in cluster 0 are cytoskeletal constituents, *Vim*, *Actb*, and *Tuba1a*, and proteins involved in cytoskeletal remodelling such as *Cfl1*, *RhoC*, and *Tagln2* (Jin et al. 2016). Pathway analysis assignments of the DEG using the GO database is shown in Fig 4.5C. Interestingly, there are few gene transcripts lost as EC transition from cluster 1 to cluster 0. These findings suggest cluster 0 EC have acquired a motile phenotype, consistent with sprouting EC.

Inspection of the DEG in cluster 0 reveals upregulation of genes that have previously been identified in angiogenic tip cells. We curated a list of endothelial tip cell genes in Fig 4.5D. *Esm1*, *Cxcr4*, *Pdgfb*, *Adm*, and *Ang2* but not *Dll4* show upregulated expression in cluster 0 EC. These data indicate that the loss of apelin decreases tumor endothelial tip cell differentiation that facilitate sprout extension.

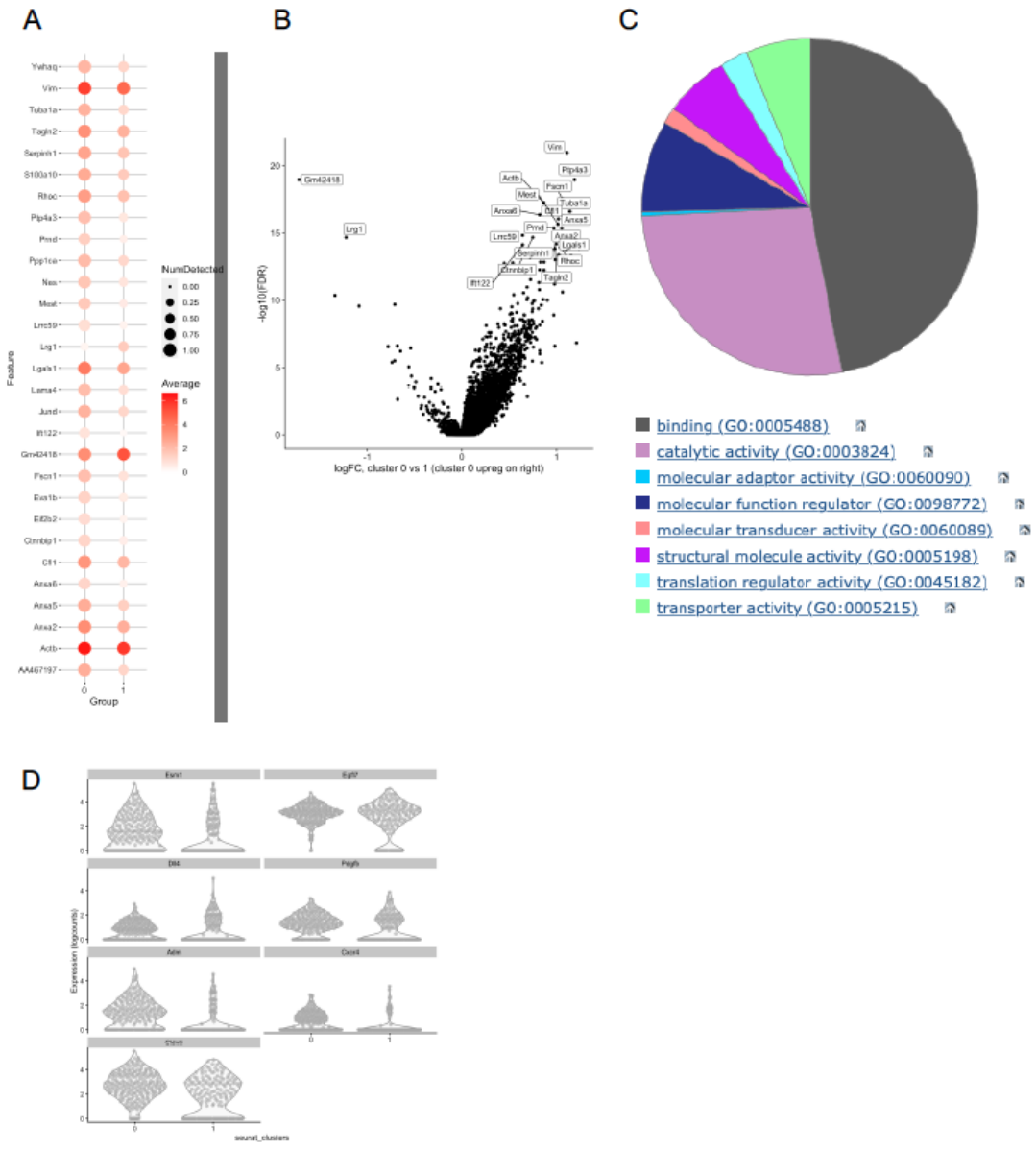


Fig. 4.5: Loss of apelin inhibits tip cell enriched gene expression in tumor EC transcriptome. A, Expression of most discriminating genes between Apln-/y vs. Ctrl mice. **B,** Volcano plot of differentially expressed genes in *Apln*$^{-/y}$ vs Ctrl mice. **C,** Panther GO analysis differentially expressed genes in *Apln*$^{-/y}$ vs Ctrl mice representing different biological processes. **D,** Violin plot of differential expression of tip cell marker gene in Apln-/y vs Ctrl mice.

4.3.4 Loss of apelin reduces sunitinib-induced hypoxic marker gene expression

Since sunitinib treatment-associated vessel pruning enhances tumor hypoxia (Conley et al. 2012, Pàez-Ribes et al. 2009), we determined effect of apelin loss on tumor hypoxia. To this end, we evaluated the expression of hypoxia marker genes, *Ndrg1* and *Glut1*, among the groups. Sunitinib treatment increases the expression of both *Ndrg1* and *Glut1* in sunitinib-treated versus vehicle-treated wild-type mice (Fig 4.6 A). Interestingly, despite reduced microvessel density, the expression of *Ndrg1* and *Glut1* were markedly reduced in *Apln*$^{-/y}$ mice under sunitinib treatment (Fig 4.6A), indicating that tumor hypoxia was reduced. Hypoxia up-regulates several pro-angiogenic growth factors, including VEGF and CXCL12 (Semenza 2014, Semenza 2012). We observed that *Vegf* and *Cxcl12* were decreased in sunitinib-treated *Apln*$^{-/y}$ versus wild-type mice (Fig 4.6B). Taken together, this data suggests that delayed tumor growth associated with perturbed apelin and VEGF pro-angiogenic microenvironmental cues reduces tumor angiogenesis and reduces overall tumor hypoxia.

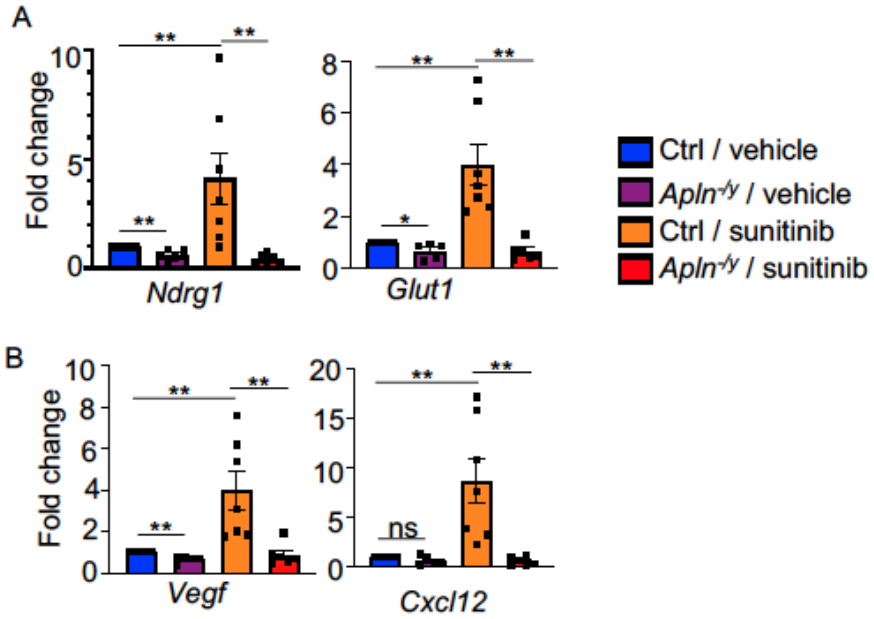


Fig. 4.6: Apelin deficiency reduces tumor hypoxia in sunitinib-treated mice. A, hypoxia responsive marker gene expression in vehicle or sunitinib treated control vs *Apln*$^{-/y}$ mice (mean ± SEM; mice n = 5-7 mice/group; $^{**}P < 0.01$ by one-way ANOVA). **B,** pro-angiogenic growth factor gene expression in vehicle or sunitinib treated control vs *Apln*$^{-/y}$ mice (mean ± SEM; mice n = 5-7 mice/group; $^{**}P < 0.01$ by one-way ANOVA).

4.4 Discussion

Angiogenesis inhibitor treatment of vascular proliferative retinopathies or cancer neovascularization, such as renal cell carcinoma (RCC), are limited by partial responses to VEGF pathway inhibitors, and the eventual escape from VEGFR pathway inhibition (Rosenfeld et al. 2006, Gasperini et al. 2012, Zuber-Laskawiec et al. 2019, Lambertini et al. 2017). Hence there is clinical interest to define the mechanisms of vascular escape from angiogenesis inhibition. Apelin is implicated in tumor neoangiogenesis, and loss of apelin reduces developmental angiogenesis (Kuba et al. 2019, Sorli et al. 2007).

Endothelial tip cell expression of apelin and the apelin G-protein-coupled receptor is thought to be a common and necessary step in neoangiogenesis. Hence, targeting critical common EC differentiation steps downstream of VEGF or alternative proangiogenic cues is attractive to deepen angiogenesis inhibition and improve initial and sustained responses to therapy.

We report the additive effect of apelin inactivation to sunitinib-mediated VEGF receptor-2 inhibition to block growth and neovascularization of two types of experimental tumors. This finding is consistent with an earlier report that found apelin loss reduced vessel density in a mammary tumor model only when combined with sunitinib(Uribesalgo et al. 2019). Further, inactivation of apelin signalling acts distinctively compared to interruption of another pro-angiogenic G-protein coupled receptor, S1PR1(Balaji Ragunathrao et al. 2019). Our analysis indicates that interruption of VEGF signalling with sunitinib perturbs tumor microvessels at several maturation stages that is dissimilar to the effect of apelin loss. Empty collagen IV+ basement membrane sleeves are increased in sunitinib-treated tumors in wild-type mice, marking regression of relatively mature microvessels, but are not a feature of tumor neovascularization in the absence of apelin. Empty basement membrane sleeve remnants after anti-VEGF AI-associated microvessel regression, may be used as a scaffold for the re-emergence of new vessels (Inai et al. 2004). Maturation of new tumor vessel sprouts to become enveloped in basement membrane is impaired in sunitinib-treated, but not apelin-deficient mice. These findings suggest the suppression of tumor vessel remodeling with apelin inactivation.

Most strikingly, tip cell emergence from tumor mother vessels is reduced in each sunitinib-treated and apelin-deficient mice, and additively reduced when both are combined. This observation is supported by the finding of reduced tip gene expression in apelin-deficient mice in the whole-tumor RNA expression analysis. Notably, the expression of the selected tip cell genes was variable and inconsistent in sunitinib-treated mice, perhaps reflecting more chaotic microvascular patterning.

This first report in apelin-deficient mice of single-cell RNAseq analysis of tumor EC revealed marked loss of a subpopulation of 'angiogenic' EC expressing tip cell markers and genes involved in cytoskeletal remodelling. Together, our data indicate that apelin produced by host cells in the tumor microenvironment is critically required for tip cell differentiation.

Tumor hypoxia and induction of hypoxia-responsive pro-angiogenic gene expression is reduced in apelin-deficient mice. A transient window of tumor vessel normalization may open up after anti-VEGF AI therapy (Matsumoto et al. 2011, Winkler et al. 2004). Apelin loss-of-function with anti-VEGF AI therapy reduced tumor vascular permeability, and tumor hypoxia (Uribesalgo et al. 2019, Zhang et al. 2016). Normalized tumor vessels facilitate homogenous oxygen and nutrient delivery and reduce hypoxic niches (Martin, Seano and Jain 2019, Viallard and Larrivee 2017). Together, our data confirm that loss of host apelin complements the effects of sunitinib to deepen the reduction in tumor vessel density, supress new vessel sprouting, and vessel remodeling that contribute to inhomogeneous oxygen and nutrient supply to the tumor.

High expression of apelin has been found to correlate with poor clinical outcome in human lung (Berta et al. 2010), head and neck (Heo et al. 2012), gastric (Feng et al. 2016), colorectal (Zuurbier et al. 2017), hepatocellular (Lee, Park and Ha 2019), renal (Tolkach et al. 2019) and breast (Uribesalgo et al. 2019). Pharmacologic inhibition of the apelin receptor reduces cholangiocarcinoma tumor angiogenesis and growth (Hall et al. 2017). Recent studies have shown that targeting host apelin in combination with AI therapy delayed experimental tumor angiogenesis and growth (Mastrella et al. 2019, Uribesalgo et al. 2019). Nevertheless, tumor invasiveness may be markedly increased in some cancers (Mastrella et al. 2019).

Loss of apelin reduces the potential of EC to express several accepted tip cell marker genes, e.g. *Pdgfb*, *Esm1* and *Cxcr4*. Interestingly, we did not observe any change in *Dll4*, a characteristic tip-specific gene that suppresses tip cell differentiation of adjacent EC (Jakobsson, Claudio and Gerhardt 2010). This data suggests that although EC from apelin-deficient mice are enriched with Dll4 expression equally compared to wild-type mice, apelin is required to achieve full tip cell potential. Moreover, persistent Dll4 expression in apelin-deficient mice may actively promote an EC phenotype associated with enhanced vessel stability.

Cytoskeletal remodelling is a key feature of motile cells, and is required for tip cell filopodia extension and sprout growth (Lamalice, Le Boeuf and Huot 2007). Differential expression of numerous genes dependent on apelin cues in the tissue

microenvironment suggest apelin signalling in the EC is functionally linked to prime EC for angiogenic sprouting. Expression of nestin, another differentially expressed gene suppressed in the apelin-deficient mice is functionally linked to cell deformability that may facilitate penetration of basement membrane and extracellular matrix (Yamagishi et al. 2019).

Together, our observations indicate that apelin inactivation complements VEGF pathway inhibition for anti-angiogenic therapy to reduce tumor growth and neoangiogenesis. Mechanistically, apelin loss decreases sunitinib-induced tumor vascular remodeling, and promotes vessel normalization. Tumor-associated EC fail to acquire the tip cell phenotype when apelin is lost.

4.5 Materials and Methods

The details of this section are described in **Chapter 2.**

4.5.1 Cell culture

B16F10 mouse melanoma and mouse Lewis lung carcinoma (LLC1) cell lines (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) with 10% FBS and 100 U/ml penicillin and 100 µg/ml streptomycin (Life Technologies). All the cells were cultured in a humidified incubator at 37^0C containing 5% CO_2. The cells were trypsinized and washed twice in 1XPBS and were resuspended in 1XPBS (without Ca2+ and Mg2+) for the implantation into mice.

4.5.2 Animal model

The generation of Apelin knockout mice (*Apln*$^{-/y}$) in the background of C57BL/6 mice was described previously (Kuba et al. 2019). Apelin is coded on X-chromosome, therefore the Apelin knockout male mice are designated as *Apln*$^{-/y}$. The APLN knockout was confirmed by genotyping by Gavin Oudit, laboratory, University of Alberta. 10 to 12 weeks of old male mice were used for the tumor study.

Mouse Lewis lung carcinoma (LLC1) or B16F10 mouse melanoma cell lines (1 X 10^6) were implanted into the subcutaneous of 12 weeks old *Apln*$^{-/y}$ or control mice. Tumor volume was measured every 3 days with a slide calipers by using the formula: π x width^2 x length/6. Vehicle or sunitinib (40mg/kg i.p. daily) was initiated when the tumor volume reached an average size of 200mm^3, and mice were euthanized when tumor

volume reached an average size >1500 mm^3. 60mg/kg pimonidazole (Hypoxyprobe^{TM}-1 kit; NPI Inc) was administrated through the tail vein 1 hour before euthanization. Primary tumor samples were harvested in RNAlater for PCR analysis and in Zinc fixative for immunohistochemical analyses.

4.5.3 Drugs

Sunitinib (Pfizer) was dissolved in 1X PBS (without Ca^{2+} and $Mg^{2+)}$ containing 0.1% DMSO at a concentration 5mg/mL. Sunitinib was kept at 4^0C and used within a week. Hypoxyprobe^{TM}-1 (NPI Inc) prepared at a concentration of 100mg/mL in 0.9% saline and kept in 4^0C.

4.5.4 RNA isolation and PCR analysis

Less than 20mg sample was homogenized in RLT lysis buffer to isolate tissue RNA using Rneasy Mini Kit (Qiagen) and 1ug of total tissue RNA was used for reverse transcription. qRT-PCR was carried out using the Mastercycler®ep realplex real-time PCR system (Eppendorf). The reaction mixture consisted of 1µl of cDNA, 1µl of 10µM primers, and 10µl of SYBR Select Master Mix (Applied Biosystems) in a total volume of 20µl. Experimental samples were first normalized to internal control HPRT, then to the control samples, and the fold change was calculated based on 2^{DDCT} method. The PCR primers used are listed in Table 2.2.

4.5.5 Immunohistochemistry

Tumor tissue samples were paraffin embedded and 5um sections were prepared for staining. After deparaffinization and rehydration, samples were subjected for antigen retrieval in citrate buffer solution (pH 6.0) for 15 min by heating at microwave. Samples were blocked by 10% normal goat serum at room temperature for 1h. Primary antibodies used are listed in Table 2.1. The antibodies CD31 (1:40), NG2 (1:100) and Col IV (1:100) were prepared in Dako diluent buffer and incubated overnight at 4^0C. Next day after washing trice in 1X TBS, slides were incubated for 1h at room temperature with secondary antibodies: FITC-conjugated goat anti-rat (1:200; ThermoFisher) and red fluorescent conjugated goat anti-rabbit antibody (1:200; ThermoFisher). After washing, slides were counterstained with DAPI and then mounted with prolong gold mounting medium.

4.5.6 Single cell isolation

First, tumor dissociation solution was prepared by adding 4mL of 10X collagenase A in 26 mL of DMEM F12, 10mL of BSA fraction V and 0.5mg/mL DNase and was kept on ice. Primary tumor was harvested and washed with cold 1XPBS. Tumor section was placed in a 10cm dish containing 5mL of dissociation buffer. Tumor tissue was minced with a scalpel into small pieces and transferred to 10mL tubes. Another 5mL of dissociation solution was added and the tubes were placed in 37^0C incubator for 20 mins rotating slowly in a Mini Tube Rotator (Fisher Scientific). The tissue suspension was passed through 70um cell strainer for twice. Cell pellet was collected and resuspended in 1mL RBC solution and incubated for 10mins at room temperature. After

centrifugation, cell pellet was resuspended in 2mL of PBS-based washing buffer containing 0.5% BSA. Cell suspension was incubated with 7.5ug/mL E-cadherin (CD34, eBioscience) antibody for 10 min to deplete tumor cells by using Dynabeads® Sheep Anti-Rat antibody (ThermoFisher Scientific). The remaining cells were used to enrich CD31-positive endothelial cells by using 7.5ug/mL Anti-CD31 antibody (eBioscience) and the EasySep™ PE Positive Selection Kit II (Stemcell Technologies). The obtained cells were resuspended in 1 mL PBS + 0.04% BSA and were used for single cell sequencing by 10X genomics (CA, USA).

4.5.7 scRNAseq data analysis

Single-cell expression data were quantified using Cell Ranger 3.0.2. Data were subject to quality control and normalized using Scater (McCarthy et al. 2017) and scran (Lun, Bach and Marioni 2016). In particular, cells were excluded from downstream analysis if fewer than 500 genes were detected, or if the percentage of counts mapping to the mitochondrial genome was outside the 0.5%-10% interval. Expression data were normalized using the scran sum factor normalization and log-transformed for downstream analysis.

A principal component analysis (PCA) representation of the data were computed using the runPCA function in Scater (default parameters). Cells were clustered using Seurat leiden clustering (Satija et al. 2015) using the top 10 principal components with a resolution of 0.5. The putative endothelial cluster was identified based on high expression of the markers *Pecam1*, *Cdh5*, and *Vwf*. Using only expression data

corresponding to cells extracted from this cluster, PCA analysis and clustering was re-run (same parameters as before) revealing 6 further sub-clusters, of which only 2 exhibited high expression of the endothelial markers as above. These 2 sub-clusters were subsequently extracted as the purified endothelial clusters. PCA analysis and clustering (with resolution 0.2) were re-run to identify endothelial cell subclusters. Differential gene expression between these clusters was performed using the find Markers function in scran (Lun et al. 2016). The biological processes of differentially expressed genes were determined by using online based Panther program.

4.5.7 Statistical analysis

Data were shown as mean ± SEM. Test for normality was done using Shapiro-Wilk test. Statistical analysis was performed using Prism 8 (Graphpad, San Diego, CA). One-way ANOVA was used to evaluate differences in more than two groups. Two-way ANOVA was used to compare differences in more than two groups with two independent variables. *P* value <0.05 were considered significant.

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Chapter 5

FGD5 regulates endothelial cell PI3 kinase-β to promote neoangiogenesis

Authors: Abul K. Azad[1], Maikel A. Farhan[1], Cameron R. Murray[2], Kunimasa Suzuki[3], Nicolas Touret[2], Gary Eitzen[4], Ronald B. Moore[5], Allan G. Murray[1]

Affiliations:

[1]Departments of Medicine, [2]Biochemistry, [3]Alberta Diabetes Institute, [4]Cell Biology, [5]Oncology and Surgery, University of Alberta, Canada.

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The following work is a continuation of our previous published work as "*Maikel A. Farhan*, Abul K. Azad*, Nicolas Touret and Allan G. Murray. FGD5 Regulates VEGF Receptor-2 Coupling to PI3 Kinase and Receptor Recycling. Arterioscler Thromb Vasc Biol. 2017 Dec;37(12):2301-2310*." I was a first co-author of this paper. In this work we showed the role of FGD5 in regulating VEGF/RTK/PI3K signaling pathway. However, the role of FGD5 in regulating other angiogenic signaling that co-operate with VEGF signaling is not known yet. In our current work, we investigate the role of FGD5 in regulating GPCR signaling via PI3 kinase-β isoform class IA PI3K. Before that, we would like to add a brief summary of the paper to highlight the importance of FGD5 in regulating VEGF/RTK/PI3K signaling pathway, paving the way for our current work

where we will present the details of the role of FGD5 in regulating CXCL12-mediated GPCR/PI3K signaling pathway.

5.1 Brief summary of FGD5 in regulating VEGF/RTK/PI3K signaling pathway

1. Loss of FGD5 inhibits VEGF-stimulated PI3K signaling pathway
2. Knockdown of FGD5 blocks angiogenic sprouting and tip cell marker genes *in vitro*
3. FGD5 is required for efficient VEGFR2 and PI3 kinase coupling in early endosomes
4. Loss of FGD5 releases VEGFR2 form early endosomes and transported to Rab7-positive late endosome
5. VEGFR2 is degraded more quickly in the absence of FGD5

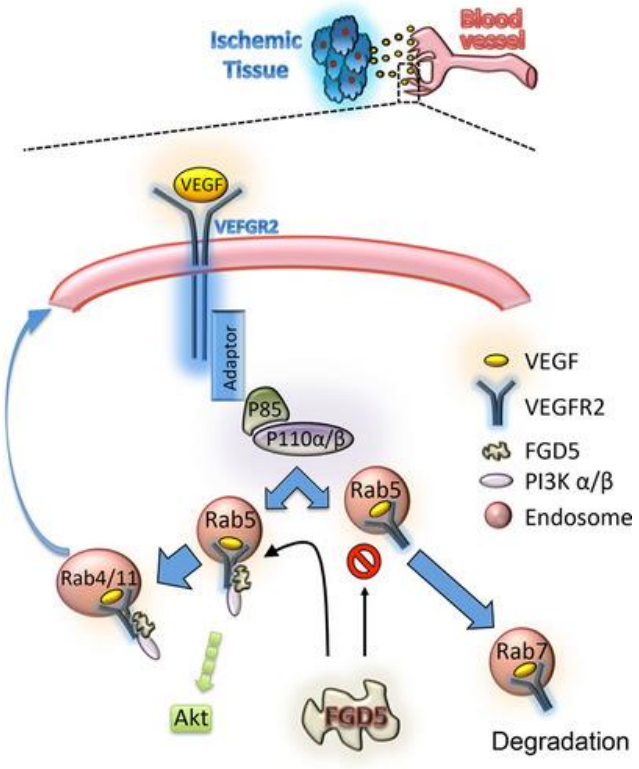

Fig. 5.1: FGD5 regulates VEGF receptor-2 coupling to PI3 kinase and receptor recycling. After activation, VEGFR2 is transported to Rab5 positive early endosomes. FGD5 binds to the activated VEGR2 and retains them to the early endosomes. In the absence of FGD5, VEGFR2 is transported to the Rab7 positive vesicles for degradation (Farhan et al. 2017b).

FGD5 regulates endothelial cell PI3 kinase-β to promote neoangiogenesis

5.2 Abstract

Angiogenesis is required in embryonic development and tissue repair in the adult. Vascular endothelial growth factor (VEGF) initiates angiogenesis, and VEGF or its receptor is targeted therapeutically to block pathological angiogenesis. Additional pro-angiogenic cues, such as CXCL12 acting via the CXCR4 receptor, co-operate with VEGF/VEGFR2 to cue vascular patterning. We studied the role of FGD5, an endothelial Rho GTP/GDP exchange factor (RhoGEF), to regulate CXCR4-dependent signals in the endothelial cell (EC**).** Patient-derived renal cell carcinomas produce a complex milieu of growth factors that stimulated sprouting angiogenesis and endothelial tip cell differentiation *ex vivo* that was blocked by EC FGD5 loss. In a simplified model, CXCL12 augmented sprouting and tip gene expression under conditions where VEGF was limiting. CXCL12-stimulated tip cell differentiation was dependent on PI3 kinase β (PI3Kβ) activity. Knockdown of EC FGD5 abolished CXCR4 signaling to PI3Kβ and Akt. Further, inhibition of Rac1, a Rho GTPase required for PI3Kβ activity, recapitulated the signaling defects of FGD5 deficiency, suggesting that FGD5 may regulate PI3Kβ activity through Rac1. Overexpression of a RhoGEF deficient, DBL domain-deleted FGD5 mutant reduced CXCL12-stimulated Akt phosphorylation, and failed to rescue PI3K signalling in native FGD5-deficient EC, indicating that FGD5 RhoGEF activity is required for FDG5 function. Endothelial expression of mutant PI3Kβ with an inactivated Rho binding domain confirmed that CXCL12-stimulated PI3K activity in EC requires Rac1-GTP co-regulation. Together, this data identifies the role of FGD5 to generate Rac1-GTP to regulate pro-angiogenic CXCR4-dependent PI3Kβ signaling in EC.

5.3 Introduction

Angiogenesis is critical for embryonic development and postnatal tissue repair such as the response to ischemic brain or heart injury (Hayashi et al. 2003, He et al. 2017). Moreover, neoplastic tumor growth recruits neovascularization, using similar hypoxia-induced growth cues, notably vascular endothelial growth factor (VEGF) (De Palma, Biziato and Petrova 2017). VEGF-driven tumor angiogenesis is targeted therapeutically in cancers such as renal cell carcinoma that respond poorly to conventional chemotherapy, and potentiates the effects of immunotherapeutic anti-cancer strategies (Motzer et al. 2020, Bergerot et al. 2019). However, other growth factors such as CXCL12 acting on the endothelial cell (EC) CXCR4 G-protein-coupled receptor (GPCR), complement VEGF, modulate developmental or postnatal neovascularization by directing vascular patterning and endothelial cell differentiation (Marin-Juez et al. 2019, Das et al. 2019, Kim et al. 2017, Takabatake et al. 2009, Tachibana et al. 1998, Hultgren et al. 2020), and may mediate resistance to anti-VEGF angiogenic treatment (Ma et al. 2018, Duda et al. 2011).

Phosphoinositide 3-kinases (PI3 kinase) are a family of lipid kinases that lead to the 3'-phosphorylation of phosphatidylinositol-4,5-bisphosphate to initiate membrane recruitment and activation of downstream protein kinase mediators, such as mTOR and Akt, that have fundamental roles in cell proliferation, adhesion, migration, and survival (Manning and Toker 2017). The PI3 kinase-α and -γ isoforms are coupled to receptor tyrosine kinase activation, and are modulated by Ras-GTP binding. The PI3 kinase-β and -δ isoforms are functionally coupled to GPCR activation. However, PI3-kinase-β

activity is uniquely modulated by Rho GTP binding proteins (Fritsch et al. 2013a). In vascular EC, PI3 kinase and Akt activation are critical for growth factor-stimulated blood vessel formation and angiogenic sprouting, demonstrated in both gain- and loss-of-function models *in vivo* (Lee et al. 2014, Graupera et al. 2008, Hamada et al. 2005).

FGD5 is a Rho GTP binding protein GTP/ GDP exchange factor (RhoGEF), expressed in vascular endothelial cells (Gazit et al. 2014, Ho et al. 2003). The protein contains a tandem DBL-PH RhoGEF domain, with a c-terminal FYVE and a second PH domain (Eitzen et al. 2019). Loss of FGD5 in mice is embryonic-lethal at the time of vascular patterning, and inactivation is linked to impaired microvascular network remodeling in development (Gazit et al. 2014, Cheng et al. 2012). FGD5 is involved in vascular endothelial growth factor receptor (VEGFR2) trafficking and function (Kurogane et al. 2012, Farhan et al. 2017a). However, it is unknown if FGD5 is required to mediate pro-angiogenic GPCR signals in EC.

Here we describe participation of endothelial FGD5 in cancer- and CXCL12-induced angiogenic sprouting. We link FGD5 activity to generate Rac1-GTP to regulate the PI3 kinase-β response to CXCL12/ CXCR4 GPCR stimulation.

5.4 Results

5.4.1 Knockdown of FGD5 decreases sprouting angiogenesis in endothelial cell (EC) co-cultured with patient derived renal cell carcinoma (PD-RCC) samples

Previous work identified a role for FGD5 in vascular remodeling during development *in vivo*, proangiogenic sprout development in *in vitro* models, and mechanistically linked these functional defects to altered VEGF/ VEGFR2 signal transduction in ECs (Kurogane et al. 2012, Nakhaei-Nejad et al. 2012, Gazit et al. 2014, Farhan et al. 2017a). However, it is unclear if FGD5 contributes to vascularization in the adult.

First, we sought to determine if FGD5-dependent effects contribute to angiogenic sprouting in response to growth factors released by human tumors, as a model for angiogenesis in the adult. Human patient-derived renal cell carcinoma (PD-RCC) tissue samples were obtained fresh from surgical resection, and used to stimulate angiogenic sprouting of HUVEC spheroids *ex vivo* in co-cultures. We identified 4 PD-RCC samples that elicited endothelial sprouting *ex vivo* among 8 consecutive PD-RCC tissue samples (Fig. 5.2A, B). In comparison to the non-angiogenic PD-RCC samples, the angiogenic PD-RCCs elicited sprouts with characteristic filopodia, and expression of tip endothelial genes (Fig. 5.2A-C).

We examined the effect of depletion of endothelial FGD5 on angiogenic sprouting in co-cultures with angiogenic PD-RCC. HUVEC were pre-treated with scrambled control or small interfering RNA (siRNA) against FGD5. We observed that FGD5 knockdown inhibited sprout number, filopodia extensions, and expression of tip cell genes (Fig.

5.2A-C). This indicates that endothelial FGD5 is required for optimal angiogenic sprouting in response to complex pro-angiogenic cues produced by human tumors.

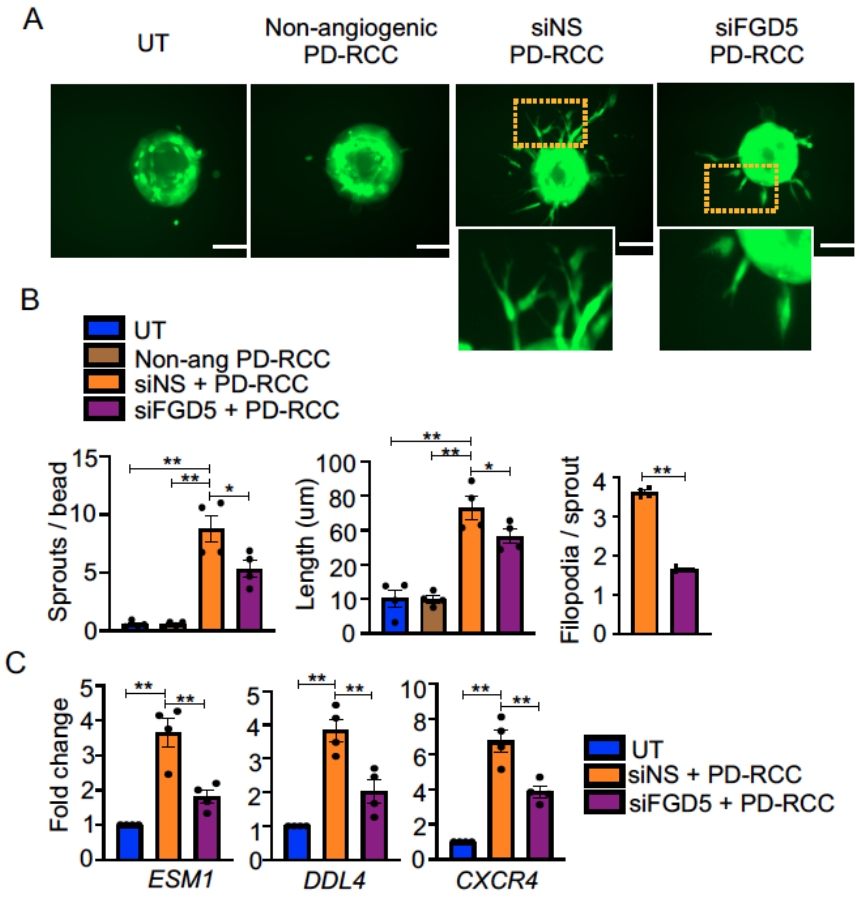

Fig. 5.2: FGD5 knockdown inhibits PD-RCC-stimulated angiogenesis. Human umbilical vein endothelial cells (HUVECs) were transfected with control non-specific siRNA (siNS) or siRNA against FGD5 (siFGD5), mounted on Cytodex beads, then embedded in 3D fibrin gels as described in Methods. Freshly harvested patient-derived renal cell carcinoma (PD-RCC) samples were minced and added to the cultures. **A,** Representative images of EC sprouts and filopodia (insets) after 18 h of co-incubation; scale bar 95μm. **B,** Quantification of the number and length of sprouts per bead (mean ± SEM, n = 4 independent experiments, P^* <0.05, P^{**} <0.01 by student *t* - test), and the number of tip cell filopodia (n = 4 independent experiments, P^{**} <0.01 by one-way

ANOVA). **C,** Expression of tip cell marker genes by qPCR (mean ± SEM; n = 8 independent experiments, *P** <0.05, *P*** <0.01 by one-way ANOVA)

5.4.2 CXCL12 stimulation increases the angiogenic effect of VEGF

Although VEGF is recognized as a dominant pro-angiogenic cue secreted by cancer and tumor stromal cells, additional pro-angiogenic molecules, such as the chemokines CXCL12 and CXCL7, also contribute and may participate in cancer that escapes from anti-angiogenesis inhibitor therapy directed against the VEGF/ VEGF receptor tyrosine kinase pathway (Azad et al. 2020a). *In vitro*, stromal cell-derived CXCL12 has been shown to require mTOR complex-2 to support angiogenic sprouting (Ziegler et al. 2016). We determined the effect of CXCL12 alone, and in combination with suboptimal VEGF, to stimulate angiogenic sprouting from HUVEC spheroids *in vitro*. We observed that treatment with CXCL12 alone stimulated occasional sprout formation, but did not induce tip gene expression (Fig. 5.3A-C). However, CXCL12 co-operated with suboptimal VEGF to induce sprout formation and tip gene expression more robustly than VEGF alone (Fig. 5.3A-C). Further, the CXCL12-stimulated expression of Dll4 elicited robust expression Notch receptor-dependent reporter genes, *HES1* and *HEY1*, in the EC (Fig. 5.3C). In agreement with previous observations, we found VEGF stimulation increases endothelial expression of CXCR4, the CXCL12 receptor, suggesting a positive-feedback loop to promote angiogenesis (Fig. 5.4). These data indicate that sprouting angiogenesis in the setting of limiting VEGF availability may be augmented by co-stimulation with ligands for endothelial pro-angiogenic GPCRs.

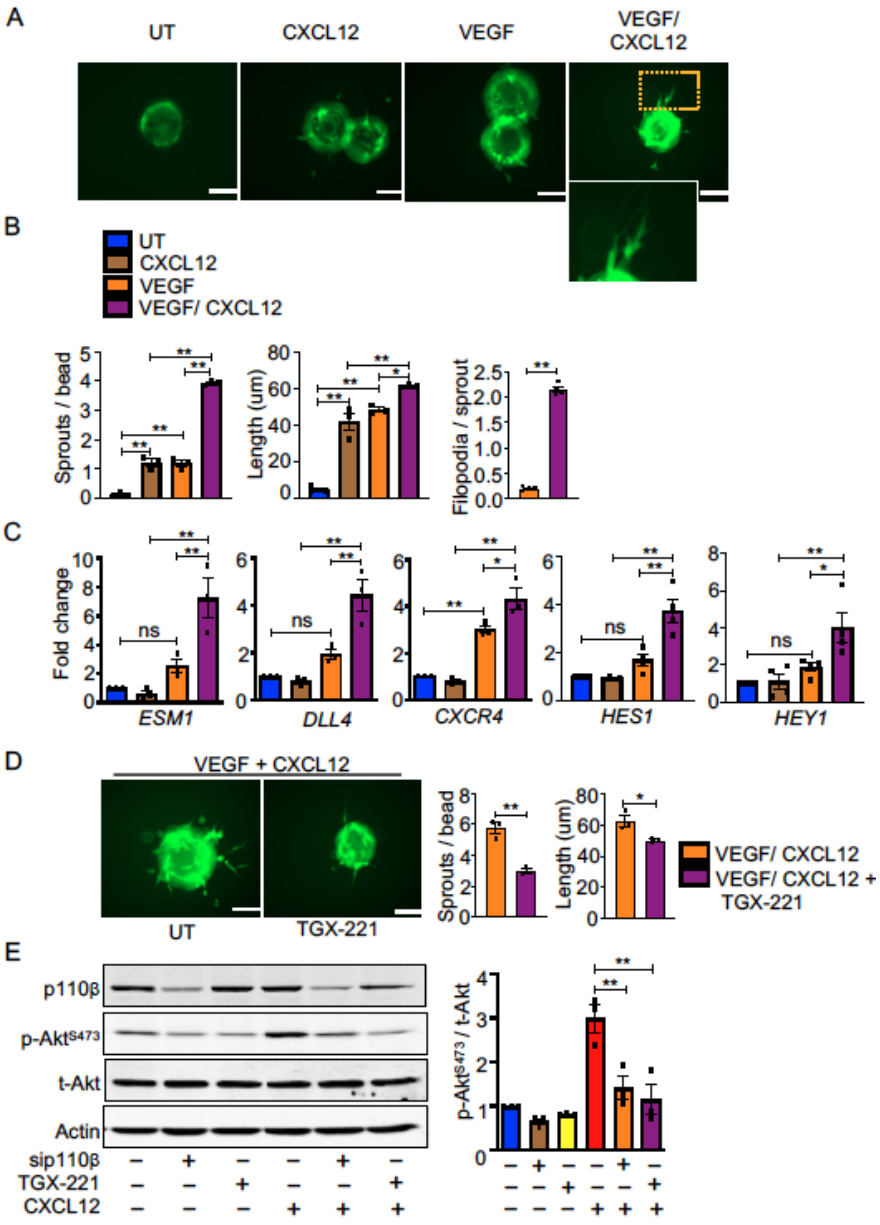

Fig. 5.3: CXCL12 stimulation increases the angiogenic effect of suboptimal VEGF. HUVECs were treated with 15ng/mL VEGF for 4h, mounted on Cytodex beads, embedded in 3D fibrin gels, and cultured with growth media supplemented with vehicle, 15 ng/ml VEGF, 100ng/ml CXCL12, or with both VEGF and CXCL12. **A,** Representative images of EC sprouts and tip cell filopodia (inset) after 18h of incubation; scale bar 95μm. **B,** Quantification of the number and length of the sprouts, and the tip cell filopodia (mean ± SEM; n = 3 independent experiments, *P** <0.05, *P***

<0.01 by one-way ANOVA). **C,** Expression of tip cell marker genes quantified by RT-qPCR (mean ± SEM; n = 3 independent experiments, *P** <0.05, *P*** <0.01 by one-way ANOVA). **D,** CXCL12-stimulated sprouting is dependent on PI3 kinase-β activity. Representative images of EC sprouting as in **A**. EC were pretreated with TGX-221 (100nM). Quantification of the number and length of the sprouts (right panels; mean ± SEM; n = 3 independent experiments, *P** <0.05, *P*** <0.01 by one-way ANOVA). **E,** ECs were transfected with control or p110β siRNA, or pre-treated with TGX-221 (100nM) to inactivate PI3 kinase-β, then stimulated with CXCL12 (50ng/mL) for 10mins. Lysates were immunoblotted as shown. Quantification of p-Akt (right panel; mean ± SEM; n=3 independent experiments, **P* <0.05 by one-way ANOVA).

5.4.3 CXCL12-stimulate PI3K activation is PI3Kβ dependent

Whereas the VEGF receptor-2 is known to recruit PI3 kinase-α to mediate pathway activation, as reported by Akt phosphorylation, GPCRs may use the PI3 kinase-α or-β isoforms. We find sprouting and tip cell differentiation in response to suboptimal VEGF with CXCL12 stimulation is sensitive to PI3 kinase-β inhibition (Fig. 5.3D). To further evaluate PI3 kinase-β use by CXCL12-stimulated EC, we pre-treated EC with siRNA against *PIK3CB*, or the PI3 kinase-β-selective small molecule inhibitor, TGX221, then stimulated the EC with CXCL12. We observed that either *PIK3CB* knockdown or PI3 kinase-β inhibition markedly inhibited CXCL12-stimulated Akt phosphorylation (Fig. 5.3E). PI3 kinase-β inhibition with AS252424 had little effect on CXCL12-stimulated Akt phosphorylation (Fig. 5.5).

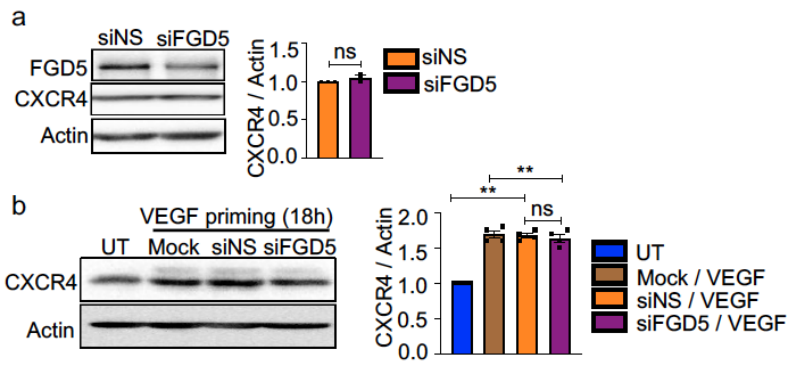

Fig. 5.4. Knockdown of FGD5 does not alter CXCR4 expression in EC. HUVECs were transfected with non-silencing RNA (siRNA) or siRNA targeting FGD5 (siFGD5) then incubated overnight without growth supplement to indicate the basal level of CXCR4 under resting conditions. **A,** Representative image of FGD5, CXCR4, and Actin Western blot in HUVECs, and the quantitation of CXCR4, n = 3 independent experiments. **B,** Representative image of CXCR4 after VEGF stimulation, and Actin Western blot in HUVECs, and the quantitation of CXCR4, n = 3 independent experiments, ***P* <0.01 by one-way ANOVA.

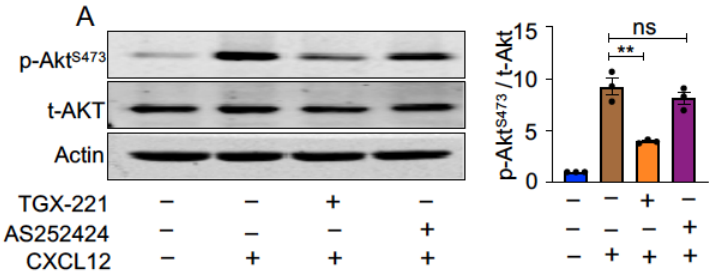

Fig. 5.5: CXCL12 stimulated p-Akt activation depends on p110β, but not on p110γ. HUVECs were pre-incubated with either with 100nM of p110β inhibitor TGX-221 or 100nM of p110γ inhibitor AS252424 for 1h then treated with 50ng/mL CXCL12 for 10min. **A,** Representative Western blotting to check p-Akt expression level. **B,** Quantification of p-Akt. Mean ± SEM; n = 3 independent experiments, ***P* <0.01 by one-way ANOVA.

5.4.4 FGD5 regulates CXCL12-stimulated CXCR4/PI3K signaling

Since FGD5 is known to participate in VEGF receptor trafficking and PI3 kinase-α signal transduction, we examined if FGD5 loss perturbed CXCL12 signal transduction. Endothelial FGD5 was depleted by siRNA, then EC were stimulated with CXCL12. Treatment of HUVEC cultures with FGD5 siRNA markedly depleted FGD5 detected by western blot (Fig. 5.6A). We observed a reduction in CXCL12-stimulated Akt phosphorylation in EC treated with FGD5 siRNA *vs* scrambled siRNA (Fig. 5.6A). We found that EC FGD5 knockdown did not alter abundance of CXCR4, the receptor for CXCL12 (Fig. 5.4). To confirm the specificity of this effect, we depleted native FGD5 with a 3'UTR-directed siRNA, then expressed a wild-type siRNA-insensitive FGD5 transgene. Compared to the empty-vector transfection, FGD5-transfection rescued CXCL12-stimulated Akt phosphorylation in cells deficient in native FGD5 (Fig. 5.6B). This data indicates that FGD5 is required for pro-angiogenic PI3 kinase-β-dependent signals elicited by the CXCL12/ CXCR4 GPCR.

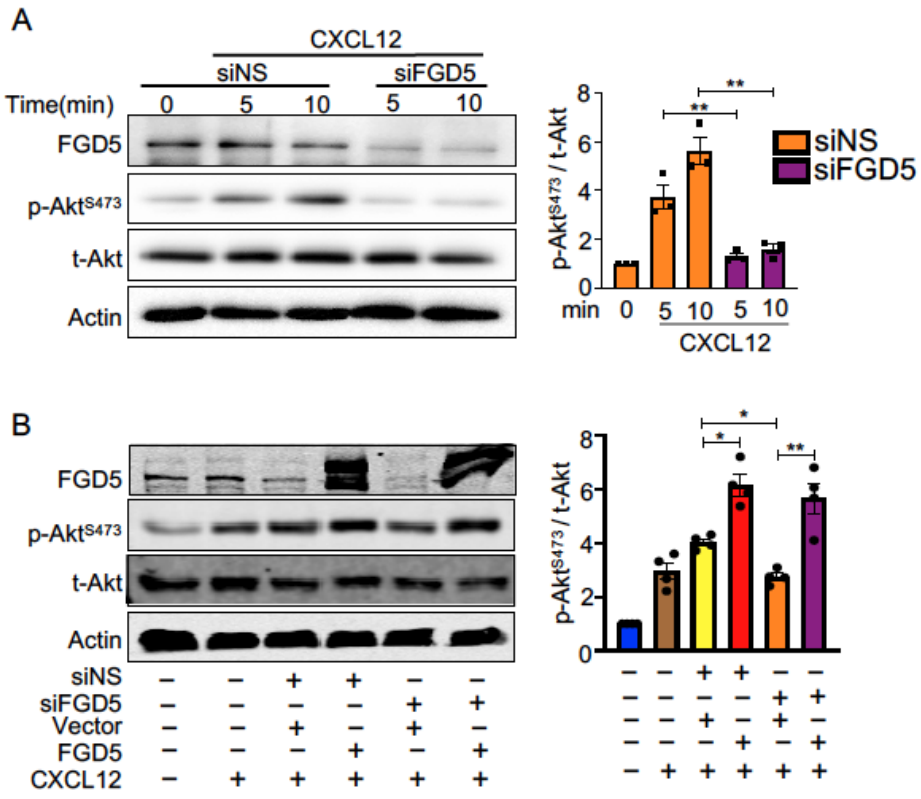

Fig. 5.6: Knockdown of FGD5 blocks CXCL12-stimulated PI3 kinase signaling. A, HUVECs were transfected with control (siNS) or siRNA against FGD5 (siFGD5). ECs were stimulated with CXCL12 (50ng/mL) for 10mins. Lysates were immunoblotted for p-Akt to evaluate PI3K activity. Quantification of p-Akt (right panel; mean ± SEM; n = 3 independent experiments, ***P* <0.01 one-way ANOVA). **B,** FGD5 knockdown-induced defective signalling was rescued by FGD5 transgene overexpression. 3'UTR FGD5 siRNA was used to knockdown native FGD5 as in 3A. Vector or FGD5 transgene was overexpressed in siNS- or siFGD5- treated cells. EC were then stimulated with CXCL12 (50ng/mL) for 10mins. Representative immunoblot of p-Akt. Quantification of p-Akt (right panel; mean ± SEM; n = 4 independent experiments, **P* <0.05, ***P* <0.01 by one-way ANOVA.

5.4.5 FGD5 acts as a functional Rho-GEF for RAC1 to control PI3Kβ activity

To determine the mechanism by which FGD5 facilitates endothelial PI3 kinase-β activity, we studied the effect of monomeric Rho-GTPase inhibitors. FGD5 RhoGEF activity has been attributed to Cdc42, but recent work using the truncated RhoGEF DBL-PH domains from FGD5, identified that FGD5 acts as an exchange factor for Rac1 in preference to Cdc42 *in vitro* (Park et al. 2019). First, EC were pre-treated with the Rac1 inhibitor, EHT-1864 (10μM) or carrier, then stimulated with CXCL12. We found the Rac1 inhibitor markedly blunted CXCL12-stimulated Akt phosphorylation *vs* the carrier control treatment (Fig. 5.7A). In contrast, the Cdc42 inhibitor, ML-141 (5μM), had little effect (Fig. 5.7B). These data indicate that the function of FGD5 RhoGEF activity in PI3 kinase pathway signaling involves Rac1-GTP.

To confirm that the RhoGEF activity of FGD5 is required for CXCL12-stimulated PI3 kinase-β signaling in EC, we tested the effect of expression of a mutant FGD5, lacking the RhoGEF DBL domain. Using siRNA, native FGD5 was knocked down in HUVEC, and an FGD5 wild-type or DBL domain-deletant transgene was expressed (Fig. 5.8). The EC were stimulated with CXCL12. We found the DBL domain-deletant acted as a dominant-negative inhibitor of native FGD5 in ECs treated with non-silencing siRNA (Fig. 5.7C). Further, the DBL domain-deletant failed to rescue defective CXCL12-stimulated Akt phosphorylation on the background of native FGD5 knockdown (Fig. 5.7C). The $FGD5^{\Delta DBL}$ deletant also impaired CXCL12-stimulated repair of an endothelial monolayer (Fig. 5.7D). Together, these data indicate FGD5 RhoGEF activity to generate

Rac1-GTP is required for CXCL12-stimulated activation of endothelial PI3 kinase-β signaling and cytoskeletal remodelling.

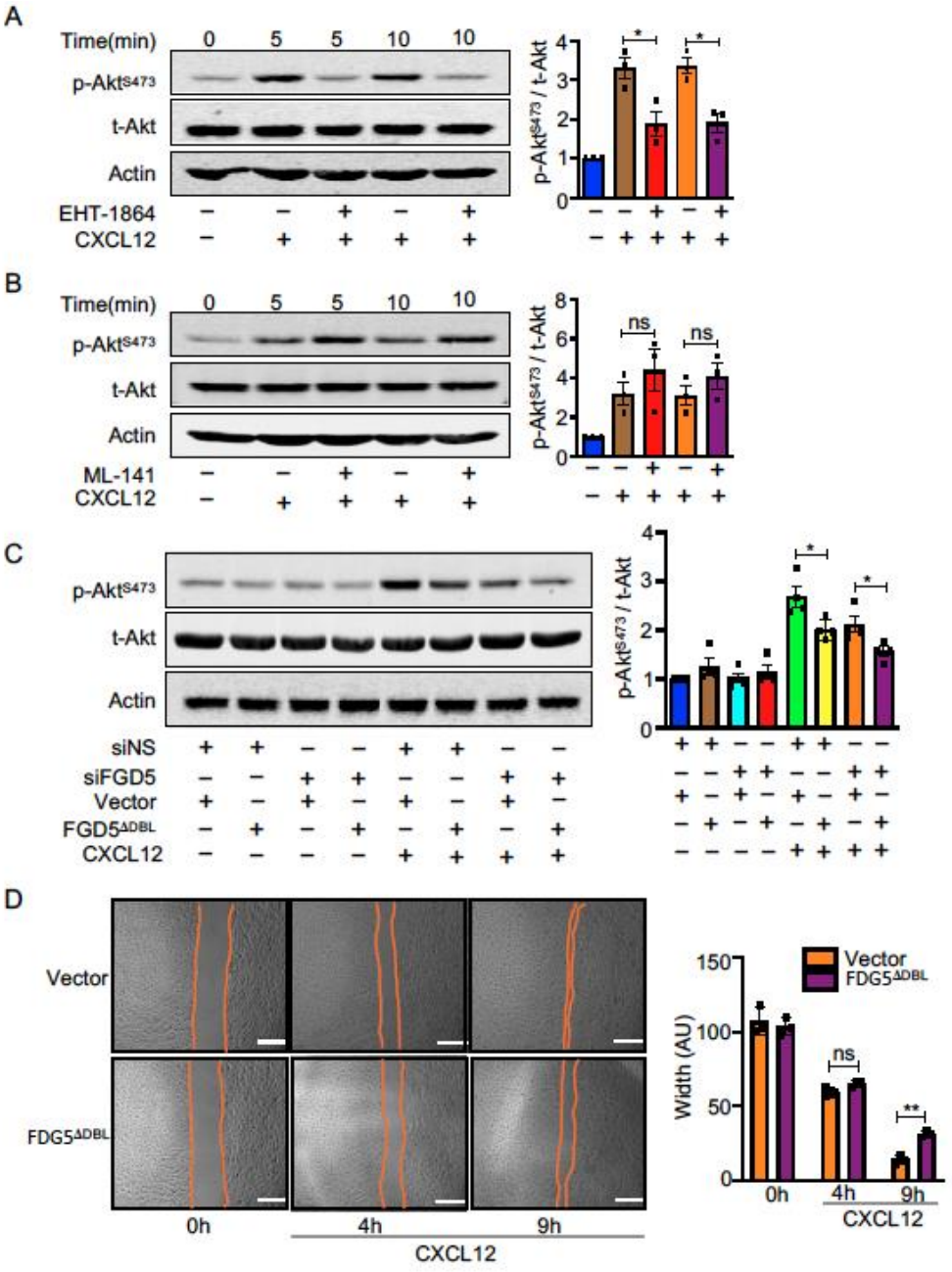

Fig. 5.7: CXCL12-stimulated activation of PI3 kinase-β requires Rac1 and FGD5 RhoGEF activity. HUVECs were pre-incubated with the Rac1 inhibitor, EHT-1864 (10 uM) **(A)**, or CDC42 inhibitor, ML-141 (5uM) **(B)** for 1h, then stimulated with CXCL12 (50

ng/mL) for the time as indicated. Lysates were immune-blotted for p-Akt to evaluate PI3K activity. Quantification of p-Akt for Rac1 inhibitor (**A**, right panel) and CDC42 inhibitor (**B**, right panel); mean ± SEM; n = 3 independent experiments, **P* <0.05 by one-way ANOVA. **C,** ECs were transfected with control or FGD5 siRNA, then vector or FGD5$^{\Delta DBL}$ transgene was overexpressed in siNS or siFGD5 cells. EC were stimulated with CXCL12 (50ng/mL) for 10min and lysates were immunoblotted. Quantification of p-Akt (right panel; mean ± SEM; n = 4 independent experiments, **P* <0.05, ***P* <0.01 by one-way ANOVA). **D,** Representative images of EC monolayer wound healing; scale bar 200μm. ECs were transfected with vector or FGD5$^{\Delta DBL}$ plasmids. A scratch was made across a confluent EC monolayer, then EC were treated with CXCL12 (100ng/mL). Quantification of the wound at the indicated timepoints was done using ImageJ. Wound healing was impaired in FGD5$^{\Delta DBL}$ EC (right panel; mean ± SEM; n = 3 independent experiments, **P* <0.05 by two-way ANOVA)

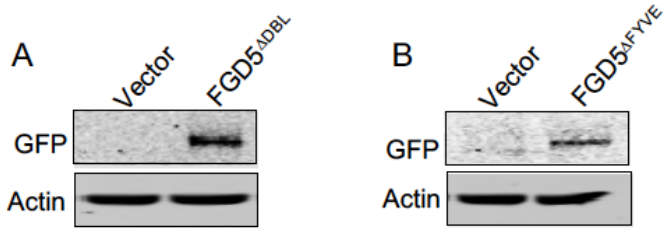

Fig. 5.8: Overexpression of GFP-FGD5$^{\Delta DBL}$ and GFP-FGD5$^{\Delta FYVE}$ in HUVECs. HUVECs were transiently overexpressed with empty vector or GFP-FGD5$^{\Delta DBL}$ **(A)** or GFP-FGD5$^{\Delta FYVE}$ **(B)**. The overexpression was confirmed by Western blotting by using anti-GFP antibody.

The PI3 kinase-β isoform, unlike PI3 kinase-α, binds and is modulated by Rac1 and Cdc42-GTP in fibroblasts (Fritsch et al. 2013b). To directly determine if FGD5-generated Rac1-GTP regulates PI3 kinase-β activity downstream of CXCL12 stimulation in EC, we tested the effect of mutant PI3 kinase-β, carrying a Rho binding domain (RBD) point mutation to abolish the Rac1-GTP interaction. EC were treated with siRNA to deplete native *PIK3CB*, and transfected to express wild-type or RBD-mutant *PIK3CB*. The EC were stimulated with CXCL12. We found that RBD-mutant PI3 kinase-β did not inhibit native PI3 kinase-β in CXCL12-stimulated cells pre-treated with control siRNA, but also did not augment CXCL12-stimulated Akt phosphorylation as the wild type transgene did in these cells (Fig. 5.9A). Further, expression of wild-type, but not RBD-mutant, PI3 kinase-β rescued Akt phosphorylation after native FGD5 knockdown (Fig. 5.9A). These data indicate full CXCL12-stimulated PI3 kinase-β activity is dependent on FGD5-generated Rac1-GTP.

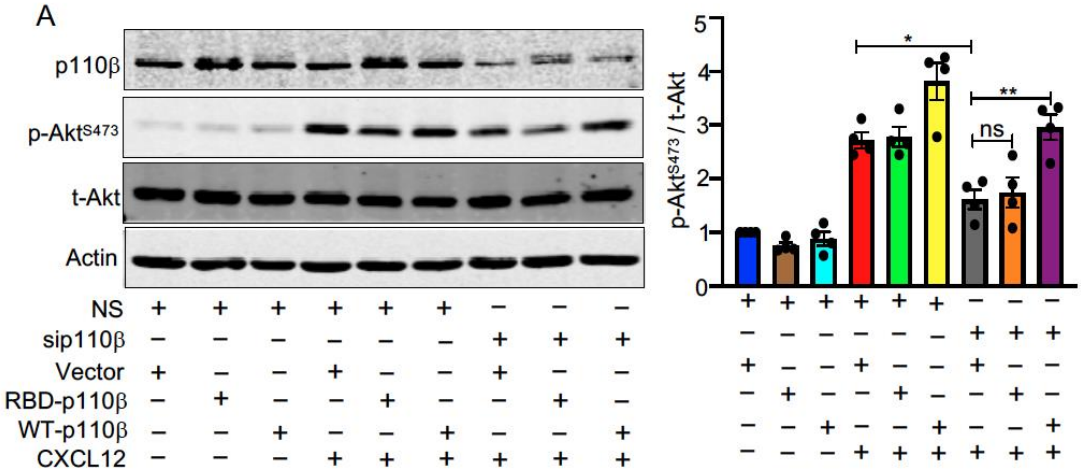

Fig. 5.9: The PI3 kinase-β RBD regulates CXCL12-stimulated activity. HUVECs were at first transfected with control or 3'UTR p110β siRNA. Vector or Ras binding

domain mutated p110β (RBD-p110β) or wild type p110β (WT-p110β) transgenes were overexpressed in siNS or sip110β cells. ECs were stimulated with CXCL12 (50ng/mL) for 10min, and lysates were immunoblotted for p-Akt as in 2E. Quantification of p- Akt (right panel; mean ± SEM; n = 4 independent experiments, *P <0.05, **P <0.01 by one-way ANOVA).

5.4.6 FYVE domain deleted FGD5 functions as a dominant negative mutant

Finally, we sought to determine the role of the FYVE domain of FGD5 in CXCL12-stimulated PI3 kinase-β activation. FYVE domains often target proteins to endosomes, a site where VEGF-stimulated PI3 kinase-α activity sustains downstream signals. We tested the effect of expression of a deletion-mutant of FGD5, lacking the FYVE domain. EC were treated with siRNA to deplete native *FGD5*, and transfected to express empty vector or the FGD5$^{\Delta FYVE}$ transgene. When ECs were stimulated with CXCL12, we found FGD5$^{\Delta FYVE}$ acted as a dominant-negative inhibitor of native FDG5 in ECs treated with scrambled siRNA, and failed to rescue Akt phosphorylation in ECs deficient in native FGD5 (Fig. 5.10A).

We tested the effect of FGD5$^{\Delta FYVE}$ expression on CXCL12-stimulated scratch wound repair of an endothelial monolayer. HUVEC were transfected to express the FGD5$^{\Delta FYVE}$ transgene, then re-plated at confluence. The monolayer was scratched, and evaluated for wound closure as shown in Fig. 5.10B. Expression of the dominant-negative FGD5$^{\Delta FYVE}$ impaired CXCL12-stimulated monolayer repair (Fig. 5.10B).

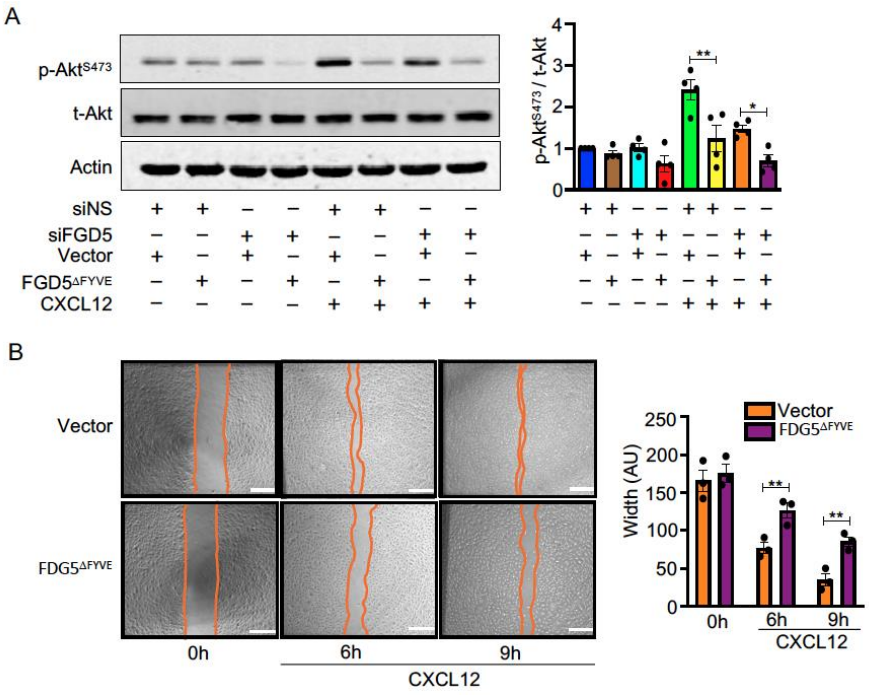

Fig. 5.10: FYVE domain-deleted FGD5 functions as a dominant negative mutant. **A,** HUVECs were transfected with control or FGD5 siRNA. Vector or a FGD5$^{\Delta FYVE}$ transgene was overexpressed in the siNS or siFGD5 cells. EC were stimulated with CXCL12 (50ng/mL) for 10min then immunoblotted. Quantification of p-Akt (right panel; mean ± SEM; n=4 independent experiments, *P <0.05, **P <0.01 by one-way ANOVA). **B,** Representative images of EC monolayer wound healing; scale bar 200μm. ECs were transfected to express empty vector or FGD5$^{\Delta FYVE}$ transgene. A scratch was made across a confluent EC monolayer, then EC were treated with CXCL12 (100ng/mL). Quantification of the wound at the indicated timepoints was done using ImageJ. Wound healing was impaired in FGD5$^{\Delta FYVE}$ EC (right panel; mean ± SEM; n = 3 independent experiments, *P <0.01 by two-way ANOVA)

Finally, to determine if the FYVE domain directed FDG5 to endosomes, we studied native and FGD5$^{\Delta FYVE}$ subcellular distribution in EC. HUVEC were transfected to express GFP-tagged wildtype or FGD5$^{\Delta FYVE}$ transgenes, then stimulated with CXCL12 for 10 minutes and immunostained for EEA1, a marker of the early endosomal compartments. Confocal images of ECs were obtained (Fig. 5.11) and we analyzed the recruitment of GFP-FGD5 by measurement of the ratio of FGD5 on the EEA1^{+} endosomes to the level in the cytosol. We found native and FGD5$^{\Delta FYVE}$ enriched at the plasma membrane. Native FGD5 also co-localized with EEA1^{+} vesicles, but deletion of the FGD5 FYVE domain did not abolish endosome localization (Fig 6.10). These data indicate that the FYVE domain of FGD5 is required for CXCL12-stimulated PI3 kinase-β Akt phosphorylation and endothelial motility. However, the FYVE domain does not appear to be essential for either plasma membrane or endosomal localization of FGD5.

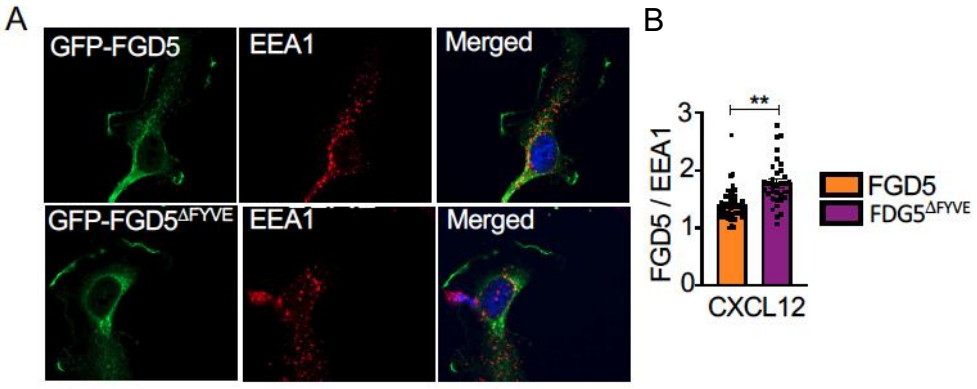

Fig 5.11. FGD5$^{\Delta FYVE}$ localizes FGD5 to early endosomes. HUVECs were transfected with GFP-FGD5 or GFP-FGD5$^{\Delta FYVE}$, then stimulated with CXCL12 (50ng/mL) for 10min. **A**, Representative Immunofluorescence images showing GFP colocalization with the early endosome marker, EEA1 (red); scale bar 20μm. **B,** Quantification of the median intensity of GFP in the EEA1+ endosomes vs the cytosol of the transfected cell was

performed using Matlab. At least 30 cells were taken for analysis pooled from 3 independent experiments (mean ± SEM; n=4 independent experiments, $^{**}P < 0.01$ by student *t* - test).

5.5 Discussion

The molecular mechanisms in the EC that underlie the integration of signals for neovascularization or vascular repair in the adult remain to be fully defined. We examined the function of the endothelial-restricted Rho GTP/GDP exchange factor, FGD5, in EC stimulated with freshly-isolated patient-derived renal cancer cells as a pro-angiogenic stimulus. Pro-angiogenic PD-RCC cells generate a complex milieu of pro-angiogenic growth factors, including VEGF and CXCL12 (Azad et al. 2020a). We found that FGD5 loss impaired tumor-stimulated angiogenic sprouting and expression of pro-angiogenic tip cell genes in EC spheroid culture *ex vivo*.

We have previously characterized FGD5 regulation of the VEGF receptor tyrosine kinase -2 (Farhan et al. 2017a). While pro-angiogenic signaling from VEGFR-2 is well established, the role and regulation of downstream signaling from the complementary ligand CXCL12 and its G-protein-coupled receptor, CXCR4, is less understood. We show that CXCL12 complements suboptimal VEGF concentrations to promote both sprout number and expression of angiogenic tip EC genes *in vitro*. This is consistent with the observation that CXCL12 knockout is associated with reduced eye angiogenesis and simplified vascular networks in the heart, gut, and kidney during

development (Tachibana et al. 1998, Takabatake et al. 2009, Cavallero et al. 2015, Ivins et al. 2015). CXCL12 contributes to motility, and EC tip cell differentiation.

Previous work identified recruitment of an endothelial PI3 kinase-β-dependent mechanism to elicit angiogenesis escaping from VEGFR-2 inhibition (Azad et al. 2020b). To examine this finding further, we determine that CXCR4 activation of PI3 kinase activity to phosphorylate the downstream effector, Akt, used the p110β catalytic isoform. A minor redundant component of CXCL12-stimulated PI3 kinase activity was attributed to the PI3 kinase-δ isoform, whereas PI3 kinase-α inhibition had no effect on CXCL12-stimulated pathway activation. CXCL12/ PI3 kinase-β appears to function in parallel to VEGF receptor tyrosine kinase/ PI3 kinase-α, but alone is insufficient to elicit tip cell differentiation in spheroids cultured *in vitro*.

Endothelial PI3 kinase-β is not required for developmental angiogenesis (Graupera et al. 2008), but may be recruited when receptor tyrosine kinase-stimulated PI3 kinase-α is limiting or lost (Schwartz et al. 2015). The effect of inactivation of tumor stromal PI3 kinase-α has been reported as an increased density/ hyper-sprouting phenotype (Soler et al. 2013). Genetic inactivation of the counter-regulatory phosphatase, PTEN, or the downstream PI3 kinase-inhibited effector, FoxO1, in mouse EC induces a hyper-sprouting phenotype (Wilhelm et al. 2016, Hamada et al. 2005). These data suggest that phosphorylation and inactivation of FoxO1 by an alternate PI3 kinase in the setting of limited PI3 kinase-α or an upstream receptor, e.g. VEGFR-2, activity may function redundantly to support EC sprouting and tip differentiation.

PI3 kinase-α activity is co-regulated by Ras monomeric GTP binding protein association, and loss of binding in p110α mutated to inactivate Ras binding decreases vascular density (Murillo et al. 2014). The beta PI3 kinase isoform also demonstrates complex regulation by upstream input (Bresnick and Backer 2019). Among these, inputs from GPCRs are mediated via association with β/γ heterotrimeric G-proteins and Rho GTPase proteins (Fritsch et al. 2013b, Houslay et al. 2016). In leukocytes, which share FGD5 expression with EC, β/γ co-regulation dominates (Houslay et al. 2016), whereas in fibroblasts, the RhoGEF DOCK180 has been identified to regulate PI3 kinase-β (Fritsch et al. 2013b). Our data identify novel upstream regulation of PI3 kinase-β by FGD5 in EC, acting downstream of the pro-angiogenic GPCR CXCR4. It is unclear if FGD5 is similarly coupled downstream of other EC GPCRs, such as S1PR1, that initiate qualitatively different vessel-stabilizing cues. VEGFR-2 stimulation is reported to increase PI3 kinase-β association with the receptor via the p85 regulatory subunit, but functionally, VEGFR-2 appears to elicit PIP3,4,5-dependent Akt1 activity almost exclusively via PI3 kinase-α (Graupera et al. 2008, Lee et al. 2014).

The activity of FGD5 on Rho GTPase protein exchange has been controversial. Initial work identified FGD5-dependent Cdc42-GTP increased in VEGF-stimulated EC(Kurogane et al. 2012). More recent study shows FGD5 preferentially elicits GTP exchange on Rac1 *in vitro* (Park et al. 2019). Our data indicate that FGD5 RhoGEF activity is required to elicit CXCL12-stimulated PI3 kinase-β activity in EC, since a FGD5 DBL domain-deletant both acted as a dominant-negative, and failed to rescue native

endothelial FGD5 loss. Further, small molecule inhibition of Rac1, but not Cdc42, blocked CXCL12-stimulated Akt phosphorylation. Moreover, the functional coupling of FGD5 to PI3 kinase-β was supported by the finding that that mutated-RBD PI3 kinase-β, defective in Rac-GTP binding, phenocopied the FGD5 DBL domain-deletant. Finally, rescue of native PI3 kinase-β loss with the PI3 kinase RBD mutant fails to fully rescue CXCL12-stimulated monolayer wound healing. Thus, taken together we identify a novel regulatory pathway for angiogenesis involving FGD5 generation of Rac1-GTP to promote pro-angiogenic PI3 kinase-β-mediated CXCR4 cues.

The FYVE domain of FGD5 may target the PI3 kinase-β / FGD5 complex to move from the plasma membrane to the endosome to sustain a signal (Eitzen et al. 2019). We find the FGD5 FYVE-domain deletant, like the DBL domain-deletant, acts as a dominant negative and does not rescue CXCL12-stimulated Akt phosphorylation after the loss of native FGD5 in the EC. Loss of the FYVE domain also delays CXCL12-stimulated monolayer wound repair. However, we did not identify FYVE domain-dependent movement of FGD5 to the endosome after CXCL12 stimulation. The FYVE domain may be required to maintain the conformation of FGD5 to enable full function. The observations suggest endosomal membrane activation of PI3 kinase-β activity is not required, and is consistent with observations that Akt1 phosphorylation mainly occurs at the plasma membrane(Liu et al. 2018).

In summary we show that FGD5 Rho GEF activity on Rac1 regulates CXCL12/ CXCR4 -stimulated PI3 kinase-β activity in EC. FGD5 is required for optimal CXCL12-stimulated EC movement, angiogenic sprout formation, and tip cell differentiation.

5.6 Materials and methods

The details of materials and methods are available in **Chapter 2.**

5.6.1 Reagents

M199 media and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific. Endothelial cell growth supplement (ECGS) was purchased from BD Biosciences. VEGF-A was obtained from Peprotech, CXCL12 from R&D, Cdc42 and Rac1 inhibitors (EHT-1864, ML-141 respectively) from Tocris, and TGX-221 from Cayman Chemical. Hiperfect, non-silencing short interfering RNA (siRNA), FGD5 silencing siRNA and CXCR4 silencing siRNA were purchased from Qiagen Inc. Anti-phospho-Akt^{S473}, anti-Akt, anti-p110β and anti-Actin were from Cell Signaling Technology. Anti-FGD5 was obtained from Protein Tech. Cytofect EC transfection kit was purchased from Cell Applications.

5.6.2 Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated as described previously (Chakrabarti and Davidge 2012). HUVECs were grown at 37^0C with 5% CO_2 in M119

medium supplemented with 20% FBS, 1% ECGS, 100 U/ml penicillin and 100 µg/ml streptomycin. Where indicated, EC were serum-starved for 5 hours before CXCL12 stimulation. All cells used in the experiments were used at passage 3-5, and taken from both male and female infants.

5.6.3 Angiogenesis

A 3D angiogenesis assay *in vitro* was done as previously described (Nakatsu and Hughes 2008). Briefly, Ecs were labeled with CellTracker Green (Life Technologies). Cytodex beads were coated with Ecs (~400 cells/bead) and cultured for 4 hours in (M199, 8% FBS). The beads were washed and suspended in fibrin (2 mg/mL). Then angiogenesis growth media (M199, 10% FBS, 15 ng/ml VEGF and/or 100 ng/ml CXCL12) was added on top. For PD-RCC stimulated angiogenesis, 1 ml of 10% FBS medium or minced 150 mg fragments of a PD-RCC sample in 1mL medium was added on top of the gel. At least 30 beads per treatment from each experiment were imaged after 18-20 hours incubation in 5% CO_2 at 37^0C, using a 20X objective lens and a CCD camera-equipped inverted fluorescence microscope (Leica, Concord, ON). Scoring was done using OpenLab (PerkinElmer; Waltham, MA).

5.6.4 RNA interference and plasmid overexpression

N-terminal GFP-labelled FGD5 (Genecopoeia) cDNA was used as a template to PCR-amplify fragments of FGD5 without the DBL/PH or FYVE domains. The fragments were annealed and used as templates to PCR-amplify the deletant constructs. The deletants

were subcloned into the LZRSpBMN vector. The constructs were confirmed by sequencing.

ECs at 75% confluence were transfected with either 50nM non-silencing (siNS) or specific siRNA using Hiperfect transfection reagent according to the manufacturer's instructions. The next day, EC were transiently transfected with empty-vector or GFP-FGD5 or GFP-FGD5$^{\Delta DBL}$ or GFP-FGD5$^{\Delta FYVE}$ or RBD-mutated p110β (a gift from Dr. Thomas Roberts, Dana Farber Cancer Institute) plasmids using the *Cytofect™ EC* transfection kit. The following day, EC were serum-starved and stimulated with 50ng/mL CXCL12 for 10 minutes. Protein expression was evaluated by western blot.

5.6.5 Wound healing assay

EC cells were transiently transfected with empty vector or FGD5$^{\Delta FYVE}$. The following day, a scratch was made across a confluent EC monolayer using a sterile pipette tip. CXCL12-driven cellular migration to cover the scratch area was monitored at the indicated time points. Images were taken by 5X objective lens and a CCD camera-equipped inverted microscope (Leica, Concord, ON). Data represent the distance of cellular migration (the difference in size between the time zero scratch area and the scratch area at each time point).

5.6.6 Immunofluorescence Microscopy

Cells were fixed with 3% formaldehyde for 10 minutes. Cells were blocked and permeabilized with 3%BSA and 0.1% Triton-X100 for 30 minutes and then incubated with anti-EEA1 (BD Bioscience) overnight at 4^0C, processed for immunofluorescence,

and visualized using a spinning disk confocal microscope (WaveFX system, Quorum Technologies, Puslinch, ON, Canada) with a 63X 1.42 NA objective and similar parameters across all the conditions. Enrichment of GFP-FGD5 on the $EEA1^+$ endosomes was determined using custom Matlab scripts (The Mathworks, Natick, MA). Images were background-subtracted and both the $EEA1^+$ endosomes and the outline of the transfected cells were segmented using the Otsu thresholding method. FGD5 enrichment on the endosomes was defined as the ratio of its mean intensity in the $EEA1^+$ mask divided by the mean intensity in the cell mask outside the endosomes. Data represent 30 cells in each group, pooled from three independent experiments.

5.6.7 Western blot

HUVEC monolayers were washed once with ice cold PBS and then lysed immediately on ice by RIPA buffer (Thermo Scientific) supplemented with protease inhibitor cocktail, phosphatase inhibitor cocktail 2 and 3 (Sigma). The protein concentration in each extract was measured by DC protein assay (BioRad). The extracts, containing equal amounts of protein, were fractionated by SDS-PAGE, then the separated proteins were electro-transferred onto nitrocellulose membranes (BioRad). Each membrane was incubated with a specific primary antibody at 4^0C in blocking buffer (5% BSA/TBS-Tween-20). Protein bands were detected using HRP-conjugated secondary antibodies and visualized by Odyssey (LiCor Biosciences).

5.6.8 RNA extraction and qPCR

Total RNA from 3D angiogenesis assay was extracted using Trizol and cDNA was synthesized by using the QuantiTect Reverse Transcription Kit (Qiagen) following the manufacturer's instructions. RT-qPCR was carried out as described (Azad et al. 2020a).The qPCR primers used are listed here:

hESM1: F: 5' GGTGGACTGCCCTACACT 3', R: 5' GTCGTCGAGCACTGTCCTCTT 3'

hCXCR4: F: 5'GTCATGGGTTACCAGAAGAAACTGA 3', R: 5' AGAGGAGGTCGGCCACTGA 3'

hDLL4> F: 5' TGGGTCAGAACTGGTTATTGGA 3', R: 5' CTGCAGATGACCCGGTAAGAG 3'

5.6.9 Statistical analyses

Data were shown as mean ± SEM. Test for normality was done using Shapiro-Wilk test. Statistical analysis was performed using Prism 8 (Graphpad, San Diego, CA). Student t - test was used to measure the differences between two groups. One-way ANOVA was used to evaluate differences in more than two groups. Two-way ANOVA was used to compare differences in more than two groups with two independent variables. *P* value <0.05 were considered significant. Each independent experiment was done at least three times.

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Chapter 6

Discussion and future directions

One of the promising strategies for cancer treatment is to target angiogenesis, the process of new blood vessel formation, to treat many cancer types. Since VEGF is the main driver of angiogenesis, consequently, most of the anti-angiogenic drugs are focused on blocking the VEGF-signaling pathway. Monoclonal antibody bevacizumab targets VEGF-A alone, while aflibercept, a fusion protein, can neutralize VEGF-A, B and PLGF-mediated angiogenesis (Zirlik and Duyster 2018). On the other hand, many of the RTKs, such as sunitinib and sorafenib, can target multiple pro-angiogenic signals including VEGF, FGF and PDGF pathways (Qin et al. 2019). Therefore, it is expected that the use of RTKs would increase the therapeutic benefits by effectively blocking these multiple angiogenic pathways (Qin et al. 2019). And yet, an initial response to AI therapy is not durable, and often times tumors become resistance to anti-angiogenic drug therapy due to the participation of multiple escape mechanisms. Accumulating evidence from clinical and pre-clinical studies show that several alternative pro-angiogenic growth factors such as FGF, apelin and CXCL12/SDF-1 are released after AI therapy and contribute to escape from VEGF-blockade (Batchelor et al. 2007, Saylor, Escudier and Michaelson 2012, Zuurbier et al. 2017). Therefore, targeting alternative pro-angiogenic pathway(s) is critical for the development of more effective anti-angiogenic therapy.

In this study, we tested the hypothesis that GPCR/PI3K signaling plays a critical role as an alternative pathway for tumor angiogenesis. We first showed the importance of

p110β isoform of PI3K as a downstream target of GPCR signaling in tumor angiogenesis. Second, we investigated the role of apelin as GPCR ligand in tumor angiogenesis. Finally, we showed that EC-specific FGD5 as a potential target for anti-angiogenic therapy as it regulates PI3K/Akt activity in EC coming through both VEGF/RTK and GPCR/PI3K pathways. Targeting multiple pathways of angiogenesis is particularly important in clinical setting, as recent clinical trials show that combining AI drugs (axitinib, cabozantinib) with checkpoint inhibitors such as avelumab and nivolumab lead to better clinical outcome than either of the treatment alone (Motzer et al. 2020, Bergerot et al. 2019, Motzer et al. 2019).

6.1 EC-specific p110β regulates tumor angiogenesis in parallel with VEGF signaling

RCC is a highly angiogenic cancer and represents one of the top 10 prevalent cancer types worldwide with over quarter of the patients developing metastatic spread by the time they are diagnosed (Siegel, Miller and Jemal 2020, Posadas, Limvorasak and Figlin 2017). The small molecule inhibitors such as sunitinib and axitinib are used as standard of care for mRCC with improved clinical outcome (Motzer et al. 2017, Rini et al. 2011). The anti-angiogenic effect of RTKs has been reported in multiple pre-clinical studies and showed this anti-angiogenic effect is mediated mainly by blocking the VEGF and PDGF receptors signaling pathways in endothelium with the concomitant reduction in sprouting angiogenesis and tumor microvascular densities (Hao and Sadek 2016, Mendel et al. 2003, Baffert et al. 2006, Inai et al. 2004). However, cancer becomes resistance to RTKs and other AI drugs over time and progress with re-emergence of

vascularization. Clinical evidences show that several VEGF-independent pro-angiogenic factors including CXCL12, HGF, FGF and apelin are upregulated in patients treated with AI therapy, suggesting the development of therapy-induced acquired resistance via the involvement of alternative angiogenic signaling pathways (Kopetz et al. 2010, Porta et al. 2013, Zuurbier et al. 2017). Here we tested the ability of PD-RCC samples to elicit angiogenesis in an *ex vivo* HUVEC spheroid assay and found that a subset of these PD-RCC samples were able to induce angiogenesis. We found that the expression of VHL was lost in PD-RCC angiogenic group compare to the non-angiogenic PD-RCC group. This is in agreement with the finding that RCC is mainly driven by the mutations or loss of function in VHL tumor suppressor gene that, in normal condition, regulates hypoxic pathway by controlling the activation of HIF transcription factors (Kim and Zschiedrich 2018, Baldewijns et al. 2010). HIF in turns, in one hand, induces the expression of diverse angiogenic factors including VEGF, FGF and PDGF that works through RTK/PI3K (Krock, Skuli and Simon 2011). On the other hand, the HIF-mediated expression of CXCL12 and apelin can work through GPCR/PI3K signaling to stimulate angiogenesis (Krock et al. 2011, Baldewijns et al. 2010, Kryczek et al. 2005).

Our *ex vivo* HUVEC spheroid assay with PD-RCC provides a unique model to evaluate the angiogenic growth factors expressed in PD-RCC samples, thus providing an opportunity to identify more aggressive angiogenic tumors from tumors with less angiogenic potential. Indeed, our analysis of growth factors expression revealed higher levels of VEGF, CXCL12 and apelin in PD-RCC angiogenic samples in comparison to the PD-RCC samples that failed to induce angiogenesis. Among these, CXCL12 and

apelin act via the cognate GPCR receptors CXCR4 and APJ, respectively (Kuba et al. 2019, Takabatake et al. 2009). Studies have shown that both CXCL12/CXCR4 and apelin/APJ signaling pathways play important role in vascular development and pathological angiogenesis (Kuba et al. 2019, Kang et al. 2013, Takabatake et al. 2009, Unoki et al. 2010). In endothelium, PI3K/Akt is the major signaling pathway for most angiogenic cues and is critically required for the growth, survival and proliferation ECs (Graupera and Potente 2013, Soler, Angulo-Urarte and Graupera 2015). P110β signals downstream of both VEGF/VEGFR2 and CXCL12/CXCR4, however, VEGF/VEGFR2 signaling is p110α dominant (Soler et al. 2015, Guillermet-Guibert et al. 2008). *In vitro*, we found that CXCL12-stimulated CXCR4/PI3K signaling in EC is mainly mediated through p110β isoform of PI3K, while VEGF-stimulated RTK/PI3K signaling depends on p110α. Therefore, we hypothesized that inhibition of p110β in EC would reduce the effect of PD-RCC-induced angiogenesis. Indeed, inhibition of a p110β by TGX-221 reduced the number of PD-RCC-induced angiogenic sprouting and tip cell marker genes compared to the vehicle treated PD-RCC angiogenic samples. Interestingly, we did not observe reduction in any of the growth factor expression itself under TGX-221 treatment. Together our data indicate the EC p110β is involved in tumor angiogenesis in parallel with VEGF-pathway.

We evaluated the effect of EC-specific loss of p110β in tumor angiogenesis and tumor growth *in vivo*. Since VEGF is the primary signaling pathway for angiogenesis, we used sunitinib to block VEGF/RTK signaling. We found that mice deficient in EC-specific p110β delayed primary tumor growth of LLC1 and B16F10 slightly in vehicle treated

group. Treatment with sunitinib alone delayed the tumor growth than the vehicle treated control group, however, the tumor growth was markedly reduced in EC-βKO mice in combination with sunitinib treatment. Histological samples from mRCC patients treated with RTKs such as sunitinib and pazopanib showed an increased in tumor grade and Ki67-positive tumor cells (Sharpe et al. 2013). Interestingly, we observed that tumor growth delayed in EC-βKO plus sunitinib treatment was associated with an increase cleaved caspase-3 positive apoptotic tumor cells and a reduction in Ki67-positive proliferating tumor cells. Therefore, our data suggest that blocking EC-βKO in combination with VEGF/VEGFR2 signaling might reduce the tumor growth and AI drug treatment associated tumor aggressive phenotype.

Tumor microenvironment produces a complex set of pro-angiogenic growth factors many of which, such as CXCL12 and apelin, can stimulate tumor vascularization independent of VEGF-signaling (Kryczek et al. 2005, Helker et al. 2020). Pre-clinical studies showed that tumor vessels regression with TKIs is associated with inhibition of formation of new tumor vessels and at the same time reduction of immature vessels (Mancuso et al. 2006, Hao and Sadek 2016, Osusky et al. 2004). Sunitinib is a multitargeted TKI which has been shown to delay tumor growth with the concomitant regression of tumor vessels by blocking VEGF/VEGFR2 signaling, however, sparing the any potential VEGF-independent angiogenic pathway (Motzer et al. 2017, Hao and Sadek 2016). Our data showed that the tumor vascular density was reduced in sunitinib treated mice compared to the vehicle treated control mice. The combination of EC-βKO and sunitinib further decreased in the background of EC-βKO mice. In addition, we

found that sunitinib-treated EC-βKO mice markedly reduced the tumor vessels sprouting of the larger vessels and tip cell marker genes expression. We further validated our observation by marked reduction in the level of angiopoietin-2 protein expression in EC-βKO mice combining with sunitinib treatment. Taken together, our data indicate that GPCR/PI3K through p110β participates in tumor angiogenesis along with the canonical VEGF/PI3K signaling.

The anti-angiogenic drugs aim to reduce tumor vasculatures to limit nutrient and oxygen supplies in an effort to shrink tumor primary growth (Veeravagu et al. 2007, Vasudev et al. 2013). However, one of the major problems associated with excessive vessels pruning by AI therapy is an increase in tumor hypoxia and the subsequent development of more aggressive and metastatic tumor (Chang and Lai 2020, Jain 2014). In our current study, we found that treatment with sunitinib increased the intra-tumoral hypoxia and hypoxic responsive marker genes such as *Ndrg1* and *Glut1*. This is consistent with multiple pre-clinical studies which show that sunitinib treatment exacerbates tumor hypoxia and aggressiveness (Ebos and Kerbel 2011, Pàez-Ribes et al. 2009). Interestingly, we found that loss of EC p110β markedly reduced the sunitinib induced hypoxia, and the expression of hypoxic marker genes such *Ndrg1* and *Glut1*. Reduced hypoxia in EC-βKO mice was further accompanied by the reduction of pro-angiogenic VEGF and CXCL12 expression, suggesting a sustained inhibition of angiogenesis in EC-βKO under sunitinib treatment.

Accumulating evidence shows that hypoxia is one of the major causes for the development of acquired resistance associated with the AI drugs (Chang and Lai 2020, Jain 2014). For example, apart from its pre-dominant role in inducing angiogenesis, hypoxic micro-environments also select more aggressive tumor cell types, promotes metastasis and chemotherapeutic resistance by inducing EMT (Tam, Wu and Law 2020, Bao et al. 2012). In agreement with this, we found that sunitinib treatment significantly upregulated the expression of EMT marker genes including *Twist1*, *Zeb1*, *Slug* and *Snail1*, and we further confirmed this by detecting *Zeb1* and *Slug* at protein level. Interestingly, we found that loss of EC p110β in combination with sunitinib treatment prevented EMT. Since EMT is involved in many steps of tumor metastatic cascade (Lambert and Weinberg 2021), we explored the metastatic potential of B16F10 in EC-βKO mice. We found that inactivation of EC p110β in combination with sunitinib markedly reduced the tumor metastasis accompanied by a reduction in tumor angiogenesis and hypoxia, however, promoting the tumor micro-vessel maturation. Interestingly, tumor specific overexpression of p110β mutant that abolishes Gβγ binding has been shown to inhibit multiple steps of metastatic cascade *in vivo*, thus reinforcing the role of p110β in regulating tumor metastasis (Khalil et al. 2016). Together, our data suggest that simultaneous inhibition of GPCR/PI3K and RTK/PI3K signaling favour alleviation of tumor hypoxia and prevents EMT and metastasis.

To tackle the problem of AI drug-induced hypoxia and the subsequent tumor progression and the development of more aggressive tumor phenotype, theory of tumor vessel normalization has been proposed, a concept mainly advanced by Jain and his

collogues (Jain 2014, Jain 2003). Normalization of tumor vessels is characterized by well coverage of tumor vasculatures by pericytes such as PDGFRβ and neural glial antigen 2 (NG2), and collagen IV positive basement membrane, thereby reducing the vessels tortuosity and leakiness, while increasing the perfusion (Jain 2003, Carmeliet and Jain 2011). The TKIs sunitinib and sorafenib target both the VEGF-signaling in EC and PDGFRβ signaling on pericytes, implying that TKIs can interfere with vessels maturity by inhibiting the PDGFRβ-mediated pericytes recruitment to the tumor vessels (Ellis and Hicklin 2008, Hellberg, Ostman and Heldin 2010). However, pre-clinical studies show variable results in terms of pericytes coverage after TKIs treatment depending on the tumor model used (Welti et al. 2012, Tong et al. 2004, Shaheen et al. 2001). We found that sunitinib treatment slightly increases the number of tumor vessels with pericytes coverage compared to vehicle treated LLC1 bearing mice. However, inactivation of p110β in combination with sunitinib markedly increased the number of tumor vessels, which were well-covered by pericytes markers such as NG2 and PDGFRβ. We further confirmed that the EC-βKO plus sunitinib treatment led to improved tumor vessel functionality by measuring the increased tumor vessels perfusion in EC-βKO mice. Regarding the fate of the pericytes under AI treatment, McDonald's group showed that the pericytes form regressing vessel moved to the vessel that had not regressed after AI treatment (Baffert et al. 2006). In our model, the fewer blood vessels in EC-βKO plus sunitinib treated mice thus might have better chance for improved stability.

Another important phenomenon observed after AI drugs treatment is the presence of empty basement membranes, representing the existence of tumor vessels that have been regressed after AI treatment (Baffert et al. 2006, Inai et al. 2004). We found that inactivation of EC p110β reduces the number of empty basement sleeves compared to the control group. Treatment with sunitinib markedly increases the presence of the empty basement membrane. However, we found that loss of EC p110β significantly reduces the sunitinib treatment-induced empty basement membranes. In addition, we observed that combining sunitinib and EC-βKO increases the number of collagen positive tumor vessels, indicating an increase in more mature vessels. Of note, the presence of empty basement sleeves can function as scaffolds to support the further regrowth of tumor vasculatures (Inai et al. 2004, Baffert et al. 2006). Taken together, our data suggest that inactivation of EC p110β in combination with VEGFR2 blockade decreases the immature tumor vessels turnover, thereby leading to a sustained inhibition of tumor angiogenesis.

A recent report also indicates the blood vessel normalization function of p110β inactivation in pericytes associated with low PI3K activity, albeit the study was conducted in mice retina, not in tumor vasculatures (Figueiredo et al. 2020). Graupera *et. al.* showed that inactivation of p110β in mural cells promoted early vessels maturation in mice retina, whereas the loss of the counter-regulatory phosphatase (PTEN) of PI3K signaling, resulted in an immature vessels growth (Figueiredo et al. 2020). Surprisingly, they did not observe any effect of mural cell p110α deletion in vessels maturation. Mural cell specific loss of p110β-mediated vascular maturation was

associated the decrease proliferation of mural cells, representing more quiescent vascular phenotype. In our current study, we found that inhibition of EC-specific p110β in combination with sunitinib reduces angiogenesis and heterogenous tumor vascular densities, suggesting that inactivation of p110β in such a setting might increase the number of mature tumor vessels, thus alleviating the AI therapy induced hypoxia.

In summary, targeting VEGF-signaling alone in an effort to block vessels growth might not be the therapy of choice as it exacerbates hypoxia, metastasis and more aggressive tumor behaviours (Itatani et al. 2018, Maj, Papiernik and Wietrzyk 2016). Instead, co-targeting parallel angiogenic pathway might have better potential to achieve the maximum benefits of anti-angiogenic therapy. We show that inhibition of GPCR/PI3K signaling by blocking p110β isoform of PI3K prevents PD-RCC released growth factors driven sprouting angiogenesis. Our findings demonstrate that EC-specific inactivation of p110β in combination with sunitinib decreases primary tumor growth and tumor metastasis vs sunitinib treatment alone. The density of the tumor vasculature and tip cell gene expression is reduced, but tumor oxygen delivery is normalized. Therefore, inhibition of endothelial p110β may be useful as adjuvant therapy with sunitinib, and may facilitate delivery and/or response of the tumor to conventional chemo- and immunotherapeutic agents.

6.2 Apelin is the dominant pro-angiogenic GPCR ligand that participates in tumor angiogenesis

Apelin is an endogenous peptide for GPCR. Higher expression of apelin has been found to corelate with poor clinical outcome in certain cancer types including in breast, colorectal and oral squamous cell carcinomas (Zuurbier et al. 2017, Heo et al. 2012, Uribesalgo et al. 2019). Previous studies showed the association of upregulation of apelin/APLNR signaling in RCC (Tolkach et al. 2019, Zhang et al. 2017). In our published data, we found that apelin is highly expressed in PD-RCC samples with angiogenic potential compared to the non-angiogenic group. Furthermore, we showed that inhibition of CXCR4/PI3K signaling by blocking EC-specific p110β in mice decreases tumor growth accompanied by a marked reduction in tumor angiogenesis. Since apelin is a pro-angiogenic GPCR ligand, we posit that apelin is a dominant agonist to activate CXCR4/PI3K signaling and angiogenesis. Interestingly, recent studies have shown that targeting apelin in combination with AI therapy delayed tumor angiogenesis and tumor growth (Uribesalgo et al. 2019, Mastrella et al. 2019). However, the exact role of apelin in regulating tumor angiogenesis and endothelial cells behaviour remains relatively unclear.

In this study we evaluated the growth of LLC1 and B16F10 implanted subcutaneously in *Apln*$^{-/y}$ or WT control mice. Similar to our earlier project, we blocked VEGF-signaling with RTK sunitinib and found that overall tumor growth delayed in *Apln*$^{-/y}$ mice compared to the sunitinib treatment alone. We found that there were small changes in tumor growth in apelin knockout mice in comparison to the wild type control mice when treated

with vehicle alone. However, inhibition of host apelin in combination with sunitinib treatment delayed the growth of subcutaneously implanted LLC1 and B16F10 primary tumors.

Recent studies show that the efficacy of targeting apelin depends on the tumor models used. For example, Uribesalgo *et. al.* showed that loss of apelin alone in mice delayed the growth of breast and lung cancer (Uribesalgo et al. 2019). Further, in their tumor model they found that treatment with sunitinib increased the dissemination of tumor cells from primary tumor site to the lung, confirming the previous observations of increased metastasis associated with AI therapy (Ebos and Kerbel 2011, Pàez-Ribes et al. 2009). However, they showed that the loss of host apelin prevented metastasis associated with AI therapy (Uribesalgo et al. 2019). On the other hand, Mastrella *et. al.* showed that implantation of glioma in apelin knockout mice did not change the tumor volume compared to their respective control group, however, the tumor invasiveness was markedly increased in apelin knockout mice (Mastrella et al. 2019). They attributed this pro-invasive phenotype in apelin KO mice due to the upregulation of APLNR in the invasive zone. Subsequently, the glioma growth was markedly reduced when they inhibited apelin/APLNR signaling in combination with anti-VEGF therapy. These studies suggest that loss of apelin in combination with AI therapy is better than any of the intervention alone.

Apelin is a pro-angiogenic growth factor, highly expressed in ECs and regulates developmental and tumor angiogenesis (Kuba et al. 2019, Sorli et al. 2007). We found

that the loss of apelin alone decreases the tumor vessel densities in comparison to the vehicle treated control mice. Sunitinib treatment markedly reduced the vascular densities compared to the vehicle treated control group. Inhibition of host apelin in combination with sunitinib therapy further reduced the tumor vessel densities in comparison to the sunitinib treated control mice. The presence of EC sprouts from larger vessels indicate an active ongoing state of angiogenesis (Baluk, Hashizume and McDonald 2005, Baluk et al. 2003). The TKIs and other AI drugs blocks the formation new vessels by inhibiting the formation vessels sprouting from the larger vessels (Inai et al. 2004). We found that angiogenic sprouting in larger vessels was reduced in vehicle treated *Apln*$^{-/y}$ mice in comparison to control mice. Treatment with sunitinib reduces vessel sprouting, which was further decreased in the background of apelin knockout mice. We extended our data to show that the decreased in vascular densities and sprouting in *Apln*$^{-/y}$ were accompanied by a concomitant reduction in tip cell specific marker genes expression. AI therapy is associated with the regression of existing vessels, however, leaving behind the empty basement membrane sleeves, which can be used as a support for the re-emergence of new vessels (Inai et al. 2004). We found that loss of apelin reduces the fraction of sunitinib-induced empty basement sleeves and the immature tumor vessels, indicating the suppression of tumor vessels remodeling in *Apln*$^{-/y}$ mice when combined with AI therapy. Together, our data suggest that inhibition of host apelin with sunitinib not only reduces the tumor vessel densities, but also participates in supressing the formation of new vessels by inhibiting tip cell sprout and vessels turn over.

Apelin/APLNR signaling plays important role in developmental angiogenesis (Cox et al. 2006, Kasai et al. 2008). It has been shown that loss of apelin in zebrafish prevents tip cell phenotype and sprouting angiogenesis (Helker et al. 2020). The expression of apelin remains relatively low in adults, however, its expression is upregulated in tumor EC (Uribesalgo et al. 2019, Sorli et al. 2007). Our data show that loss of apelin inhibits tumor angiogenesis and tip cell marker genes. Therefore, we sought to understand the role of apelin loss in EC tip cell morphology. We compared the single cell EC transcriptome associated with sunitinib or *Apln*$^{-/y}$ tumor micro-environment. Unsupervised algorithmic analysis identified 2 distinct populations of EC in the tumors. We found that loss of apelin reduces the potential of EC transcriptome to express tip cell marker genes, especially the expression of *Cxcr4*, *Pdgfb* and *Adm*, supporting our observations that loss of apelin prevents EC tip cell differentiation and sprouting angiogenesis.

During angiogenesis, the VEGF and Notch signaling dynamically cooperate for the selection of tip and stalk cells (Jakobsson, Bentley and Gerhardt 2009, Hellström et al. 2007). In the presence of VEGF, the Notch ligand Dll4 is highly expressed in EC tip cell, which in turn activates Notch signaling in the adjacent cell. Notch activation within the adjacent cell inhibits the tip cell differentiation, consequently leading to acquire stalk cell phonotype. Lack of endothelial Dll4 or Notch signaling has been shown to result in hyper-sprouting phenotype (Hellström et al. 2007). Interestingly, in our tumor EC transcriptome analysis, we did not observe any changes in Dll4 expression in apelin deficient vs control group. Our data suggest that although the EC is equally enriched

with Dll4 expression in apelin deficient mice and control mice, the cooperation of apelin is required to achieve full potential to initiate sprouting angiogenesis. On the other hand, the presence of Dll4 in apelin deficient mice may further ensure ECs to acquire stalk cells phenotypes. This is in agreement with the observation that inhibition of Dll4 in zebrafish resulted in hyper-sprouting phenotypes, and this increased sprouting was blocked when apelin signaling was inhibited, suggesting that apelin is required in regulating Notch-mediated sprouting angiogenesis (Helker et al. 2020).

Previous studies have shown that loss of apelin/APLNR signaling prevents AI therapy induced metastasis and improves efficacy of AI therapy by improving tumor vessels functionality (Mastrella et al. 2019, Uribesalgo et al. 2019). In agreement with these studies, we found that a higher fraction of tumor vessels was covered by pericytes in vehicle treated apelin deficient mice compared to the control mice. Sunitinib treatment further increases the pericytes coverage in apelin deficient mice. The maturity and stability of tumor vessels are reflected by facilitating homogenous oxygen and nutrients delivery, and the subsequent reduction in tumor hypoxia (Martin, Seano and Jain 2019, Viallard and Larrivée 2017). We found that the expression of hypoxic genes such as *NDRG1* and *Glut1* were markedly reduced in *Apln*$^{-/y}$ mice with sunitinib treatment. In addition, the pro-angiogenic hypoxia-sensitive *Vegf* and *Cxcl12* expression were also decreased in *Apln*$^{-/y}$ plus sunitinib group in comparison to the sunitinib treatment alone. Together, our data suggest that inactivation of apelin along with AI inhibits tumor angiogenesis and the turnover of immature vessels, consequently having a population of mature vessels. However, we need to further validate our observation of vessels

maturity in apelin knockout mice by determining the features of improved vessels functionality such as an increase in vessel perfusion and a decrease in vascular leakiness.

In summary, our data show that inactivation of host apelin in combination with VEGF-signaling inhibition reduces experimental tumor growth and tumor angiogenesis. Most importantly, loss of host apelin perturbs tumor associated EC transcriptome to acquire tip cell phenotypes. Further, our data show that loss of apelin reduces the tumor hypoxia, but increases tumor vessels maturity. Our studies with EC-βKO and *Apln*$^{-/y}$ mice repeatedly show that GPCR/PI3K signaling is involved in tumor vascularization, suggesting that targeting GPCR-signaling along with VEGF inhibition will have better therapeutic potential to control tumor angiogenesis (Fig. 6.1).

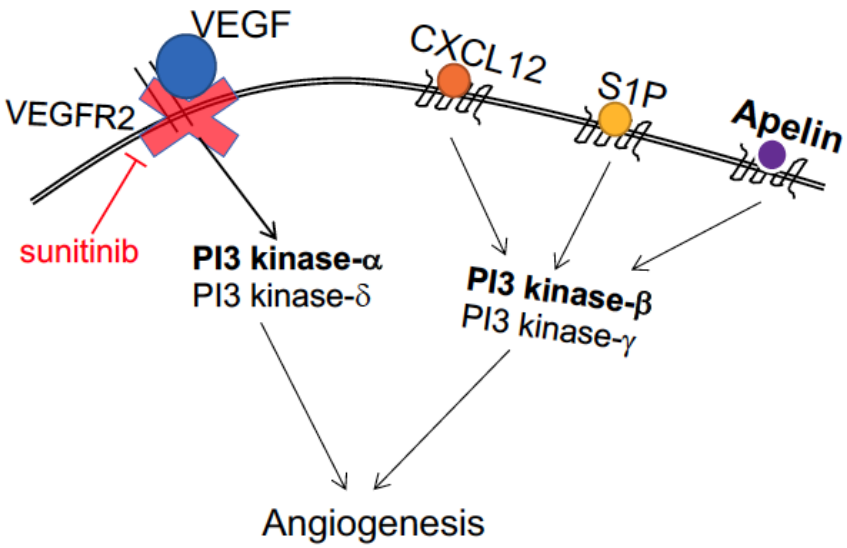

Fig 6.1: A schematic representation of pro-angiogenic GPCR signaling in tumor angiogenesis alternative to the typical VEGF-RTK signaling.

6.3 FGD5 regulates parallel proangiogenic signalings coming through both RTK/PI3K and CXCR4/PI3K pathways

First, we showed that inactivation of p110β blunts GPCR/PI3K signaling and inhibits the PD-RCC induced angiogenesis *ex vivo*, as well as tumor angiogenesis *in vivo*. Second, we demonstrated that apelin being a GPCR ligand also participates in tumor angiogenesis and inhibition of host apelin prevents the tumor EC transcriptome from acquiring tip cell enriched gene expression. Both of our works with p110β and apelin show the importance of parallel angiogenic signaling along with VEGF-pathway in tumor vascularization. Thus, identifying EC-specific target that would effectively inhibit multiple pro-angiogenic signaling cues coming through GPCR/PI3K and VEGF/RTK is critical for the development of more effective anti-angiogenic therapy.

Earlier, we showed that PD-RCC samples secrete several GPCR ligands including CXCL12 and apelin. Mice deficient in CXCL12 or its receptor CXCR4 die *in utero* or shortly after birth due to abnormal vascular development in the gut, however, without any apparent vascular abnormalities in heart or brain (Tachibana et al. 1998, Nagasawa et al. 1996). The embryonic kidneys from CXCL12 or CXCR4 knockout mice also show abnormal glomeruli vascular development, indicating that CXCL12/CXCR4 axis plays a critical role in organ-specific vascular development (Takabatake et al. 2009, Ara et al. 2005). In addition, CXCL12 has been shown to recruit EPC to the tumor vessels, thus facilitating tumor vascular development (Aghi et al. 2006). However, it is largely unclear whether CXCL12 is directly involved in angiogenesis by inducing EC tip cell differentiation or just facilitate EC polarization and migration during angiogenesis. Here,

using CXCL12 as a single stimulus for angiogenesis, we found that CXCL12 alone was not enough to drive angiogenesis, rather priming of EC with suboptimal dose of VEGF was required to stimulate CXCL12-mediated angiogenesis. CXCL12 alone did not increase the expression of tip cell marker genes, but it augmented the tip gene expression when combined with low dose VEGF. Our data indicate that during the tumor AI therapy when the VEGF-signaling is blocked, CXCL12 could still drive the angiogenesis even in the presence of residual amount of VEGF. This is consistent with previous findings that VEGF primes the EC by inducing the expression of CXCR4, the GPCR ligand for CXCL12, and CXCL12 then stimulates the EC migration (Stratman, Davis and Davis 2011, Kryczek et al. 2005).

Previously, we showed that FGD5 as an endothelial-restricted Rac-GEF regulates VEGF-stimulated PI3K signaling pathway (Farhan et al. 2017, Park et al. 2021). However, the role of FGD5 in regulating GPCR signaling is not known yet. In our current work, we have exploited a 3D *ex vivo* angiogenesis assay, similar to our previous experiment with p110β inhibition, co-culturing HUVEC and PD-RCC together. We showed that loss of FGD5 in EC decreases number and lengths of PD-RCC induced sprouting angiogenesis accompanied by inhibiting the expression of tip cell marker genes. Being an EC-restricted molecule, we found that loss of function of FGD5 in EC prevents CXCL12-stimulated GPCR/PI3K signaling. However, this does not exclusively reveal whether defects in PD-RCC induced angiogenesis in FGD5 deficient cells is associated with its meddling of GPCR signaling, since loss of FGD5 can also interfere with VEGF signaling as well. Previous studies have shown that p110β is the

predominant subunit of PI3K that regulates signal coming from GPCR, however, p110β and p110γ might have redundant function when stimulated with GPCR ligands (Guillermet-Guibert et al. 2008, Backer 2010). We observed that inhibition of p110β, but not p110γ reduced the CXCL12-stimulated Akt activation in EC, suggesting that p110β is the major PI3K isoform that couples to CXCR4/PI3K signaling in EC. In line with this observation, we found that inhibition of p110β reduces the EC angiogenic sprouting synergistically induced by low dose VEGF and CXCL12. Together, our data showed that inhibition of p110β in EC has similar signaling and angiogenic sprouting defects as in FGD5 deficient EC, suggesting that both FGD5 and p110β are working downstream of GPCR signaling.

Among PI3K class I isoforms, PI3Kβ is uniquely regulated by members of the Rho GTPase family members such as Cdc42 and Rac1, whereas the other PI3K isoforms are regulated by Ras (Fritsch et al. 2013, Rodriguez-Viciana et al. 1996). Despite evidences that Rac1 and Cdc42 can mediate PI3Kβ activity downstream of GPCRs, we found that p110β signaling in EC is Rac1 dependent. Since Rac1 regulates p110β activity by binding to its Ras binding domain (RBD), we used RBD-mutated p110β to inhibit Rac1 mediated activation. Indeed, our result showed that overexpression of RBD-mutated p110β failed to rescue defective PI3K signaling in p110β deficient EC after CXCL12-stimulation. In line with this, the preferential activation of p110β by Rac1 has been identified in mouse embryonic fibroblasts (MEF) (Cizmecioglu et al. 2016). In particular, Rac1 is required for p110β localization to the membrane raft where it becomes activated by the GPCR agonists (Cizmecioglu et al. 2016).

In its GDP bound form, Rac1 remains inactive and requires GEFs to be activated by switching GDP for GTP (Etienne-Manneville and Hall 2002). Since inhibition of Rac1 demonstrates the same signaling defects as in FGD5 loss after CXCL12 treatment, and FGD5 contains a RhoGEF domain, we conceived of a model in which FGD5 functions GEF for Rac1 to regulate p110β activation and cytoskeletal remodeling. Our observation showed that overexpression of DBL domain, the Rho-GEF domain, deleted FGD5 decreases CXCL12-stimulated Akt activation, indicating a dominant negative function of DBL mutated FGD5. In addition, $FGD5^{\Delta DBL}$ overexpression failed to rescue defective Akt signaling in FGD5 deficient cells. Functionally, we observed that $FGD5^{\Delta DBL}$ delayed EC migration in a wound healing assay. Together, these data suggest that FGD5 functions as a RhoGEF for Rac1 in EC. This is consistent with a recent finding where it has been shown in an *in vitro* GEF enzyme assay that FGD5 preferentially binds to Rac1 and activates it (Park et al. 2021). However, it should be noted that earlier work has identified FGD5 as RhoGEF to increase CDC42-GTP activity in EC after VEGF-stimulation (Kurogane et al. 2012).

GPCR signaling primarily thought to happen at plasma membrane and is quickly desensitized and endocytosed by β-arrestin (Eichel and von Zastrow 2018, Magalhaes, Dunn and Ferguson 2012). However, emerging evidences show the importance of GPCR endocytosis and trafficking to early endosomal compartment to maintain sustained GPCR signaling (Eichel and von Zastrow 2018, Tsvetanova, Irannejad and von Zastrow 2015). FGD5 contains a FYVE domain and the studies have shown that

FYVE domain containing proteins bind to PI3P on the surface of early endosomes (Lee et al. 2005, Kutateladze and Overduin 2001). We found that overexpression FYVE domain deleted FGD5 decreases CXCL12-stimulated Akt activation and failed to rescue defective Akt signaling in FGD5 deficient EC. In addition, we found that FYVE domain deleted FGD5 delayed CXCL12-stimulated EC wound healing. However, we did not observe FYVE domain dependent translocation of FGD5 to the early endosomes. Our data suggest that the FYVE domain is required to retain full functionality of FGD5 to activate p110β independent of endosomal translocation. This is in agreement with the aforementioned study where it has been shown that Rac1-dependent plasma membrane localization of p110β is required for its activation by GPCRs (Cizmecioglu et al. 2016).

In summary, the FGD5 is downstream of GPCR signaling, and the DBL and FYVE domains are critical in regulating GPCR/PI3K signaling. We show that the FGD5 functions as a RhoGEF for Rac1 to regulate CXCL12-stimulated p110β activity in EC (Fig. 6.2).

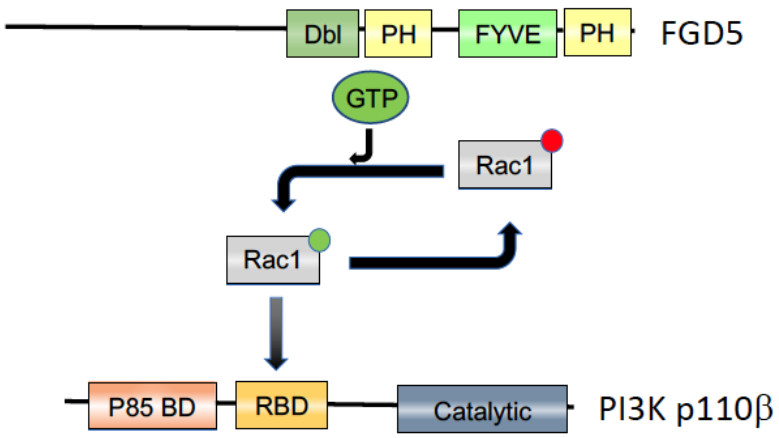

Fig. 6.2: A schematic representation of FGD5 as a GEF for Rac1 in EC to regulate p110β activity.

6.4 Summary of the findings

1) PD-RCC samples secrete several pro-angiogenic growth factors including VEGF, apelin and CXCL12. Pharmacological inhibition of p110β in EC prevents CXCL12- and PD-RCC-induced sprouting angiogenesis. EC-specific deletion of p110β delays tumor growth and angiogenesis *in vivo*. These data indicate that alternative pro-angiogenic signaling operates in parallel with VEGF-signaling.

2) Inactivation EC-p110β in combination with sunitinib promotes tumor vessel maturity of the remaining vessels and alleviates hypoxia, prevents EMT and metastasis. These data suggest that targeting EC-p110β may be useful as an adjuvant therapy with AI drugs, and may facilitate delivery and/or response of the tumor to conventional chemo- and immune checkpoint inhibitors therapies.

3) Targeting GPCR ligand apelin in combination with sunitinib delays primary tumor growth accompanied by a reduction in tumor vascular densities and angiogenesis, while at the same time improving the features of normal mature vessels. This observation suggests that blockade of apelin/APLNR signaling contributes to most of the effect of p110β inactivation in EC to limit tumor growth and tumor angiogenesis.

4) Loss of apelin substantially prevents tumor EC transcriptome from acquiring tip cell enriched gene expression. These data indicate that apelin as a major GPCR agonist might be targeted specifically at EC to achieve better anti-angiogenic efficacy than the ubiquitously expressed p110β.

5) FGD5 is an EC specific Rac-GEF that regulates CXCL12-stimulated Rac1 dependent p110β activation. FGD5 controls CXCL12-mediated sprouting angiogenesis and EC migration. Our data suggest that FGD5 is involved in regulating PI3K/Akt activity coming from both VEGF/VEGFR2 and CXCL12/ GPCR receptors, thus can be a potential target for the development more effective anti-angiogenic therapy (Fig. 6.3).

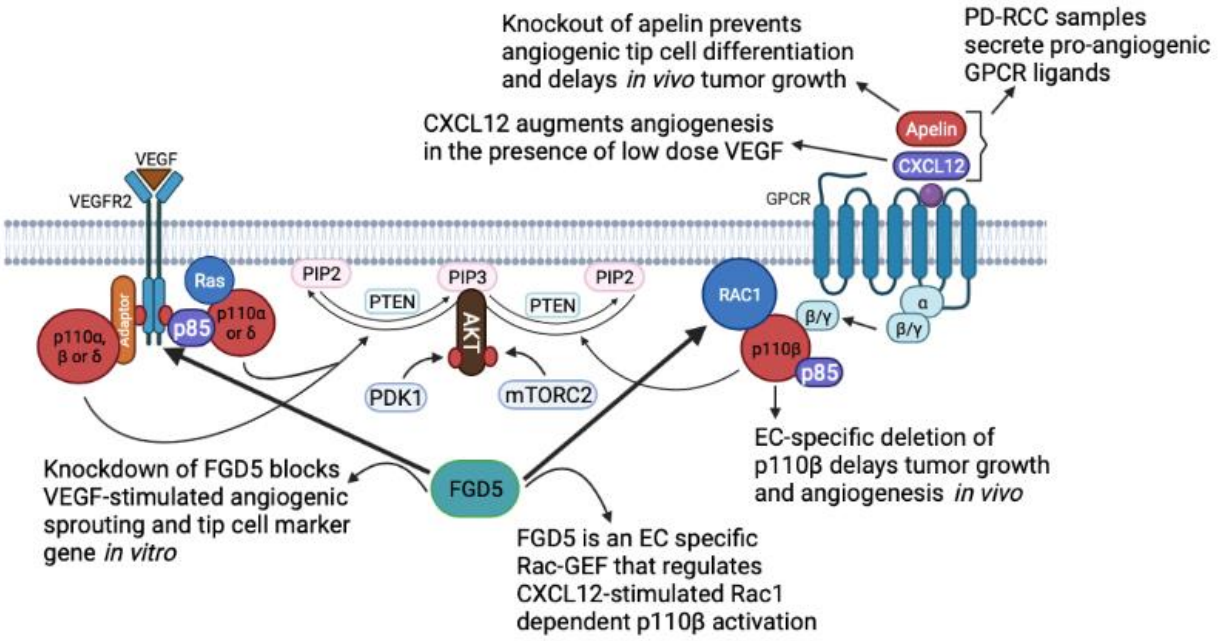

Fig. 6.3: GPCR signaling works in parallel with VEGF/VEGFR2 signaling in tumor angiogenesis

6.5 Future directions

1) To further characterize the role of apelin in tumor vessels normalization

We found that loss of apelin reduces tumor angiogenesis and hypoxic marker genes, and increases the stability of the remaining tumor vasculatures after sunitinib treatment. However, we need to confirm our observation of tumor hypoxia by pimonidazole staining, and tumor vessels normalization by determining the vessel perfusion, dextran leakage and trafficking of leukocytes infiltration status in tumors. Further, confirmation of vessel normalization will be evaluated by determining vessel diameter, vascular permeability, and edema by intravital imaging.

2) To determine the role of apelin deficiency in metastasis

Our data indicate that loss of apelin reduces tumor hypoxia and promotes vessels normalization, suggesting that loss of apelin might impede aggressive tumor EMT and subsequent metastasis. A spontaneous metastasis model using B16F10 cell line to determine the effect of apelin loss could be exploited. B16F10 cell line will be implanted in *Apln*$^{-/y}$ and control mice, once the tumor size reach to 500mm3, tumor will be surgically excised, and the tumor metastasis will be determined after 30 days.

3) To determine the role of tumor-associated fibroblast transcriptome isolated from apelin knockout mice

We have isolated tumor stromal cells and the samples have been processed for single cell RNA sequencing. Our initial observation shows that majority of cell population are cancer associated fibroblasts (CAFs). We will investigate the implication of apelin deficiency in CAFs transcriptome, as it is well validated that CAFs play diverse functions in tumor progression including in angiogenesis, extracellular matrix (ECM) remodeling, modulation of immune systems and metastasis.

4) To determine the role of FGD5 in tumor growth and angiogenesis *in vivo*

We observation showed that FGD5 regulates both RTK/PI3K and GPCR/PI3K signaling, thus making it a potential target for anti-angiogenic therapy. We will investigate the role of EC-specific FGD5 in tumor growth and angiogenesis. Currently, we are breeding Cdh-Cre^{ERT2} X FGD5$^{flox/flox}$ mice to generate Cdh-Cre^{ERT2}- FGD5$^{flox/flox}$ mice. This

tamoxifen inducible EC-specific FGD5 deleted mice will be used for further experiment to examine tumor angiogenesis.

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