Hypoxia-induced TIMAP Upregulation Promotes Tumor Angiogenesis

by

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Abstract

Angiogenesis, the process of forming new blood vessels from a pre-exciting vasculature, is an essential component of embryonic development, tissue remodelling, wound healing, and tumor growth. This process is strictly regulated by a vast array of cellular signalling pathways involving kinases, phosphatases, growth factors and cytokines. Hypoxia, which is a hallmark of tumor growth, is a key trigger of angiogenesis. During tumorigenesis, neovascularization is a critical element for the tumor to promote its growth and progression. In rapidly expanding solid tumors, cancer cells in the center of the tumor become hypoxic as they grow further away from the original organ's vasculature. In these hypoxic cancer cells, hypoxia induces several signalling cascades and stimulates release of pro-angiogenic factors which promote angiogenesis to overcome the hypoxic microenvironment and continue to survive. Several anti-angiogenic drugs that target specific angiogenic signalling pathways have proven clinically useful, although tumors frequently develop resistance to this approach. This is due to the substantial complexity and redundancy of the signalling pathways that regulate tumor angiogenesis. Therefore, thoroughly understanding these mechanisms may help to develop better anti-angiogenic approaches that more effectively inhibit tumor growth and progression by interfering with the disordered angiogenesis.

TIMAP (TGF-β-Inhibited Membrane-Associated Protein) is an endothelial cell (EC)-predominant pro-angiogenic protein that promotes EC proliferation and enhances *in vitro* angiogenic sprout formation. However, its angiogenic role in the *in vivo* setting remains unknown. Here we demonstrate that TIMAP is necessary for mouse tumor growth and

angiogenesis and elucidate the mechanisms that regulate TIMAP expression in EC. We found that hypoxia, the principal stimulus of tumor angiogenesis, raises TIMAP levels in mouse lung tissues and in cultured EC. We found that repression of TIMAP expression is mediated through activation of the EC-specific Activin Receptor-Like Kinase-1 (ALK1) SMAD1/5/8 pathway and that hypoxia stimulates TIMAP expression by attenuating BMP-9-induced ALK1-mediated TIMAP inhibition. The TGF-β signalling molecule Bone Morphogenetic Protein-9 (BMP-9) potently and selectively activates the EC ALK1 SMAD1/5/8 pathway and significantly reduces TIMAP abundance. HIF-α activation and ALK1 inhibition both impede BMP-9-stimulated SMAD1/5/8 phosphorylation, and both increase EC TIMAP levels. Furthermore, we also found that the angiogenic growth factors VEGF and IGF-1 induced by HIF-α activation in hypoxic tumor cells, also enhance EC TIMAP expression by attenuating the ALK1 pathway signalling, although they also induced EC TIMAP expression through ALK1 pathway independent effects. These findings indicate that TIMAP has critical pro-angiogenic effects in EC, which may point to a new potential target for inhibition of tumor angiogenesis.

Preface

This study represents original work of Salah Aburahess unless specified otherwise. This thesis consists of unpublished work for which the manuscript has been submitted to a peer-reviewed journal. Figure 1.10 represents work I did as a co-author for a published paper *(Xin Wang, Marya Obeidat, Laiji Li, Phuwadet Pasarj, Salah Aburahess, Charles F. B. Holmes, and Barbara J. Ballermann. TIMAP inhibits endothelial myosin light chain phosphatase by competing with MYPT1 for the catalytic protein phosphatase 1 subunit PP1cβ. J Biol Chem 294 (36) 13280-13291).*

I am responsible for the data collection and analysis in this thesis, the preparation of the submitted manuscript, and the literature review in chapter 2.

Dr. Barbara Ballermann supervised and coordinated the research and participated in manuscript preparation and editing, as well as the data analysis.

The lab mentor Laiji Li, PhD, provided expert technical support, helped design some experiments and assisted with the data collection. Dr. Li also prepared some buffers and reagents and was responsible for animal husbandry.

Parnian Alavi, a PhD candidate, performed the immunofluorescence microscopy of tumor tissues (Figure 3.2 A). The immunofluorescence microscopy of control and hypoxic mouse lung tissue sections (Figure 3.3 B) was carried out by Marya Obeidat, PhD. Aashiq Hussain, PhD, was responsible for RNA isolation, cDNA synthesis and qRT-PCR experiments (Figures 3.4 E, 4.1 F, 4.2 E, 4.4 F).

Nadia Jahroudi, PhD, provided the control and hypoxic mouse tissues and was involved in the concept formation.

All animal experiments and procedures were carried out according to the guidelines approved by the Canadian Council for Animal Care (CCAC), and the animal protocol was approved by the University of Alberta Animal Care and Use Committee (protocol AUP00000222).

Dedication

With sincere gratefulness and warm regards, I dedicate my dissertation work to my first teachers in life, my parents.

أبورخيص عبدالله امطير أبورخيص رحمه محمد محمد التونسي

Thank you for being the source of my strength, wisdom, and inspiration.

To my wonderful wife, Fatema Haman, and my beloved children Ansam, Anas, and Ayham, who all have been a tremendous constant source of support and encouragement throughout the whole program.

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Table of Contents

Abstract ii

Preface iv

Dedication vi

Acknowledgments vii

Table of Contents x

List of Tables xiv

List of Figures xv

List of Abbreviations xvii

Chapter 1 General introduction 1

1.1 Angiogenesis 1

1.1.1 Sprouting angiogenesis 1

1.1.2 Intussusceptive angiogenesis 2

1.2 Blood vessel maturation 5

1.3 Tumor angiogenesis 6

1.4 Hypoxia-induced angiogenesis 7

1.5 Angiogenic growth factors 10

1.5.1 VEGF signaling pathway 10

1.5.2 IGF-1 signaling pathway 16

1.5.3 FGF signaling pathway 19

1.5.4 EGF signaling pathway 22

1.6 PI3K/AKT signaling pathway 26

1.7 Growth factor receptors crosstalk during angiogenesis 30

1.8 Therapeutic growth factor inhibitors 31

1.9 Transforming growth factor (TGF)-β superfamily 32
1.9.1 TGF-β Signaling pathway 38
1.9.2 BMP-9 Signaling pathway 39
1.9.3 The effect of hypoxia on the TGF- β signaling pathway 42
1.10 TIMAP 44
1.10.1 TIMAP regulation by TGF- β1 45
1.10.2 TIMAP is a myosin phosphatase targeting subunit (MYPT) 46
1.10.3 TIMAP cellular localization 47
1.10.4 TIMAP phosphorylation and interaction with other proteins 48
1.10.5 Role of TIMAP in angiogenesis 51
1.11 Summary 52
1.12 Thesis hypothesis and objectives 53
Chapter 2 Materials and Methods 54
2.1 Reagents 54
2.2 Immunoblot assay antibodies 55
2.3 Animals experiments 58
2.3.1 TIMAP deficient mice 58
2.3.2 Tumor cell line 58
2.3.3 Mouse tumor cell implantation 59
2.3.4 Mouse Hypoxia 59
2.4 Immunofluorescence Microscopy (IF) 61
2.5 Cell culture 62
2.6 *In vitro* hypoxia 62
2.7 HUVEC/E0771 co-culture 63

2.8 Conditioned media 65
2.9 Western blot analysis 65
2.9.1 Sample preparation 65
2.9.2 Gel electrophoresis 66
2.9.3 Protein transfer 66
2.9.4 Blocking 66
2.9.5 Antibody incubation 67
2.9.6 Protein detection and visualization 68
2.9.7 Western blot loading control 70
2.10 TIMAP knockdown and siRNA transfection 73
2.11 RNA isolation, cDNA synthesis and quantitative real-time PCR (qRT-PCR) 73
2.12 Statistical analysis 74
Chapter 3 Hypoxia-induced TIMAP Upregulation Promotes Tumor Angiogenesis 75
3.1 Introduction 75
3.2 Results 80
3.2.1 TIMAP depletion impairs E0771 mammary tumor growth 80
3.2.2 TIMAP deletion in mice reduces tumor angiogenesis. 83
3.2.3 Chronic hypoxia upregulates mouse pulmonary endothelial TIMAP abundance 85
3.2.4 Angiogenic growth factors induce EC TIMAP expression 87
3.2.5 Growth factor-induced TIMAP expression is mainly mediated by VEGF & IGF-1 91
3.3 Discussion 93
Chapter 4 Hypoxia and growth factors antagonize ALK1-induced TIMAP inhibition

..... 98
4.1 introduction 98
4.2. Results 101
4.2.1. Hypoxia antagonizes serum-mediated TIMAP repression 101
4.2.2. BMP-9 mimics serum-induced TIMAP repression 103
4.2.3. Hypoxia raises TIMAP levels by antagonizing the ALK1 pathway 106
4.2.4. Growth factors raise TIMAP level, in part, by inhibiting the ALK1 pathway .. 109
4.3. Discussion 112
Chapter 5 General discussion and future directions 116
5.1 Introduction 116
5.2 Mechanism of TIMAP Function 116
5.3 Mechanism of TIMAP regulation 120
5.4 TIMAP in breast cancer 126
5.5 The potential role of TIMAP in tumor metastasis 128
5.6 Proposed future Experiments 131
5.7 Summary 134

List of Tables

Table 1: TGF-β superfamily of receptors……35

Table 2: TGF-β superfamily ligand/receptors complexes and their R-SMAD pathway...36

Table 3: List of reagents……..…….…….....…54

Table 4: List of primary antibodies ……..…….55

Table 5: List of secondary antibodies ……...……...….56

List of Figures

Figure 1.1: Schematic representation of the process of sprouting angiogenesis…....…3

Figure 1.2: Schematic diagram of the intussusceptive angiogenesis……..……...4

Figure 1.3: Oxygen-dependent regulation of HIF-1α…..……..……..9

Figure 1.4: Schematic illustration of the VEGF signaling pathway……..…..…..12

Figure 1.5: Schematic illustration of the IGF-1 signaling pathway…...…….....…18

Figure 1.6: Schematic illustration of the FGF signaling pathway.……..…….21

Figure 1.7: Schematic illustration of the EGF signaling pathway.…….....…..…….25

Figure 1.8: Schematic illustration of the PI3K/AKT signaling pathway……..……...29

Figure 1.9: Canonical and non-Canonical TGF-β signaling pathways……..…...37

Figure 1.10: Inhibition of TIMAP reduces MLC2 phosphorylation……..……...50

Figure 2.1: TIMAP expression in endothelial and E0771 cells.…..……..…..…….....60

Figure 2.2: E0771/HUVEC co-culture……..……...…….…….....64

Figure 2.3: Western blot stripping blocking procedure……...…….…….....69

Figure 2.4: Dynamic range for housekeeping protein quantification by WB ……....71

Figure 3.1: Reduced tumor growth in TIMAP deficient mice…..…….…….....82

Figure 3.2: Impaired tumor angiogenesis in TIMAP deficient mice…..…..…….....84

Figure 3.3: Increased TIMAP abundance in response to chronic hypoxia.…….....86

Figure 3.4: Angiogenic growth factors raise TIMAP protein levels…..…….....89

Figure 3.5: TIMAP expression in response to distinct EC growth factors…..……....92

Figure 4.1: Hypoxia antagonizes serum-mediated TIMAP inhibition mice…..……...102

Figure 4.2: BMP-9 inhibits TIMAP expression……..……...…..…..……...105

Figure 4.3: Hypoxia upregulates TIMAP abundance by attenuating ALK1 pathway signalling……...…..……...108

Figure 4.4: Growth factors stimulate TIMAP expression, in part, by antagonizing the ALK1 pathway……..…..……....111

Figure 5.1: Model illustrating the effects of HIF-1α, growth factors and ALK1 pathway on EC TIMAP expression……..122

Figure 5.2: Diagram illustrating TIMAP functions and mechanism of regulation…...…135

List of Abbreviations

ACVR1	Activin A receptor type I
AKT	Protein kinase B
ALK	Activin receptor-like kinase
AMH	Anti-Mullerian Hormone
AMHR	Anti-Mullerian Hormone Receptor
ANG1	Angiopoietin-1
BAMBI	BMP and Activin membrane-bound inhibitor
BM	Basement membrane
BMP	Bone morphogenetic protein
BMPR	Bone morphogenetic protein receptor
CM	Conditioned medium
Dll-4	Delta-like-4
DMEM	Dulbecco's Modified Eagle Medium
EBM-2	Endothelial cell growth basal medium
EC	Endothelial cells
ECM	Extracellular matrix
EDG1	Endothelial differentiation sphingolipid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGM-2	Endothelial cell growth medium-2
ERK	Extracellular signal-regulated kinase
ERM	Ezrin, Radixin, Moesin

ESM-1	Endothelial-specific molecule 1
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
GDF	Growth and differentiation factor
GF	Growth factor
GSK3β	Glycogen synthase kinase 3β
HDAC	Histone deacetylase
hGEC	Human glomerular endothelial cells
HHT	Hereditary Hemorrhagic telangiectasia
HIF-1α	Hypoxia-inducible factor-1α
hLEC	Human lung endothelial cells
HSPG	Heparan sulfate proteoglycan
HUVEC	Human umbilical vein endothelial cells
IF	Immunofluorescence
IGF-1	Insulin-like growth factor-1
IGFBP	Insulin-like growth factor binding protein
IGR-1R	Insulin-like growth factor-1 receptor
IRS	Insulin receptor substrate
I-SMAD	Inhibitory SMAD
KO	Knockout
LAMR1	Laminin receptor-1
MAPK	Mitogen-activated protein kinase

Merlin	Moesin-Ezrin-Radixin like protein
MLC2	Myosin light chain 2
MLCK	Myosin light chain kinase
mTORC2	Mammalian target of rapamycin complex 2
MYPT	Myosin phosphatase targeting subunit
ODD	Oxygen-dependent degradation
PAH	Pulmonary arterial hypertension
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDGFRβ	Platelet-derived growth factor receptorβ
PDH	Prolyl hydroxylase domain
PDK1	Phosphoinositide-dependent kinase-1
PECAM	Platelet endothelial cell adhesion molecule-1
PH	Pleckstrin homology
PI	Phosphoinositide
PI3K	Phosphotidylinositide-3 kinase
PIP2	Phosphatidylinositol (4,5) bisphosphate
PIP3	Phosphatidylinositol (3,4,5) triphosphate
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
PP1	Protein phosphatase 1

PP1cβ	Protein phosphatase-1 beta catalytic subunit
PPH	Primary Pulmonary hypertension
PTEN	Phosphatase and tensin homologue
R-SMAD	Regulatory SMAD
RT-qPCR	Quantitative real-time polymerase chain reaction
RTK	Receptor tyrosine kinase
S1P	Sphingosine 1-phosphate
SH2	Src-homology-2
SHC	Src-homology collagen
siRNA	Small interfering RNA
SMC	Smooth muscle cell
Ser/Thr	Serine/ Threonine
TGF-β	Transforming growth factor β
TIMAP	TGF-β-inhibited membrane associated protein
TKI	Tyrosine kinase inhibitor
TEER	Trans-endothelial electrical resistance
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VHL	von-Hippel Lindau
WB	Western blot
WT	Wild-type

Chapter 1

General introduction

1.1 Angiogenesis

Angiogenesis is defined as the process of new blood vessel formation from a pre-existent vasculature[1-3]. It enables gas exchange and appropriate nutrient and mediator supply to expanding tissues[1-3]. Angiogenesis is an essential process during growth and development and in numerous physiological and pathological conditions[4-7]. This process of neovascularization is accomplished by EC sprouting, proliferation, and vessel formation (sprouting angiogenesis) or by splitting of an existing blood vessel into two new vessels (splitting or intussusceptive angiogenesis)[8-10].

1.1.1 Sprouting angiogenesis

Sprouting angiogenesis involves the extension of EC sprouts from the pre-existing blood vessels[11-13]. The vascular endothelium, which lines the vasculature from the heart to the smaller blood vessels and capillaries, is normally quiescent with a low cell turnover[14, 15]. It plays a crucial role during sprouting angiogenesis[14-16]. In response to angiogenic stimuli, EC sprouting begins with a breakdown of EC intercellular junctions, vessel dilatation and basement membrane degradation[17-20]. The angiogenic stimuli then induce EC proliferation and migration to form tube-like structures of the new primitive vascular network[21-23]. The EC that will form the front of sprouts undergoes a phenotypic switch becoming motile tip cells with long thin finger-like cellular projections called filopodia which guide the developing vascular sprout through the extracellular matrix (ECM)[24-26]. Filopodia secrete proteolytic enzymes to degrade and invade the ECM and migrate toward the gradient of the angiogenic cues[27-29]. The migratory tip cells are

followed by proliferating EC cells known as stalk cells that form the body of the sprout[30-32]. While tip cells do not proliferate, stalk cells continue to proliferate to elongate the growing sprout and form the vascular lumen[29, 32, 33]. Finally, the recruitment of pericytes and smooth muscle cells and the production of a new ECM provide further vascular stability and maturation of the nascent blood vessels[34-36] (Figure-1.1).

1.1.2 Intussusceptive angiogenesis

Intussusceptive angiogenesis, also known as splitting or non-sprouting angiogenesis, is a process in which intravascular tissue pillars spread from the wall toward the lumen of a pre-existent blood vessel (intussusception), thus dividing the vessel into two new vessels[37-39]. The distinctive feature of intussusceptive angiogenesis is the formation of transmural tissue pillars, but the detailed mechanism of pillar formation is not fully understood[39]. Several studies speculate that the formation of intramural pillars is initiated by local degeneration of the basement membrane and inward extension of EC (inverse sprouting), creating a bridge between opposite vessel walls followed by infiltration of connective tissue cells and collagen deposition[39-41](Figure 1.2). Sprouting angiogenesis often precedes splitting angiogenesis, and together the two processes synergistically participate in the formation of a neovasculature in both physiological and pathological conditions[42-45]. Compared to the sprouting angiogenesis, intussusceptive angiogenesis is faster and more efficient and plays a central role in vascular network expansion by enhancing vascular branching and remodeling[46-48].

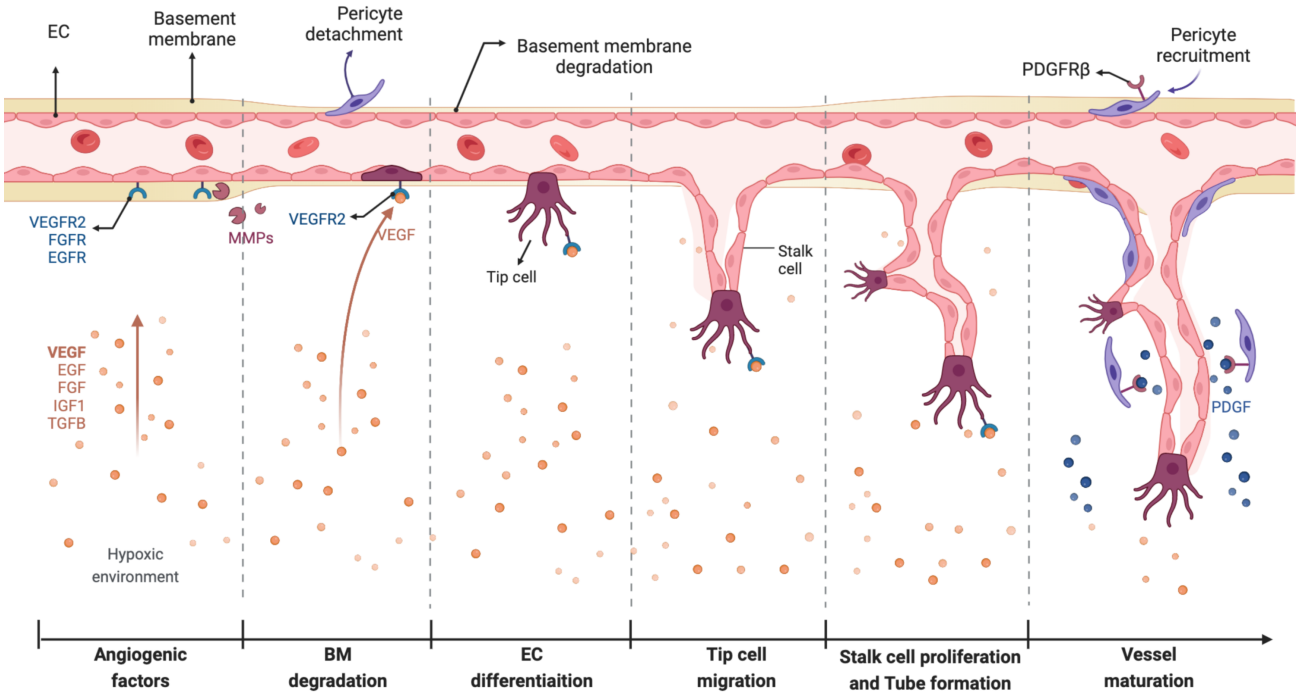

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Figure 1.1: Schematic representation of the process of sprouting angiogenesis

EC sprouting is initiated by detachment of pericytes, breakdown of the basement membrane and activation of EC in response to angiogenic stimuli. Activated EC differentiate into tip and stalk cells. Tip cells migrate toward a concentration gradient of angiogenic factors, whereas stalk cells proliferate to elongate the sprout. Stabilization and maturation of the newly formed vasculature are mediated by the recruitment of mural cells.

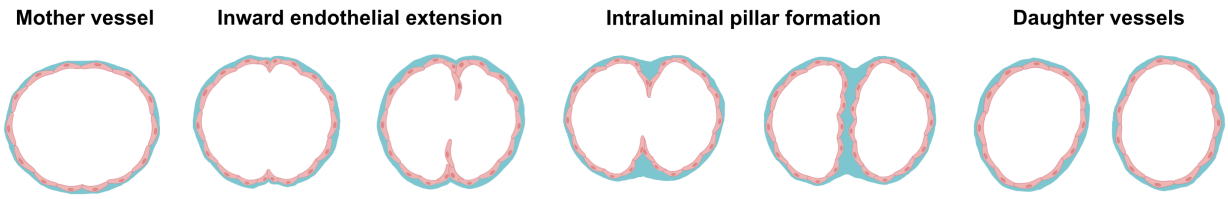

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Figure 1.2: Schematic diagram of the intussusceptive angiogenesis

Splitting angiogenesis is mediated by the development of intramural tissue pillars, which eventually divide the blood vessel into two new vessels. This process is initiated by local degradation of the basement membrane and intraluminal extension of the vascular endothelium, which is supported by connective tissue infiltration and collagen deposition.

1.2 Blood vessel maturation

The final step of angiogenesis involves stabilization and maturation of the newly formed immature blood vessels[35, 49, 50]. This involves recruitment of mural cells (pericytes and smooth muscle cells), synthesis of a new basement membrane and a surrounding matrix, as well as the establishment of tissue-specific characteristics of the EC[49-51]. This process is sophisticated and precisely regulated by multiple growth factors and signaling molecules which interact with one another to mediate vascular maturation[49]. Several studies have shown that platelet-derived growth factor subunit B (PDGFB) plays a critical role during vessel maturation[49-51]. During the later stages of angiogenesis, sprouting EC produce PDGFB to recruit PDGFRβ expressing mural cells[51]. Deletion of PDGFB or PDGFRβ in mice is embryonically lethal and associated with leaky, hemorrhagic, dilated vessels due to inadequate mural vascular coverage[51-53]. Similarly, injecting postnatal mouse pups with anti- PDGFRβ neutralizing antibodies impedes the recruitment of mural cells to the developing retinal vasculature[54]. The angiopoietin-Tie (Ang/Tie) signaling pathway also plays an important role in the later stages of angiogenesis and vascular remodeling[55]. The angiopoietins are family of growth factors which bind and stimulate phosphorylation of tyrosine kinase Tie receptors expressed primarily in EC[55, 56]. There are four identified angiopoietins, Ang1, Ang2, Ang3, and Ang4, which are all ligands for the Tie2 receptor[55]. Angiopoietins bind and activate the Tie2 receptor, which in turn forms a complex with the Tie1 receptor and results in the activation of downstream signaling cascades such as PI3-Kinase/AKT and ERK pathways[57]. Among the four known angiopoietins, Ang1 and Ang2 are the main angiopoietins involved in vascular stability and maturation[58]. Ang1 is primarily expressed by mural cells, and Ang1/Tie2 signaling promotes stabilization and maturation of the nascent vasculature[59, 60]. In contrast, Ang2

is almost exclusively expressed by EC and antagonizes Ang1-mediated Tie2 activation to mediate vessel destabilization and enhance angiogenesis[58, 60]. Genetic deletion of Ang1 or Tie2 is associated with severe hemorrhagic vascular defects due to significant reduction in mural vascular coverage and results in embryonic lethality[61, 62]. The endothelial differentiation sphingolipid G-protein-coupled receptor-1 (EDG1, S1PR), receptor for sphingosine-1-phosphate (S1P), is also crucial for vessel maturation since its deletion in mice results in a phenotype similar to that observed in Ang1/Tie2 knockout mice[63]. The S1P/EDG1 signaling is necessary for recruitment and activation of the cell-cell adhesion molecule N-cadherin and the consequent development of tight junction between EC and mural cells[64]. The EC-mural cell interaction results in the activation of the TGF-β pathway, which inhibits EC proliferation and migration, induces differentiation of EC, promotes differentiation of mesenchymal cells to mural cells, and stimulates the production and accumulation of the new basement membrane[51, 65, 66].

1.3 Tumor angiogenesis

Most tumors are characterized by uncontrolled and excessive cellular proliferation[67]. The rapid growth is associated with an increased metabolism and high oxygen demand[67]. Neovascularization is essential to ensure an adequate supply of oxygen, nutrients, and mediators to the growing tumor[68, 69]. As tumors continue to expand, cancer cells in the center become more and more hypoxic as they grow further away from the pre-existing organ's vasculature[70-72]. Hypoxic cancer cells stimulate several signaling cascades and release pro-angiogenic factors to promote angiogenesis to overcome the hypoxic microenvironment and continue to survive[71, 73]. However, due to the aggressive proliferative nature of the neoplastic cells and the associated overexpression of

angiogenic factors, tumor-induced vascularization is profoundly different from the vasculature produced by normal angiogenesis[50, 74]. Tumor blood vessels are morphologically abnormal, functionally immature and characterized by irregular branching[50, 75]. The stability and integrity of the developing vessels are provided by the recruitment and positioning of mural cells around EC junctions to tighten the gaps between ECs and the proper deposition of the basement membrane[65, 76, 77]. Tumor vessels are often improperly covered with mural cells, with poorly structured vascular basement membrane[50, 78, 79]. Lack of pericyte and smooth muscle cell coverage, and insufficient production and loose attachment of basement membrane, results in a tortuous, disorganized and hyperpermeable vasculature due to the formation of poorly aligned and loosely attached vascular EC monolayers[50, 79, 80].

1.4 Hypoxia-induced angiogenesis

Inadequate tissue oxygenation, termed hypoxia, is the fundamental drive for angiogenesis in many physiological and pathological conditions[81-83]. Rapidly proliferating tissues become hypoxic when they grow further away from the vasculature, and their oxygen demand exceeds their supply[82-84]. For example, during the early embryonic growth, a hypoxic tissue environment is necessary for adequate placental development[85-87]. Hypoxia-induced activation of angiogenesis is also involved in the pathogenesis and development of numerous health disorders[81 82, 88]. A low oxygen concentration activates the hypoxia-inducible factor (HIF) pathway, which, in turn, stimulates the expression of numerous angiogenic genes that initiate and propel the angiogenic process[89-91]. Under normal oxygen concentration, HIF-1α has a very short half-life of about 5 minutes, as it is continuously synthesized and then rapidly ubiquitinated and subsequently degraded at a

constant rate by the 26S proteasome[92, 93]. Oxygen abundance augments the hydroxylation of the HIF-1α at proline residues in its oxygen-dependent degradation (ODD) domain by oxygen-sensitive prolyl hydroxylases[89, 94]. This hydroxylation enhances the interaction of HIF-1α with the tumor suppressor Von Hippel Lindau protein which recognizes hydroxylated HIF-1α and enhances its proteasomal degradation[89, 94]. In the presence of normal oxygen levels, the transcriptional activity of HIF-1α is also inhibited by the oxygen-sensitive asparaginyl hydroxylase factor inhibiting HIF[94, 95]. Hydroxylation of HIF-1α further impedes its association with the CBP/P300 transcriptional coactivators, thereby inhibiting HIF-1α transactivation[94, 95]. Reduced oxygen levels diminish HIF-1α hydroxylation and reduce its association with the Von Hippel Lindau protein, markedly lowering its rate of degradation[92, 96]. Accumulated HIF-1α then translocates into the nucleus and dimerizes with its partner HIF-1β, which is non-oxygen sensitive, constitutively expressed and localized to the nucleus[92, 97, 98]. The resulting HIF-1 heterodimers recruit transcriptional coactivators and bind hypoxia-responsive elements to stimulate HIF-responsive gene promoters[92, 99] (Figure 1.3).

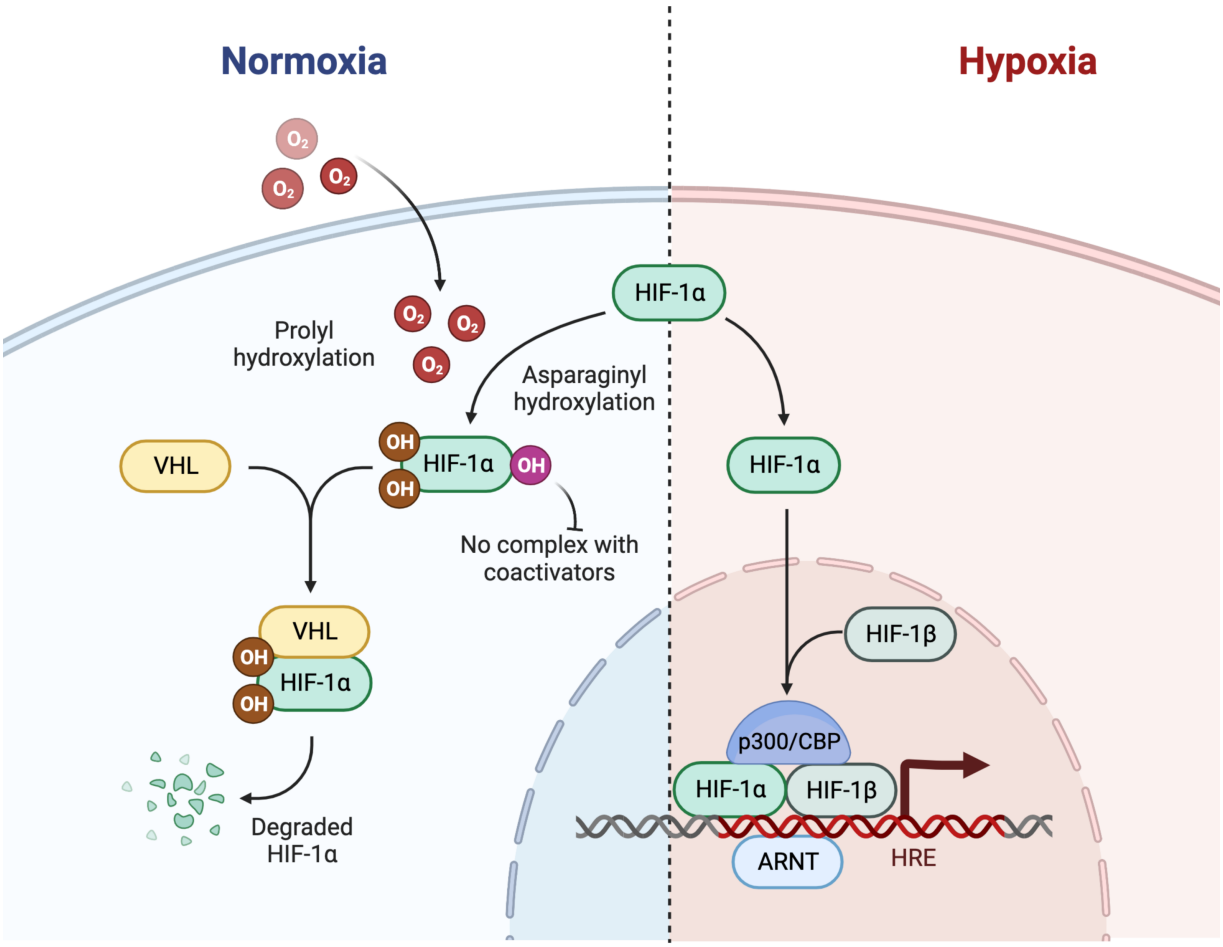

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Figure 1.3: Oxygen-dependent regulation of HIF-1α

When the oxygen concentration is normal, HIF-1α hydroxylation by the oxygen-sensitive prolyl hydroxylase mediates its interaction with Von Hippel Lindau protein which facilitates its rapid proteasomal degradation. Oxygen also augments asparaginyl hydroxylation of HIF-1α, which interferes with its binding to transcriptional coactivators. Reduction in the oxygen concentration impedes HIF-1α hydroxylation, reduces its degradation, and promotes its nuclear translocation. In the nucleus, HIF-1α forms a heterodimer with HIF-1β and binds the hypoxia-responsive element along with other coactivators to induce the transcription of target genes.

1.5 Angiogenic growth factors

The mechanisms by which angiogenesis expands the primitive embryonic or pre-existent vascular network to enhance blood supply to the developing and growing tissues and organs are extremely sophisticated and complex [100, 101]. Depending on the nature and stage of angiogenesis, the angiogenic process is mediated by various growth factors, cytokines and signaling molecules whose interplay regulates the proliferation and recruitment of different cell types involved in blood vessel formation and maturation[102, 103]. The proliferation of EC and the formation of angiogenic sprouts is a crucial first step during angiogenesis[30, 31]. A great variety of growth factors, their receptors and intracellular signaling pathways play a fundamental role to regulate EC proliferation and sprouting angiogenesis[104]. *In vitro* studies have revealed that the angiogenic growth factor vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), insulin-like growth factor-1 (IGF-1) and epidermal growth factor (EGF) are all required for EC survival and proliferation[105-107] and commonly used in endothelial growth media[108]. Although EC proliferation is essential during angiogenesis, EC apoptosis and induction of phenotypic changes also play a critical role in lumen formation and vascular remodeling and maturation[109-111]. In the sections that follow, several signaling cascades that regulate cellular proliferation, promote angiogenesis, and are relevant to the work in this thesis are described in detail[104, 112].

1.5.1 VEGF signaling pathway

The VEGF family of proteins consists of seven identified proteins which include VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and the placental growth factor (PlGF)[113]. VEGF binds its specific VEGF receptors, VEGFR1, VEGFR2, and VEGFR3,

which are expressed mainly by EC[114-116]. VEGFR1 and VEGFR2 are primarily expressed by the vascular endothelium, whereas VEGFR3 is predominantly expressed by lymphatic EC[116-118]. The VEGF is produced by numerous types of cells, including tumor cells[119], macrophages[120] and other blood cells[121], and its function is not restricted to the vascular system[122, 123]. The VEGFRs are transmembrane receptor tyrosine kinases that can be found as monomers or dimers, whereas VEGF is secreted as dimers formed by connecting two monomers by disulfide bounds and binds at least two (homomeric or heterodimeric) VEGFRs[115, 124]. Although VEGFR dimerization is required to initiate the VEGF signaling cascade, VEGF can bind a VEGFR monomer that subsequently recruits and dimerizes with another VEGFR monomer or binds to already available VEGFR dimers[124, 125]. The VEGF/VEGFR binding leads to receptor homodimerization or heterodimerization and results in transactivation and tyrosine autophosphorylation of the intracellular domain of the VEGFR and subsequent triggering of various downstream signaling cascades involved in the angiogenic program[115, 126, 127] (Figure-1.4).

VEGF is considered the most potent proangiogenic growth factor responsible for the majority of EC angiogenic responses during angiogenesis, such as vascular permeability and cellular proliferation, survival, and migration[127-129]. During angiogenesis, the angiogenic effects of other growth factors are partly mediated by enhancing the expression of VEGF[130-133]. Moreover, VEGF also stimulates the expression of other angiogenic growth factors and cytokines to potentiate the angiogenic process[134].

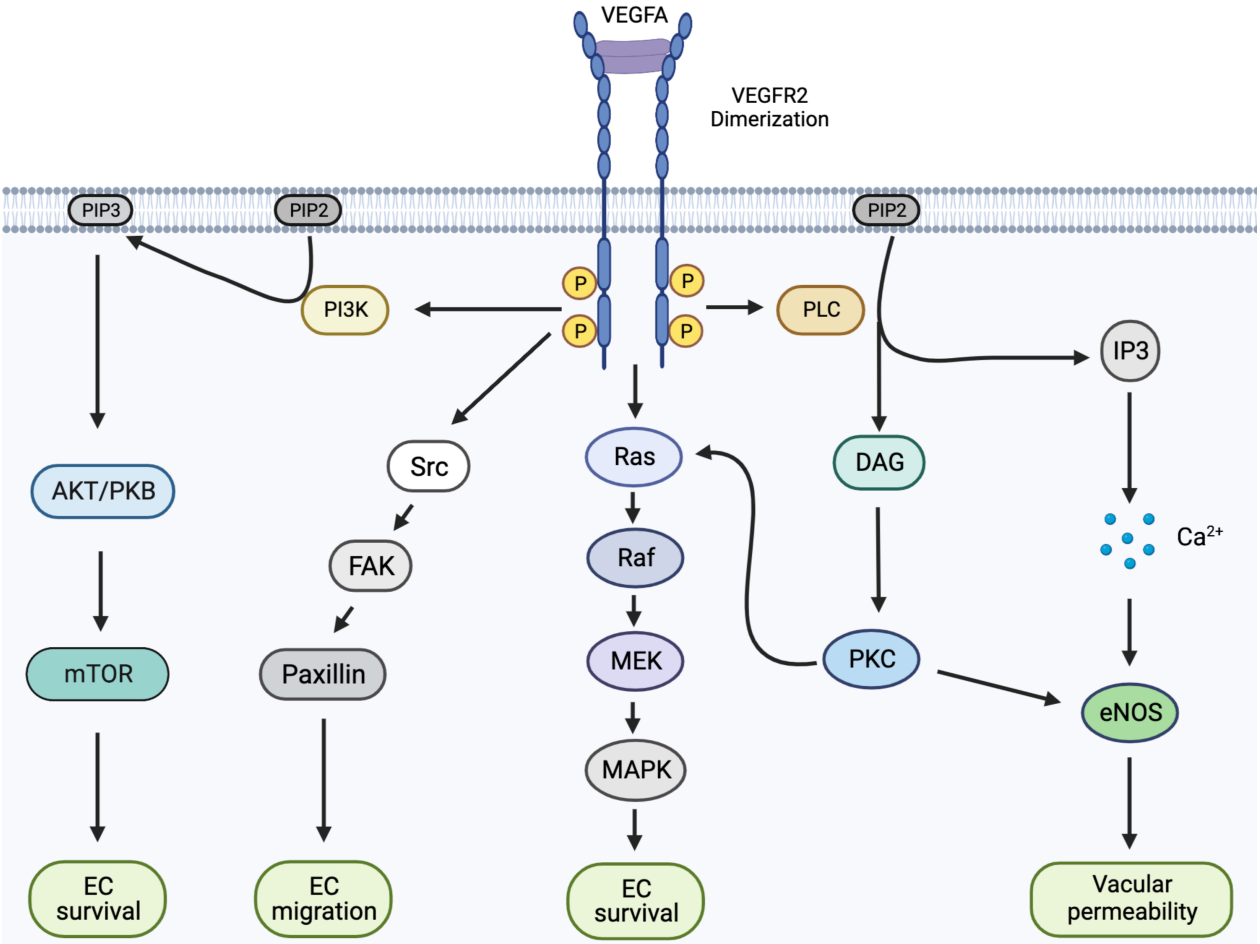


Figure 1.4: Schematic illustration of the VEGF signaling pathway

The binding of VEGF to its VEGFR2 induces receptor dimerization and autophosphorylation of specific tyrosine residues on the receptor. Several SH2-domain containing proteins are recruited, phosphorylated, and activated by binding the tyrosine phosphorylated residues on the receptor. This results in the activation of diverse downstream proliferative and survival signaling pathways to stimulate several EC responses that mediate angiogenesis, such as enhanced EC proliferation, survival, and migration, as well as increased vascular permeability.

Members of the VEGF family have different affinity and selectivity for each of the three types of the VEGFRs[126, 135-137]. Therefore, the VEGF signaling pathway is very sophisticated and has been shown to be involved in regulating various physiological functions as well as pathological disorders[123 127, 138]. The VEGFA, also called VEGF or vascular permeability factor, is the most potent and specific mitogen for the vascular EC that can bind VEGFR1 or VEGFR2 and its expression is markedly increased during the development of several types of tumors[139 68, 128]. Although VEGFR1 has a higher affinity for VEGF, VEGFR2 has higher kinase activity and stimulates a wide range of downstream signaling pathways required for angiogenesis[115, 140]. VEGFA/VEGFR2 signaling is, therefore, the most well-studied signaling pathway that mediates most of the EC responses involved in both physiological and pathological angiogenesis, including tumor angiogenesis[116, 127, 136, 138]. The expression of VEGF and its specific receptors is increased in many solid tumors which require angiogenesis, and its expression level is directly related to the tumor size and its degree of angiogenesis[68, 135, 136]. During tumorigenesis, hypoxia, the main driver of tumor angiogenesis, upregulates the expression of VEGF and its endothelial receptors through HIF-α stabilization and HIF pathway activation[138, 141, 142]. Mouse tumor models showed that using anti-VEGF antibodies normalizes tumor vasculature and results in a significant reduction in tumor size[136, 143].

Sprouting angiogenesis involves selection of tip and stalk EC, formation of angiogenic sprout, migration of tip cells and proliferation of stalk cells[144]. The proangiogenic VEGF gradient stimulates the phenotypic switch of the previously quiescent vascular EC into tip cells to initiate angiogenic sprouting[145]. Activated tip cells breakdown the vessel wall, degrade the basement membrane, develop long filopodia and acquire a migratory phenotype to invade tissues, lead and guide the endothelial sprout[28,

[144] The stalk cells are highly proliferative, follow the tip cells as they migrate, elongate the sprout, and establish adherent tight junctions to generate the nascent vascular lumen[28, 144, 146, 147]. Inhibiting EC VEGF signaling impairs formation of filopodia and reduces tip cell migration and sprout progression[28], whereas stimulating quiescent EC with VEGF induces filopodia formation and enhances tip cell migration and angiogenic sprouting[148].

The endothelial tip-stalk cell specification is not a permanent switch, but a dynamic process where EC compete and shuffle between tip and stalk cells[149]. During the process of angiogenesis and angiogenic sprouting, the development of new tip cells and occasionally converting some tip cells back into stalk cells or vice versa is required to enhance the branching and the anastomosis as the nascent vasculature expand[149, 150]. The tip cell selection depends on the expression level of VEGFRs on the EC surface, and cells with greater VEGFR2 and lesser VEGFR1 levels have more potential to switch into tip cells and maintain the leading positions[149]. The VEGF signaling pathway cooperates with the Notch signaling pathway in coordinating the balance of tip-stalk cell phenotypic switching during angiogenesis[144, 151-153].

The Notch signaling pathway is activated when cell surface Notch ligands interact with transmembrane Notch receptors expressed on the adjacent cells[154]. Notch ligand-receptor binding results in receptor proteolysis and release of the receptor's intracellular domains, which then translocate into the nucleus to regulate target gene expression[155-157]. There are five known Notch ligands that exist in two different families called Jagged (Jag-1 & 2) and Delta-like (Dll1, 2, & 4) ligands[158] and four transmembrane receptors (Notch1-4)[159]. Notch receptors 1 & 4 and four Notch ligands (Dll1 &4 and Jag 1& 2) are expressed by the vascular endothelium and play essential roles at various stages of vascular development[160].

During sprouting angiogenesis, Notch signaling forms a negative feedback loop with VEGF signaling to tightly regulate sprout tip-stalk cell selection[161]. VEGF stimulation enhances Dll4 expression in tip cells which, in turn, activates Notch signaling in stalk cells, reducing VEGFR2 and increasing VEGFR1 expression and consequently inhibiting its switch into tip cells and retaining stalk cell phenotype[144]. Reduced Notch signaling in tip cells, in contrast, results in higher expression of VEGFR2 and lower expression of VEGFR1 to maintain its tip cell phenotype[149]. Interestingly, stalk cells express high levels of Jag1, which competes with Dll4 for the Notch receptor binding and, therefore, antagonizes the Dll4-Notch signaling-mediated tip cell phenotype switch inhibition and indirectly promotes tip cell phenotype[162-164]. This Dll4-Jag Notch signaling needs to be tightly controlled to achieve normal angiogenesis characterized by a distinctive pattern of tip cells alternated by stalk cells and, therefore, proper branching points and appropriate vascular network formation[165-167]. However, how Dll4- and Jag1-mediated activation of Notch receptors is balanced and how the specificity of ligand-receptor signaling is synchronized is still under investigation[168].

1.5.2 IGF-1 signaling pathway

Insulin-like growth factor-1 (IGF-1), also known as somatomedin C, is a polypeptide hormone that consists of two distinct α & β chains linked together by disulfide bonds[169, 170]. IGF-1 is mainly produced by the liver but can also be secreted by numerous other tissues as well as tumors[171, 172]. The level of IGF-1 is mainly regulated by the growth hormone, which induces its expression and stimulates its release into the circulation[172, 173]. As much as 99% of IGF-1 in circulating blood is bound to insulin-like growth factor binding proteins (IGFBPs), and just a very small amount is found as an unbound form[174, 175]. Six distinct IGFBPs have been identified, though most of the IGF-1 in the circulation is bound to IGFBP-3[172, 175]. Free IGF-1 has a very short half-life (around 10 minutes) but binding of IGFBPs to the IGF-1 prevents its degradation and prolongs its half-life to 12-15 hours[173, 175]. However, since the unbound form of the IGF-1 is the only active molecule, its binding to IGFBPs reduces its biological bioavailability to the target tissues[173, 175]. The IGFBPs also function as transporter molecules that carry the IGF-1 to its recipient cells, and they regulate IGF-1 binding to cellular receptors[176, 177]. Depending on the subtype, IGFBPs either augment or impede the binding of IGF-1 to its specific receptors[172, 178]. The IGF-1 receptor (IGF-1R) is a heterotetrametric transmembrane receptor tyrosine kinase composed of 2 ligand binding α-subunits and 2 catalytic β-subunits[171, 179]. It is expressed by most cells, including EC[171, 172, 179]. Upon binding the IGF-1 ligand, the IGR-1R undergoes conformational changes and activation through autophosphorylation on multiple tyrosine residues in its β-chain[170, 173, 180]. Activated IGF-1R receptor recruits and Tyr-phosphorylates several adaptor proteins, including insulin receptor substrates (IRS1-4) and Src-homology collagen (SHC) proteins[173, 181, 182]. Phosphorylated tyrosine residues in these adaptor proteins are then recognized by Src-homology-2 (SH2) domain-

containing signaling molecules, which in turn, activate downstream signaling cascades[172, 173, 183]. These include the PI3K/Akt/mTOR and the mitogen-activated protein kinase (MAPK) pathways that inhibit apoptosis, promote survival, and stimulate cell proliferation, and migration[170, 172, 173, 183] (Figure-1.5).

Since IGF-1 is synthesized by many tissues and the IGF-1R is expressed by numerous cell types, it is evident that IGF-1 function is not restricted to the process of angiogenesis[172]. In fact, IGF-1R signaling is involved in regulating many biological processes and is dysregulated during the pathogenesis of several pathological disorders[184, 185]. Nonetheless, the IGF-1R is expressed in EC of both micro-and macro-vasculature, and the IGF-1/IGF-1R signaling pathway plays a major role during angiogenesis[171, 172]. In this regard, animal models have demonstrated that global or EC-specific deletion of IGF-1R is associated with disordered angiogenesis[186-188]. Also, antibody-targeted inactivation of the IGF-1R significantly reduced embryonic lung vascularization[189]. The pathological retinal neovascularization during the development of diabetic retinopathy is also associated with overexpression of the IGF-1 and EC-specific knockout of IGF-1R reduced retinopathy-associated neovascularization in mice[188]. During the development of various tumors, the expression of IGF-1 and its specific receptor is significantly upregulated and associated with poor prognosis, enhanced tumor progression and metastasis[177, 179, 182, 190, 191]. It has been shown that IGF-1 enhances tumor angiogenesis directly and indirectly by upregulating other angiogenic growth factors such as VEGF[131, 175, 192]. Inhibiting IGF-1R signaling using IGF-1 neutralizing antibodies or anti-IGR-1R specific antibodies that prevent ligand-receptor binding not only reduces VEGF-induced angiogenesis but also has VEGF-independent anti-angiogenic activity[193-195].

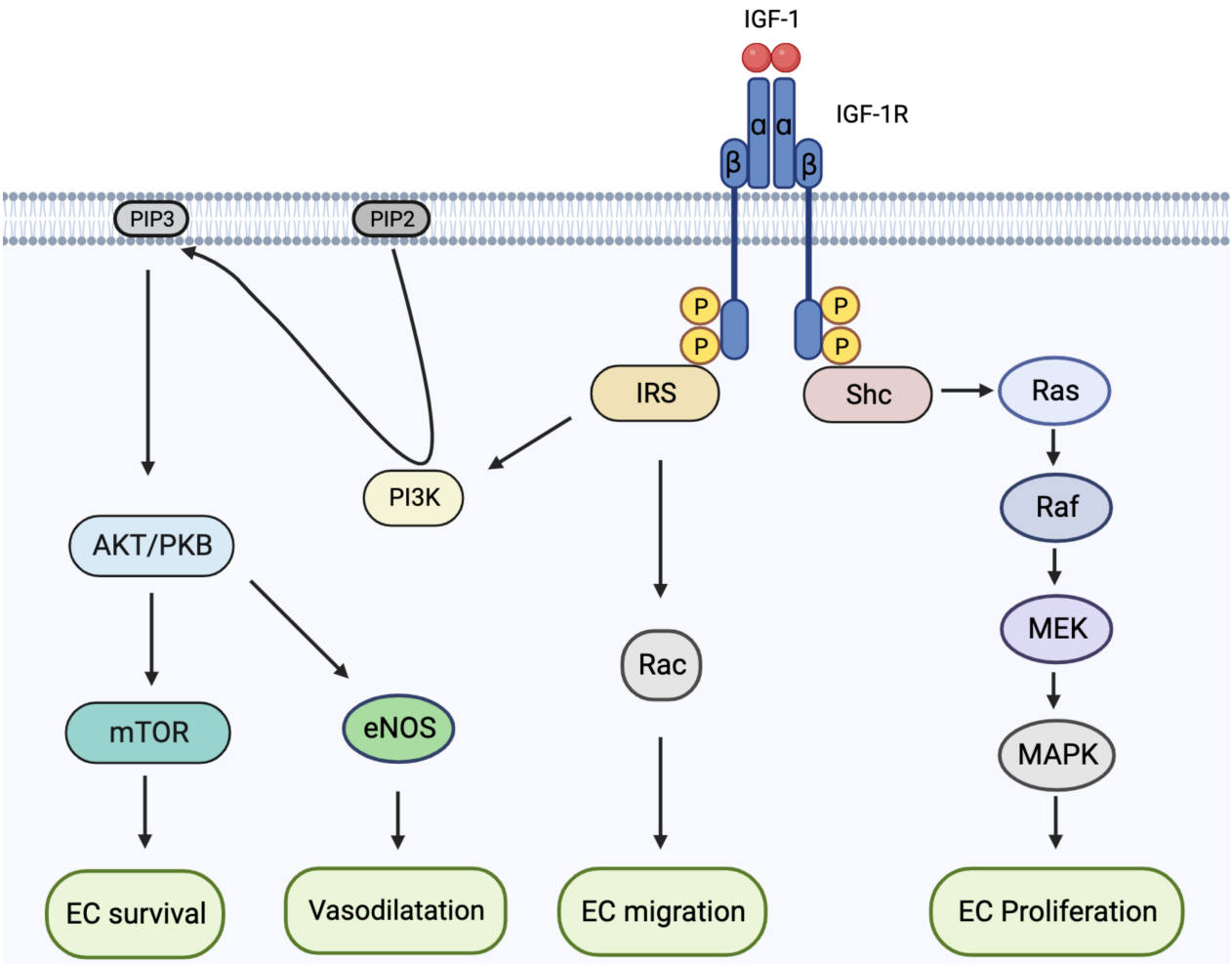


Figure 1.5: Schematic illustration of the IGF-1 signaling pathway

The binding of IGF-1 to its specific receptor tyrosine kinase IGF-1R triggers various downstream signaling pathways. Upon ligand binding, the IGF-1R undergoes autophosphorylation and subsequently stimulates the phosphorylation of multiple adaptor proteins, including the insulin receptor substrates (IRS) and the Src-homology collagen proteins (SHC). Once phosphorylated, adaptor proteins recruit and activate various signaling molecules, thereby initiating the activation of several survival and proliferative signaling pathways that support angiogenesis.

1.5.3 FGF signaling pathway

The FGF superfamily consists of 22 identified growth factors that exert their function by binding and activating their specific FGF receptors FGFR)[196-198]. Four signaling receptor tyrosine kinases (FGFR 1-4) have been identified in addition to the FGFR5, which lacks an intracellular tyrosine domain[199-201]. The FGFRs are monomeric transmembrane receptors, each with an extracellular ligand binding domain, a transmembrane anchoring domain, and an intracellular catalytic domain[202-204]. Unlike other dimeric growth factors, the FGFs are monomeric proteins that require cofactors to bind and activate their cognate receptors[205-207]. Both FGF ligands and the FGFRs have heparan sulfate (HS) or Klotho protein binding motifs that bind cell surface heparan sulfate proteoglycans (HSPGs) or Klotho protein coreceptors, respectively[208-210] The presence of these coreceptors stabilizes the ligand-receptor complex, modulates the FGF signaling, localizes their effect to specific target cells and protects FGF from proteolytic degradation[211-214]. Binding two FGF molecules to their specific tyrosine kinase receptors induces receptor dimerization and results in autophosphorylation of specific tyrosine residues of the intracellular receptor domains[203, 215]. These subsequently recruit and activate several downstream signaling protein[203, 216]. The activated FGFR also tyrosine phosphorylates a number of adaptor proteins that serve as docking sites to recruit and activate additional signaling pathways[203, 217] (Figure-1.6). Activation of the FGFR transduces signaling cascades predominantly through the Ras/Raf-MEK-MAPK, PI3K/Akt, and the PLCγ pathways[208, 218].

The FGFs and their specific receptors are expressed by many different cell types, and the FGF/FGFR signaling plays a fundamental role in regulating a wide range of cellular functions involved in several physiological and pathological conditions[207, 218]. The FGFs

are also angiogenic molecules that play essential roles during vascular development and angiogenesis by mediating proliferation, migration, and differentiation of vascular endothelium[219-222]. EC express several members of the FGF family, but angiogenesis is mainly mediated by the acidic (aFGF or FGF1) and basic (bFGF or FGF2)[223-225]. Similarly, EC express multiple subtypes of FGFRs, but the angiogenic response is mostly mediated by FGFR1 and FGFR2[226-228]. Genetic deletion of FGFR1 or FGFR2 in mice results in embryonic lethality, and studies have revealed that these receptors play critical roles in the later stages of the vascular development and angiogenesis[229-232]. The FGF/FGFR signaling pathway also plays an essential role during the development, growth, and progression of many tumors, in part by enhancing tumor angiogenesis[233-235]. During the development of certain tumors, the expression of FGF and its receptors is significantly upregulated and is required for appropriate tumor vascularization[236-238]. There is also an interplay between FGF and VEGF during angiogenesis[239-241]. In this regard, FGF enhances EC VEGF expression, promoting tumor angiogenesis[239-243]. Moreover, selective FGF inhibition impedes tumor growth and angiogenesis and augments the antiangiogenic efficacy of VEGF inhibitors[244].

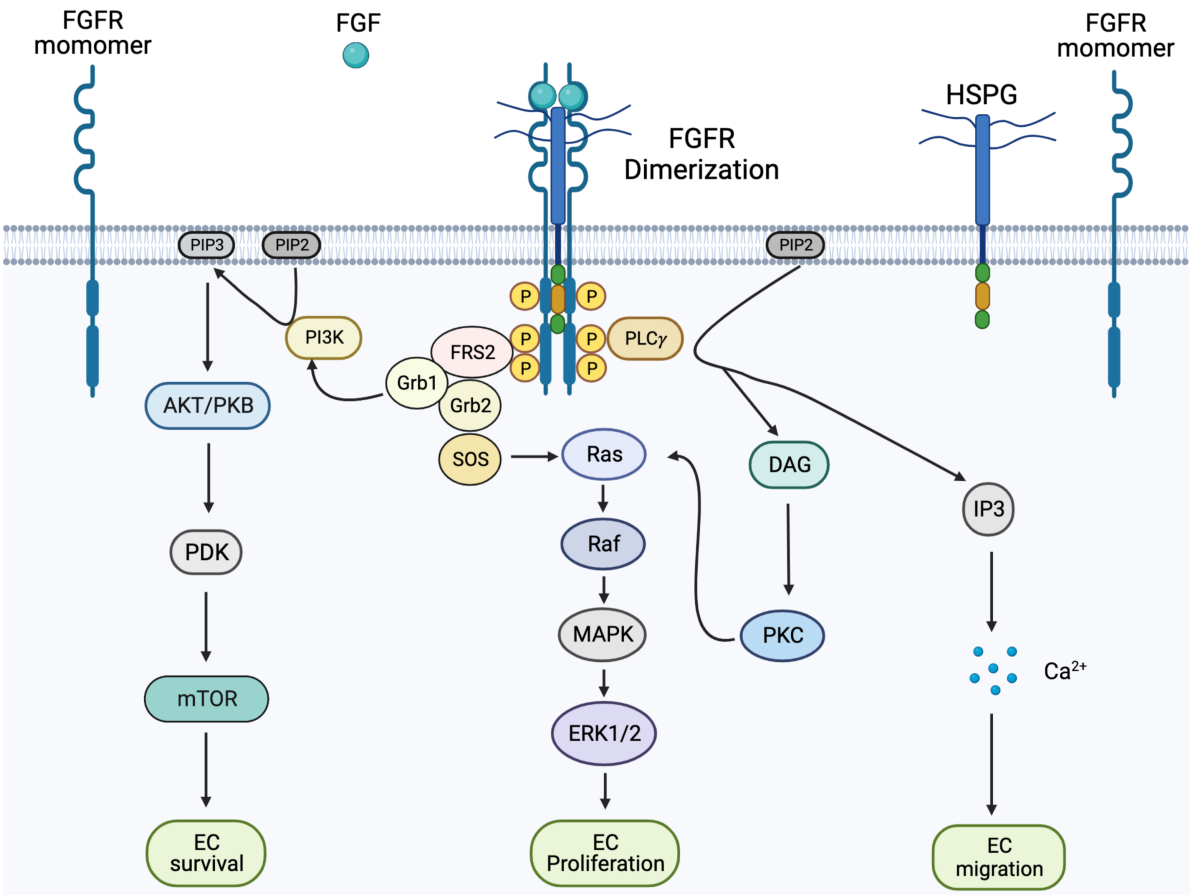


Figure 1.6: Schematic illustration of the FGF signaling pathway

HSPG-mediated binding of FGF to its specific receptor induces receptor homodimerization and autophosphorylation of multiple specific tyrosine residues in the cytoplasmic domain. Activated FGFR stimulates the phosphorylation and activation of several downstream signaling molecules. FGFR activation also recruits and phosphorylates various adaptor proteins, which stimulate additional signaling molecules and activate several signaling pathways involved in cell proliferation, survival, and migration.

1.5.4 EGF signaling pathway

Epidermal growth factor (EGF) is one member of the EGF superfamily of peptide growth factors that regulate many essential biological functions involved in cellular growth and development[245-247]. EGF is a potent mitogen that regulates cellular growth, proliferation, survival, migration, and differentiation[248-251]. It is produced by several cell types and exerts its function by binding to specific EGF receptors (EGFR), also expressed by many different cells[252-254]. The EGF ligands are synthesized as precursor proteins embedded in the plasma membrane[255-257]. Proteolysis by metalloproteases then releases soluble EGF molecules to bind their specific receptors[255, 256, 258]. The EGFR, also called ErbB1 or HER1, is a member of the larger ErbB/HER family of receptor tyrosine kinases that share a similar structure and can bind multiple members of the EGF family of proteins[257, 259-261]. These receptors are membrane-bound glycoproteins comprised of an extracellular ligand-binding domain, a membrane-spanning domain, and a cytoplasmic domain with intrinsic tyrosine kinase activity[262-264]. Ligand binding to the EGFR induces conformational modifications of its extracellular domain, causing receptor homo- or heterodimerization with additional EGFR[215, 265-267]. Receptor dimerization then triggers autophosphorylation of multiple tyrosine residues in the cytoplasmic domain that become docking sites resulting in the recruitment and phosphorylation of multiple adaptor proteins[215, 268, 269]. These then activate downstream signaling cascades to regulate cellular responses[270-272]. The nature of the signaling cascades initiated by the activated EGFR depends on the type of the adaptor proteins, and the signaling molecules recruited to the phosphorylated intracellular domain tyrosine residues[247, 273, 274]. Similar to the other receptor tyrosine kinases, activation of the EGF/EGFR signaling results in the initiation of proliferative and survival signaling cascades, including the Ras/Raf/MAPK, PI3K/AKT,

and PLCγ pathways[275-277]. The activated EGFR can also translocate into the nucleus, where it binds specific promotor regions and function as a transcription cofactor inducing the expression of various genes[278-281] (Figure 1.7).

The EFGR is considered to be a proto-oncogene whose expression is often upregulated during tumor development[282-286]. Gain of function mutations in the EGFR encoding genes results in overproduction of EGFR and EGF/EGFR hyper-signaling with subsequent overactivation of the downstream proliferative signaling pathways that promote tumor development and progression[287-289]. Although the EGF is not a potent angiogenic growth factor, activated EGFR signaling during tumorigenesis enhances tumor angiogenesis by increasing VEGF secretion in the tumor microenvironment[105, 290-292]. Studies have revealed that the level of EGFR expression is directly correlated with advanced tumor stage and invasiveness, the level of distant metastatic spread, and the risk of recurrence and predicts poor patient survival[293-296]. Selective tyrosine kinase inhibitors and monoclonal antibodies that target EGFR attenuate its downstream signal transduction and inhibit tumor growth and proliferation[297-299]. Unfortunately, the efficacy of these inhibitors is not sustainable as most tumors develop resistance and escape the targeted receptor inhibition[300-302]. Drug resistance usually develops as a result of acquired EGFR mutations and genetic alterations that reduce the binding of inhibitors to the target receptors[303-305]. The development of new mutations in the EGFR downstream signaling molecules can also produce EGF independent activation, contributing to the development of treatment resistance to the EGFR inhibitors[306-308]. The expression level and the signaling activity of other receptor tyrosine kinases can also increase during the development of resistance to EGFR target inhibition[309-311]. Activation of such alternative receptor tyrosine kinases then stimulates proliferative pathways and reduces the

dependence of tumor cells on EGFR-mediated signaling[310-312]. This explains the complexity of the tyrosine kinase receptor signaling and indicates the need for inhibitors that target multiple key signaling molecules downstream of the growth factor receptors.

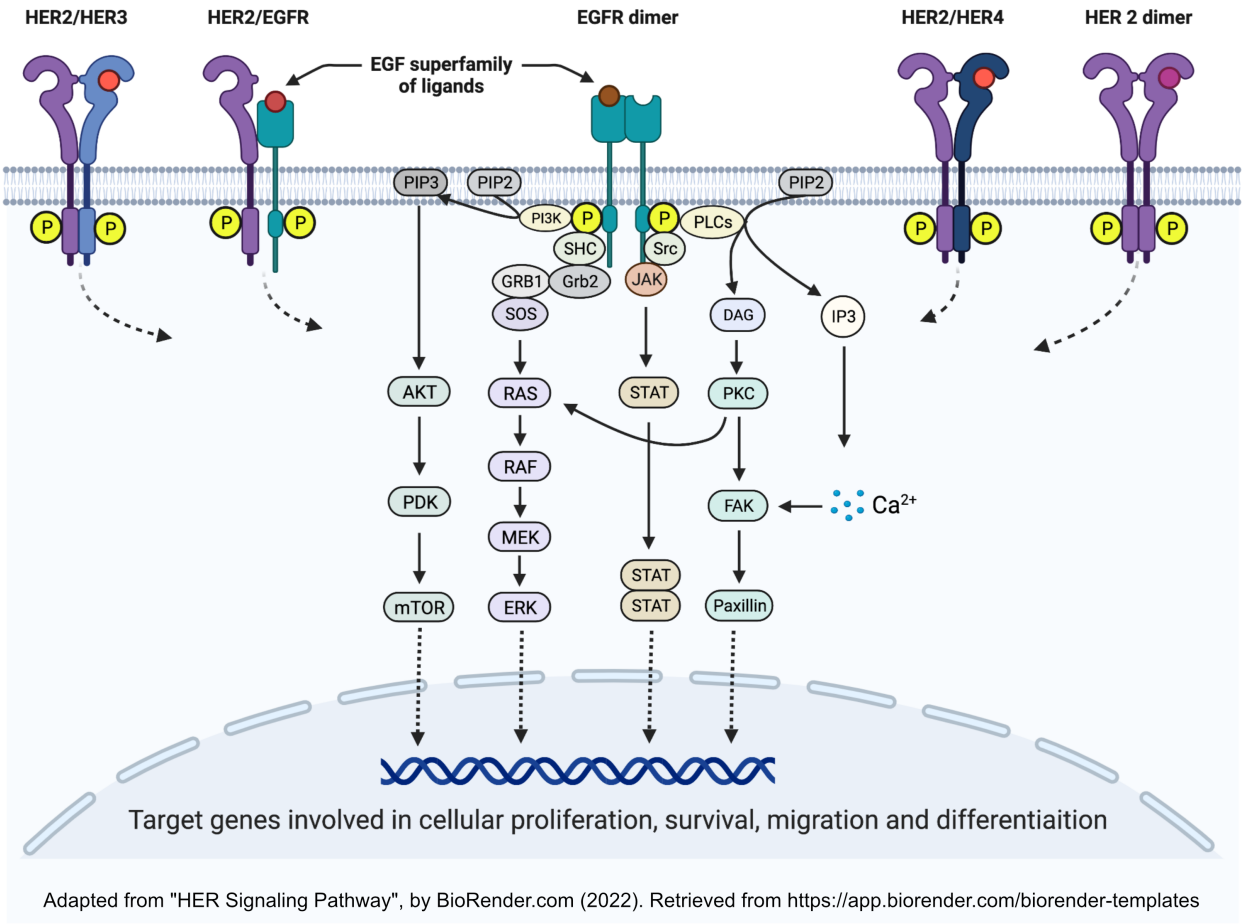


Figure 1.7: Schematic illustration of the EGFR signaling pathway

The binding of the EGF superfamily of ligands to their cognate EGF receptor family induces a conformational change in the receptor extracellular domain and facilitates its dimerization with another EGFR. The EGF family of receptors can be activated by multiple ligands in the EGF superfamily, and the ligand/receptor binding induces receptor homo- or heterodimerization and subsequent autophosphorylation of its internal domain tyrosine residues. Phosphorylated tyrosine residues serve as docking sites to recruit and activate multiple adaptor proteins. Activation of these adaptor proteins phosphorylates various signaling molecules and triggers several downstream signaling pathways.

1.6 PI3K/AKT signaling pathway

Cellular responses to growth factors are mediated by activation of cell surface tyrosine kinase receptors which transmit the signals to the nucleus to regulate the expression of a wide variety of target genes[313-315]. Binding of growth factors to their specific tyrosine kinase receptors induces receptor dimerization, autophosphorylation and activation of their intracellular kinase domains[316-318]. Through the phosphorylation of multiple tyrosine residues, each receptor tyrosine kinase (RTK) can stimulate various intracellular signaling cascades[216, 319]. However, different RTKs also utilize identical signaling pathways to modulate many cell functions in response to growth factors[320-322]. The PI3K/Akt signaling pathway is one of the most important intracellular signaling cascades triggered downstream of activated tyrosine kinase receptors[323-325]. PI3 kinases are plasma membrane-associated lipid kinases that regulate a wide variety of cellular processes[326-328]. PI3K/Akt is a major angiogenic signaling pathway that plays an essential role in normal vascular growth and development[329-331]. There are 3 classes of PI3K (Class I, II and III), with class I being the most well-studied PI3K isoform involved in vascular biology[332-334]. Class I PI3K is heterodimeric protein that consists of the catalytic p110 subunit bound to the regulatory p85 subunit[335-337]. Upon tyrosine kinase receptor activation, the regulatory subunit of PI3K binds directly to the phosphorylated tyrosine receptors causing activation of the P110 catalytic subunit[338-340]. Activated PI3Ks then convert phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3, 4, 5-trisphosphate (PIP3), which recruits protein effectors to the plasma membrane resulting in activation of multiple downstream signaling cascades[341-343]. PIP3 recruits and activates Pleckstrin homology (PH) containing proteins such as the Ser/Thr protein kinase B (PKB/AKT) and its activating phosphoinositide-dependent kinase 1 (PDK1)[344-346]. The

binding of PIP3 to AKT frees its kinase domain and enables PDK1-mediated AKT phosphorylation at Thr 308[347-349]. Additional phosphorylation at Ser 473 residue by the mammalian target of rapamycin complex 2 (mTORC2) results in the full activation of AKT[347, 350]. During angiogenesis, the activated AKT stimulates the phosphorylation of diverse downstream proteins involved in EC proliferation, survival, and migration, as well as tip cell differentiation[351-353]. The PI3K/AKT pathway is also involved in the regulation of the endothelial barrier function and junctional integrity[354-356]. Genetic deletion of the PI3K p110 catalytic subunit is associated with impaired angiogenesis and results in embryonic lethality due to severe vascular defects[357]. In tumors, VEGF-mediated PI3K/AKT pathway activation is the major signaling event that drives angiogenesis[358-360]. Genetic deletion or pharmacological inhibition of the PI3K p110 subunit in mice significantly reduces tumor growth and vascularization[361-364].

The activity of the PI3K/Akt pathway signaling is also regulated by the tumor suppressor phosphatase and tensin homologue (PTEN), which dephosphorylates PIP3 to PIP2[365-367] (Figure1.6). PTEN, therefore, restricts over activation of PI3K downstream signaling, and inhibition of PTEN results in EC hyperproliferation, disordered angiogenesis, and impaired vascular maturitation[368-370]. Homozygous PTEN knockout in mice results in early embryonic lethality, and its EC-specific heterozygous deletion enhances tumor growth and angiogenesis[371-373]. In humans, loss-of-function mutations in PTEN result in the development of Cowden syndrome, a rare genetic disorder that causes the growth of hamartomas and increases the risk of developing malignant tumors[374-376].

One of the mechanisms that regulate the activity of PTEN is the phosphorylation of specific Ser/Thr residues in its C-terminus[377-379]. Phosphorylation on these sites by specific kinases controls localization, molecular interactions and proteasomal degradation

of PTEN[380-382]. It turns out that TIMAP is one of the critical regulators of PTEN phosphorylation in EC[383]. TIMAP promotes EC proliferation and angiogenesis by augmenting the growth factor-stimulated AKT activation, and this effect is mediated at least partly through PTEN inhibition[383]. TIMAP enhances the S370 PTEN inhibitory phosphorylation[383]. Since PTEN reduces AKT and PDK1 recruitment to the plasma membrane by converting PIP3 to PIP2, it follows that inhibition of PTEN by TIMAP increases AKT phosphorylation and activity by increasing the availability of PIP3 at the EC membrane[383, 384] (Figure 1.8).

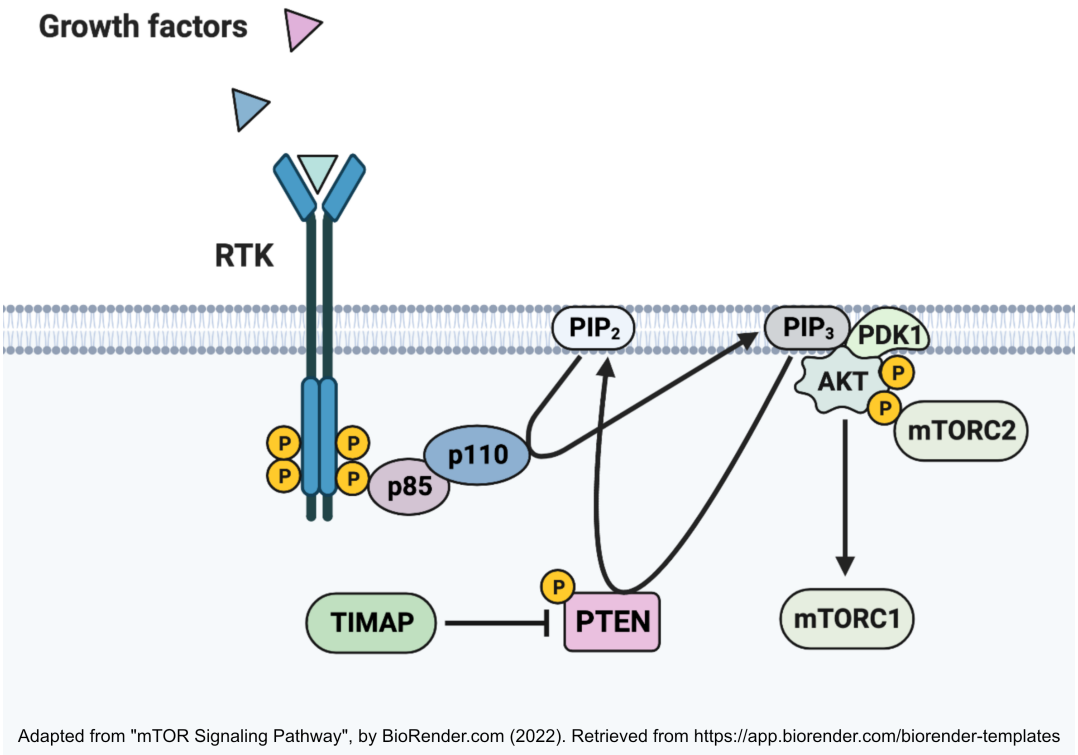


Figure 1.8: Schematic illustration of the PI3K/AKT signaling pathway

The binding of growth factors to their specific transmembrane tyrosine kinase receptors results in the recruitment and activation of PI3K, which then converts PIP2 to PIP3 at the plasma membrane. PIP3 localizes AKT and its activating kinase PDK1 to the cell membrane, thus facilitating the phosphorylation of AKT by PDK1. Additional phosphorylation of AKT is mediated by mTORC2, which results in the complete activation of AKT. The activated AKT then stimulates mTORC1 and triggers various downstream survival pathways. PTEN negatively regulates the activity of the PI3K/AKT signaling pathway by reconverting PIP3 to PIP2.

1.7 Growth factor receptors crosstalk during angiogenesis

Angiogenesis is a complex multistep process accomplished by synergistic and tightly regulated interplay between diverse array of signaling cascades that work coordinately to mediate various cellular responses involved in the different stages of the angiogenic process[385-387]. Numerous angiogenic growth factors and receptors play fundamental roles in initiating and supporting the whole process of angiogenesis, from starting the formation of the initial sprout to cellular proliferation to mature blood vessel formation[102, 388, 389]. As already described, growth factors mediate their angiogenic responses by binding and activating cell surface tyrosine kinase receptors to transduce signals and trigger multiple downstream proliferative and survival pathways[390-392]. It is important to recognize that signaling molecules during *in vivo* angiogenesis are not isolated, but instead, they interact with each other, and form sophisticated signaling networks[66, 314, 393, 394]. Activation of similar signaling molecules by distinct growth factor receptors facilitates the interconnection between their downstream signaling pathways[395-397]. Despite the apparent similarity in signaling responses to different growth factors at each stage of angiogenesis, the expression level of distinct growth factors varies, as does the magnitude and duration of each stimulus[313, 398, 399]. While VEGF is the main and probably the most potent and well-studied angiogenic growth factor that regulates most aspects of the angiogenic process, other growth factors can stimulate angiogenesis independently[122, 138, 145]. However, it turns out that their angiogenic activity also depends on enhancing the expression and angiogenic activity of the VEGF[130, 400, 401]. Reduction in the activity or the expression of VEGF or its receptor can be compensated for by increased expression and activity of alternative growth factors[402, 403], and growth factor receptors

can augment the activity of other tyrosine kinase receptors by enhancing the production of their own ligands or inducing the expression of their own downstream signaling effectors[404]. Therefore, crosstalk between growth factor receptors provides robustness of their angiogenic activity, and the presence of alternative pathways offers a backup reserve if there is dysfunction of one of the pathways[403, 405]. This redundancy explains one of the mechanisms of drug resistance in anti-angiogenic cancer treatment where tumors overcome target inhibition of selective growth factor receptors by over-activating alternative growth factor signaling pathways[406, 407].

1.8 Therapeutic growth factor inhibitors

Dysregulated angiogenic growth factor signaling pathways are associated with various diseases, for instance, diabetes, macular degeneration and particularly cancer[408, 409]. During the development of rapidly growing tumors, VEGF and other angiogenic growth factors provide angiogenic cues for tumor vascularization[69, 410, 411]. The growth of most tumors is associated with enhanced VEGF expression by the tumor and stromal cells, and inhibition of tumor angiogenesis by suppressing VEGF-signaling can powerfully impede tumor growth[412-415]. Due to the significance of VEGF signaling in cancer growth and development, various drugs that target VEGF signaling pathway have been approved to treat different types of tumors[416-418]. The principal aim of angiogenic inhibitors is to impair tumor growth by blocking the development of new tumor blood vessels[419-421]. Animal tumor models revealed that angiogenesis inhibitors deter tumor growth by reducing tumor vascular density[422-424]. Although the inhibitors of angiogenesis have evident clinical benefits, the response to therapy is usually not durable as tumors develop drug resistance[425-427]. It has been shown that

tumors escape selective angiogenesis inhibitors by upregulating the production of alternative pro-angiogenic growth factors[428, 429]. For instance, treating tumors with VEGF inhibitors induces the expression of different alternative pro-angiogenic growth factors, such as FGF and PDGF, to reactivate the angiogenic response and resume tumor growth[403, 407, 430]. Indeed, elevated serum levels of other growth factors are found in cancer patients receiving anti-VEGF treatment[431, 432]. Studies showed that in various tumor types, the VEGF-independent tumor angiogenesis could be mediated by different growth factors and combining the anti-VEGF therapy with other growth factor inhibitors can enhance the treatment efficacy[433-435]. Therefore, identifying and targeting pro-angiogenic growth factors and effectors that are upregulated upon VEGF inhibition are being studied to improve the efficiency of anti-angiogenic therapy[433-435].

1.9 Transforming growth factor (TGF)-β superfamily

Signaling via TGF-β superfamily pathways is critical for the maturation of new blood vessels and for maintaining the quiescence of EC[436]. Removal of such quiescent-inducing signals also plays an important role in facilitating angiogenesis[436]. The TGF-β superfamily consists of several ligands that bind and activate heteromeric Ser/Thr kinase receptor[437-439]. In addition to the TGF-β group of ligands, the TGF-β superfamily also includes other diverse regulatory proteins such as bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), activins, inhibins and anti-Mullerian hormone (AMH)[440-442]. All TGF-β superfamily ligands are produced as dimeric latent precursor proteins that must be cleaved by proteases and secreted before they can function as active ligands[443-445]. Despite the great variety of TGF-β ligands, there are only three classes of TGF-β receptors, namely the type-I TGF-β receptors (TβRI), the ligand-binding type-II TGF-β

receptors (TβRII) and auxiliary type-III receptors (TβRIII)[446-448]. In mammals, there are seven distinct TβRI, also called activin-like kinases (ALK1-7), and five types of TβRII in addition to the coreceptors endoglin and betaglycan are known[449-451] (Table-1). TβRI and TβRII are structurally related, each consisting of an extracellular N-terminal ligand binding domain, a transmembrane region and cytoplasmic C-terminal Ser/Thr kinase domain[451]. The accessory TGF-β coreceptors endoglin and betaglycan are transmembrane glycoproteins, each consisting of a large ectodomain, a transmembrane region and short non-catalytic cytoplasmic tail, and function to potentiate ligand/receptor binding and subsequent TGF-β pathway signaling[452, 453]. Endoglin and ALK1 are predominantly expressed by EC, while other TGF-β receptors are expressed by many other cell types [454-456]. To initiate signaling, TGF-β superfamily ligands bind the constitutively active type-II receptors, which then dimerize with type-I receptors and induce their phosphorylation[457, 458]. Together, they form active signalling heterodimers with Ser/Thr kinase activity[440, 457, 458]. Each TGF-β superfamily member binds a unique combination of TβRI and TβRII, and the activated ligand-receptor complex stimulates phosphorylation and activation of the downstream receptor-regulated SMADs (R-SMADs) SMAD1/5/8 or SMAD2/3[459-462] (Table-2). Phosphorylated R-SMADs form a complex with the common mediator SMAD (SMAD4/Co-SMAD) and translocate into the nucleus, where they regulate the transcriptional activity of target genes[461, 463].

After TGF-β stimulation, the inhibitory SMAD6 and SMAD7 (I-SMADs) are rapidly upregulated and negatively regulate the TGF-β-induced SMAD-mediated signaling[464-466]. I-SMADs compete with R-SMADs for the TβRI and Co-SMAD, thus reducing R-SMAD activation and nuclear translocation[467, 468]. The binding of I-SMADs to TβRI and other

SMADs also enhances their ubiquitination and subsequent proteasomal degradation[469-471].

In EC, TGF-β superfamily ligands activate the two distinct pathways via ALK1/SMAD1/5/8 and ALK5/ SMAD2/3 pathways, and the signalling equilibrium between these two pathways regulates EC behaviour during angiogenesis[472-474]. The net outcome of the TGF-β signaling pathway during angiogenesis depends on multiple factors, such as the specific ligands, their concentration, and the nature and stage of angiogenesis [475-477]. In addition to the SMAD (canonical) pathway, the TGF-β superfamily of ligands can also transduce signaling through SMAD-independent (non-canonical) pathways to regulate a wider range of cellular functions[478-481] (Figure 1.9). Although the TGF-β receptors are well-known as Ser/Thr kinases, they can also be phosphorylated on specific tyrosine residues, which serve as docking sites to recruit and activate tyrosine kinase signaling molecules[482-484]. Among the non-SMAD pathways that can be activated by TGF-β receptors are the PI3K/AKT and MAPK pathways[485-487]. Interestingly, SMAD phosphorylation and activation are also regulated by other signaling pathways, for instance, PKC, ERK and other enzymes involved in the MAPK pathway[488-490]. This crosstalk between SMADs and other signaling molecules enhances the diversification and versatility of TGF-β cellular responses[491-493].

Table 1 TGF-β superfamily of receptors

TGF-βRI Activin-like kinases	TGF-βRII	TGF-βRII
ALK1 (ACVRL1)	TGF-βRII	Endoglin
ALK2 (ACVR1/ACTR-1)	BMPRII	Betaglycan
ALK3	ACTRIIA	
ALK4	ACTRIIB	
ALK5	AMHRII	
ALK6		
ALK7 (ACVR1C)		

Table 2 TGF-β superfamily ligand/receptor complexes and their R-SMAD pathways

Ligand	TGF-βRI	TGF-βRII	R-SMADS
TGF-β	ALK1 ALK2	TGF-βRII	SMAD1/5/8
	ALK5		SMAD2/3
BMP	ALK1 ALK2 ALK3 ALK6	BMPRII	SMAD1/5/8
	ALK2	ACTRIIA/B	SMAD1/5/8
	ALK4 ALK5 ALK7		SMAD2/3
Activin/inhibin	ALK2	ACTRIIA/B	SMAD1/5/8
	ALK4		SMAD2/3
GDFs	ALK6	ACTRIIB	SMAD1/5/8
	ALK4 ALK5		SMAD2/3
	ALK6	BMPRII	SMAD1/5/8
	ALK5		SMAD2/3
AMH	ALK2 ALK3 ALK6	AMHR1	SMAD1/5/8

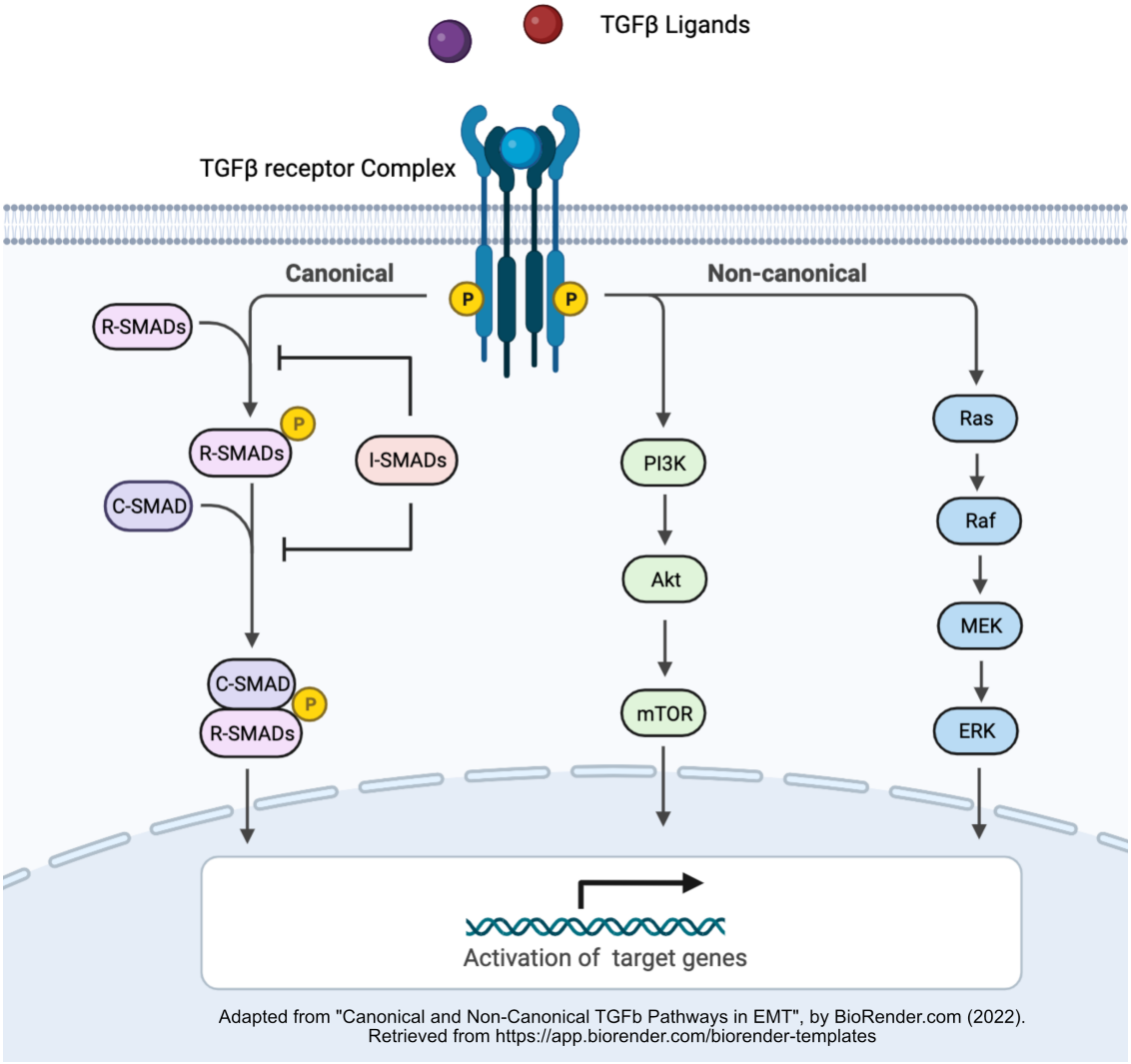


Figure 1.9: Canonical and non-canonical TGF-β signaling pathways

Ligand-induced phosphorylation of TGF-β receptor Ser/Thr residues triggers the phosphorylation of R-SMADs, which subsequently bind the Co-SMAD and translocate into the nucleus to induce transcription of target genes (Canonical pathway). The I-SMADS reduces R-SMAD activation and nuclear translocation. Phosphorylation of TGF-β receptor tyrosine residues recruits and activates several tyrosine kinase signaling molecules and triggers proliferative and survival pathways, including MAPK and PI3K/AKT (Non-canonical pathway).

1.9.1 TGF-β Signaling pathway

TGF-β family of signaling proteins are members of the broad TGF-β superfamily of multifunctional peptides and cytokines that share similar structure and function[494-496]. The TGF-β protein ligands and their receptors are involved in diverse cellular processes, including metabolism, proliferation, migration, apoptosis, and differentiation and their signaling dysfunction is associated with the development of a wide variety of diseases, including vascular disorders and cancer[497-501]. In mammals, three isoforms of TGF-β have been identified, including TGF-β1, TGF-β2, and TGF-β3[502-504]. Their sequences are ~80% conserved at the amino acid level, and they signal through the same TGF-β receptor complexes[505-507]. Despite their similarity, genetic deletion of each specific TGF-β isoform shows that they are non-redundant, and each results in embryonic lethality[508-513]. Furthermore, there are intrinsic functional variations between these isoforms with different and sometimes opposing biological effects[505, 514, 515]. TGF-β ligands have high affinity for TβRII which forms a complex with TβRI and TβRIII but do not directly interact with TβRI[516]. In most cells, ALK5 is highly abundant TβRI, whereas in EC, signaling via ALK1 predominates, though ALK5 is also expressed and can be activated in EC[516]. Although TGF-β preferentially activates ALK5 receptors, it can also signal via the ALK1 pathway[516]. During angiogenesis, TGF-β signaling pathways play critical roles to regulate EC proliferation, blood vessel morphogenesis and maturation[517]. Since TGF-β1 was the first TGF-β superfamily ligand discovered, it is also the most well-studied member of this family of proteins[518]. Early studies showed contradictory results regarding the role of TGF-β1 in angiogenesis, in that TGF-β signaling was found to mediate both angiogenic and angiostatic cellular responses depending on the environmental context, the type of ligand/receptor complex and its interaction with other angiogenic pathways [441, 519, 520]. In

EC, TGF-β1 can activate the ALK1/SMAD1/5/8 or the ALK5/SMAD2/3 pathways, which have opposing effects on EC proliferation and angiogenesis[521]. Although *in vitro* studies revealed that TGF-β1 treatment inhibits EC proliferation and induces apoptosis, the presence of TGF-β1 is required for angiogenesis in the *in vivo* setting[522]. The expression of TGF-β1 in mouse tissues gradually increases during embryonic and postnatal life[523], and its homozygous knockout results in embryonically lethal defective vasculogenesis[513]. In humans, loss of function mutations in endoglin, ALK1 or SMAD4 give rise to disordered angiogenesis and the vascular malformations of hereditary hemorrhagic telangiectasia (HHT)[524-526]. During tumorigenesis, TGF-β1 stimulates angiogenesis indirectly by regulating the expression and the signaling activity of other angiogenic factors such as VEGF[522]. Furthermore, the non-canonical TGF-β signaling results in the activation of proliferative and survival pathways such as the PI3K/AKT and the MAPK[487]. By contrast, in some tumors, TGF-β1 signaling seems to impair tumor growth and angiogenesis by inducing the expression of angiogenic inhibitors[527, 528]. Thus TGF-β1 triggers a complex signaling network that can promote or inhibit tumor angiogenesis depending on the cellular context in the tumor microenvironment[491, 529-531].

1.9.2 BMP-9 Signaling pathway

The bone morphogenetic proteins (BMPs) form a subgroup of the TGF-β superfamily of ligands that bind TGF-β receptors and transduce signals through SMAD and non-SMAD pathways[532-534]. Although BMPs were initially identified for their roles as osteogenic signaling molecules that induce ectopic bone formation and regulate bone and cartilage repair and regeneration, it is now known that they also play essential roles in embryonic development and homeostasis of many other tissues[535-537]. The BMPs

constitute nearly one third of the TGF-β superfamily, with more than 20 recognized members that signal via BMP receptors[533]. In the canonical signaling pathway, BMPs bind to the Ser/Thr kinase type I and type II receptors, and the presence of coreceptors potentiate receptor binding and signaling activity[538]. With the exception of BMP-1, which does not belong to the TGF-β superfamily, BMP family members are classified into subgroups based on their structural similarities, function and affinity to distinct type-1 receptors[539-541]. The four BMP binding type-1 receptors (ALKs), also called type-1 BMP receptors, include ALK-1, ALK-2 (ACVR1), ALK-3 (BMPR1A) and ALK-6 (BMPR1B)[539]. There are three BMP-activated type-2 receptors, also called type-2 BMP receptors, namely BMPRII, ACTRIIA, and ACTRIIB[539]. The BMPRIA, BMPRIB, and BMPRII are specific to the BMPs, whereas other Type-1 and type-2 BMP receptors can function as receptors for other TGF-β superfamily of ligands[539]. In contrast to TGF-β ligands which have high affinity for the type-2 receptor and preferentially activate the SMAD2/3 pathway, BMP ligands bind both type-1 and type-2 receptors with different affinities and activate the SMAD1/5/8 pathway[542]. In addition to the I-SMADs, which negatively regulate the intracellular signaling, the BMPR activity is also inhibited extracellularly at the cell membrane by the BMP and Activin membrane-bound inhibitor (BAMBI)[537]. The expression of BAMBI reduces ligand-induced signaling by impeding the formation of signaling receptor complexes[537]. The BAMBI is structurally similar to the type-1 receptors, but it is considered a pseudo-receptor for BMP ligands as it lacks the intracellular Ser/Thr kinase domain[543]. The binding of BAMBI to various type-1 and type-2 receptors inhibits the activation of their downstream signaling pathways[543]. The signaling activity of the BMPR is also regulated in the extracellular space by antagonists that bind BMPs and prevent the interaction with their cognate receptors[543]. The expression of intra- and

extracellular regulatory inhibitors is upregulated by BMPs and thus creates negative feedback loops that modulate the functional activity of the BMP signaling pathway[537]. By contrast, the presence of specific coreceptors and other protein potentiators enhances the ligand-receptor interaction and augments BMP signaling[537]. Although BMP-1 is not a TGF-β superfamily ligand, it functions as a metalloprotease that enhances BMP signaling activity by releasing active BMPs from their latent precursor proteins[544]. The BMPs are currently well recognized as important angiogenic molecules, and several studies have highlighted their roles as critical modulators of angiogenesis[545]. BMP-9, which is also called growth differentiation factor-2 (GDF-2), is recognized as the most potent physiological ligand for the endothelial ALK1 receptor[546]. In EC, BMP-9 binds with high affinity to ALK1 and endoglin in association with BMPRII and strongly induces SMAD1/5/8 pathway activation[547]. BMP-9-mediated endothelial signaling is an essential regulator of angiogenesis and plays an important role in the maintenance of vascular quiescence[548]. Activation of the BMP-9/ALK1/SMAD1/5/8 pathway inhibits EC proliferation and migration and induces tube-like formation[549]. Perturbations of the EC BMP-9/ALK1-signaling are associated with various vascular diseases, including hereditary hemorrhagic telangiectasia (HHT), also known as Rendu-Osler disease, and Pulmonary Arterial Hypertension (PAH)[524, 550, 551]. The HHT is an autosomal dominant disease characterized by disordered angiogenesis and formation of abnormal fragile blood vessels in multiple organs as well as skin and mucous membranes[552]. HHT results from impaired EC BMP-9 signaling due to loss of function mutations in endoglin (HHT type-1), ALK1 (HHT type-2) or SMAD4 (Combined juvenile polyposis-HHT syndrome)[550, 553]. It has been shown that the EC ALK1 activation antagonizes VEGF signaling and results in antagonistic effects on angiogenesis[554]. Conversely, impairment of ALK1 signaling leads to overactivation of

VEGF signaling and results in the development of the abnormal excessive fragile vasculature associated with the HHT[555]. Hereditary PAH is another rare but serious autosomal dominant vascular disorder characterized by high pulmonary arterial pressure that results in severe right-side heart failure[556]. Loss of function mutations in BMP-9, its receptors or downstream signaling molecules can all lead to PAH[551]. The most prevalent mutation leading to hereditary PAH reduces BMPRII function though mutations in ALK1, endoglin, BMP-9 or SMAD4, which can all result in enhanced EC proliferation and consequent pulmonary vascular obliteration, in turn raising pulmonary vascular resistance leading to PAH[557, 558]. Disturbances in the BMP-9/ALK1 signaling are also reported during the development of some tumors[547]. Similar to TGF-β1, BMP-9 has been reported to act either as a pro- or antitumorigenic signaling molecule in a cellular-context dependent manner[559, 560]. Some studies have demonstrated that the expression of BMP-9 is significantly decreased during tumorigenesis and that BMP-9-induced ALK1 activation attenuates angiogenesis and impairs tumor growth[561-563]. However, other studies reported that BMP-9 expression is significantly increased during tumor development and that activation of the BMP-9/ALK1 pathway signaling induces EC proliferation and stimulates tumor angiogenesis[564-566]. Therefore, the precise mechanisms of how BMP-9 regulates tumor angiogenesis depends on several factors, including tumor type and remains largely unknown[567].

1.9.3 The effect of hypoxia on the TGF- β signaling pathway

How the TGF-β superfamily of growth factors impact angiogenesis and vascular development and homeostasis in response to hypoxia is only partially understood[518]. During angiogenesis, hypoxia is the main driver of the EC phenotypic switch that produces

guiding tip and proliferating stalk cells, as well as other components of the angiogenic process[84]. Under hypoxic conditions, oxygen deprivation and HIF pathway activation modulate the expression of TGF-β superfamily cytokines and their receptors as well as their signaling activity[84]. As already described, in the endothelium, TGF-β superfamily ligands exert their effects by activating ALK1 or ALK5 receptors to stimulate SMAD1/5/8 or SMAD2/3 pathways, respectively[521]. During tumorigenesis, the hypoxic tumor microenvironment leads to increased intracellular HIF-α abundance but also increases TGF-β1 level within tumors[568, 569]. Several studies have demonstrated the crosstalk between TGF-β and HIF pathways[569, 570]. Importantly, TGF-β1 was found to raise HIF-1α levels by inhibiting the expression of prolyl hydroxylase, thus reducing HIF-1α degradation[568]. The increase in HIF-1α abundance in response to TGF-β1 was then found to be responsible for TGF-β-induced VEGF expression[84]. Hypoxia also induces a SMAD2/3-driven increase in TGF-β2 and TβRII transcription and induces expression of proteolytic enzymes that release active TGF-β2 from its latent precursors[571]. Hypoxia has also been shown to stimulate SMAD2/3 nuclear translocation in HUVEC[572]. It turns out that there is a direct binding of HIF-1α to ALK5[569] as well as SMAD3[572]. Hence, SMAD3/ HIF-1α complexes are believed to jointly activate promoters that enhance the transcription of cMyc[572] and TGF-β2[570]. Thus, TGF-β2 and HIF-1α seem to stimulate the expression of each other[569, 570]. TGF-β enhances the accumulation of HIF-1α and activates HIF target genes even when O_2 concentrations are in the normal physiological range[569, 573]. It has also been reported that ALK5 activation induces HIF-1α accumulation and that HIF-1α, in turn, enhances SMAD2/3 phosphorylation and their interaction with SMAD4 augmenting TGF-β-stimulated ALK5 signaling[573-575]. This type of synergistic crosstalk between HIF-

1α and TGF-β1and TGF-β2 activated pathways is now well-documented for the ALK5 receptor[569].

Much less is known about crosstalk between the BMP-stimulated ALK1/SMAD1/5/8 and hypoxia. Experimental evidence indicates that BMP-2 signaling via SMAD1/5/8 is significantly reduced under hypoxic conditions in glioblastoma cells[576, 577]. Also, the expression of the EC-predominant BMPRII, the BMPRIa, and SMAD1/5/8, as well as SMAD1/5/8 phosphorylation, are reduced by hypoxia in the lung *in vivo* and in cultured pulmonary EC[578, 579]. Studies in cultured EC showed that increasing the O_2 concentration from 1 to 20 % enhanced SMAD1/5/8 phosphorylation even without BMP addition[576]. This effect was mimicked by HIF-1α silencing, which augmented SMAD1/5/8 phosphorylation even under hypoxic conditions[576]. Therefore, the reduction in SMAD1/5/8 phosphorylation in response to hypoxia seems to be mediated by HIF-1α stabilization[576]. Although HIF-1α colocalizes with the p-SMAD1/5/8 in the nucleus, the molecular mechanisms by which HIF-1α inhibits SMAD1/5/8 function and how crosstalk between the BMP/ALK1 and the HIF-1α pathway operates are poorly understood[580].

1.10 TIMAP

The TGF-β superfamily pathways play an essential role in regulating the proliferation and differentiation of EC during angiogenesis and vascular development[436]. For instance, *in vitro* studies revealed that TGF-β1 is required for the formation of capillary-like structures by cultured EC[581]. Also, during kidney development in rats *in vivo*, TGF-β1 was found to be necessary for the differentiation of the glomerular endothelium, in that neutralizing TGF-β1antibody prevented the formation of the thin fenestrated glomerular EC, essential for the massive fluid flux that underlies glomerular filtration[582]. The

formation, full differentiation, and maturation of the newly formed vasculature in other organs also require a coordinated balance between the ALK1 and ALK5 signaling cascades of the TGF-β pathways[473]. The Ballermann laboratory explored the TGF-β1 downstream targets that might be involved in regulating TGF-β EC responses[583]. Representational difference analysis was carried out by harvesting mRNA from cultured primary bovine glomerular EC with or without TGF-β1 treatment[583]. By defining the mRNA species that were either powerfully repressed or induced, the lab identified a previously unknown transcript strongly downregulated by TGF-β1[583]. After developing the full-length cDNA clone and defining the sequence, they found a novel transcript that encodes a protein containing a C-terminal CAAX motif that function as a prenylation site to target the protein to the plasma membrane[583]. Therefore, they named this novel protein "TGF-β-inhibited membrane-associated protein" (TIMAP)[583]. Although TIMAP is predominantly expressed in EC, it is also expressed in the central nervous system and hematopoietic cell lines[583, 584]. TIMAP is highly expressed in the lung, spleen, kidney, adrenal glands, and testis but its expression in other organs is low or undetectable[583, 584].

1.10.1 TIMAP regulation by TGF- β1

As already described, the TGF-β superfamily of ligands bind and activate cell surface receptor Ser/Thr kinases with subsequent activation of intracellular SMAD signaling molecules[585]. In EC, TGF-β1 ligand/receptor binding triggers activation of two distinct downstream signaling cascades, the ALK1/SMAD1/58 or the ALK5/SMAD2/3[521]. Phosphorylation of these SMADs (R-SMADs) enhances their binding to SMAD4 (Co-SMAD), facilitating their nuclear translocation[469]. In the nucleus, the R-SMADs/Co-SMAD heterocomplex interacts with multiple transcriptional regulators at the SMAD-sensitive

promotor regions to regulate the transcription of target genes[586]. It has been demonstrated that the R-SMAD-Co/SMAD complex interacts with various transcriptional regulators, including transcriptional cofactors, such as FAST, and transcriptional repressors, such as Ski, SnoN, and TGIF[586]. It turns out that TGF-β-dependent TIMAP repression does not involve enhanced degradation or a change in TIMAP mRNA stability[583]. TGF-β-mediated TIMAP inhibition is mediated by the interaction of the R-SMAD/Co-SMAD complex with transcriptional repressors and the recruitment of corepressors and histone deacetylase (HDAC) into the SMAD responsive promoter[583]. However, whether the inhibition of TIMAP by TGF-β1 is mediated downstream of ALK1/SMAD1/5/8 or ALK5/SMAD2/3 has not been determined so far.

1.10.2 TIMAP is a myosin phosphatase targeting subunit (MYPT)

The human TIMAP protein, also known as PPP1R16B, consists of 567 amino acid residues which form a protein with a molecular weight of approximately 64 kDa[587]. The TIMAP protein contains a protein phosphatase-1 (PP1)-binding motif and five ankyrin repeats in its N-terminal domain as well as a C-terminal CAAX box[583, 588]. Structural homology evaluation showed that the domain structure of TIMAP is similar to that of the myosin phosphatase targeting subunits (MYPTs)[589]. The MYPTs form holoenzymes with the Ser/Thr protein phosphatase-1β catalytic subunit (PP1cβ)[588]. Like other PP1c regulatory subunits, the MYPTs bind and regulate the activity, substrate specificity, and cellular localization of Ser/Thr phosphatase activity[588]. Based on the amino acid sequence, all members of the MYPT family share the RVxF (KVSF in TIMAP) PP1c-binding domain located in the N-terminal domain, followed by several ankyrin repeats which form a region for additional protein-protein interactions[589]. The MYPTs also contain

multiple phosphorylation sites in their C-terminal domain, which reduces MYMPT/PP1c phosphatase activity upon phosphorylation[588]. TIMAP can bind all three isoforms of the PP1c (PP1cα, β, and $\gamma 1$,$\gamma 2$), but in EC, endogenous TIMAP specifically binds the PP1cβ[589]. Point mutations in the KVSF motif of TIMAP prevent TIMAP-PP1cβ interaction and abolish TIMAP-PP1cβ-associated phosphatase activity[590]. Although it was expected that TIMAP/PP1cβ would exhibit phosphatase activity against the myosin light chain 2 (MLC2)[589], it turns out that, in cells, TIMAP strongly suppresses the PP1cβ-induced MLC2 dephosphorylation and, therefore, increases the levels of MLC2 phosphorylation, enhancing myosin activity[591]. Consistent with these observations, I have been able to show that TIMAP deletion in mice or its silencing in cultured EC reduces the level of MLC2 phosphorylation[591] (Figure 1.10), and other members of this lab demonstrated that the effect of TIMAP on MLC2 hyperphosphorylation in EC is due to competition between TIMAP and MYPT1 for the PP1cβ subunit[591]. TIMAP has a stronger affinity for PP1cβ than other MYPTs[591]. TIMAP expression in EC, therefore, reduces the association of PP1cβ with MYPT1 leading to loss of MYPT1/ PP1cβ mediated MLC2 dephosphorylation and MYPT1 degradation[591]. TIMAP, therefore, is a powerful myosin phosphatase inhibitor in EC[591].

1.10.3 TIMAP cellular localization

The C-terminus of MYPTs contains a leucine zipper domain that mediates dimerization and protein-protein interactions[588]. TIMAP and its closest relative MYPT3 C-terminus lack this domain[583, 588]. Instead, the extreme C-terminus of TIMAP and MYPT3 consists of a CAAX box which enhances their association with the plasma membrane[583, 588, 592]. The CAAX box motif at the C-terminus of CAAX box proteins refers to an amino

acid sequence where C is cysteine, AA are two aliphatic residues, and X is an undefined amino acid and depends on the substrate specificity of these proteins[593]. The CAAX motif results in C-terminal prenylation of the protein and its translocation to the plasma membrane[593]. Prenylation of CAAX box proteins not only regulates their subcellular location but also their interaction with other proteins[593]. Prenylation of the CAAX box by farnesyl transferase localizes TIMAP to glomerular EC filopodia and enhances filopodia formation[583]. It has been shown that both TIMAP and farnesyl transferase interact with the adaptor protein RACK1, and this interaction is required for the association of TIMAP with plasma membrane[594]. RACK1 depletion or point mutations in the CAAX box reduce TIMAP plasma membrane localization and enhance its nuclear localization[594]. Although TIMAP has a nuclear localization motif, the regulatory mechanisms of nuclear translocation and the function of TIMAP in the nucleus have not been explored[583].

1.10.4 TIMAP phosphorylation and interaction with other proteins

It has been demonstrated that phosphorylation of certain amino acid residues in the central regulatory domain by Ser/Thr kinases regulates the function of MYPTs[592, 595]. Phosphorylation of MYPTs induces conformational changes that reduce the association of the holoenzyme components, their targeting function, subcellular localization, and the associated phosphatase cactivity[592, 596]. Unlike other MYPTs, which contain inhibitory phosphorylation sites in their C-terminal domain, reducing their myosin phosphatase activity, TIMAP and MYPT3 lack this inhibitory phosphorylation domain[597, 598]. Instead, TIMAP is phosphorylated by protein kinase A (PKA), which serves as a priming kinase to facilitate additional phosphorylation of TIMAP by GSK-3β[590]. TIMAP phosphorylation by PKA/GSK-3β on Ser333/Ser337 markedly lowers its affinity for PP1cβ[590]. TIMAP can also

be phosphorylated by PKCα, and phosphorylation of TIMAP has been shown to regulate TIMAP/PP1cβ association, which enhances the TIMAP-plasma membrane association[599-601]. Intriguingly, TIMAP is auto-dephosphorylated by its associated PP1cβ and point mutation in the KVSF motif, which abolishes the binding of TIMAP to PP1cβ, enhances TIMAP phosphorylation[590]. Several studies have demonstrated that TIMAP mediates the interaction of PP1cβ with several protein substrates to regulate their phosphorylation level and physiological activity[594, 602-605]. The non-integrin laminin receptor-1 (LAMR1) is a direct TIMAP binding partner, and both colocalize at the plasma membrane[602]. TIMAP binds LAMR1 via the Ankyrin repeat 4, and this interaction regulates the phosphorylation of LAMR1[602]. In regard to the work in this thesis, it is of great interest that expression of LAMR1 increases in proliferating EC during angiogenesis and plays an important role in tumor growth and metastasis[606, 607]. TIMAP has also been shown to bind the cytoskeletal linker proteins Ezrin-Radixin-Moesin (ERM) proteins as well as the Moesin-Ezrin-Radixin like protein (Merlin) collectively involved in regulating cell proliferation, migration and filopodia formation as well as the formation of endothelial cell-cell junctions and barrier function[601, 605, 608, 609]. The activity and subcellular localization of these proteins are regulated by their phosphorylation state, and TIMAP has been shown to enhance endothelial barrier function by regulating the phosphorylation of cytoskeletal proteins[605, 608].

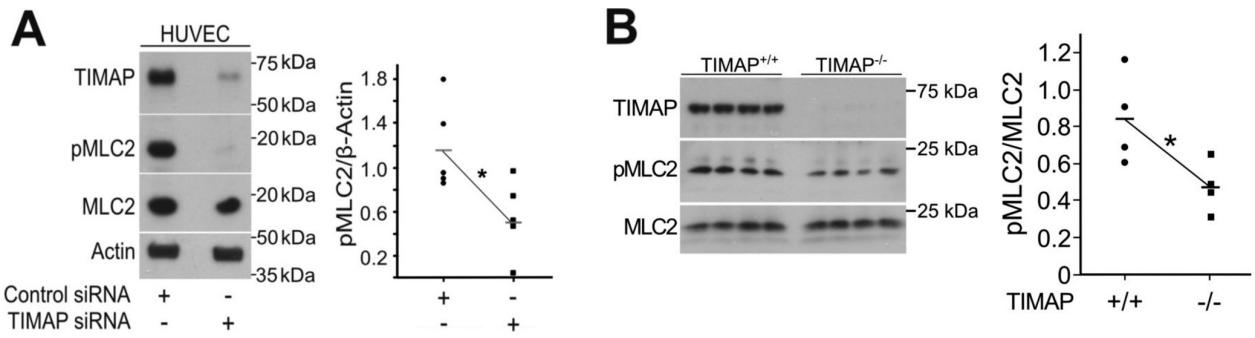


Figure-1.10: Inhibition of TIMAP reduces MLC2 phosphorylation

A. Cultured HUVEC were transfected with control or TIMAP-specific siRNA. Left panel, representative WB analysis. Right panel, densitometric analysis (● = control siRNA, ■ = TIMAP specific siRNA, n = 5 independent experiments, $p < 0.01$). **B.** Whole-lung lysates from WT ($TIMAP^{+/+}$) and age-matched TIMAP-deficient ($TIMAP^{-/-}$). Left panel, WB analysis for TIMAP, pMLC2, and MLC2. Each lane represents lysate from a distinct mouse. Right panel, densitometric quantification of pMLC2/MLC2 (● = WT mice, ■ = TIMAP KO mice, n = 4/group; *, $p < 0.05$).

1.10.5 Role of TIMAP in angiogenesis

Identification of TIMAP as an EC-predominant protein and target of TGF-β transcriptional downregulation suggested the possibility that TIMAP might be involved in the regulation of EC angiogenic responses mediated by TGF-β signaling pathways[583, 610]. In response to diverse angiogenic stimuli, vascular EC are activated and differentiate into tip and stalk cells, degrade the extracellular matrix, proliferate, and migrate to form tubular angiogenic sprouts of the nascent new blood vessels[149, 611, 612]. TIMAP directly interacts with molecules involved in angiogenesis, such as LAMR1 and MLC2, and regulates their phosphorylation and physiological activity[591, 602]. It turns out that siRNA-mediated silencing of TIMAP in cultured EC attenuates their proliferation and survival and impairs the *in vitro* formation of angiogenic sprouts in an *in vitro* sprouting assay[383]. TIMAP silencing profoundly reduces AKT activation, and conversely, TIMAP overexpression results in a dramatic increase in AKT phosphorylation[383]. These effects are associated with reduced EC apoptosis and enhanced EC proliferation[383]. The TIMAP-induced hyperphosphorylation and activation of AKT in EC appear to be mediated through inhibitory phosphorylation of the tumor suppressor PTEN[383]. It is well-established that loss of function mutation in PTEN results in enhanced cell proliferation and increases the risk of development of several tumors, and PTEN inactivation during tumorigenesis stimulates tumor growth and angiogenesis[613-615]. This further suggests that TIMAP could play an important role during tumor development which relies on angiogenesis for growth and dissemination.

1.11 Summary

Angiogenesis is an essential component of physiological and pathological tissue expansion and has been targeted as a therapeutic approach in diseases where excessive blood vessel growth is deleterious, including tumors[101]. Tumor angiogenesis requires EC activation and proliferation to form angiogenic sprouts that migrate toward less vascularized hypoxic regions within growing tumors[142]. The process of angiogenesis is principally mediated by tumor hypoxia which stimulates a vast array of signaling pathways that regulate multiple components of the angiogenic process[616]. Most of the angiogenic effects of hypoxia are mediated by HIF-1α stabilization, which, together with its partner HIF-1β, forms an active heterodimer that alone or in conjunction with other activators and repressors controls the transcriptional activity of various genes involved in angiogenesis[617]. The hypoxic tumor microenvironment induces the expression of angiogenic growth factors that, in turn, regulate EC activation to initiate sprout formation, survival, proliferation, migration, and differentiation[71, 72, 617]. Angiogenesis inhibitors that target specific EC signaling pathways reduce tumor vascularization, induce tumor vessel maturation, and impair excessive tumor growth[69]. TIMAP is predominantly expressed in EC and regulates endothelial responses involved in angiogenesis, such as proliferation, survival, and migration[383, 583]. TIMAP silencing induces apoptosis, reduces survival and proliferation of EC, and impairs *in vitro* sprouting angiogenesis[383]. As a PP1c regulatory subunit in the MYPT family, TIMAP enhances MLC2 phosphorylation and regulates myosin-dependent actin cytoskeleton reorganization during cell proliferation, migration and endothelial spouting and vascular branching[591, 618-620]. In addition, TIMAP promotes endothelial cell-cell junctional integrity, and its silencing reduces vascular barrier function[608]. In EC, TIMAP is negatively regulated by TGF-β pathway activation, but

whether TIMAP is a downstream target of the ALK1/SMAD1/5/8 pathway or ALK5/SMAD2/3 pathway is not known[583]. Given these diverse functions of TIMAP in EC, it is attractive to postulate that TIMAP plays an important role in promoting *in vivo* angiogenesis and that its expression is induced by pro-angiogenic stimuli. This project aims to explore the potential role of TIMAP in tumor angiogenesis and investigate the regulatory mechanism of TIMAP expression in EC.

1.12 Thesis hypothesis and objectives

Hypothesis: TIMAP is a pro-angiogenic molecule that promotes *in vivo* angiogenesis. In EC, TIMAP expression is induced by pro-angiogenic stimuli.

Objective 1. Using the syngeneic E0771 breast cancer model in C57BL/6 wild-type and TIMAP knockout mice, examine the effects of TIMAP deletion *in vivo* on tumor growth and angiogenesis.

Objective 2. Investigate the effect of hypoxia on TIMAP expression in EC and explore potential mechanisms of hypoxia-induced TIMAP regulation

Objective 3. Define the TGF-β downstream signaling pathways that regulate EC TIMAP expression.

Chapter 2

Materials and Methods

2.1 Reagents

Reagents are prepared and used as per the manufacturer's instructions.

Table 3 List of reagents

Reagent	Company	Catalogue #
rh BMP-9	R&D systems, Minneapolis, MN	3209-BP-010/CF
rh VEGF	R&D systems, Minneapolis, MN	293-VE
rh IGF-1	R&D systems, Minneapolis, MN	291-G1
rh VEGF	Lonza, Walkersville, MD, USA	CC-4114B
rh IGF-1	Lonza, Walkersville, MD, USA	CC-4115B
rh FGF-1	Lonza, Walkersville, MD, USA	CC-4113A
rh EGF-1	Lonza, Walkersville, MD, USA	CC-4317B
Roxadustat (FG-4592)	Selleckchem, Houston, TX, USA	S1007
ALK1 inhibitor (K02288)	Selleckchem, Houston, TX, USA	S7359
ALK5 inhibitor (SB505124)	Selleckchem, Houston, TX, USA	S2186
VEGFR2 inhibitor (Sunitinib)	Selleckchem, Houston, TX, USA	S7781
IGF-1R inhibitor (PPP)	Selleckchem, Houston, TX, USA	S7668

2.2 Immunoblot assay antibodies

Table 4 List of primary antibodies

Primary antibodies			
Antibody	**Clonality**	**Species**	**Concentration**
Anti-GAPDH	Polyclonal	Rabbit	1:10000
Anti-SMAD1	Polyclonal	Rabbit	1:1000
Anti-SMAD5	Polyclonal	Rabbit	1:1000
Anti-p-SMAD1/5/8	Polyclonal	Rabbit	1:1000
Anti-SMAD2/3	Polyclonal	Rabbit	1:1000
Anti-p-SMAD2	Polyclonal	Rabbit	1:1000
Anti-p-SMAD3	Polyclonal	Rabbit	1:1000
Anti-HIF-1α	Monoclonal	Rabbit	1:1000
Anti-TIE2	Monoclonal	Mouse	1:200
Anti-ESM-1	Polyclonal	Rabbit	1:1000
Anti-ALK1	Polyclonal	Goat	1:200
Anti-CD31	Monoclonal	Rat	1:100

Table 5 List of secondary antibodies

Secondary Antibodies			
Antibody	Clonality	Species	Concentration
Anti-rabbit	Polyclonal	Goat	1:15000
Anti-mouse	Polyclonal	Goat	1:0000
Anti-goat	Polyclonal	Donkey	1:5000
Alexa Fluor™ 488 anti-Mouse	Polyclonal	Goat	1:1000
Alexa Fluor™ 488 anti-rat	Polyclonal	Donkey	1:1000

Polyclonal rabbit anti-TIMAP IgG was generated and characterized as previously described[583]. This antibody is used at a concentration of 1:400, specifically recognizes the 67-kDa endogenous TIMAP, knocked down by TIMAP-specific siRNA and absent in TIMAP knockout mouse tissues[591] (Figure 1.10, 2.1 & 3.2 D). Anti-GAPDH (Cat # 2118s), anti-SMAD1 (Cat # 9743S), anti-SMAD5 (Cat # 9517), anti-p-SMAD1/5/8 (Cat # 9511S), anti-SMAD2/3 (Cat # 3102), anti-p-SMAD2 (Cat # 3101S), and anti-p-SMAD3 (Cat # 9520S) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-HIF-1α [EPR16897] (Cat # ab179438) and anti-TIE2, (Cat # ab24859) were purchased from Abcam (Cambridge, UK). Anti-ESM-1 antibody (Cat # MBS2006250) was purchased from MyBioSource (San Diego, CA, USA). Anti-hALK1 antibody (Cat # AF370) and anti-CD-31 antibody (Cat # DIA-310) were purchased from R&D systems (Minneapolis, MN, USA).

Horseradish Peroxidase (HRP)-Conjugated goat anti-rabbit (Cat # 111-035-003), goat anti-mouse (Cat #115-035-003) and donkey anti-goat (Cat # 705-035-003) IgG secondary antibodies were purchased from Jackson Immuno Research Laboratories (West Grove, PA). Immunofluorescence secondary antibody Alexa Fluor 488 goat anti-mouse (Cat # A11008) and Alexa Fluor 488 donkey anti-rat IgG (H+L) (Cat # A-21208) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). For WB, primary and secondary antibodies were diluted to the required concentration (Tables 4 & 5) in a western blocker purchased from Sigma (Cat # W0138).

2.3 Animals experiments

All animal experiments and procedures were carried out in compliance with the guidelines of, and approval by the University of Alberta Animal Care and Use Committee (protocol AUP00000222).

2.3.1 TIMAP deficient mice

Mice that do not express the TIMAP protein due to a global deletion of exon 4 in the TIMAP gene were obtained from Dr. Conrad C. Bleul[621]. Our lab then backcrossed the mutation onto the C57BL/6J (Stock # 000664, the Jackson Laboratory, Bar Harbor, ME, USA) background for more than ten generations. The genotype was determined by PCR using genomic DNA, and the absence of TIMAP protein in TIMAP knockout ($TIMAP^{-/-}$) mice was confirmed by WB.

2.3.2 Tumor cell line

The murine mammary adenocarcinoma (E0771) cell line, syngeneic for C57BL/6 mice, was kindly provided by Dr. David Brindley[622] and maintained in Dulbecco's modified Eagle's medium (DMEM, Cat # D5796, Sigma, Burlington, MA, USA) supplemented with 10% fetal bovine serum (FBS, Cat # 12483-20, ThermoFisher Scientific) and 1% antibiotics (penicillin 10,000 units/ml, streptomycin 10,000 units/ml, Cat # 15140-122, ThermoFisher Scientific), at 37°C in a humidified 5% CO_2 incubator. For mouse injection, cells in the logarithmic phase of growth were harvested with 0.05% trypsin-EDTA (Cat # 25300-062, ThermoFisher Scientific), centrifuged, washed, and re-suspended in sterile Dulbecco's Phosphate Buffered Saline (DPBS, Sigma, Cat # D8537) at a concentration of 1×10^7/ml and kept on ice prior to injection. Of note, we did not delete TIMAP in the

E0771 cells, as these cells do not express endogenous TIMAP (Figure 2.1). Effects of TIMAP deletion are, therefore, due to deletion in the host.

2.3.3 Mouse tumor cell implantation

Mice were anesthetized with an intraperitoneal injection of 50-90 mg/kg of Euthanyl (Sodium pentobarbital, 240 mg/ml, Bimeda MTC Animal Health Inc., Cambridge, ON), and the E0771 cells were inoculated subcutaneously into the mammary fat pad of 5 age-matched (8 - 10 weeks old) pairs of $TIMAP^{+/+}$ and $TIMAP^{-/-}$ female mice. Mice in each pair were injected with the same number (1×10^6/100µl) of cells, from the same culture, at the same time and in the same body area (right fourth mammary gland). The general mouse health, body weight and tumor size were evaluated every three days. Tumor length (L) and width (W) were measured using externally applied calipers, and tumor volume (V) was calculated using the following equation: $V = (L\times W^2)/2$[623]. The tumors were harvested when their main diameter (the square root of the product of the two perpendicular diameters) reached 15 mm and were either fixed with 4% formaldehyde and embedded in paraffin or kept unfixed and placed into “Optimal Cutting Temperature” (OCT, Cat # 4583, Sakura, Finetek, USA) for further sectioning and analysis.

2.3.4 Mouse Hypoxia

Mouse lung tissues from control and hypoxic mice were generously provided by N. Jahroudi[624]. Those mice had been exposed to chronic hypoxia in BioSpherix hypoxia chamber (Parish, NY, USA) for 35 days. The oxygen level was gradually reduced from 18 to 10% for the first two weeks and then maintained at 10% for three weeks, whereas the control mice were kept for the same period in room air (21% O_2).

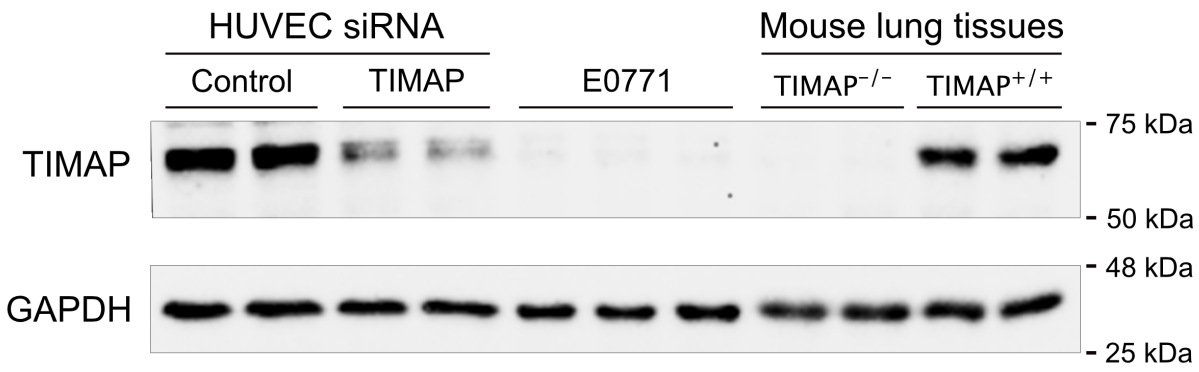


Figure 2.1 TIMAP expression in endothelial and E0771 cells

WB of TIMAP relative to GAPDH in lysates from HUVEC, E0771 cells and from lung tissue lysates of $TIMAP^{+/+}$ and $TIMAP^{-/-}$ mice. HUVEC were transfected with control or TIMAP-specific siRNA and harvested 48 hours later. E0771 cells were cultured in 10% FBS DMEM medium and harvested after 48 hours of seeding. Lung tissues were collected from TIMAP wild-type ($TIMAP^{+/+}$) and TIMAP deficient ($TIMAP^{-/-}$) mice and lysed in RIPA buffer.

2.4 Immunofluorescence Microscopy (IF)

Paraffin-embedded sections of lung tissues were placed in a 60°C preheated oven for 2 hours and then deparaffinized by immersion in three changes of xylene, 100%, 95%, 75%, and then 50% ethanol and finally placed in running water. For antigen retrieval, sodium citrate buffer (10 mM Sodium citrate, 0.05% Tween 20, pH 6.0) was used, and the slides were blocked for one hour at room temperature using a blocking solution [5% fish gelatin and 20% serum from the species host of secondary antibody in the Dako antibody diluent solution (Cat # S3022, Agilent Technologies Inc, Santa Clara, Ca, USA)] in a humid chamber, and then incubated with rat anti-mouse CD-31 antibody overnight at 4°C. The slides were then rinsed three times with TBST (Tris 20 mM pH 7.5, NaCl 150 mM, Tween 20.01% (v/v)) for 15 minutes, incubated with Alexa Fluor 488 donkey anti-rat secondary antibody for 1 hour at room temperature in a humid chamber. The sections were then washed with TBST three times for 15 minutes and stained with DAPI (4',6-diamidino-2-phenylindole) to visualize the nuclei. Slides were then washed with TBST for 15 minutes, mounted using ProLong Gold antifade reagent (Cat # P36934, Invitrogen), covered with a coverslip and kept overnight at room temperature in a dark place. The absence of staining when the primary antibody is omitted was used as a negative control.

Cryo-embedded sections of tumor tissues were permeabilized in 0.2% Triton X-100 in G-PBS (1.12g Glycine in 500ml PBS) for 15 minutes at room temperature. The samples were blocked in 10% normal donkey serum (Cat # 017-000-121, Jackson Immuno Research, West Grove, PA USA) for 1 hour at room temperature, then incubated with anti-CD31 antibody overnight in the cold room, followed by incubation with the Alexa Fluor 488 Goat anti-mouse IgG secondary antibody at room temperature for 1 hour. the Immunofluorescence images of both lung and tumor tissues were taken using EVOS

Microscope (AMF5000, ThermoFisher). At least three random images were taken from each slide, and the staining density was analyzed using Image J software.

2.5 Cell culture

Human umbilical vein endothelial cells (HUVEC), freshly isolated from human umbilical cords, were obtained from A. Murray[625]. Primary human glomerular Microvascular ECs (hGEC, Cat # cAP-0004) were purchased from Angio-Proteomie (Boston, MA), and human lung Microvascular EC (hLEC, Cat # CC-2527) were purchased from Lonza (Lonza Walkersville, MD). For all experiments, EC (passages 3–4) were seeded on plates precoated with Quick Coating Solution (Cat # cAP-01, Angio-Proteomie) using angiogenic growth factor-enriched (VEGF 2 ng/ml, IGF-1 5 ng/ml, FGF 10 ng/ml, EGF 5 ng/ml) medium (EGM-2, Cat #CC3202, Lonza). This medium is prepared by adding supplements (Cat #, CC4147, Lonza) and penicillin/streptomycin (1%) to the EBM-2 basal medium (Cat #, CC3356, Lonza). The supplements include 5% FBS, hydrocortisone, ascorbic acid, and gentamycin. In experiments with growth factor-deficient medium, only the growth factors and FBS were omitted. For all experiments, sub-confluent (~80%) EC cultures were used and preconditioned by incubation with EBM-2 lacking growth factors but containing 1% FBS for 24 hours. E0771 cells were cultured in DMEM full growth medium.

2.6 *In vitro* hypoxia

To induce hypoxia, EC were placed into a humidified BioSpherix hypoxia chamber (ProOx c21, BioSpherix) at 1% O_2 for 1 hour or 18 hours while control cells were maintained in a conventional incubator (21% O_2) with 5% CO2 at 37ºC for the same period. To stabilize HIF-α, EC were treated with 1 µl/ml Roxadustat 1 µM (Selleckchem,

Houston, TX, USA, S1007) or vehicle (DMSO 1 µl/ml). Stabilization of HIF-1α in hypoxia- or Roxadustat-treated cells was confirmed by WB.

2.7 HUVEC/E0771 co-culture

To culture EC with E0771 cells, we used transwell inserts with 0.4 µm pore-size membranes to allow the movement of the media but not the cells between the two chambers. E0771 cells were seeded and grown in 10% FBS DMEM medium on the top surface of the transwell membranes suspended over chambers to full confluence. HUVEC were cultured on the bottom of separate wells (6-well plates) in EGM-2 medium to ~80% confluence, followed by 24 hours of incubation in EBM-2 containing 1% FBS. The media were then discarded, and three E0771 membrane inserts, washed three times with EBM-2, were transferred to the upper chambers of 3 wells containing HUVEC in the lower chamber. Empty, washed transwells were placed above HUVEC of 3 separate wells and served as control (Figure 2.2). Fresh EBM-2 was placed into all upper and lower champers. The co-cultured cells were then kept in the incubator for 24 hours, washed three times with cold PBS and harvested with hot 2X Laemmli buffer (Cat # 1610737, BioRad) containing 0.05% beta-mercaptoethanol.

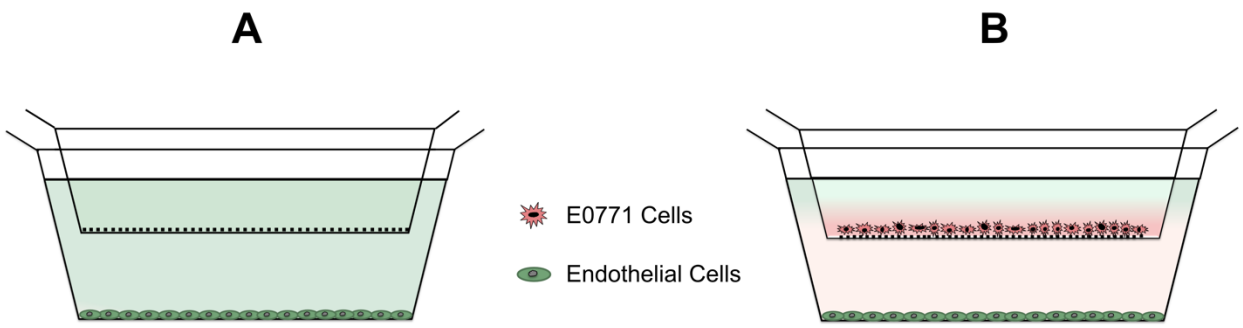


Figure 2.2 E0771/HUVEC co-culture

E0771 cells were seeded onto transwells in DMEM containing 10% FBS and allowed to become confluent. HUVEC were seeded in EGM-2 in separate 6-well plates, grown to ~80% confluency and then switched to EBM-2 medium (no serum, no growth factors) for 24 hours. Both cell cultures were then washed three times with EBM-2, followed by co-culture or control culture in EBM-2 for an additional 24 hours. **A.** Control HUVEC without E0771 co-culture. **B.** E0771 cells on transwell inserts suspended over HUVEC in EBM-2 medium.

2.8 Conditioned media

E0771 cells were cultured in 10 cm dishes containing DMEM supplemented with 10% FBS plus 1% penicillin/streptomycin and grown until full confluence. The medium was then discarded, and the E0771 cells were washed three times with EBM-2 medium. They were then cultured for an additional 24 hours in a fresh EBM-2 medium free of growth factors and FBS. The same quantity of EBM-2 media free of growth factors and FBS were simultaneously incubated in empty 10 cm culture dishes and used as control media. At the end of the 24 hours period, the media were collected, purified from cells and debris using Millex-GV Syringe filters with 0.22 µm pore size (Cat #: SLGV033RS, Sigma) and stored at -80 for further use.

2.9 Western blot analysis

2.9.1 Sample preparation

*C*ells were placed on ice, washed twice with cold PBS and harvested using hot 2X Laemmli buffer (Biorad) containing 0.05% β-Mercaptoethanol (Sigma-Aldrich, M6250). For tissue lysates, mouse organs and tumors were isolated, immediately placed on dry ice and cut on a glass slide. For each sample, approximately 50 mg of tissues were transferred into Eppendorf tubes. The remaining tissue was stored at -80ºC. The tissue samples were then cut into smaller pieces and homogenized in cold RIPA buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate) containing complete protease (Cat # 4693116001, Sigma) and phosphatase (Cat # 4906837001) inhibitors using TissueLyser (Qiagen) for 3 minutes at 25 HZ twice. The homogenized tissues were then rotated at 4ºC for 10 minutes, followed by centrifugation at 14,000 RPM also at 4ºC for 10 minutes. The supernatants were then collected, placed in new

Eppendorf tubes, and mixed with the same volume of hot 4X Laemmli buffer (Cat # 1610747, BioRad) containing 0.05% β-Mercaptoethanol and stored at -80 ºC. Both cell and tissue lysates were heated at 95 ºC and frequently vortexed for 10 minutes and centrifuged at 12000 g for 2 minutes before loading onto the gels.

2.9.2 Gel electrophoresis

Solubilized tissue and cell lysate proteins were separated by SDS PAGE using 8%, 10%, or 8-16% gradient polyacrylamide gels (Cat # XP08165BOX, ThermoFisher) at 4ºC at 50 V for a few minutes until proteins entered the separating gel followed by 150 V until the dye front reached the bottom of the gel.

2.9.3 Protein transfer

Proteins were transferred onto polyvinylidene difluoride membranes (PVDF, Cat # IPVH00010, Sigma) overnight (18 to 24 hours) at 35-40 V at 4ºC. For total protein determination and loading normalization, post-transfer membranes were incubated with No-Stain Protein Labeling Reagent (Cat # A44449, ThermoFisher Scientific) prepared according to the manufacturer's protocol, photographed using the iBright 750 imager (ThermoFisher Scientific) and analyzed by the ImageJ software (NIH, USA).

2.9.4 Blocking

To reduce the non-specific binding of antibodies to the membrane proteins, post-transfer membranes were incubated in a western blocker (Cat # WO138, Sigma) with gentle shaking overnight (6-12 hours) at 4ºC. Blotting membranes have high affinity for proteins, and sufficient blocking is necessary to avoid non-specific binding of the antibody to the membranes[626]. The laboratory had previously generated anti-TIMAP antibodies in

several rabbits. Our TIMAP-3α antibody recognized TIMAP on WB of lung lysates from wild type but not TIMAP$^{-/-}$ mice. However, a prominent non-specific band running just above TIMAP was observed in lysates from both TIMAP$^{+/+}$ and TIMAP$^{-/-}$ mice, as illustrated below (Figure 2.3). This contaminating band made the quantification of TIMAP abundance challenging. I found that longer overnight (~18 hours) blocking at 4ºC is more efficient to reduce non-specific binding than blocking for one hour at room temperature. Furthermore, I found that stripping/re-blocking of the membranes essentially eliminated the non-specific bands and significantly enhanced the specificity of the anti-TIMAP-3α antibody. We subsequently identified the more specific TIMAP-7β antibody, which does not necessitate the complex blocking/stripping procedure. Although many experiments were initially done with the TIMAP-3α antibody, they were repeated with the TIMAP-7β antibody, with consistent results. All WB shown in chapters 3 and 4 were done with the TIMAP-7β antibody.

2.9.5 Antibody incubation

After blocking, the membranes were washed three times with TBST for 15 minutes at room temperature and incubated with primary antibodies diluted in western blocker with gentle shaking overnight at 4°C. Then the membranes were washed three times with TBST for 15 minutes at room temperature to remove unbound primary antibodies and incubated with HRP-conjugated secondary antibodies specific to the species of the primary antibody diluted in western blocker (Sigma) with gentle shaking for 1 hour at room temperature.

2.9.6 Protein detection and visualization

Finally, membranes were washed three times with TBST for 15 minutes to remove residual unbound secondary antibodies, rinsed with distilled water for 5 minutes, incubated with Enhanced Chemiluminescence (ECL) solution (Cat # 45000875, Sigma) prepared according to the manufacturer's protocol for 3 minutes and visualized using the iBright750 imager.

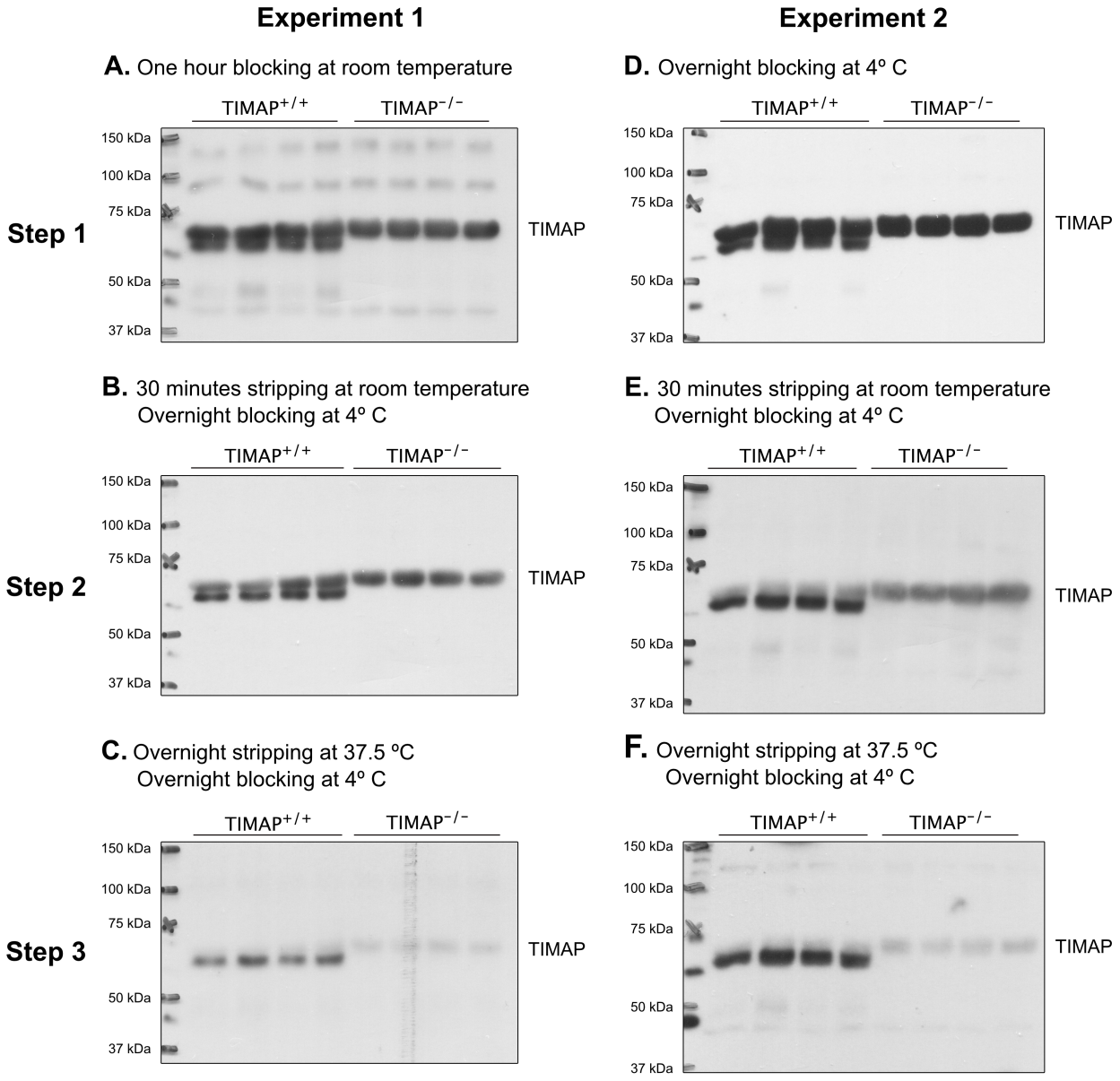
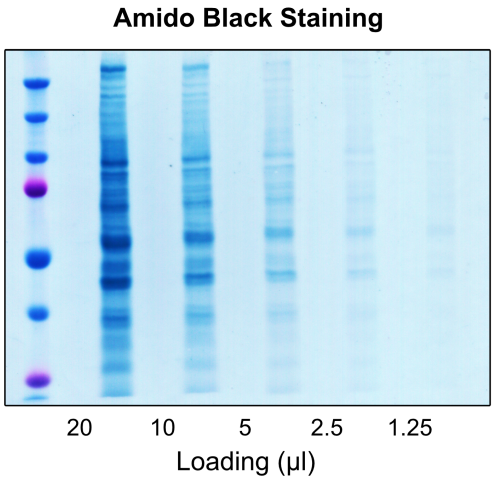


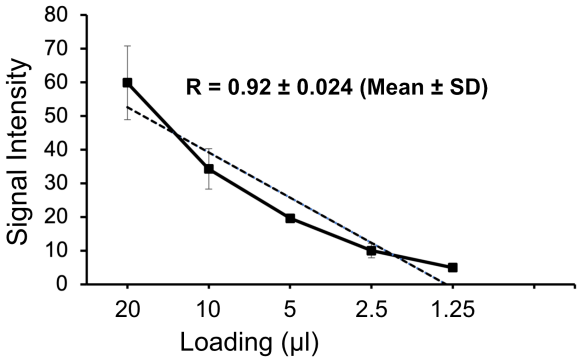
Figure 2.3 Western blot stripping blocking procedure

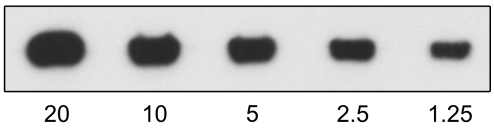
Procedure development to reduce non-specific staining of WB with TIMAP antibodies using lung tissue lysates from TIMAP$^{+/+}$ and TIMAP$^{-/-}$ mice. Differences in the procedure are explained above each blot. For all blots, the primary and secondary antibody concentrations, as well as the development and imaging of the blots, were identical.

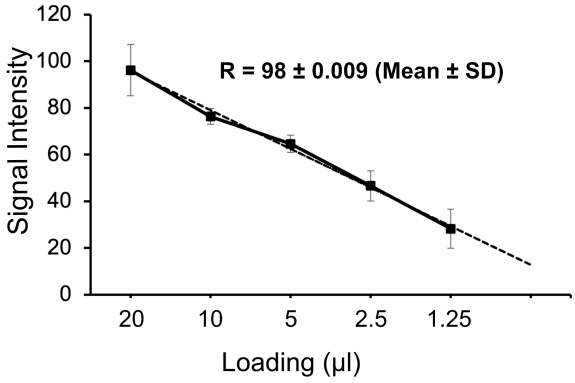
2.9.7 Western blot loading control

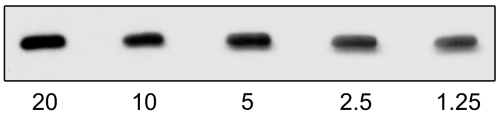
Western blotting is generally used to evaluate the change in abundance of certain proteins under different treatment conditions[627-629]. For quantification to be relatively accurate, loading controls on the same blot must be used to account for variability that could be introduced by differences in cell density and other experimental variations such as sample loading or uneven protein transfer[630, 631]. Furthermore, it is important that band densities for both, the experimental target and loading control, fall within their respective dynamic range. Some cytoskeletal proteins like tubulin and actin are commonly used as loading controls. These are often referred to as housekeeping proteins and are assumed to be expressed at constant levels[632]. Another common housekeeping protein currently used as a loading control is GAPDH[632]. For these to be adequate, they must not be affected by experimental procedures or differences. The best method to ensure that the loading control remains constant is to compare its signal to the total protein stain on the same membrane. In fact, evidence suggests that housekeeping proteins do not always reflect actual protein loading determined by total protein staining[633]. Nonetheless, several studies suggest that specific housekeeping proteins are appropriate WB loading controls for certain tissues[634-636]. I investigated which of the three housekeeping proteins would best serve as loading control for our experiments. Serial dilutions of the same EC lysate were evaluated by densitometry of Amido Black total protein stains and WB for β-actin, β-tubulin, and GAPDH, followed by linear regression analysis. The GAPDH signal most closely fits a straight line over a nearly 20-fold range of dilutions, suggesting that the signal is in the dynamic range (Figure 2.4). Therefore, I used GAPDH as the loading control for all experiments reported in chapters 3 and 4.

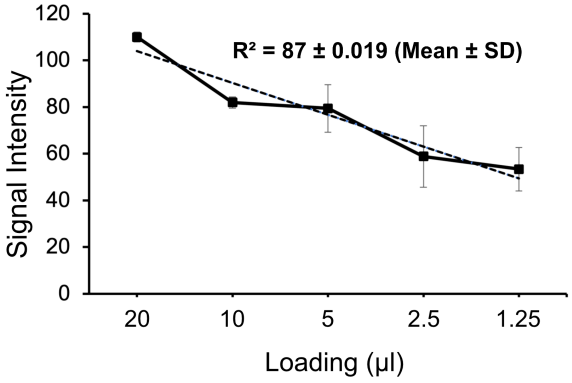


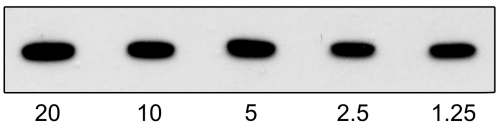


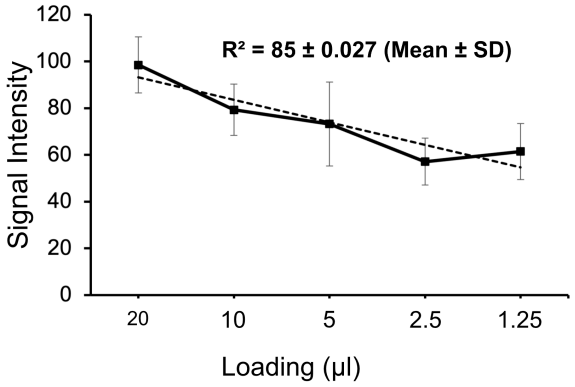












R² Values of three independent experiments				
Experiments	Amido Black	GAPDH	β-Actin	β-Tubulin
Exp-1	0.9478	0.9884	0.8530	0.8469
Exp-2	0.9046	0.9830	0.8697	0.8364
Exp-3	0.9067	0.9691	0.8900	0.8791
Mean	0.92	0.98	0.87	0.85
SD	0.024	0.009	0.019	0.027

Figure 2.4 Dynamic range for housekeeping protein quantification by WB

Serial HUVEC cell lysate dilutions were prepared and separated by SDS PAGE. The proteins were transferred onto PVDF membranes, stained with Amido Black and then subjected to WB analysis with anti-GAPDH, -tubulin, and -actin antibodies. The intensity of the bands was quantified using the Image J software, followed by linear regression curve fitting using Excel. The R-value indicates the goodness of fit, and the mean R values for three independent experiments are shown in the table.

2.10 TIMAP knockdown and siRNA transfection

To silence TIMAP expression in cells, HUVEC were cultured in 60-mm culture dishes containing EGM-2 media supplemented with 1% penicillin/streptomycin for 24 hours until they reached ~70% confluence. On the next day, the media were replaced with 2 ml of fresh EBM-2 media free of antibiotics to which 400 µl of Opti-MEM™ (Cat # 31985-070, Thermo Fisher Scientific) containing 12 µl control (10µM, Cat # sc-37007, Santa Cruz Biotechnology, Dallas, TX, USA) or TIMAP specific (10µM, Cat #: sc-76669, Santa Cruz Biotechnology) siRNAs, and 12 µl Lipofectamine™ 3000 (Cat # L3000001, Thermo Fisher Scientific) were added. Five hours later, each 60-mm culture dish received additional 2 ml EGM-2 supplemented with 10% FBS and free of antibiotics and cells were harvested after 48 hours of siRNA addition and stored at -20 Cº.

2.11 RNA isolation, cDNA synthesis and quantitative real-time PCR (qRT-PCR)

RNA was isolated using TRIzol™ (Cat # 15596026, Thermo Fisher Scientific) from HUVEC cultured in 60 mm culture dishes. Using QuantiTect Reverse Transcription Kit (Cat# 205311, Qiagen), one µg of total RNA from each sample was then reverse transcribed into cDNA in 20µl reaction volume. The cDNA samples were then diluted 100 times using SYBR Green Master Mix (Cat #4309155, Thermo Fisher Scientific) containing specific primers, synthesized by Integrated DNA Technologies (IDT, Coralville, IA, USA) for TIMAP (Hs.PT.58.38695502) and HPRT1 (Hs.PT.58v.45621572, IDT). Quantitative real-time PCR was carried out using a 7500 ABI Thermocycler (Thermo Fisher Scientific) according to the manufacturer's instructions. Standard curves were used to calculate the TIMAP mRNA abundance relative to that of control HPRT1 mRNA, and the data were analyzed with the StepOne software (Applied Biosystems, Thermo Fisher Scientific).

2.12 Statistical analysis

Each experiment was independently repeated a minimum of three times, and the data were presented as the mean ± *SD*, and 'n' indicates the number of independent experiments. When comparing two groups, statistical analyses were carried out with a two-tailed unpaired *t*-test. One-way analysis of variance (ANOVA) with Tukey's multiple comparison tests using GraphPad PRISM 9 software (San Diego, CA, USA) was used when three or more groups were compared. $p < 0.05$ was considered statistically significant.

Chapter 3

Hypoxia-induced TIMAP Upregulation Promotes Tumor Angiogenesis

3.1 Introduction

Angiogenesis is the process of formation of new blood vessels from pre-existing ones followed by vessel stabilization and maturation[637]. The proliferation of the vascular EC is essential for the formation and extension of the angiogenic sprouts to form the nascent vasculature[638]. The endothelial tip cells at the front of the angiogenic sprout dictate the direction and layout of the new vascular plexus, whereas the endothelial stalk cells proliferate to extend the sprouts[639]. Angiogenesis is an essential process that occurs throughout life in both health and disease, for instance, during embryonic vascular network formation, tissue remodeling, and tumor growth and development[640-642]. Physiological angiogenesis is essential for blood vessel formation and modelling during the embryonic vascular system development, damage repair during vascular injury and wound healing, and placental vascular establishment[642-645]. The regulation of vascular EC proliferation by growth factors and inhibitors is essential for establishing a normal vascular network and inhibiting pathological angiogenesis[31]. Dysregulated EC proliferation results in disordered angiogenesis and the formation of abnormal vascularization during the development of several pathological conditions such as diabetic retinopathy and malignancy[646-648].

Cancer is currently a major health problem around the globe and is considered the second driving cause of death worldwide[649-651]. In Canada, cancer is the most common cause of death, and according to the recent cancer statistics, it is expected that approximately 50% of Canadians will develop cancer in the course of their life, and around 25% of the total deaths will be due to cancer[652-654]. Solid tumors, which rely heavily on

angiogenesis to grow and spread, are the most common type of cancer and are responsible for the majority of cancer deaths[655-657]. These tumors are characterized by rapid uncontrolled cellular hyperproliferation and growth and require the formation of new blood vessels to fulfill the high metabolic demands of the proliferating cancer cells[658-660]. During tumor growth, the excessive proliferation of cancer cells results in the development of tumor hypoxia which drives angiogenesis to supply the growing tumors with oxygen and nutrients and enhance their progression and dissemination[71]. Tumor hypoxia stabilizes and activates hypoxia-inducible factor (HIF) proteins, which are transcription factors that induce the expression of downstream target genes to overcome the hypoxic environment[92, 617]. During tumorigenesis, activation of HIF proteins induces the transcription of angiogenic factors to stimulate vascular EC sprouting into the hypoxic tumor tissue[661, 662]. The fact that solid tumors rely on angiogenesis to survive and expand has led to the discovery of numerous therapeutic anti-angiogenic drugs to inhibit tumor growth[68, 663]. Tumor angiogenesis has been targeted as a therapeutic approach for solid tumors, and inhibitors that target specific angiogenic molecules have been approved to treat cancer[419, 420, 664, 665]. So far, most inhibitors of angiogenesis in clinical practice rely on interruption of the VEGF pathway[666-668].

The process of angiogenesis is regulated by a complex array of angiogenic factors, including growth factors, angiopoietins, and the bone morphogenetic and the TGF-β superfamily of proteins[102]. The TGF-β superfamily is composed of several ligands that bind and activate heteromeric serine/threonine kinase receptor complexes[669]. The receptor complexes are composed of specific serine/threonine kinase type-I receptors, ligand-binding type-II receptors, and auxiliary type-III receptors[447, 669]. Among these, the type I Activin-like kinase 1 (ALK1) receptor, and the type III endoglin receptor are restricted

to EC. Upon ligand binding, type-II receptors dimerize and recruit type-I receptors to form signalling heterotetramers with serine/threonine kinase activity which stimulate phosphorylation of SMAD1/5/8 or SMAD2/3[447, 669]. Phosphorylated SMADs form a complex with SMAD4 and translocate into the nucleus, where they regulate the transcription of target genes[447, 669]. In EC, the ALK1 and ALK5 pathways, the former via SMAD1/5/8 and the latter via SMAD2/3, antagonize each other, and the signalling equilibrium between these two pathways regulates EC behaviour during angiogenesis[473]. The net outcome of TGF-β signaling during angiogenesis depends on multiple factors such as the specific ligands, their concentration, and the nature and stage of angiogenesis[447, 670]. *In vivo*, TGF-β signalling pathways play a crucial role during angiogenesis, blood vessel morphogenesis and maturation[436, 521]. In this regard, expression of TGF-β1 in mouse tissues gradually increases during embryonic and postnatal life[523] and its homozygous knockout results in embryonically lethal defective vasculogenesis[513]. In humans, loss of function mutations in endoglin, ALK1 or SMAD4 give rise to disordered angiogenesis and the vascular malformations of hereditary hemorrhagic telangiectasia[526, 671]. Mutations in BMPR2, a type II receptor that recruits ALK1 in EC, result in enhanced EC proliferation, pulmonary vascular obliteration and raise pulmonary vascular resistance and the development of pulmonary arterial hypertension[558, 672].

TIMAP is a pro-angiogenic protein predominantly expressed in proliferating EC and developing vascular endothelium and strongly inhibited by the TGF-β 1[583]. *In vitro* studies revealed that TIMAP deficiency reduces EC proliferation, increases apoptosis, and impairs sprouting angiogenesis[383]. Enhanced TIMAP expression has also been demonstrated in human breast cancer, where it is a marker of poor outcomes. Based on

its domain structure, TIMAP is classified as a member of myosin phosphatase targeting subunits (MYPT), a family of proteins that bind the serine/threonine protein phosphatase 1β catalytic (PP1c β) subunit to form the myosin phosphatase holoenzyme (MYPT/PP1cβ) that regulate myosin activity[591]. Unlike the other MYPT family members, which are PP1c β activators that target PP1cβ to dephosphorylate MLC2, TIMAP is a potent competitive inhibitor of PP1cβ toward MLC2[591]. TIMAP/PP1cβ complex enhances non-muscle myosin activity by reducing the rate of myosin light chain-2 (MCL2) dephosphorylation[591]. During tumor angiogenesis, the formation of filopodia at the tip cells, EC proliferation and migration, as well as branching of the newly formed blood vessels, require enhanced myosin activity and contractility[619, 673, 674]. The activity of endothelial Ser/Thr protein phosphatases, which reduce myosin activity by increasing the rate of dephosphorylation of its regulatory MLC2, are also markedly reduced during the development and progression of many cancers, either by reducing their expression or increasing the expression of their endogenous inhibitors[675].

It has been shown that TIMAP undergoes prenylation at its CAAX motif, which results in its association with the plasma membrane[583]. In EC, TIMAP directly interacts with the non-integrin LAMR1 and regulates its phosphorylation[602, 609]. LAMR1 has been found to be required for *in vitro* capillary formation and *in vivo* angiogenesis, and its level is upregulated during EC proliferation and angiogenesis[676-678].

TIMAP-induced PP1cβ inhibition also results in hyperphosphorylation and activation of the AKT that, in turn, signals EC proliferation[383]. Inhibition of PP1cβ enhances the inhibitory phosphorylation of PTEN, a tumor suppressor that reduces phosphorylation and activity of the AKT signaling cascade[383]. Akt is a Ser/Thr kinase that promotes survival, induces proliferation and migration of EC and stimulates angiogenesis[351, 679-681].

The activation of Akt is mediated by phosphorylation of its Ser 473 and Thr 308 residues[682]. The Ser473 residue in the AKT regulatory domain is phosphorylated by mTORC2, while the Thr 308 in the AKT kinase domain is phosphorylated by PDK1[682-684]. Phosphorylation of AKT takes place at the plasma membrane and is induced by PI3K-induced generation of PIP3[349]. In response to the activation of receptor tyrosine kinases at the plasma membrane, activation of PI3K stimulates the conversion of PIP2 to PIP3, which recruits AKT and its activating kinase PDK1, hence facilitating the phosphorylation of AKT by PDK1[684]. Additional phosphorylation of the AKT is mediated by mTORC2, which results in the full activation of AKT[685]. Constitutively active AKT stimulates excessive abnormal angiogenesis, and its deletion in mice is associated with impaired fetal vascular development[686-688]. The tumor suppressor PTEN reduces AKT activation by dephosphorylating PIP3 to PIP2, and deletion of PTEN in mice leads to hyperproliferation of EC and results in the development of disordered angiogenesis[365, 689, 690]. It has been previously shown that the effect of TIMAP on the endothelial angiogenic spouting *in vitro* is mediated by inhibiting PTEN and subsequent AKT hyperphosphorylation and activation[383]. Both TIMAP and PTEN colocalize at the tip of the EC projections, and as a PP1βc targeting subunit, TIMAP enhances the inhibitory phosphorylation of PTEN[383].

These observations indicate that TIMAP could be a critical regulator of angiogenesis, and we, therefore, sought to determine the effects of TIMAP deletion on tumor angiogenesis. We utilized a murine breast cancer model to investigate whether *in vivo* angiogenesis also requires TIMAP and used an *in vitro* culture model to determine the mechanism of TIMAP regulation in angiogenic EC. We found that TIMAP knockout in mice impedes tumor growth and reduces tumor vascularization. Mechanistically, we demonstrated the potent angiogenic inducer hypoxia, as well as the angiogenic growth

factors VEGF and IGF-1, which are produced by hypoxic tumor cells, induce TIMAP expression in EC.

3.2 Results

3.2.1 TIMAP depletion impairs E0771 mammary tumor growth

Previous work identified TIMAP as a proangiogenic protein that enhances proliferation and promotes survival of EC, and its silencing profoundly reduced sprouting angiogenesis *in vitro*[383]. The growth of most tumors depends on angiogenesis and most solid tumors require the formation of new blood vessels for their ongoing growth, and expansion[691]. Therefore, to investigate the role of TIMAP during *in vivo* angiogenesis, we first determined whether deletion of TIMAP in mice alters *in vivo* tumor growth. We used the E0771 mouse breast tumor model and compared tumor angiogenesis in TIMAP deficient mice ($TIMAP^{-/-}$) and their wild-type control littermates ($TIMAP^{+/+}$). We subcutaneously injected E0771 into the mammary glands of 5 age-matched (8–10-week-old) pairs of $TIMAP^{+/+}$ and $TIMAP^{-/-}$ female mice and evaluated the tumor development over time (Figure 3.1 A). We observed that during the first 7-10 days after injection of E0771 cells, tumors were similar in size in $TIMAP^{+/+}$ and $TIMAP^{-/-}$ mice, but tumor growth rate then diverged and was significantly slower in $TIMAP^{-/-}$ compared to $TIMAP^{+/+}$ mice (Figure 3.1 B). In all five mouse pairs, the tumor size first reached the experimental euthanasia endpoint (15 mm tumor diameter) in the $TIMAP^{+/+}$ mouse, while $TIMAP^{-/-}$ tumors remained much smaller (Figure 1 B) and regressed in 2 of the 5 $TIMAP^{-/-}$ (individual data not shown). Moreover, we regularly observed ulceration, bleeding, and necrosis of the skin overlying the tumors in $TIMAP^{-/-}$ but not in $TIMAP^{+/+}$ mice (Figure 3.1 C). At the time of euthanasia, we noticed that, compared to $TIMAP^{-/-}$ mouse tumors, which tended to be pale and less vascular, tumors of $TIMAP^{+/+}$ mice appeared fleshy and highly

vascular (Figure 3.1 D & E). When the tumors were excised, their mean diameter (size) and weight were measured, and both were significantly lower in TIMAP$^{-/-}$ compared to TIMAP$^{+/+}$ mice (Figure 3.1 F & G). These findings indicate that TIMAP supports tumor growth and suggest that lack of TIMAP in the host mice results in tumor necrosis.

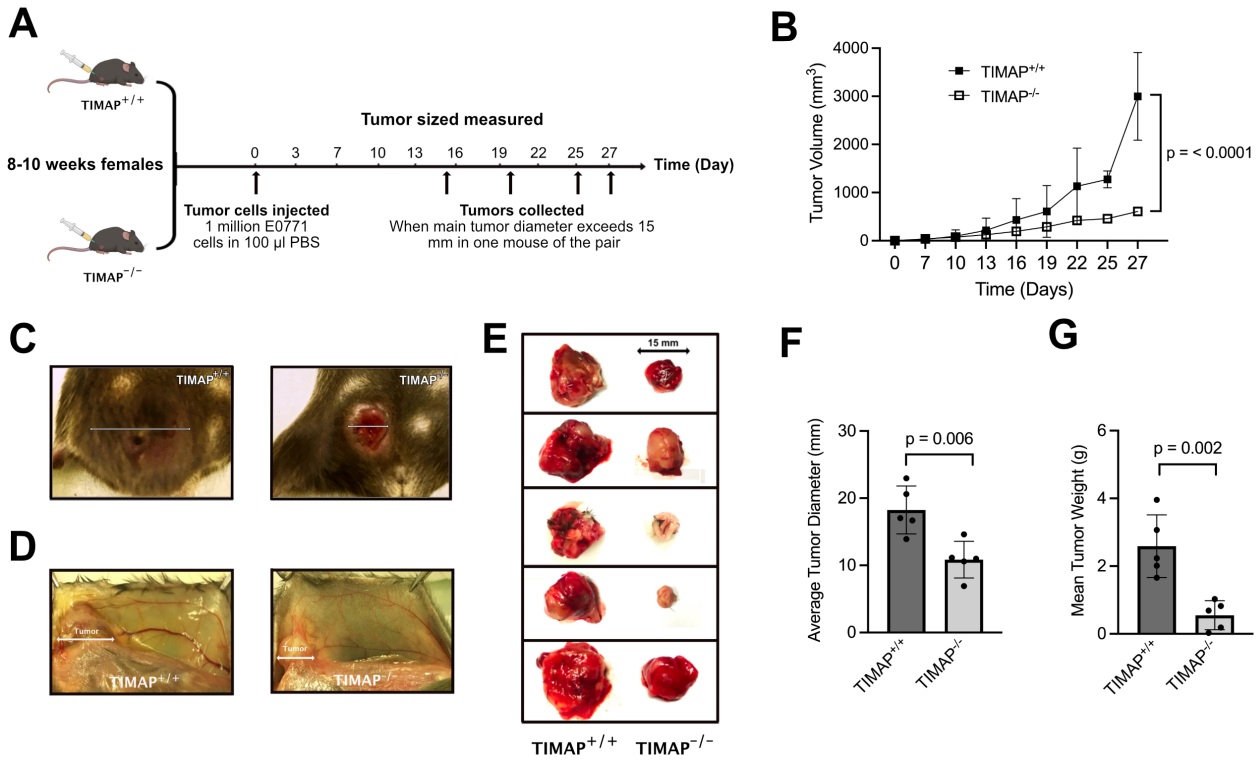


Figure 3.1 Reduced tumor growth in TIMAP deficient mice

A. Protocol. E0771 cells were injected into the mammary fat pad of 8–10-week-old female TIMAP$^{+/+}$ and TIMAP$^{-/-}$ mouse pairs, tumor growth was evaluated over time, and mice were euthanized when the tumor volume reached 15 mm in one mouse of each pair (1 pair each euthanized on days 15, 20, 25 and 2 pairs on day 27). **B**. Tumor growth rate in TIMAP$^{+/+}$ (■) and TIMAP$^{-/-}$ ( ) mice (n = 5 pairs on days 0-13, n = 4 pairs on day 19, n = 3 pairs on days 22 & 25, n = 2 pairs on day 27, Mean +/− SD). **C**. Necrotic Skin ulceration in a TIMAP$^{-/-}$ mouse shown with its paired TIMAP$^{+/+}$ control just prior to euthanasia. White lines = tumor diameter. **D**. Blood vessels surrounding tumors in one mouse pair. The white lines show the approximate tumor size. **E**. Tumors after excision from TIMAP$^{+/+}$ and TIMAP$^{-/-}$ mouse pairs. **F**. Average tumor diameter after excision (n = 5 pairs, p = 0.006). **G**. Wet tumor weight after excision (n = 5 pairs, p = 0.002). Statistical analysis was by two-way ANOVA (B) or Student *t*-test (F & G).

3.2.2 TIMAP deletion in mice reduces tumor angiogenesis.

The findings that TIMAP deficiency in EC impairs *in vitro* sprouting angiogenesis and its deletion *in vivo* reduced tumor growth suggested that TIMAP stimulates *in vivo* angiogenesis and supports tumor growth. The finding that tumors of $TIMAP^{-/-}$ mice became necrotic and then involuted might be due to insufficient tumor angiogenesis. To determine whether reduced tumor growth in $TIMAP^{-/-}$ mice was due to insufficient tumor angiogenesis, we next determined the vascular density in tumors from $TIMAP^{+/+}$ and $TIMAP^{-/-}$mice by evaluating tumor tissue EC markers. The tumor vessel density was evaluated by IF microscopy for PECAM-1 and WB analysis of tumor tissue for Tie2 and ESM-1. We found that the average PECAM-1 IF staining density was significantly less in tumors of $TIMAP^{-/-}$ than in $TIMAP^{+/+}$ mice (Figure 3.2 A). Similarly, the abundance of both Tie2 (Figure 3.2 B) and ESM-1 (Figure 3.2 C) were lower relative to GAPDH in tumor tissue lysates from $TIMAP^{-/-}$ than $TIMAP^{+/+}$ mice. Lung tissues were harvested from $TIMAP^{-/-}$ and $TIMAP^{+/+}$ mice, and TIMAP abundance was analyzed by WB to ensure that TIMAP is absent in $TIMAP^{-/-}$ mice (Figure 3.2 D). These findings indicate that there were less EC and, therefore, fewer blood vessels and less angiogenesis in the mammary tumors of $TIMAP^{-/-}$ mice than in $TIMAP^{+/+}$ mice.

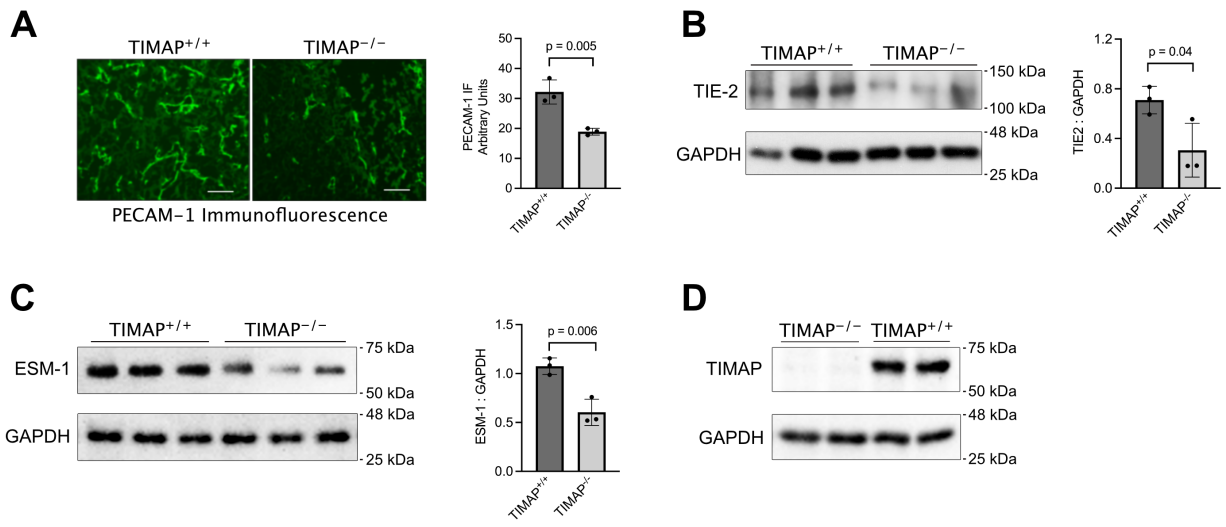


Figure 3.2 Impaired tumor angiogenesis in TIMAP deficient mice

A. PECAM-1 IF immunoreactivity in tumor tissues from one pair of TIMAP$^{+/+}$ and TIMAP$^{-/-}$ mice (left panel) and quantitative analysis of PECAM-1 immunoreactivity in tumor tissues from three pairs of TIMAP$^{+/+}$ and TIMAP$^{-/-}$ mice (right panel, $p = 0.005$). **B, C:** WB analysis and densitometric quantification of tumor tissue lysates from three pairs of TIMAP$^{+/+}$ and TIMAP$^{-/-}$ mice for Tie2 (**B**, $p = 0.04$) and ESM-1 (**C**, $p = 0.006$). **A−C:** n = 3 pairs because tumors in two TIMAP$^{-/-}$ mice had regressed, and insufficient tissue was available for analysis. **D.** WB of TIMAP and GAPDH in mouse lung tissue lysates from TIMAP$^{-/-}$ and TIMAP$^{+/+}$ mice.

3.2.3 Chronic hypoxia upregulates mouse pulmonary endothelial TIMAP abundance

During tumorigenesis, tumor hypoxia is the principal driver of angiogenesis that support tumor growth and progression by upregulating several angiogenic pathways in EC[84]. Since TIMAP is a pro-angiogenic EC protein required for *in vivo* and *in vitro* angiogenesis[383], we next postulated that TIMAP is one of the angiogenic molecules that might be upregulated under hypoxic conditions. To determine the effect of hypoxia on EC TIMAP abundance, TIMAP levels were evaluated in lung tissues from control and chronically hypoxic mice. Tissues from a previously published study[624] were used for analysis. In wild-type mice, the TIMAP abundance in whole lung lysates was significantly higher after 35 days of chronic hypoxia compared to that in control mouse lung lysates (Figure 3.3 A). Furthermore, TIMAP IF microscopy of the lung tissue sections also showed that TIMAP immunoreactivity is localized to the lung EC (Figure 3.3 B). This demonstrates that chronic hypoxia *in vivo* results in TIMAP upregulation in pulmonary EC.

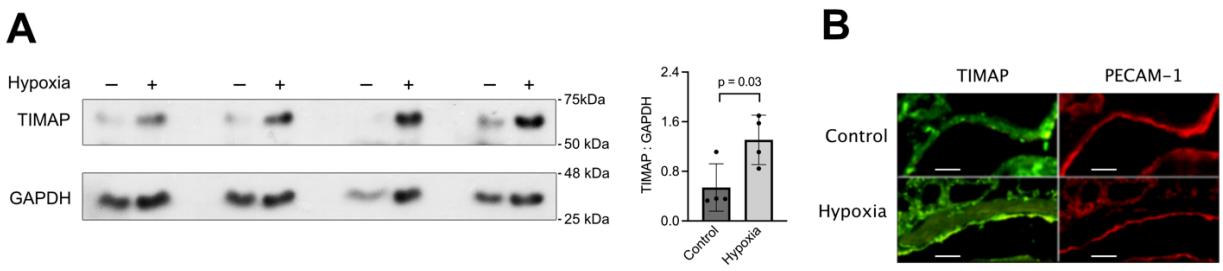


Figure 3.3 Increased TIMAP abundance in response to chronic hypoxia

A. WB analysis and densitometric quantification of TIMAP abundance in lung lysates from control (-) and chronically hypoxic (+) mice exposed to 35 days of progressive hypoxia. (n= 4 mouse pairs, p = 0.030). Each lane represents a distinct mouse, and the loading control is the whole lung lysate GAPDH. **B.** Localization of TIMAP (green) and the PECAM-1 (red) immunoreactivity in lung EC of control and hypoxic mice.

3.2.4 Angiogenic growth factors induce EC TIMAP expression

Tumor hypoxia activates the HIF pathway in cancer and other cell types, which results in transcription, synthesis, and release of angiogenic growth factors that stimulate nearby vascular EC proliferation and sprouting angiogenesis[73, 692]. *In vitro* studies also showed that hypoxia and HIF-1α activation stimulate the expression of angiogenic growth factors in cancer cells[693, 694]. The E0771 mammary tumor cell line is characterized by high proliferation capacity in culture[695], and similar to some other tumors[696-698], we observed very high levels of HIF-1α expressed by these cells even when they are grown under normal (21%) oxygen concentration (Figure 3.4 A). Therefore, we hypothesized that enhanced EC TIMAP expression under hypoxic tumor environment is mediated at least partly by the effect of cancer cell angiogenic growth factors. To investigate our proposed hypothesis, we evaluated TIMAP abundance in HUVEC that were either cultured alone or co-cultured with E0771 cells in growth factor- and serum-free EC basal medium (EBM-2^{TM}) for 18 hours (Figure 3.4 B). Alternatively, growth factor- and serum-free EBM-2 was conditioned by E0771 cells and this conditioned medium was added to quiescent HUVEC, followed by evaluation of TIMAP levels. Growth factor- and serum-free EBM-2 not exposed to E0771 cells but otherwise treated identically was used as control (Figure 3.4 C). We observed that HUVEC co-cultured with E0771 cells and those treated with E0771-conditioned medium were more confluent compared to their controls (Data not shown). In HUVEC co-cultured with E0771 cells in growth-factor- and serum-free medium, TIMAP abundance was significantly higher than in HUVEC grown in the absence of E0771 cells (Figure 3.4 B). Similarly, when HUVEC were cultured in E0771 cell-conditioned medium, TIMAP levels were higher than in HUVEC treated with control medium (Figure 3.4 C). To further support our hypothesis, we investigated whether the angiogenic growth factors can

directly augment TIMAP abundance in cultured EC. HUVEC (Figure 3.4 D & E), hLEC (Figure 3.4 F), and hGEC (Figure 3.4 G), were grown in growth factor- and serum-free (EBM-2) medium with or without the addition of endothelial growth factors (VEGF, aFGF, IGF-1, EGF) for 18 hours and TIMAP levels were evaluated by WB. We found that TIMAP abundance was significantly higher in EC that were grown in growth factor-enriched relative to growth factor-deprived media. Finally, RT-qPCR analysis revealed that TIMAP mRNA levels were significantly higher in HUVEC provided with growth factor-supplemented medium for 4 hours compared to HUVEC grown in growth factor-free medium (Figure 3.4 E). These results imply that the cancer cell-EC crosstalk during tumor angiogenesis is mediated at least partly by the angiogenic growth factors that upregulate EC TIMAP at the mRNA level.

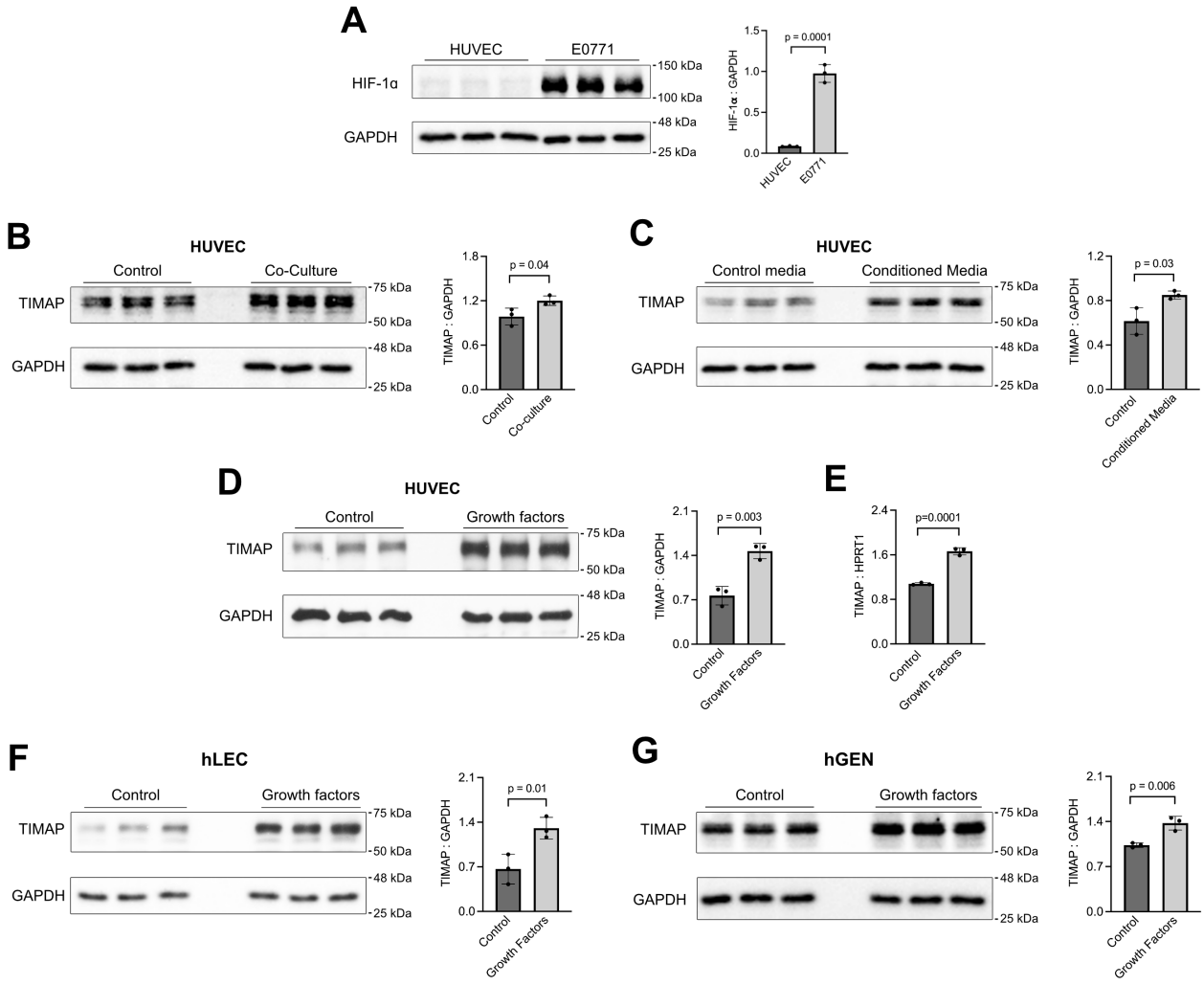


Figure 3.4 Angiogenic growth factors raise EC TIMAP protein levels

A. WB and densitometric quantification of HIF-1**α** in lysates from HUVEC and E0771 cells grown under non-hypoxic conditions for 18 hours (n = 3 distinct experiments, *p* = 0.0001). **B.** WB and densitometric analysis of TIMAP in lysates from HUVEC ± E0771 co-culture (n = 3 distinct experiments, *p* = 0.04) HUVEC were cultured alone or co-cultured with E0771 for 24 hours. **C.** WB and densitometric analysis of TIMAP level in lysates from HUVEC cultured in E0771-conditioned or control media for 18 hours (n = 3 distinct experiments, *p* = 0.03). **D.** WB and densitometric analysis of TIMAP level in lysates from HUVEC cultured in growth factor-free (EBM-2) or growth factor supplemented (VEGF 2

ng/ml, IGF-1 5 ng/ml, FGF 10 ng/ml, EGF 5 ng/ml) medium for 18 hours (n = 3 distinct experiments, p = 0.006). **E.** Quantitative RT-qPCR of TIMAP mRNA relative to HPRT1 in HUVEC cultured in EBM-2 or growth factors-enriched medium for 4 hours (n = 3, p = 0.0001). **F, G:** WB and densitometric analysis of TIMAP in lysates from human lung Microvascular EC (hLEC) (**F**) and primary human glomerular microvascular ECs (hGEC) (**G**) cultured in EBM-2 or growth factors-enriched medium (VEGF 2 ng/ml, IGF-1 5 ng/ml, FGF 10 ng/ml, EGF 5 ng/ml) for 18 hours (n = 3, p = 0.01 and 0.006, respectively).

3.2.5 Growth factor-induced TIMAP expression is mainly mediated by VEGF & IGF-1

Since we found that growth factor-enriched medium significantly enhanced EC TIMAP expression in cultured EC, we next determined the effect of the individual growth factors on the EC TIMAP abundance. The EC growth medium (EGM-2) contains a combination of VEGF, IGF-1, FGF and EGF, and therefore, we cultured EC in EBM-2 with or without the addition of the individual growth factors VEGF, IGF-1, FGF or EGF for 18 hours. WB analysis of the EC lysates revealed that TIMAP levels are significantly increased in EC treated with VEGF and IGF-1 compared to control, growth factor- and serum-free conditions (Figure 3.5 A & B). FGF and EGF also raised TIMAP abundance (Figure 3.5 C & D), but less than that observed with VEGF and IGF-1 at the concentrations used. Interestingly, the tyrosine kinase receptor (RTK) inhibitor Sunitinib, which inhibits VEGF- but not FGF or EGF receptors[699], in combination with the IGF-1R inhibitor Picropodophyllin abolished the effect of the E0771 cell-conditioned medium on EC TIMAP abundance (Figure 3.4 E). These results suggest that the effects of growth factor-supplemented EC culture medium and E0771 cell-conditioned medium on the EC TIMAP expression are mainly mediated by VEGF and IGF-1. The data also support the hypothesis that angiogenic growth factors released by cells in which HIF-1α is stabilized can induce TIMAP expression in EC.

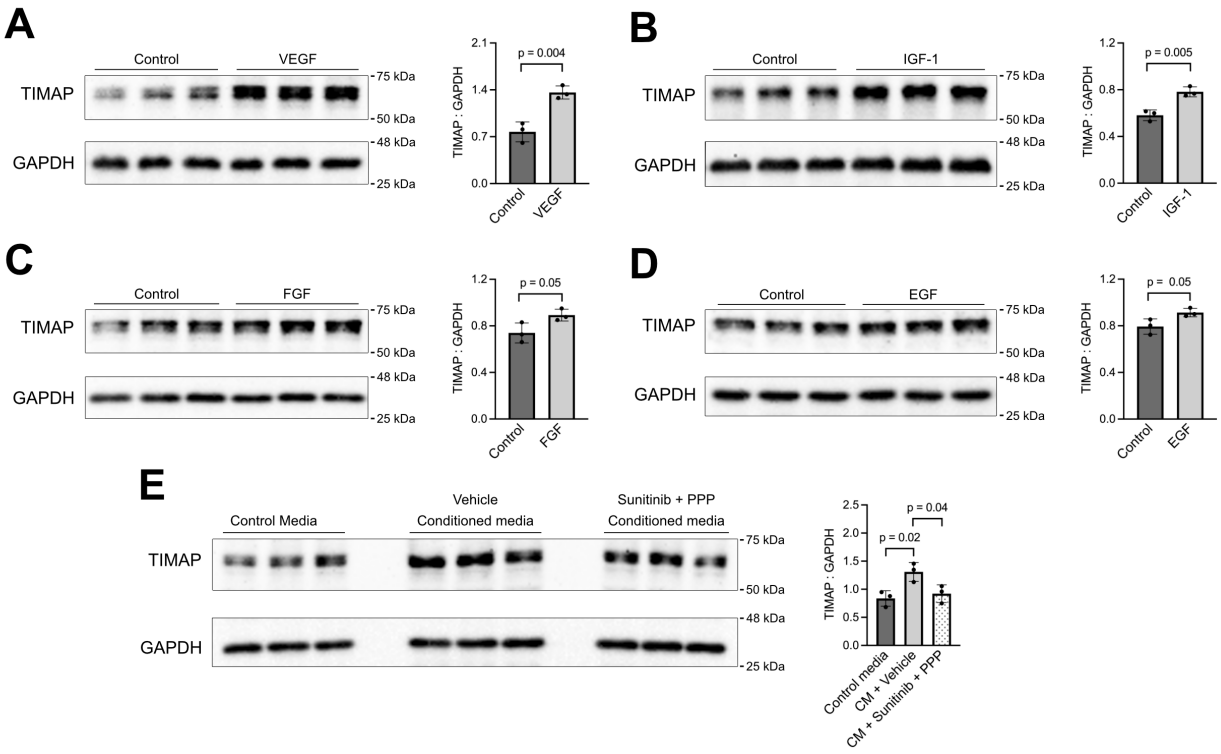


Figure 3.5 TIMAP expression in response to distinct EC Growth factors

A-D: WB and densitometric analysis of TIMAP relative to GAPDH in lysates from HUVEC cultured in growth factor-free (EBM-2) medium with or without the addition of individual growth factors for 18 hours (Each, n = three distinct experiments). **A.** VEGF 100 ng/ml or vehicle ($p = 0.004$). **B.** IGF-1 100 ng/ml or vehicle ($p = 0.005$). **C.** FGF 100 ng/ml or vehicle ($p = 0.05$). **D.** EGF 100 ng/ml or vehicle ($p = 0.05$). **E.** WB and densitometric analysis of TIMAP relative to GAPDH in lysates from HUVEC cultured in E0771-conditioned or control medium with the addition of Sunitinib and Picropodophyllin or vehicle for 18 hours (n = three distinct experiments, $p = 0.02$, one-way ANOVA with post-hoc Tukey's multiple comparisons).

3.3 Discussion

This component of my work establishes that TIMAP expression *in vivo* is an important component of tumor angiogenesis, at least in the mouse breast cancer model. Since TIMAP is also upregulated in lung EC of mice exposed to chronic hypoxia, it follows that a hypoxic microenvironment is a likely stimulus for enhanced TIMAP expression. The findings in this part of the work also indicate that angiogenic growth factors, produced in the hypoxic tumor microenvironment, provide stimulus for TIMAP expression.

Growth of the E0771 mammary tumors in TIMAP$^{-/-}$ mice during the first 7-10 days was similar to that observed in the wild-type littermate controls, as determined by externally applied calipers, so the differences in the initial establishment of the tumors did not account for the final differences in tumor size and weight. However, beginning in the second week, all tumors in the TIMAP$^{-/-}$ mice grew more slowly than those in their paired controls, and in two TIMAP$^{-/-}$ mice, the tumors regressed. Also, in the TIMAP$^{-/-}$ mice, tumors tended to ulcerate, with bleeding and skin necrosis at the tumor site (Figure 3.1 C), consistent with tumors that outstrip their blood supply during rapid expansion. All tumors in the TIMAP$^{-/-}$ mice were smaller than those in wild-type controls at the study endpoint (Figure 3.1 B-E), and the tumor micro-vessel density was substantially lower in tumors of TIMAP$^{-/-}$ mice (Figure 3.2 A). Also consistent with inadequate tumor vascularization in the TIMAP$^{-/-}$ mice were the findings of large blood vessels supplying tumors in the wild-type, but not in the TIMAP$^{-/-}$ mice (Fig. 3.1 D), and lower levels of the EC markers Tie-2 and ESM-1 in whole tumor lysates from TIMAP$^{-/-}$ mice (Fig. 3.2 B & C).

Reduced tumor angiogenesis in the mouse breast cancer model is consistent with previous work which reported that TIMAP silencing reduces the proliferation and survival of cultured EC and impairs *in vitro* sprout formation[383]. The pro-angiogenic effect of TIMAP

in cultured EC was due to reduced PTEN-mediated AKT inhibition. Whether the same mechanism drives TIMAP-dependent tumor angiogenesis *in vivo* remains to be shown. It was also recently shown that TIMAP is highly expressed in human breast cancer and high levels of TIMAP expression are associated with poor patient survival[700]. However, whether TIMAP is overexpressed in the EC of human breast cancer still needs to be determined. It turns out that the E0771 mouse adenocarcinoma cells do not express TIMAP, at least in culture (Figure 2.1). In addition, all of the mice received exactly the same tumor cells, so we do not think that TIMAP in the E0771 cells played a role in tumor growth or tumor angiogenesis in this study.

Inhibition of angiogenesis has been widely used as a therapeutic approach to combat cancers, and the most commonly used angiogenesis inhibitors are agents that inhibit the EC VEGF signaling pathway either by blocking the VEGF receptor or deactivating its ligand by specific antibodies[664, 701]. The effect of VEGF inhibition on tumour angiogenesis, however, is usually not sustainable, and most tumors eventually develop drug resistance[407, 702]. It would be very interesting to determine whether treatment of patients with anti-angiogenic therapies also reduces TIMAP expression in the tumor vasculature. Since tumors can activate alternative pathways to maintain angiogenesis and support their growth[703-705], it will also be of interest to find out whether resistance to angiogenesis inhibitors is associated with growth factor independent expression of TIMAP. If this were the case, targeting of TIMAP could theoretically prevent resistance to anti-angiogenic therapies. It has also been shown that a combination of anti-angiogenic therapies can offer enhanced clinical effectiveness than using single remedies[706-708], so targeting of TIMAP in combination with other anti-angiogenic therapies could also be helpful.

At this time, it is still unclear whether impaired tumor vascularization in TIMAP$^{-/-}$ mice is due to a reduction in EC tip cell formation, stalk cell proliferation, or both. TIMAP is a myosin phosphatase inhibitor that enhances MLC2 phosphorylation *in vivo* and in cultured cells[591], and TIMAP enhances EC proliferation and survival through its effects on AKT and PTEN phosphorylation[383]. Filopodia formation in angiogenic tip cells, EC proliferation, migration and vessel branching all require enhanced myosin activity[674, 709], and AKT activity is critical for stalk cell survival[710]. TIMAP could therefore act in all these processes. Ulceration, bleeding, and necrosis of tumors in TIMAP$^{-/-}$ mice might reflect greater tumor hypoxia and/or disordered angiogenesis with an abnormal, less stabilized vasculature. During the late phase of angiogenesis, recruitment of pericytes is required for stabilization and maturation of new vascular walls[711]. During tumorigenesis, the aggressive proliferation of neoplastic cells and the excessive production of angiogenic factors results in the development of disorganized, poorly mature vasculature[74]. Therefore, further experiments to evaluate the morphological changes and abundance of pericytes in tumor tissues will be needed to investigate whether TIMAP plays a role in vessel maturation.

During tumorigenesis, tumor hypoxia generated due to rapid cellular proliferation is the main stimulus that drives tumor angiogenesis[72]. Hypoxic cells synthesize and release pro-angiogenic growth factors to stimulate neighbouring EC proliferation and sprouting into the hypoxic tissue[84, 712-714]. Since tumor-driven angiogenesis is principally mediated by hypoxia which, in turn, induces the synthesis of angiogenic growth factors by tumor and stromal cells[84, 714, 715], we determined whether TIMAP is upregulated during hypoxia *in vivo*. TIMAP is predominantly expressed by EC, and lung tissue contains large numbers of EC that line the macro-and microvasculature[716]. TIMAP abundance in lung

lysates of wild-type mice subjected to chronic hypoxia was significantly higher than in control mice (Figure 3.3 A), and TIMAP was localized to the pulmonary endothelium (Figure 3.3 B), evidence that hypoxia *in vivo* raises TIMAP levels. It could be argued that we should have tried to correlate *in vivo* tumor hypoxia levels with TIMAP expression in the tumor vessels of wild-type mice, but such correlations are very difficult to quantify. We chose instead to use the more controlled approach of chronic hypoxia. Even so, since this component of the work could not rule out the possibility that the increase in pulmonary TIMAP abundance reflects an increase in EC number, and influences of blood flow and pressure could not be ruled out, additional work in cultured cells was done.

Tumor hypoxia is a well-known angiogenic stimulus and activation of HIF-α in hypoxic cells induces the transcription of angiogenic growth factors, which, in turn, stimulate angiogenic sprouting by neighbouring blood vessels into the hypoxic tissue[84]. In cultured cancer cells, hypoxia-induced HIF-1α stabilization also leads to a substantial increase in the expression of VEGF as well as IGF-1 and the IGF1 receptor[694]. We found that the E0771 cells express high levels of HIF-1α even in the absence of hypoxia (Figure 3.4 A) and that these cells release mediators that induce TIMAP expression in EC even in the absence of hypoxia (Figure 3.4 B & C). Angiogenic growth factor-stimulated TIMAP expression was observed in 3 distinct EC subtypes derived from umbilical vein, lung and renal glomeruli, and the effect of E0771 conditioned medium on EC TIMAP expression was blocked by a combination of the VEGF inhibitor sunitinib and the IGF-1 inhibitor Picropodophyllin (Figure 3.5 E), so it is safe to conclude that angiogenic growth factors raise EC TIMAP abundance even in the absence of hypoxia. We think that VEGF and IGF-1 are likely to be the main players, but we recognize that formal concentration- and

time-course experiments would have to be done to be certain. Nonetheless, since hypoxia is well-known to stimulate the expression and release of several angiogenic growth factors in combination, the experiments suggest that hypoxia-induced TIMAP upregulation in EC is mediated, at least in part, by angiogenic growth factors released from neighbouring cells in which HIF-1α is stabilized.

In summary, our findings in the E0771 tumor model reveal that TIMAP is an important contributor to EC tumor angiogenesis *in vivo*, possibly through its function as an intracellular phosphatase inhibitor. The data are interpreted to indicate that mammary tumors in TIMAP deficient mice become necrotic and then involute due to insufficient hypoxia-driven angiogenesis. In the hypoxic environment, the EC TIMAP expression is significantly enhanced, and *in vitro* experiments suggest that hypoxia-induced TIMAP upregulation is at least partly mediated by the effect on angiogenic growth factors. Together, our observations indicate that TIMAP inhibition provides a potential therapeutic approach to target tumor angiogenesis and, therefore, merits further investigation to determine whether TIMAP could be targeted in the treatment of highly vascular tumors, including renal cell carcinoma.

Chapter 4 Hypoxia and growth factors antagonize ALK1-induced TIMAP inhibition

4.1 introduction

TIMAP is a pro-angiogenic protein phosphatase regulatory subunit expressed mainly by EC[591]. It was first discovered as a protein profoundly inhibited by TGF-1β treatment in glomerular EC[583]. TGF-1β belongs to the TGF-β superfamily of proteins that bind and activate a heteromeric complex of Ser/Thr kinase receptors[717]. Generally, TGF-β receptor complexes are composed of type-I receptors, ligand-binding type-II receptors, and auxiliary type-III receptors[717]. The type-1 TGF-β receptor ALK1, and the auxiliary type-III receptor endoglin are EC-specific. Although EC also express ALK5 and other type-I TGF-β receptors. Several ligands of the TGF-β superfamily can bind and activate TGF-β receptors, but each ligand induces the formation of a unique complex of TGF-β receptors[718]. Upon ligand binding, the constitutively active type-II receptors dimerize and recruit type-I receptors to form a signaling heterotetramer with Ser/Thr kinase activity[717]. Depending on the type of ligand, the type-II receptor can recruit and activate ALK1 or ALK5 type-I receptor and stimulate SMAD1/5/8 or Smad2/3 phosphorylation, respectively[447]. Subsequently, phosphorylated SMADs form a complex with the Co-SMAD (SMAD4) to translocate into the nucleus and induce transcription of target genes[447]. It is well documented that BMP-9 has high affinity for the ALK1 receptor and strongly activates its downstream SMAD1/5/8 cascade[547]. Although TGF-β1 has higher affinity for the ALK5 receptor and preferentially activates its downstream SMAD2/3 cascade, it can also activate the ALK1/SMAD1/5/8 pathway[719]. It has been previously shown that TIMAP is a target of TGF-β1-mediated downregulation in EC[583], though whether this is downstream of the ALK1 or ALK5 pathway has not yet been explored.

Under hypoxic conditions, both TGF-β and HIF pathways play critical roles in regulating several cellular responses involved in angiogenesis[720]. Several studies demonstrated interactions between TGF-β1 and HIF-1α and showed that TGF-β1 and HIF-1α induce the expression of each other and both are significantly upregulated during hypoxia-induced tumorigenesis[569]. The crosstalk between HIF-1α and ALK5/SMAD2/3 pathways is well described, and several studies revealed that HIF-1α physically interacts with both ALK5 and SMAD2/3 and induces their expression[569, 720]. Hypoxia-induced HIF-1α stabilization has also been shown to augment TGF-β/ALK5 signalling by enhancing the phosphorylation of SMAD2/3 in endothelial and non-EC[569, 570]. HIF-1α also enhances the interaction of SMAD4 with the SMAD2/3 and their subsequent nuclear translocation[575]. In contrast, under hypoxic conditions *in vivo*, expression of BMPRII was down regulated in pulmonary EC, and hypoxia strongly attenuated SMAD1/5/8 phosphorylation *in vivo* and BMP-9-induced SMAD1/5/8 phosphorylation in cultured EC [578]. Although it has been demonstrated that the hypoxia-induced reduction of the SMAD1/5/8 phosphorylation is HIF-1α dependent[576], the crosstalk between HIF-1α and ALK1/SMAD1/5/8 is not fully understood.

The TGF-β signaling is very sophisticated as it can be activated by a wide range of ligands[721] and often interacts with other signaling pathways that play essential roles in normal development and disease[722]. Through the non-canonical pathway, ligand-induced activation of TGF-β receptors triggers a variety of non-SMAD intracellular signaling pathways to regulate a wide array of cellular functions[487]. In addition to the well-defined Ser/Thr phosphorylation, both type-1 and type-2 TGF-β receptors undergo phosphorylation on multiple tyrosine residues and subsequently regulate the activation of

the ERK MAPK pathway[484, 723, 724]. The PI3K/AKT pathway is another SMAD-independent signaling pathway triggered downstream of some activated TGF-β receptors[725]. Immunoprecipitation experiments revealed that the TGF-β receptors indirectly interact with the P85 regulators subunit[726], and the Ser/Thr kinase activity of the TGF-β receptor is required for TGF-β-induced PI3K/AKT pathway regulation[727]. Since the PI3K/AKT and the ERK MAPK pathways are also activated by many growth factors that signal through tyrosine kinase receptors, these signaling pathways are considered to be the links that mediate crosstalk between TGF-β and other growth factor signaling pathways[722]. Several studies have illustrated that the signaling interplay between TGF-β and other growth factors regulates numerous biological functions depending on cellular context[441]. During tumorigenesis, hypoxia-induced angiogenesis is partly mediated by enhanced expression of angiogenic growth factors such as VEGF and IGF-1[728-731]. Under physiological conditions, TGF-β has cytostatic effects by antagonizing the proliferative pro-survival cellular responses induced by the PI3K/AKT pathway[732]. However, during tumorigenesis, tumor hypoxia enhances the expression of TGF-β[733] and its signaling integration with other growth factors drives tumor growth and progression[734-736]. TGF-β modulates the expression of numerous genes to regulate a variety of cellular responses involved in angiogenesis and carcinogenesis[518, 734]. In EC, TIMAP is one of the genes strongly suppressed by TGF-β[583] and plays an essential role in regulating EC survival and *in vitro* angiogenesis[383]. As illustrated in chapter 3, we found that TIMAP is regulated by growth factors and its expression *in vivo* is significantly enhanced by chronic hypoxia. Here, we sought to explore mechanisms of hypoxia and growth factors induced TIMAP expression in EC. We found that TIMAP expression in EC is suppressed by the ALK1, not the ALK5 pathway and that hypoxia and angiogenic growth factors induce EC TIMAP expression by

inhibiting the ALK1 pathway. While ALK1 inhibition accounted fully for the direct effect of hypoxia on EC TIMAP expression, angiogenic growth factors also stimulated TIMAP expression through additional ALK1-independent pathways.

4.2. Results

4.2.1. Hypoxia antagonizes serum-mediated TIMAP repression

Since we had observed increased TIMAP levels in lung EC of chronically hypoxic mice (Figure 3.3 A & B), we explored whether hypoxia could alter EC TIMAP abundance by acting directly on EC. HUVEC were first pre-conditioned in growth factor-free EBM2 containing 1% serum followed by 18 hours in hypoxic (1% O_2) or control (21% O_2) conditions. At the time the cells were placed into the hypoxia chamber, the medium was either changed to fresh EBM2 with 1% FBS or left unchanged. Hypoxia consistently raised TIMAP abundance when the EC were provided with a fresh medium containing 1% FBS (Figure 4.1 A). By contrast, no change in TIMAP abundance was observed if cells were not provided with a fresh FBS-containing medium (Figure 4.1 B). Similarly, HIF-α stabilization with the prolyl hydroxylase inhibitor Roxadustat raised TIMAP levels in EC in the presence of fresh 1% FBS (Figure 4.1 C) but not without fresh FBS (Figure 4.1 D). Fresh medium containing 1% FBS alone reduced the EC TIMAP protein abundance (Figure 4.1 E) and TIMAP mRNA levels (Figure 4.1 F) compared to controls not provided with fresh 1% FBS. These results indicate that HIF-α stabilization, either in response to hypoxia or prolyl hydroxylase inhibition, blocks an inhibitory effect of serum on TIMAP expression.

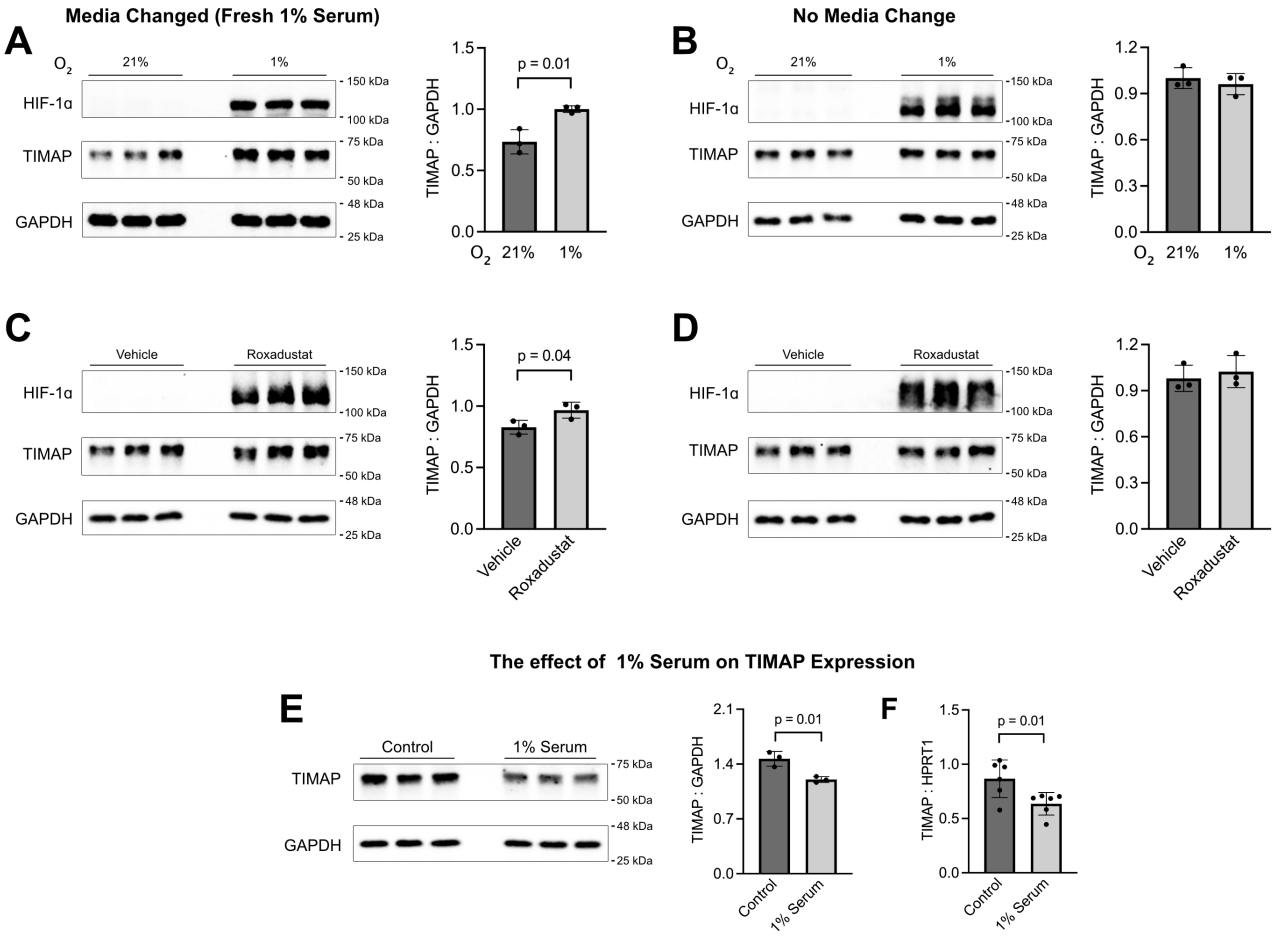
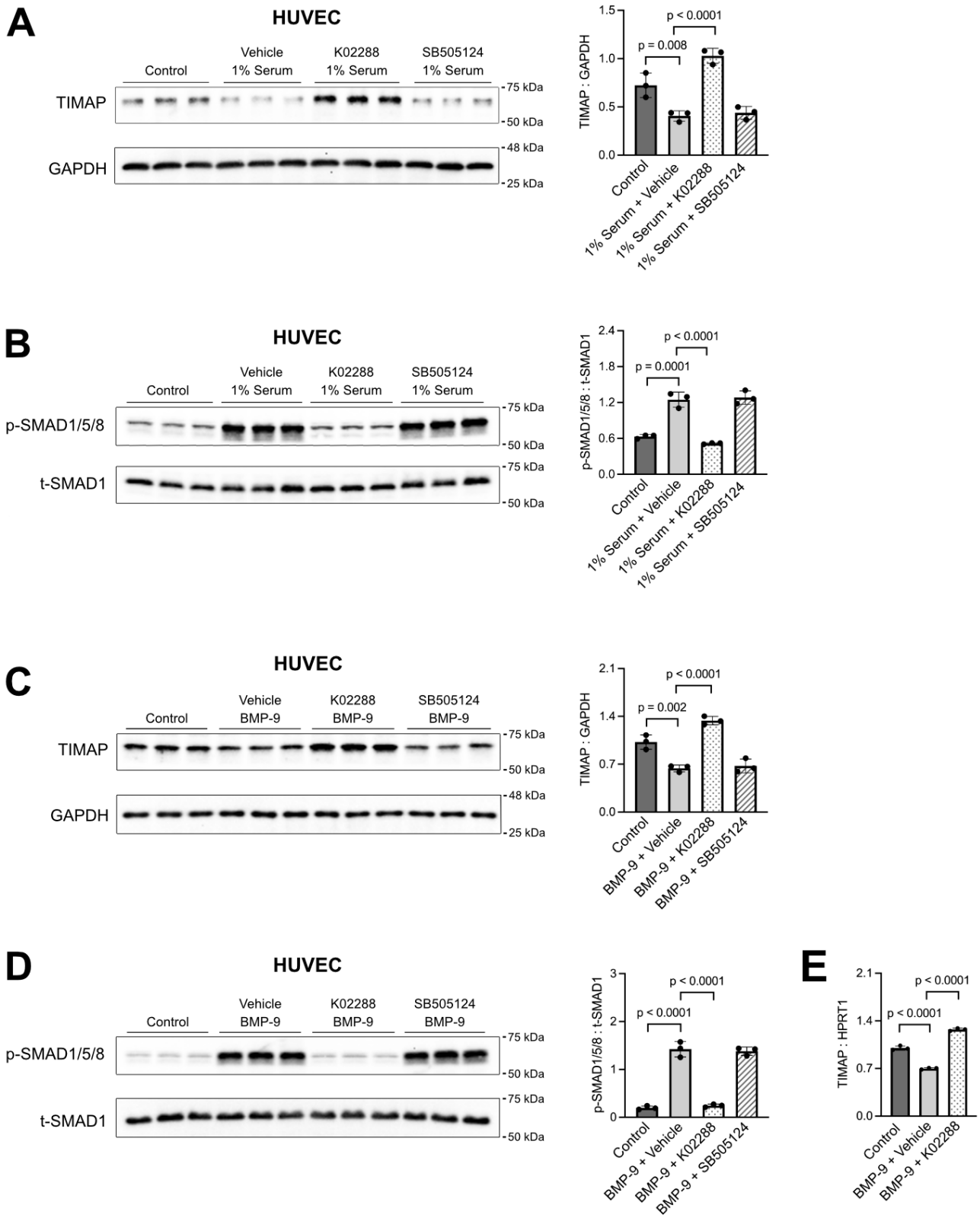


Figure 4.1 Hypoxia antagonizes serum-mediated TIMAP inhibition

WB analysis and densitometric quantification of the EC TIMAP abundance relative to the whole cell lysate GAPDH **A.** HUVEC exposed to 21% or 1% O_2 in the presence of fresh 1% FBS for 18 hours (p = 0.01). **B.** HUVEC exposed to 21% or 1% O_2 without medium change for 18 hours. **C.** HUVEC treated with 1 μM Roxadustat or vehicle in the presence of fresh 1% FBS for 18 hours (p = 0.04) **D.** HUVEC treated with Roxadustat 1 μM or vehicle without medium change for 18 hours. **E.** HUVEC cultured in growth factor-free media with or without fresh 1% FBS for 18 hours (p = 0.01). **A−E.** n= 3 distinct experiments each. **F.** Quantitative RT-PCR of TIMAP in HUVEC ± fresh 1% FBS for 4 hours (n = 6 distinct experiments, p = 0.01).

4.2.2. BMP-9 mimics serum-induced TIMAP repression

Since serum contains several TGF-β ligands[548, 737] and TIMAP is strongly repressed by TGF-β pathway activation[583], we next investigated whether serum downregulates EC TIMAP by activating a specific TGF-β pathway. Exposure of EC to FBS as low as 1% significantly reduced TIMAP protein levels in EC, an effect that was completely blocked by the ALK1 inhibitor K02288 but not by the ALK5 inhibitor SB505124 (Figure 4.2 A). Fresh 1% FBS also stimulated SMAD 1/5/8 phosphorylation, and this was inhibited by K02288 but not SB505124 (Figure 4.2 B). Not shown, fresh 1% FBS had no effect on SMAD 2/3 phosphorylation in these cultured HUVEC. Since BMP-9 is the main TGF-β family ligand in serum capable of activating the ALK1/SMAD1/5/8 but not ALK5/SMAD2/3 pathway[548], we evaluated the effect of BMP-9 on EC TIMAP abundance and SMAD phosphorylation. BMP-9 significantly reduced TIMAP abundance (Figure 4.2 C) and stimulated SMAD1/5/8 phosphorylation (Figure 4.2 D). As expected, inhibition of the ALK1, but not the ALK5 pathway, significantly raised TIMAP abundance in BMP-9 treated cells (Figure 4.2 C) and blocked BMP-9-induced SMAD1/5/8 phosphorylation (Figure 4.2 D). BMP-9 also reduced TIMAP mRNA levels, an effect that was reversed by K02288 (Figure 4.2 E). Finally, BMP-9-mediated inhibition of TIMAP expression was not restricted to HUVEC but was also observed in hLEC and hGEC (Figure 4.2 F & G). The data indicate that activation of the ALK1/SMAD1/5/8 pathway by BMP-9 inhibits TIMAP expression.


A
HUVEC
Control
Vehicle 1% Serum
K02288 1% Serum
SB505124 1% Serum
TIMAP
GAPDH
75 kDa
50 kDa
48 kDa
25 kDa
TIMAP : GAPDH
p = 0.008
p < 0.0001
Control
1% Serum + Vehicle
1% Serum + K02288
1% Serum + SB505124
B
HUVEC
p-SMAD1/5/8
t-SMAD1
p-SMAD1/5/8 : t-SMAD1
p = 0.0001
p < 0.0001
C
HUVEC
Vehicle BMP-9
K02288 BMP-9
SB505124 BMP-9
TIMAP : GAPDH
p = 0.002
p < 0.0001
BMP-9 + Vehicle
BMP-9 + K02288
BMP-9 + SB505124
D
HUVEC
p-SMAD1/5/8 : t-SMAD1
p < 0.0001
E
TIMAP : HPRT1

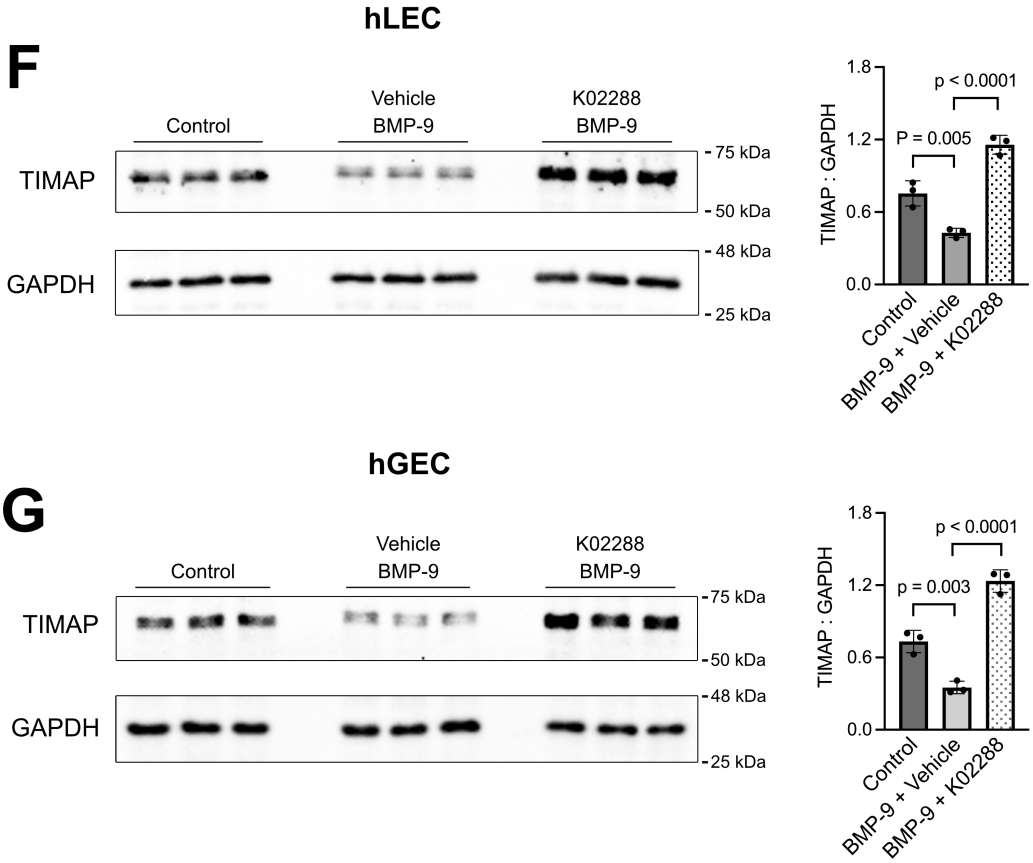
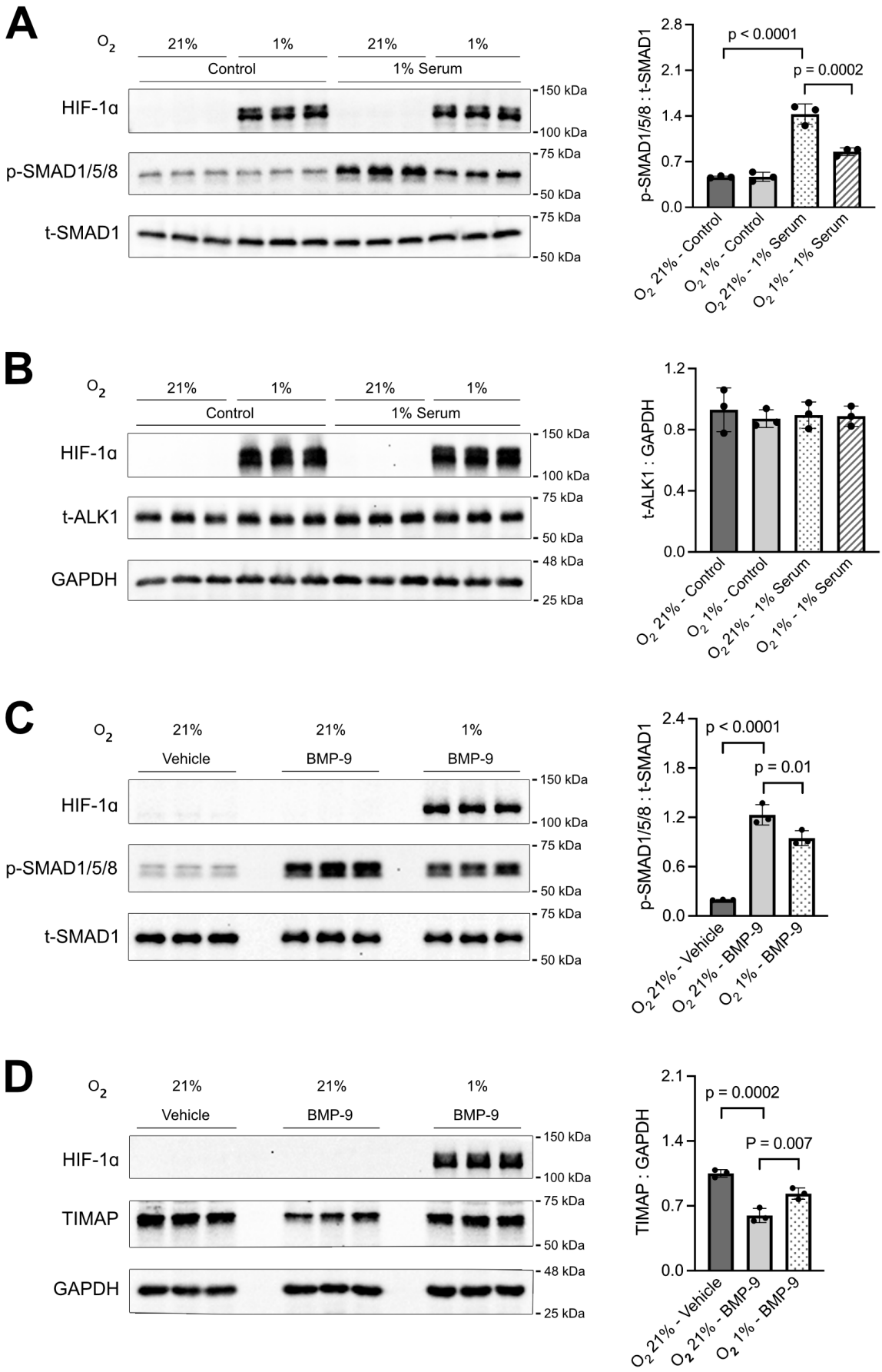


Figure 4.2 BMP-9 inhibits TIMAP expression

A, B: WB and densitometric analysis of TIMAP (**A**) and p-SMAD1/5/8 (**B**) in lysates of HUVEC cultured in growth factor-free medium ± 1% fresh FBS and 1μM K02288 (ALK1 inhibitor), 1μM SB-505124 (ALK5 inhibitor) or vehicle. **C, D:** WB and densitometric analysis of TIMAP (**C**) and p-SMAD1/5/8 (**F**) in HUVEC treated with 5 ng/ml BMP-9 or vehicle and 1μM K02288, 1μM SB-505124 or vehicle. **E.** Quantitative RT-qPCR analysis of TIMAP expression in HUVEC treated with 5 ng/ml BMP-9 or vehicle ± 1μM K02288 or vehicle for 4 hours. **F, G.** WB and densitometric analysis of TIMAP in hLEC **(F**) or hGEC (**G**) treated with 5 ng/ml BMP-9 or vehicle ± 1μM K02288 or vehicle. For all WB, the ratio of p-SMAD1/5/8 : total SMAD was determined 1 hour after treatment initiation and the ratio of TIMAP : GAPDH 18 hours after treatment initiation. **A−G**: n = 3 three distinct experiments each, statistical analysis was by one-way ANOVA with post-hoc Tukey's multiple comparisons.

4.2.3. Hypoxia raises TIMAP levels by antagonizing the ALK1 pathway

Since hypoxia-induced TIMAP upregulation is serum-dependent (Figure 4.1), and FBS-induced TIMAP inhibition is mediated by the ALK1 pathway (Figure 4.2), we next determined whether hypoxia antagonizes the ALK1 signalling cascade. Hypoxia significantly attenuated FBS-induced SMAD1/5/8 phosphorylation without altering the abundance of total SMAD1 (Figure 4.3 A) or the ALK1 receptor (Figure 4.3 B). Similarly, hypoxia significantly reduced BMP-9-stimulated SMAD1/5/8 phosphorylation (Figure 4.3 C) and partially reversed the BMP-9-mediated reduction in TIMAP abundance (Figure 4.3 D). Stabilization of HIF-α with the prolyl hydroxylase inhibitor Roxadustat similarly blunted BMP-9-induced SMAD1/5/8 phosphorylation (Figure 4.3 E) and reversed BMP-9 dependent TIMAP inhibition (Figure 4.3 F). Therefore, stabilization of HIF-α inhibits BMP-9-induced ALK1 activation and ALK1 pathway-dependent TIMAP repression.


A
O2
21%
1%
21%
1%
Control
1% Serum
HIF-1α
p-SMAD1/5/8
t-SMAD1
150 kDa
100 kDa
75 kDa
50 kDa
75 kDa
50 kDa
p-SMAD1/5/8 : t-SMAD1
p < 0.0001
p = 0.0002
O2 21% - Control
O2 1% - Control
O2 21% - 1% Serum
O2 1% - 1% Serum
B
O2
21%
1%
21%
1%
Control
1% Serum
HIF-1α
t-ALK1
GAPDH
150 kDa
100 kDa
75 kDa
50 kDa
48 kDa
25 kDa
t-ALK1 : GAPDH
O2 21% - Control
O2 1% - Control
O2 21% - 1% Serum
O2 1% - 1% Serum
C
O2
21%
21%
1%
Vehicle
BMP-9
BMP-9
HIF-1α
p-SMAD1/5/8
t-SMAD1
150 kDa
100 kDa
75 kDa
50 kDa
75 kDa
50 kDa
p-SMAD1/5/8 : t-SMAD1
p < 0.0001
p = 0.01
O2 21% - Vehicle
O2 21% - BMP-9
O2 1% - BMP-9
D
O2
21%
21%
1%
Vehicle
BMP-9
BMP-9
HIF-1α
TIMAP
GAPDH
150 kDa
100 kDa
75 kDa
50 kDa
48 kDa
25 kDa
TIMAP : GAPDH
p = 0.0002
P = 0.007
O2 21% - Vehicle
O2 21% - BMP-9
O2 1% - BMP-9

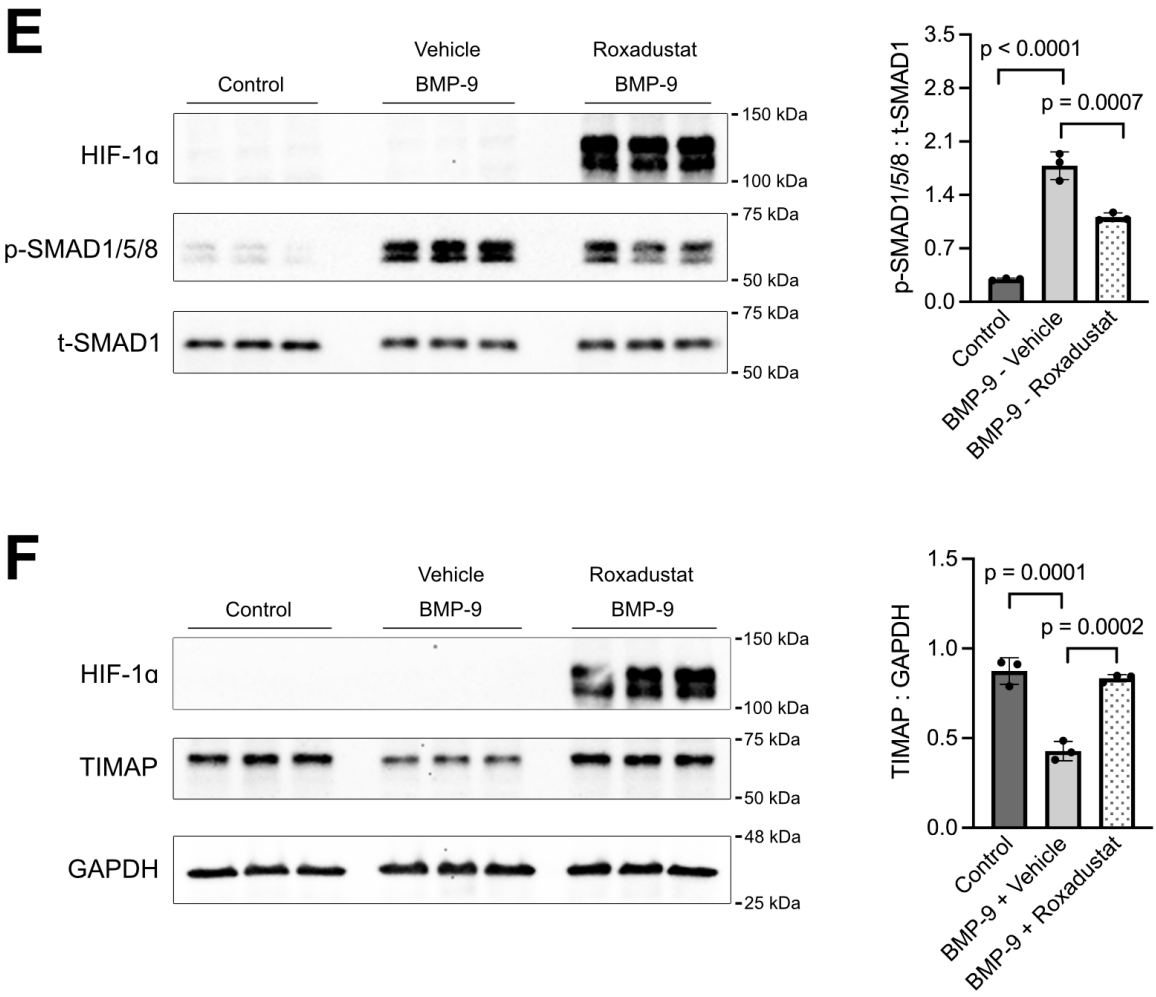
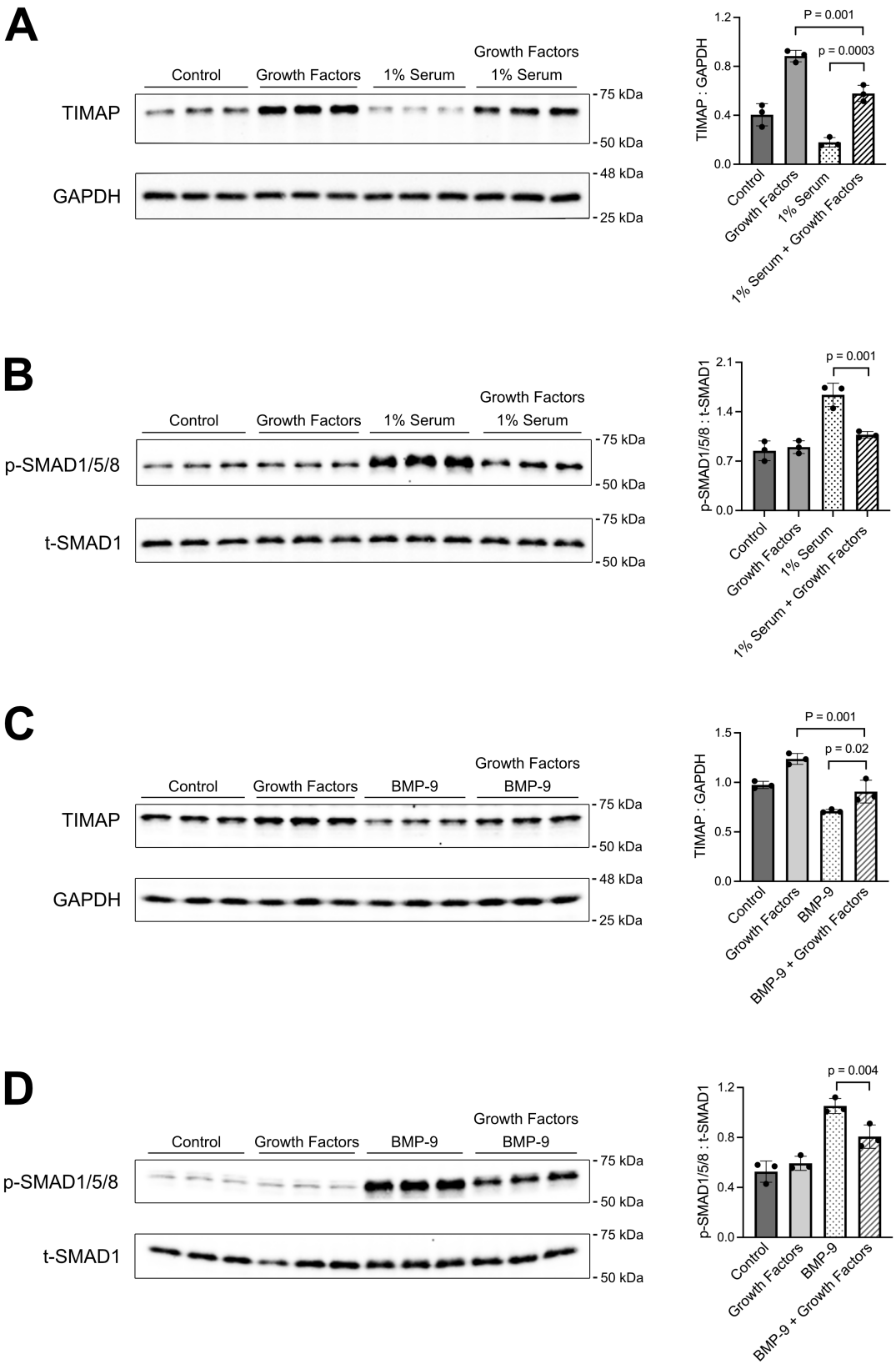


Figure 4.3 Hypoxia upregulates TIMAP abundance by attenuating ALK1 pathway signalling

A, B: WB and densitometric analysis of p-SMAD1/5/8 (**A**) and ALK1 (**B**) in HUVEC ± fresh 1% FBS, exposed to 21% or 1% O_2 for 1 hour (n = 3, one-way ANOVA with Tukey's multiple comparison tests). **C, D:** WB and densitometric analysis of p-SMAD1/5/8 (**C**) and TIMAP (**D**) in HUVEC treated with 5 ng/ml BMP-9 or vehicle and exposed to 21% or 1% O_2 for 1 hour (**C**) or 18 hours (**D**). **E, F:** WB and densitometric analysis of p-SMAD1/5/8 (**E**) and TIMAP (**F**) in HUVEC treated with 5 ng/ml BMP-9 or vehicle and 1 μM Roxadustat or vehicle for 1 hour (**E**) or 18 hours (**F**). **A − F:** n = 3 distinct experiments each; statistical analysis: one-way ANOVA with post-hoc Tukey's multiple comparisons.

4.2.4. Growth factors raise TIMAP level, in part, by inhibiting the ALK1 pathway

We next determined whether the mechanism by which angiogenic growth factors raise TIMAP levels is also due to ALK1 pathway inhibition. Similar to the effects of hypoxia, exposure of EC to angiogenic growth factor-supplemented medium antagonized the FBS-induced reduction in TIMAP abundance (Figure 4.4 A) and reduced FBS-stimulated SMAD1/5/8 phosphorylation (Figure 4.4 B). Similarly, angiogenic growth factors antagonized BMP-9-induced TIMAP repression (Figure 4.4 C) and SMAD1/5/8 phosphorylation (Figure 4.4 D). However, unlike hypoxia (Figure 4.1 A-D), growth factor supplementation increased TIMAP abundance even in the absence of serum or BMP-9 (Figure 4.4 A-C). In addition, growth factor supplementation raised TIMAP protein and mRNA abundance even in the presence of ALK1 pathway inhibition (Figure 4.4 E). Therefore, while angiogenic growth factors raise TIMAP abundance in part by opposing ALK1 pathway activation, angiogenic growth factors also induce TIMAP expression through ALK1-independent pathway(s). The findings indicate that angiogenic growth factor- and ALK1 pathway activation have antagonistic roles on EC TIMAP expression.


A
Control
Growth Factors
1% Serum
Growth Factors
1% Serum
TIMAP
GAPDH
75 kDa
50 kDa
48 kDa
25 kDa
P = 0.001
p = 0.0003
TIMAP : GAPDH
Control
Growth Factors
1% Serum
1% Serum + Growth Factors
B
p-SMAD1/5/8
t-SMAD1
p = 0.001
p-SMAD1/5/8 : t-SMAD1
C
BMP-9
BMP-9 + Growth Factors
D
p = 0.004

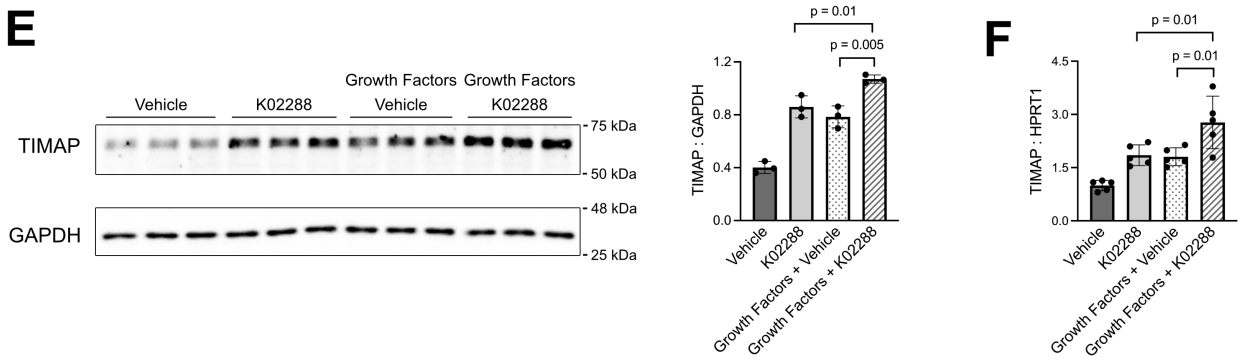


Figure 4.4 Growth factors stimulate TIMAP expression, in part, by antagonizing the ALK1 pathway

A, B: WB and densitometric analysis of TIMAP (**A**) and p-SMAD1/5/8 (**B**) in HUVEC cultured in growth factor-free or growth factor-enriched EBM2 (VEGF 2 ng/ml, IGF-1 5 ng/ml, FGF 10 ng/ml, EGF 5 ng/ml) ± fresh 1% FBS for 18 (**A**) or 1 (**B**) hour (s). **C, D.** WB and densitometric analysis of TIMAP (**C**) and p-SMAD1/5/8 (**D**) in HUVEC cultured in growth factor-free or growth factor-enriched EBM2 (VEGF 2 ng/ml, IGF-1 5 ng/ml, FGF 10 ng/ml, EGF 5 ng/ml) ± 5 ng/ml BMP-9 or vehicle for 18 (**C**) or 1 (**D**) hour (s) **E.** WB and densitometric analysis of TIMAP in HUVEC cultured in growth factor-free or growth factor-enriched EBM2 (VEGF 2 ng/ml, IGF-1 5 ng/ml, FGF 10 ng/ml, EGF 5 ng/ml) ± 5 ng/ml BMP-9 or vehicle, with or without 1μM K02288 or vehicle for 18 hours. **A−E**: n = 3 distinct experiments each. **F.** Quantitative RT-qPCR of TIMAP mRNA expression in HUVEC cultured in growth factor-free or growth factor-enriched EBM2 (VEGF 2 ng/ml, IGF-1 5 ng/ml, FGF 10 ng/ml, EGF 5 ng/ml) ± 1μM K02288 or vehicle for 4 hours (n = 5 distinct experiments). **A−F:** Statistical analysis was by one-way ANOVA with post-hoc Tukey's multiple comparisons.

4.3. Discussion

Tumor-driven angiogenesis is principally mediated by hypoxia which, in turn, induces the synthesis of angiogenic growth factors by tumor and stromal cells[84, 738]. In Chapter 3, I showed that TIMAP plays an important role in tumor angiogenesis and its deletion significantly delays the growth of mammary tumors in mice, that TIMAP is upregulated in response to hypoxia *in vivo*, and that angiogenic growth factors induce TIMAP expression in cultured EC. However, the fact that angiogenic growth factors can raise TIMAP levels did not address the question of whether hypoxia could also raise EC TIMAP abundance by acting *directly* on EC. Both hypoxia and Roxadustat raised HIF-1α levels and TIMAP abundance, but TIMAP levels only increased when the cells were simultaneously provided with fresh FBS (Figure 4.1 A−D). Since serum contains high levels of TGF-β pathway ligands, including BMP-9[548, 737], and TIMAP is a downstream target of TFG-β1-mediated repression[587], it seemed likely that hypoxia antagonizes an inhibitory effect of serum on TIMAP expression. Indeed, serum strongly induced SMAD1/5/8 but not SMAD2/3 (not shown) phosphorylation in the cultured EC and inhibited EC TIMAP expression (Figure 4.2 A, B). The ALK1 inhibitor K02288, but not the ALK5 inhibitor SB505124, completely reversed the inhibitory effect of serum on TIMAP expression and inhibited serum-stimulated SMAD1/5/8 phosphorylation (Figure 4.2 A, B). BMP-9 mimicked the effects of serum (Figure 4.2 C, D), consistent with previous reports showing that serum-induced ALK1/SMAD1/5/8 pathway activation is mediated by the BMP-9[548]. We can, therefore, conclude that TIMAP expression is inhibited when the EC-specific ALK1 pathway is activated, while no effect of ALK5 pathway inhibition on TIMAP expression was observed. Since K02288 directly binds and inhibits the kinase domain of BMP receptors[739], it is still possible that the non-canonical (non-SMAD) TFG-β pathway

could also be involved. To determine whether BMP9-mediated repression of TIMAP expression is SMAD-dependent, further studies are needed, for example through siRNA-mediated knockdown of SMAD1,5 or 8.

When we evaluated cultured EC in 21% and 1% O_2, we found that SMAD1/5/8 phosphorylation induced by serum is suppressed by hypoxia (Figure 4.3 A) and that even a relatively brief 1-hour exposure to hypoxia or Roxadustat attenuates BMP-9-induced SAMD1/5/8 phosphorylation in EC (Figure 4.3 C, E). Both hypoxia and Roxadustat partially reversed the inhibitory effect of BMP-9 on TIMAP protein abundance some 18 hours later (Figure 4.3 D, F). Since Roxadustat stabilizes HIF-α[740], it is likely that inhibition of the ALK1-SMAD1/5/8 pathway under hypoxic conditions is also mediated by HIF-α stabilization. However, the precise mechanism by which HIF activation attenuates ALK1-SMAD1/5/8 pathway signalling remains unknown. HIFs are transcription factors that translocate into the nucleus to regulate genes involved in many cellular processes[98, 741-743]. One possibility, therefore, could be transcriptional effects of HIFs, increasing inhibitory SMADs or repressing components of the ALK1-SMAD1/5/8 pathway. But we found that even 10 minutes of exposure to Roxadustat, sufficient to stabilize HIF-1α, significantly reduced SMAD1/5/8 phosphorylation (data not shown), and no change in total SMAD1 or the ALK1 receptor was observed (Figure 4.3 B). Since the inhibitory effect of HIF stabilization on SMAD1/5/8 phosphorylation is so rapid, it seems unlikely that the decline in SMAD1/5/8 phosphorylation is mediated by a transcriptional effect. It was previously reported that HIF-1α inhibits the transcriptional effects of SMAD1/5/8 and that HIF-1α is part of the same protein complex as phosphorylated SMAD1/5/8 in nuclei of hypoxic EC[580]. Also, several studies have described crosstalk between HIF-1α and the ALK5 pathway. For instance, HIF-1α abundance increased in response to TGF-β1, and hypoxia-

induced HIF-1α not only enhanced the phosphorylation of SMAD2/3 but also increased SMAD transcription[569, 570]. Intriguingly, HIF-1α was also reported to physically interact with the ALK5 receptor and to enhance its expression[569]. The possibility that HIFs interact with the ALK1 receptor to alter its kinase activity or with SMADS1/58 to reduce their phosphorylation will need to be explored in the future.

Since a direct effect of hypoxia on EC was inhibition of the ALK1 pathway, we explored the possibility that enhanced TIMAP expression in response to angiogenic growth factors might also be due to ALK1 pathway inhibition. Indeed, like hypoxia, angiogenic growth factors blunted serum- and BMP-9-induced ALK1/p-SMAD1/5/8 pathway activation, but unlike hypoxia, growth factors stimulated TIMAP expression, even in the absence of serum or BMP-9 (Figure 4.4 A-D). Hence, an ALK-1 independent effect of angiogenic growth factors on TIMAP expression also seemed to be at play. In the absence of serum and angiogenic growth factors, ALK1 pathway inhibition with K02288 still induced TIMAP expression (Figure 4.4 E & F), though an effect of K02288 on SMAD1/5/8 phosphorylation could not be detected in cells not provided with fresh serum or BMP-9 (data not shown). This finding suggests that there is a residual basal activity of the ALK1 pathway in the EC, though the possibility of an off-target effect of the inhibitor cannot be excluded. The findings that angiogenic growth factors raised TIMAP mRNA and protein abundance even in the presence of K02288 (Figure 4.4 E & F), indicate that angiogenic growth factors raise TIMAP levels through an effect that is ALK-1 independent. It might be argued that we still did not achieve full inhibition of ALK1 activity with K02288 and that the addition of angiogenic growth factors blocked some residual activity, but when we increased the K02288 concentration 5-fold (data not shown), there was no further increase in TIMAP protein or mRNA abundance and growth factors still raised TIMAP

levels above those observed with K02288 alone. Therefore, we conclude that angiogenic growth factors increase TIMAP expression both by inhibiting the ALK1 pathway and through additional direct effects.

Angiogenic growth factors and hypoxia, therefore, exert synergistic effects on EC by antagonizing the ALK1 pathway. We wondered whether hypoxia might induce angiogenic growth factor expression in the EC, resulting in autocrine growth factor-mediated ALK1 pathway inhibition. This possibility seems unlikely since the effect of hypoxia and Roxadustat on ALK1 pathway inhibition is extremely rapid. Even so, it is likely that complex crosstalk between the ALK1 pathway and angiogenic growth factor receptors is at play. Several studies illustrated crosstalk between different types of growth factors and the TGF-β signaling pathway[492]. In this regard, BMP-9-dependent ALK1 pathway activation reduces growth factor-induced EC proliferation, inhibits angiogenesis, and enhances blood vessel maturation[554, 744, 745] and activation of the ALK1 pathway is associated with reduced VEGF signalling[554, 746]. Conversely, loss of function mutation in the ALK1 receptor results in overactivation of VEGF signalling[555]. Hypoxia and angiogenic growth factors would therefore be expected to amplify the VEGF signal as they inhibit the ALK1 pathway. Still, the exact mechanisms by which angiogenic growth factors induce TIMAP expression independent of the ALK1 pathway will need further investigation.

Chapter 5

General discussion and future directions

5.1 Introduction

The work presented in this thesis demonstrates that TIMAP, a protein phosphatase 1 regulatory subunit[591], is an important component of *in vivo* tumor angiogenesis, at least in the mouse E0771 model of breast cancer. This finding supports previous work from our laboratory that showed pro-angiogenic activity of TIMAP in cultured EC[383]. The current work also shows that TIMAP expression is downregulated when the BMP9/ALK1/SMAD1/5/8 pathway is activated, and that activity of the ALK5/SMAD2/3 does not regulate TIMAP expression in EC. This finding is consistent with the original observation that TIMAP expression is strongly suppressed by TGF-β pathway activation, though that study had not defined which TGF-β pathway is responsible for the regulation of TIMAP expression in EC[583]. The current work also shows that TIMAP upregulation in EC in response to hypoxia is due to suppression of the ALK1/SMAD1/5/8, indicating that hypoxia-mediated angiogenic signaling can affect EC directly. The work also shows that growth factors, including VEGF and IGF-1, released by tumor cells[728, 747, 748], upregulate EC TIMAP expression partly by inhibiting ALK1/SMAD1/5/8 pathway activation, and partly through ALK1/SMAD1/5/8 independent effects. Hence crosstalk between the BMP-9 stimulated pathway and angiogenic growth factors cooperate in inducing TIMAP expression.

5.2 Mechanism of TIMAP Function

Exactly how TIMAP regulates the angiogenic process *in vivo* and *in vitro* still needs to be better defined. The data so far indicate that TIMAP is a powerful and competitive inhibitor of PP1cβ in EC, and this function results in a dramatic increase of MLC2, AKT

and PTEN phosphorylation[383, 591]. However, whether TIMAP/PP1cβ could function as a phosphatase against specific target(s) or whether its function is confined to its inhibitory action towards PP1cβ is still unclear. The catalytic PP1c is never found in the free state in normal cells, so indiscriminate dephosphorylation of Ser/Thr sites does not occur[749-751]. Instead, PP1c regulatory subunits, which are extremely diverse, bind and "cage" PP1c, blocking its activity against most substrates, except for the very few that are specifically targeted[591]. So, it is conceivable that TIMAP competitively inhibits PP1cβ phosphatase activity towards MLC2, PTEN and AKT as well as other targets of PP1cβ, while the holoenzyme TIMAP/PP1cβ could still simultaneously act as a phosphatase against its own specific, but currently unknown targets. Potential targets are the ERM[601] proteins and LAMR1[589, 602], but other undefined targets are also possible. Defining such substrates for TIMAP/PP1cβ is probably best done by analysis of the phospho-proteome, perhaps using tissues from wild-type and TIMAP deficient mice or alternatively using EC in which TIMAP is silenced or overexpressed.

At this point in the investigation of TIMAP function, it is clear that TIMAP serves to inhibit myosin phosphatase activity in cells by sequestering PP1cβ so it cannot bind MYPT1[591]. Therefore, the TIMAP/PP1cβ holoenzyme does not function as a conventional myosin phosphatase but as a myosin phosphatase inhibitor that increases the level of MLC2 phosphorylation[591]. Myosin activity in cells depends on the phosphorylation of its MLC2 regulatory subunit[752-754] and mediates cellular contractility and motility[755-757]. In migrating EC, TIMAP strongly localizes to the plasma membrane of EC projections[583], and it colocalizes with MLC2 and p-MLC2 in projections of migrating EC[591]. Expression of a dominant negative TIMAP in EC that cannot bind PP1cβ reduces MLC2 phosphorylation in EC[591] and increases the length and number projections in migrating EC[590]. Similarly, a

TIMAP point mutant that cannot be PKA/GSKβ phosphorylated at Ser 333/337, which has a high affinity for PP1cβ, lengthens EC projections, and cells expressing TIMAP with phosphomimic mutations at Ser333/Ser337, which binds PP1cβ less tightly, have very short, stumpy projections[590]. Those findings might point to the idea that TIMAP-mediated stimulation of myosin activity through sequestration of PP1cβ is necessary for the formation of EC projections. The finding of TIMAP in EC projections[583] and regulation of the length of projections by TIMAP[590] raises the question whether TIMAP might be expressed predominantly in EC tip cells where it might regulate the extension and retraction of exploratory projections during sprouting angiogenesis. The finding that TIMAP expression is upregulated under hypoxic conditions furthermore raises the possibility that TIMAP-dependent tumor angiogenesis might be mediated, at least partly, by increasing MLC2 phosphorylation and enhancing myosin activity. It turns out that the level of expression of protein phosphatases is upregulated during tumorigenesis either by increasing the expression of their regulatory targeting subunits or reducing the expression of their endogenous inhibitors[675, 758, 759]. However, the level of MLC2 phosphorylation tends to increase during tumorigenesis[760-762], suggesting that the activity of myosin phosphatase is reduced. This is consistent with our findings since TIMAP is a targeting subunit that strongly binds PP1cβ[591], upregulated under hypoxic conditions (tumorigenesis), increases the abundance of its associated PP1cβ and strongly stimulates MLC2 phosphorylation[591]. In our animal model, we evaluated tumor angiogenesis in wild-type and TIMAP knockout mice. We previously showed that the level of MLC2 phosphorylation in EC is significantly reduced in mice when TIMAP is deleted (Figure 1.10)[591], and here we show that tumor angiogenesis is reduced in TIMAP knockout mice. Although indirect, such data are consistent with the theory that impaired tumor

angiogenesis in TIMAP knockout mice might be related to a decline in the level of MLC2 phosphorylation. Since TIMAP deletion in mice reduces MLC2 phosphorylation[591], experiments to evaluate MLC2 phosphorylation in tumor tissues of wild-type mice, particularly in the EC of tumor micro-vessels, need to be carried out by comparing the level of MLC2 phosphorylation in blood vessels from normal mammary gland and those invading mammary tumors.

TIMAP also interacts with and regulates the phosphorylation of other angiogenic molecules in EC. At the cell membrane, TIMAP directly interacts with and reduces the phosphorylation of LAMR1 in cultured EC[602]. However, recombinant TIMAP/PP1cβ did not dephosphorylate recombinant, purified LAMR1 *in vitro*[589], so at this juncture, it is unclear whether LAMR1 is a true TIMAP/PP1cβ substrate. Enhanced expression of LAMR1 is detected in proliferating but not quiescent EC[763] and is associated with tumor angiogenesis and metastasis[764]. It is possible that LAMR1, which is a membrane-spanning protein that binds extracellular laminin, serves to localize TIMAP/PP1cβ to the plasma membrane of EC projections, and LAMR1 might regulate the TIMAP/PP1cβ interaction. Further work is needed to define the physiological role of the direct LAMR1 interaction with TIMAP/PP1cβ and to determine whether interruption of the interaction has any impact on sprouting angiogenesis. Preliminary data from the lab suggest that mutant TIMAP constructs in which the LAMR1 binding motif has been deleted preclude TIMAP localization to the plasma membrane (unpublished data), but further studies will be necessary to determine whether disruption of LAMR1/TIMAP binding alters the TIMAP/PP1cβ association.

Endogenous TIMAP also colocalizes with PTEN in EC projections, and endogenous PTEN and TIMAP can be co-immunoprecipitated from EC[383]. When

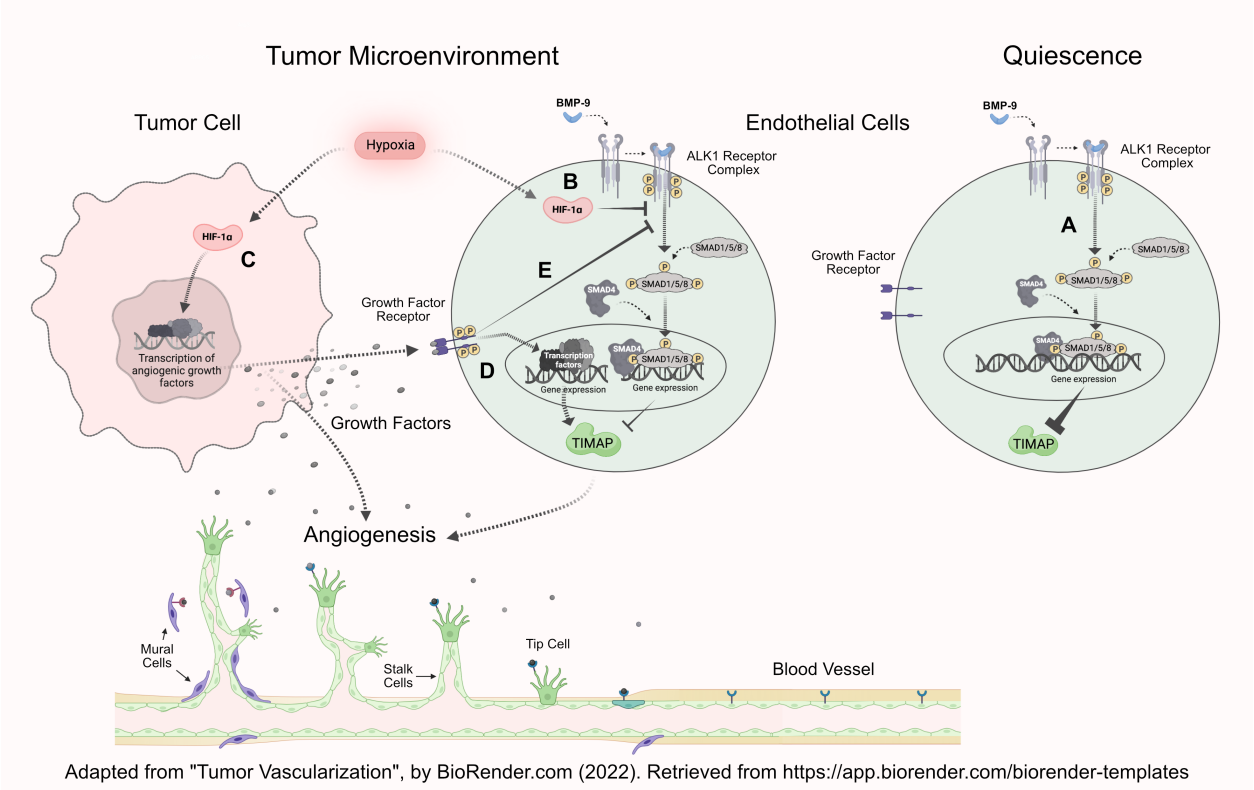
endogenous TIMAP expression in EC is silenced, there is a marked reduction in the inhibitory phosphorylation of PTEN at Ser370, without effect on activating phosphorylation sites, indicating that TIMAP knockdown activates PTEN[383]. It turns out that the PTEN inhibitor bpv (phen) [potassium bisperoxo (1,10-phenanthroline) oxovanadate] rescues the TIMAP knockdown-induced inhibition of Ser473 AKT phosphorylation[383]. Those findings, therefore, suggest that the effect of TIMAP on AKT activity is mediated through its effect on PTEN, with TIMAP overexpression reducing and TIMAP silencing enhancing PTEN activity[383]. At this point, it is still unclear whether the actions of TIMAP on PTEN and AKT phosphorylation are due to sequestration and inhibition of PP1cβ by TIMAP, or whether these actions are confined to inhibition of MYPT1/PP1cβ phosphatase activity. A way to investigate these possibilities would be to silence PP1cβ with and without silencing of MYPT1 in EC and then determine whether AKT, PTEN and MLC2 phosphorylation are enhanced, and to determine whether PP1cβ ± MYPT1 silencing change EC filopodia extension, EC proliferation and *in vitro* angiogenic sprouting.

5.3 Mechanism of TIMAP regulation

TIMAP was first reported as an EC-predominant protein strongly suppressed downstream of the TGF-β1 pathway in cultured EC, though the mechanism of TGF-β1-induced TIMAP inhibition was not explored in that study[583]. In EC, TGF-β1 can activate both ALK1/SMAD1/5/8 and ALK5/SMAD2/3 signalling cascades[765], though the EC ALK1/SMAD1/5/8 pathway is mainly activated by BMP-9[766], which has higher affinity than TGF-β1 for the EC-specific ALK1 receptor[767]. In this study, we found that TIMAP is specifically repressed downstream of the BMP-9/ALK1/SMAD1/5/8 pathway and that the ALK5/SMAD2/3 pathway is not involved. Consistent with our finding, several studies have

revealed that activation of the ALK1 pathway inhibits EC proliferation[768] and tumor angiogenesis[769], suggesting that some of these effects might be mediated through EC TIMAP inhibition. In our study, we observed that in lung EC *in vivo* and cultured EC *in vitro*, TIMAP abundance increases under hypoxic conditions. Various studies in the literature illustrate that the effect of hypoxia on EC is indirectly mediated through the release of angiogenic mediators from neighboring cells in the hypoxic microenvironment[84]. While our experiments suggest that hypoxia raises TIMAP abundance indirectly by enhancing the expression of angiogenic growth factors that induce EC TIMAP expression, further experiments revealed that hypoxia also has a direct effect on the TIMAP expression in EC. We found that the EC TIMAP expression is repressed by the BMP-9-induced ALK1/SMAD1/5/8 pathway activation, and hypoxia increases TIMAP levels by antagonizing ALK1/SMAD1/5/8 activation. Interestingly, growth factors also attenuate ALK1/SMAD1/5/8 signaling and, therefore, have dual effects on EC TIMAP expression (Figure 5.1).

Growth factors stimulate cell surface tyrosine kinase receptors and activate many downstream signaling cascades[216], including crosstalk with TGF-β pathways[770-775]. We did not dissect the exact mechanisms by which angiogenic growth factors stimulate TIMAP expression independent of the ALK1/SMAD1/5/8 pathway. Therefore, defining the mechanism of growth factor-induced TIMAP expression will require more work.



Adapted from "Tumor Vascularization", by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates

Figure 5.1: Model illustrating the effect of HIF-1α, growth factors and ALK1 pathway on EC TIMAP expression

A. Activation of the EC-specific ALK1 receptor stimulates SMAD1/5/8 phosphorylation and inhibits TIMAP expression. **B.** HIF-1α stabilization in hypoxic EC attenuates ALK1-induced SMAD1/5/8 phosphorylation. **C.** Stabilization of HIF-1α in hypoxic tumor cells induces the expression of angiogenic growth factors. **D.** Angiogenic growth factors induce TIMAP expression and stimulate angiogenesis. **E.** Growth factor-induced TIMAP expression is partly mediated by attenuating the ALK1/SMAD1/5/8/signaling pathway.

Under hypoxic conditions, HIF-1α is the master regulator of cellular responses to low oxygen concentrations[776]. Our experiments demonstrated that the EC TIMAP abundance is also enhanced in response to the hypoxia-inducible factor prolyl hydroxylase inhibitor (HIF-PHI) Roxadustat. We used Roxadustat to induce chemical hypoxia as an alternative experimental approach and showed that HIF-1α is stabilized in cells exposed to low oxygen concentration or treated with Roxadustat providing evidence that the cells are, in fact, hypoxic. It could be argued that the hypoxia-induced TIMAP upregulation might not be mediated by HIF-1α stabilization since Roxadustat targets all HIF transcription factors, including HIF-1α, HIF-2α and HIF-3α to a similar extent[777-779]. In order to confirm that the enhanced TIMAP abundance under hypoxic conditions is mediated by HIF-1α, we might have used the specific HIF-1α inhibitor YC-1[780] or silenced the HIF-1α in hypoxic EC. Nonetheless, since Roxadustat mimicked the effect of *in vitro* hypoxia, we can be certain that HIF-dependent pathways are involved. Determining the detailed mechanism of hypoxia-induced TIMAP expression will also require more work.

The finding that TIMAP is upregulated by hypoxia-induced inhibition of the ALK1/SMAD1/5/8 pathway suggests that increased TIMAP expression could also play a role in conditions where EC ALK1/SMAD1/5/8 signaling is reduced. For example, *in vivo* studies have shown that hypoxia attenuates ALK1 signaling in EC, as SAMD1/5/8 phosphorylation is significantly reduced in EC of rats exposed to chronic hypoxia[578]. Interestingly, the pathogenesis of PAH is mediated by hypoxia-induced attenuation of the BMP-9/ALK1/SMAD1/5/8 signaling pathway in vascular endothelial and smooth muscle cells (SMCs)[551, 781]. During the development of PAH, thickening of the pulmonary arteries is mediated by excessive proliferation of SMCs and subsequent abnormal muscularization

of the distal pulmonary arterioles, but there also is hyperproliferation of EC and consequent obstruction of these arterioles[781-784]. Accordingly, the therapeutic approaches to treat PAH target the pathological proliferation of SMCs and EC of the pulmonary vasculature[781]. Hence, dysregulation of TGF-β signaling pathways with a consequent deficiency of the anti-proliferative effects of the BMP-9/ALK1/SMAD1/5/8 signaling largely contributes to the deregulated vascular cell proliferation in PAH[781]. Mutations in the BMPRII are responsible for most cases of hereditary PAH[785-787], and even in the absence of BMPRII mutations, low levels of BMPRII protein are found in other forms of PAH[788-790], indicating that BMPRII-dependent signaling plays an essential role in the etiology and pathogenesis of PAH. Indeed, *in vitro* experiments revealed that silencing BMPRII in cultured EC results in its transformation into a proliferative phenotype[791]. Furthermore, selective deletion of BMPRII in EC is associated with hyperproliferating EC and SMCs and results in the development of PAH-like disease in mice[792]. Moreover, preclinical studies revealed that exogenous delivery of the wild-type BMPRII gene to replenish BMPRII insufficiency in the pulmonary vascular endothelium improves cardiovascular function in the PAH animal models[793-795].

Depending on the type of the ligand, BMPRII signaling is a critical regulator of the proliferation of both pulmonary artery EC and SMCs[781]. Circulating BMP-9 which acts primarily on EC, functions as a critical quiescence factor in pulmonary arteries[796]. Interestingly, mutations in the genes encoding several TGF-β superfamily of ligands that functionally interacts with BMPRII, including BMP-9[797-799], or the BMPRII signaling partners such as the ALK1[800-802] or endoglin[802-804] receptors are also associated with the PAH. These findings highlight the significance of the BMP-9 signaling, particularly in EC, in mediating vascular endothelial quiescence and protecting against the development of

PAH. Since BMP-9/SMAD1/5/8 pathway signaling exerts anti-proliferative effects on the pulmonary vascular endothelium[548], stimulating this pathway is expected to have a protective effect against PAH. Indeed, activation of the ALK1/SMAD1/5/8 pathway by Tacrolimus reversed the defective BMPRII signaling in pulmonary EC of patients with idiopathic PAH, reversing vascular remodeling and alleviating the cardiopulmonary dysfunction in rats with severe PAH[805]. Conversely, the BMP antagonist gremlin-1 was found to promote PAH-like pathological vascular remodeling[806-808]. In addition, hypoxia enhances gremlin-1 release by EC[809] and inhibition of gremlin-1 reduces vascular remodeling in PAH models of mice exposed to chronic hypoxia[810]. This raises the interesting question of whether TIMAP upregulation by hypoxia and Roxadustat could be mediated by gremlin-1 release from the EC, and acting in an autocrine fashion on the EC. However, we observe that hypoxia- and Roxadustat-induced inhibition of SMAD1/5/8 phosphorylation occurs within as short as 10 minutes (Data not shown), indicating that enhanced gremlin-1 expression is unlikely responsible for these rapid effects.

The large amount of data linking BMP pathway suppression to the pathogenesis of PAH, together with our findings that TIMAP expression is profoundly suppressed by BMP-9/ALK1/SMAD1/5/8 activation, that chronic hypoxia upregulates TIMAP by antagonizing this pathway in EC, and that TIMAP promotes survival and stimulates EC proliferation raises the possibility that TIMAP could be involved in the pathogenesis of PAH. If this is the case, TIMAP inhibition could have similar effects to ALK1 activation to mitigate the excessive proliferation of the EC during the development of PAH.

Although we have shown that exposing mice to chronic hypoxia results in significant upregulation of pulmonary EC TIMAP, it is uncertain whether TIMAP deficient mice could survive under prolonged hypoxic conditions. Earlier studies revealed that TIMAP

promotes pulmonary endothelial barrier function[608], and chronic hypoxia has been shown to induce pulmonary vascular constriction and results in the development of pulmonary edema[811]. It is, therefore, possible that TIMAP deficient mice would be unable to survive under hypoxic conditions as TIMAP deletion would be expected to aggravate the development of fatal pulmonary edema. Further experiments will be needed to explore the potential involvement of TIMAP in the pathogenesis of PAH and in response to chronic hypoxia.

5.4 TIMAP in breast cancer

A recent study reported that the TIMAP protein abundance is upregulated in human breast cancer and that high expression levels are associated with poor survival[700], suggesting that TIMAP expression promotes tumor progression in this common disorder[812]. The work in this thesis is consistent with the published findings in humans[700], in that E0771 breast carcinomas were much smaller in TIMAP deficient mice compared to their wild-type littermates (Figure 3.1). Since we previously found that TIMAP is predominantly expressed in EC both in culture and developing blood vessels *in vivo*[583], our lab had reported that TIMAP expression in EC is necessary for *in vitro* sprouting angiogenesis[383], and global knockout of TIMAP in mice results in a significant reduction of tumor blood vessel density *in vivo* (Figure 3.2), it seems likely that TIMAP regulates tumor angiogenesis through its effects in EC. However, in the human study[700] and in our mouse model, TIMAP was not localized to a specific compartment within the breast tumors, so it is still possible that TIMAP expression in human breast cancer is not confined to the vasculature. Although TIMAP is highly expressed in EC, it is also expressed in other cell lines such as hematopoietic cells and specifically macrophages[583, 813]. During

angiogenesis, activated macrophages participate in multiple stages of angiogenesis[120]. They secrete various growth factors involved in the initiation of angiogenesis and capillary sprouting[814-819]. Moreover, activated macrophages secrete proteolytic enzymes to degrade ECM, facilitating endothelial sprouting and guiding new vessel growth[820]. Macrophages are also involved in the recruitment of pericytes to promote vessel maturation and induce apoptosis to remodel the vasculature during the later stages of angiogenesis[821]. In our studies, it is possible that host stromal cells or host macrophages could be contributing to the *in vivo* angiogenesis response. Therefore, a limitation of the current work is that the TIMAP deletion in our animal model is global and not EC-specific. Further studies will be required to clarify this question. While EC-specific deletion of TIMAP would be one approach to deal with this problem, it would also be interesting to create transgenic mice on the TIMAP$^{-/-}$ background by introducing TIMAP transgenes under an EC-specific promoter. This approach would enable us to express wild-type and distinct TIMAP mutants in the EC *in vivo* and determine whether they rescue tumor angiogenesis. It would also be possible to create specific mutations in the TIMAP gene using the CRISPR/Cas9 approach.

Another limitation of the current study is that we have only evaluated tumor angiogenesis in a single tumor model. Although it seems unlikely that the effect of TIMAP on tumor angiogenesis is restricted to breast cancer, it still needs to be determined whether its effects hold generally for tumors in which angiogenesis is critical for progression. Probably the best way to explore this question would be to obtain human tumor tissue arrays and to stain them with TIMAP antibodies, co-labeling with EC- and macrophage markers.

5.5 The potential role of TIMAP in tumor metastasis

Most malignant tumors have the potential to metastasize and form secondary tumors in diverse locations[822]. Tumor metastasis is a complex multistep process involving entry of the tumor cell into the tumor vasculature and exit at the distant site[823]. The distinct stages are controlled by several signaling cascades[822, 824]. When tumor cells metastasize, they break off from the primary tumor, invade the extracellular matrix and migrate toward the blood or lymph vessels[825]. The tumor cells then enter the vascular lumen (intravasate) into the circulation, and some of the tumor cells that survive in the circulatory system eventually exit the vascular lumen (extravasate) into a distant organ, forming a secondary metastatic tumor[826.] Transmigration of tumor cells across the vascular endothelium during the intravasation and extravasation are essential components of tumor metastasis that depend on EC retractability and vascular barrier function[827.828, 829].

EC are not only essential for vessel sprouting during tumor angiogenesis, but they also play an important role during tumor metastasis[830.] To start, angiogenesis itself provides an entry point for cancer cells into the circulation, increasing tumor metastasis[830-832] Retraction of the ECs, reducing the EC barrier function, is required for tumor cells to cross the endothelium when they enter the circulation from the tumor (intravasation)[833] and also for tumor cells to move from the circulation into distant tissues (extravasation)[834-837]. EC retraction during tumor cell transmigration involves several signaling pathways in EC, with growing evidence that MLC2 phosphorylation and enhanced myosin activity are required[833, 838.] Several studies revealed that the EC myosin activity is enhanced during tumor angiogenesis and metastasis[618, 673, 838]. During tumor metastasis, the EC myosin light chain kinase (MLCK) activity, and therefore MLC2 phosphorylation and myosin contraction are increased to enhance tumor cell trans-endothelial migration during the

intra- and extravasation steps[833]. Moreover, during tumor development and progression, the activity of endothelial Ser/Thr protein phosphatases, which reduce myosin activity by increasing the rate of dephosphorylation of its regulatory MLC2, are markedly reduced either by reducing their expression or increasing expression of their endogenous inhibitors[675, 758, 759]. Since our lab has found that TIMAP regulates MLC2 phosphorylation in EC by inhibiting MYPT/PP1cβ phosphatase activity[591], it is attractive to postulate that TIMAP may also aid in EC retraction during tumor cell intravasation and/or extravasation. TIMAP has also been shown to promote EC barrier function[608, 609]. Generally, the EC monolayer lining all blood vessels functions as a selective barrier to all elements found in circulating blood, including circulating cells[839, 840]. Trans-endothelial cell migration for circulating white blood cells is strictly regulated at the EC level, and some tumor cells take advantage of this EC function[840-842]. Changes in the EC barrier function depend on EC cytoskeletal contractility and rearrangement of intercellular junctional proteins, which are regulated by phosphorylation and dephosphorylation[843-845]. During tumor cell trans-endothelial migration, EC contractility and adherens junction disassembly due to VE-cadherin phosphorylation cause gaps to form between the EC, facilitating the transmigration process[843].

A potential role for TIMAP in the EC barrier function has been described by others. In mature mice, TIMAP seems to protect the lung EC barrier, reducing lung edema formation in response to lipopolysaccharide (LPS)[609]. This protective effect was postulated to be due to the inhibition of PP1c-mediated ERM (**E**zrin, **R**adixin, **M**oesin) dephosphorylation, increasing ERM activity in the regulation of EC barrier function[601]. Recent studies also showed that TIMAP silencing or deletion impairs the vascular and pulmonary endothelial barrier to fluid[608]. If TIMAP protects the pulmonary endothelial

barrier, our own experiments would suggest that TIMAP upregulation in lung endothelium in response to hypoxia would be similarly protective. If the barrier to cell transmigration were similarly enhanced by TIMAP, then one would expect a greater potential for metastasis in the TIMAP deficient mice compared to their controls. However, the mechanisms that define the EC barrier to fluid flux versus cell transmigration are different, so it is difficult to predict whether TIMAP in EC of the lung or other organs would impact tumor cell transmigration.

So far, data about the role of TIMAP in the process of endothelial transmigration by immune or cancer cells have not been published. Since TIMAP is a critical regulator of myosin activity in EC[591], we expect that retraction of EC during cell transmigration processes requires TIMAP and is reduced in its absence due to myosin dephosphorylation. Since the lungs are the second most common site of cancer metastasis[846], future work should be done to determine whether TIMAP enhances or reduces the extravasation of cancer cells across the pulmonary endothelium into the lung. Finally, because hypoxia increases pulmonary tumor metastasis[847-849], taken together with our finding that hypoxia induces TIMAP expression in mouse lung ECs, investigating the effect of hypoxia on the main components of metastasis, the intra- and extravasation would seem to be a promising approach. We expect that TIMAP could facilitate tumor cell migration across vascular endothelium by modifying myosin-dependent retraction of endothelial cell-to-cell adhesions, and this process might be enhanced under hypoxic conditions through upregulation of the EC TIMAP.

So far, we have attempted to quantify E0771 pulmonary metastasis in the $TIMAP^{+/+}$ and $TIMAP^{-/-}$ mice, but there were too few metastases to come to any meaningful conclusion (data not shown). The tumors composed of E0771 cells apparently do not tend

to metastasize, so this is probably not the right model. Furthermore, since angiogenesis itself can enhance metastasis[850], whether TIMAP alters EC retraction during tumor cell intra- and extravasation would not be evident just by evaluating the number and size of metastases.

5.6 Proposed future Experiments

An approach to define the role of EC TIMAP on tumor metastasis *in vivo* would be to study $TIMAP^{+/+}$ and $TIMAP^{-/-}$ mice made chronically hypoxic or left in the normal oxygen environment. The B16F10 melanoma cells are syngeneic for C57BL/6 mice, highly aggressive, have high propensity to form pulmonary metastasis *in vivo*, and preferentially attach to lung tissue[851]. These cells are used in the well-studied subcutaneous melanoma mouse model[852] and could be used to evaluate the effect of TIMAP on the tumor cell extravasation. The B16F10 cells would be injected intravenously (tail vein) into sex-and age-matched $TIMAP^{+/+}$ and $TIMAP^{-/-}$ mice, followed by evaluation of pulmonary metastasis. To study intravasation, the tumor cells would need to be injected subcutaneously, and the appearance of tumor cells in the blood would be monitored and counted as a function of time.

To isolate the transmigration process using a cell culture model, *in vitro* intra- and extravasation assays in Boyden chambers can be used. Two chambers separated by a microporous membrane with pores 8.0 μm in diameter can be used where the tumor cells, seeded in the upper chamber, can then migrate through a pre-established EC monolayer cultured on either side of the membrane. The EC can be exposed to hypoxia or control O_2 concentrations and transfected with control or TIMAP, MYPT1, or PP1cβ specific siRNAs. Alternatively, the cells can be transduced using our adenoviral vectors with wild type

TIMAP or TIMAP mutants unable to bind PP1cβ or unable to interact with the plasma membrane due to deletion of the TIMAP prenylation site or the non-integrin laminin receptor interacting domain. The EC would be seeded onto the microporous membrane in endothelial growth medium (EGM-2) and allowed to form polarized, confluent monolayers. The formation of a continuous EC monolayer would be confirmed prior to adding B16F10 cells by measuring the trans-endothelial electrical resistance (TEER)[853-855]. To effectively attract B16F10 cells to cross EC monolayers, the lower chamber would need to contain chemoattractant, for instance, lung-conditioned medium[856].

Based on work already published in the literature, it is expected that MLC2 and VE-cadherin will be phosphorylated in EC at the sites of B16F10 transmigration[833, 857]. If the hypothesis is correct, silencing TIMAP should prevent MLC2 hyperphosphorylation, reducing cancer cell migration across the EC monolayer. It is also expected that at sites of B16F10 transmigration, the VE-cadherin cell-cell contacts will be disrupted, forming gaps. If this gap formation depends on TIMAP-stimulated myosin activity, it should be reduced when TIMAP is silenced. Conversely, it would be expected that overexpression of wild-type TIMAP will enhance MLC2 phosphorylation, B16F10 cell transmigration and VE-cadherin gap formation. Since the TIMAP effect on EC transmigration is proposed to enhance EC myosin activity by inhibiting PP1cβ of the myosin phosphatase, overexpression of the $TIMAP^{V64A/F66A}$, which cannot bind PP1cβ would be expected to produce same results as TIMAP silencing. If MYPT1 and PP1cβ silencing were found to mimic the effects of wild type-TIMAP, this would be consistent with the theory that TIMAP-dependent EC retraction and B16F10 transmigration are enhanced by inhibition of MYPT1/PP1cβ myosin phosphatase activity.

It needs to be kept in mind that others have shown that TIMAP increases the barrier function to fluid in lung EC[608]. It is, therefore, also possible that TIMAP inhibition could increase tumor metastasis and B16F10 cell transmigration, opposite to our expectations. But in either case, the experiment will be informative: if TIMAP inhibition reduces tumor metastasis, TIMAP would be a potential therapeutic target. On the other hand, if TIMAP inhibition made the metastasis worse, we would conclude that TIMAP inhibits tumor metastasis. Another possible limitation of this experiment is that in the living body, shear stress in the vascular lumen is required for the WBCs to attach and cross the vascular EC during the inflammatory response[858-860]. Lack of the shear stress in the *in vitro* model could lead to negative results. If that happens, an alternative approach of perfusable microfluidic chambers with continuous shear stress[861] would be needed.

While the metastasis experiments have not been started, they will be very important. To start, if TIMAP in distant vessels reduced tumor cell transmigration, then a therapeutic attempt to interfere with TIMAP would be ill-advised since one could enhance metastatic potential while at the same time limiting tumor angiogenesis. On the other hand, if TIMAP silencing or deletion in EC were to reduce the transmigration of tumor cells *in vivo* and *in vitro*, then the development of TIMAP inhibitors as an adjunct treatment for people with cancers could be of value. Defining the mechanistic role of TIMAP in the intra- and extravasation of tumor cells across the endothelium would be a big step forward in understanding tumor metastasis. The experiments may further define the mechanism through which lung metastasis formation is enhanced by hypoxia. If hypoxia, which induces lung EC TIMAP expression, augments lung metastasis in wild type but not in TIMAP deficient mice, then this will imply that inhibitors of TIMAP could be particularly useful in patients with chronic hypoxia, for instance, those with chronic obstructive lung disease.

5.7 Summary

TIMAP is a proangiogenic TGF-β1-inhibited protein predominantly expressed in EC, enhances their proliferation and survival, and promotes *in vitro* angiogenic sprouting[383]. In EC, TIMAP enhances AKT phosphorylation by inducing PTEN inhibitory phosphorylation[383]. Phosphorylation of ALK is partly mediated by the generation of PI(3,4,5)P3 at the plasma membrane, which recruits AKT and its activating kinase PDK1 and enhances AKT phosphorylation[862]. TIMAP, therefore, indirectly stimulates AKT phosphorylation by inhibiting PTEN and increasing PI(3,4,5)P3 availability[383]. TIMAP enhances MLC2 phosphorylation by inhibiting the PP1cβ subunit of the TIMAP/PP1cβ myosin phosphatase holoenzyme[591]. In our study, we found that TIMAP is negatively regulated by the BMP-9/ALK/SMAD1/5/8 pathway and TIMAP expression contributes to EC tumor angiogenesis *in vivo*, possibly through its function as an intracellular phosphatase inhibitor. In the hypoxic environment, TIMAP expression in EC is released from the tonic ALK1/SMAD1/5/8-mediated suppression by HIF-1α and angiogenic growth factors. Growth factors seem to raise TIMAP levels through dual ALK-1- dependent and -independent mechanisms (Figure 5.2). Taken together with previous reports that the BMP-9 abundance in human breast cancer tissue is reduced, that overexpressing BMP-9 in breast cancer cells reduces tumor growth[863], and that TIMAP is overexpressed in human breast cancer[700], our findings support the idea of stimulating the BMP9-ALK1 pathway and/or inhibiting the specific phosphatase regulatory subunit TIMAP as future therapeutic options. Finally, the fact that TIMAP is a critical regulator of several cytoskeleton and motor proteins involved in modulating cellular contractility and maintaining the endothelial gap junction integrity[589, 601, 602, 605] and the finding that TIMAP promotes endothelial barrier function[608, 609] indicate that TIMAP might also be involved in regulating the intra- and/or extravasation processes during tumor metastasis.

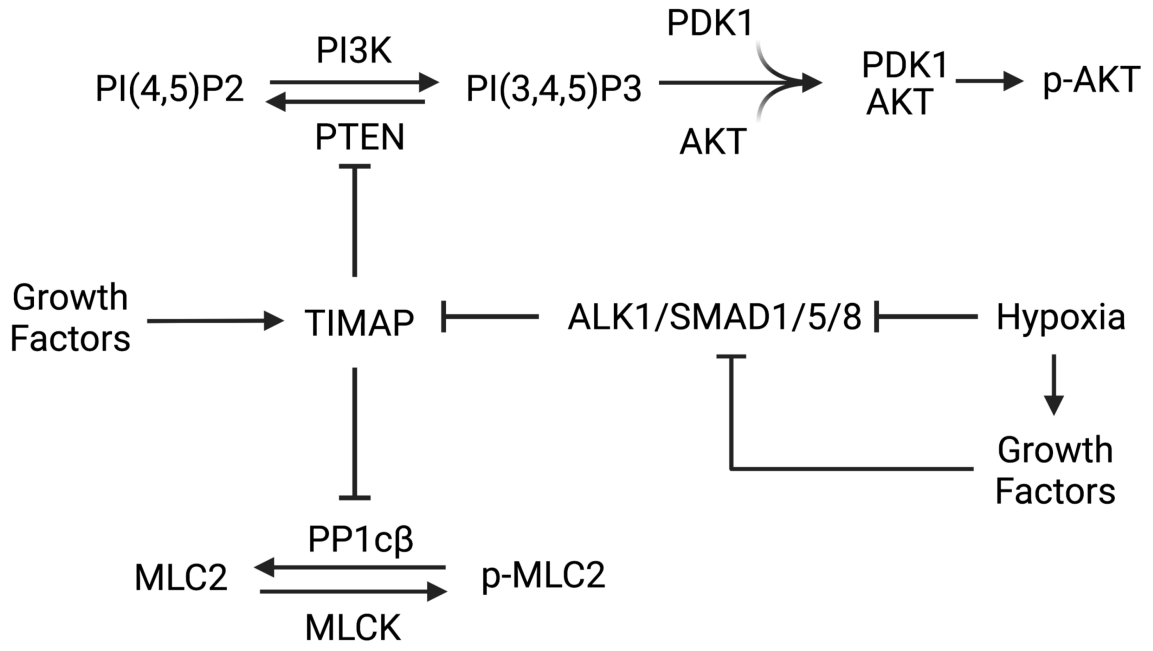


Figure 5.2: Diagram illustrating TIMAP functions and mechanism of regulation

PI3K converts PI(4,5)P2 to PI(3,4,5)P3, which recruits AKT and its activating kinase PDK1 to the plasma membrane and consequently enhances AKT phosphorylation and activation. PTEN converts PI(3,4,5)P3 to PI(4,5)P2 and subsequently reduces AKT phosphorylation and activation. TIMAP enhances AKT phosphorylation and activation by augmenting inhibitory PTEN phosphorylation. TIMAP is negatively regulated by the ALK1/SMAD1/5/8 pathway, and growth factors induce TIMAP expression, in part by attenuating EC ALK1/SMAD1/5/8 pathway signaling. Hypoxia increases TIMAP abundance by attenuating EC ALK1/SMAD1/5/8 and indirectly by augmenting the expression of growth factors. TIMAP raises MLC2 phosphorylation by inhibiting the activity of PP1cβ.

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