The role of leucine-rich alpha-2 glycoprotein 1 (LRG1) in diabetes-related ischaemic heart diseases

Chia, Rui Ning

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**The Role of Leucine-Rich Alpha-2 Glycoprotein 1
(LRG1) in Diabetes-Related Ischaemic Heart
Diseases**

Chia Rui Ning

SCHOOL OF BIOLOGICAL SCIENCES

2019

The Role of Leucine-Rich Alpha-2 Glycoprotein 1 (LRG1) in Diabetes-Related Ischaemic Heart Diseases

Chia Rui Ning

SCHOOL OF BIOLOGICAL SCIENCES

A thesis submitted to the Nanyang Technological
University in partial fulfilment of the requirement
for the degree of Doctor of Philosophy

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research done by me except where otherwise stated in this thesis. The thesis work has not been submitted for a degree or professional qualification to any other university or institution. I declare that this thesis is written by myself and is free of plagiarism and of sufficient grammatical clarity to be examined. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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Wang Xiaomeng

4th July 2019

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Date

Authorship Attribution Statement

This thesis contains research materials for a conference paper published by Karger in the Journal of Vascular Research for 3rd Joint Meeting of the European Society for Microcirculation (ESM) and European Vascular Biology Organisation (EVBO) held between 15th to 18th April 2019 in which I am listed as an author. Contents from Chapter 2, 3 and 4 is published as Chia, R. N., Song, W. & Wang, X. Role of Leucine-Rich Alpha-2-Glycoprotein 1 (LRG1) in Diabetes-Related Ischaemic Heart Diseases (abstract). Journal of Vascular Research 56(suppl 1), 1-134, doi:10.1159/000499516 (2019)

The contributions of the authors are as follows:

- I had designed the research, performed the *in vitro*, *ex vivo* and *in vivo* experiments, tabulated and analysed the raw data, organized the findings into figures and presented the materials in the conference poster session
- Senior Research Fellow, Dr Song Weihua, planned and performed the transverse aortic surgeries on diabetic mice model
- Associate Professor Wang Xiaomeng conceptualized the initial project and actively participated in the design, analysis, writing and revision of the project

.....
Chia Rui Ning

4th July 2019

.....
Date

.....
Wang Xiaomeng

4th July 2019

.....
Date

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Abbreviations

Common units

In-text abbreviation	Expanded-text form
°C	Celsius
ANOVA	Analysis of Variance
bp	Base pair
Ct	Cycle threshold
dL	<i>Deciliter</i>
E	Embryonic Stage
G	Gauge
g	Relative centrifugal force
g	Gram
kD	Kilodalton
L	Liter
mg	Milligram
mL	Milliliter
mM	Millimolar
nM	Nanomolar
rpm	Revolutions per minute
rt	Room temperature
SD	Standard deviation
SEM	Standard Error of Mean
µg	Microgram
µL	Microliter

Chemical and Reagents

In-text abbreviation	Expanded-text form
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
COL	Collagen
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DTT	Dithiothreitol
EBM	Endothelial Basal Medium-2
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGM2-MV	Endothelial Growth Medium-2 (Microvascular)
FBS	Fetal Bovine Serum
HBSS	Hanks' Balanced Salt Solution
HRP	Horse Radish Peroxidase
IB4	Isolectin B4
LDN193189	ALK1 inhibitor
LRG1	Leucine-Rich α -2-Glycoprotein 1
MEM	Minimum Essential Medium Eagle
MGO	Methylglyoxal
MTS	3-(4, 5-Dimethylthiazol-2-yl)-5-(3-Carboxymethoxyphenyl)-2-(4-Sulfophenyl)-2H-tetrazolium, Inner salt)
MWM	Molecular Weight Marker
NaCl	Sodium chloride
NBF	Neutral Buffered Formalin
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PMSF	Phenyl-Methane Sulfonyl Fluoride
qRT-PCR	Quantifying Real-time PCR

rh-LRG1	Recombinant Human LRG1
RIPA	Radioimmunoprecipitation assay
SB431542	ALK5 inhibitor
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	SDS-Polyacrylamide gel electrophoresis
STZ	Streptozotocin
TBST	Tris-Buffered Saline with 0.1% Tween 20
TGF- β	Transforming Growth Factor Beta
VEGF-A	Vascular Endothelial Growth Factor-A
VEGF ₁₆₅	Vascular Endothelial Growth Factor-165
VEGFR2	Vascular Endothelial Growth Factor Receptor-2
WGA	Wheat germ agglutinin

Others

In-text abbreviation	Expanded-text form
ACS	Acute coronary syndrome
AGE	Advance glycation end-product
ALK1	Activin receptor-like kinase-1
ALK5	Activin receptor-like kinase-5
ALKs	Activin receptor-like kinases
AMI	Acute myocardial infarction
Ang1	Angiopoietin-1
ARBs	Angiotensin receptor blocker
BMPs	Bone morphogenetic proteins
CABG	Coronary artery bypass graft
CADs	Coronary artery diseases
CD31	Cluster of differentiation 31
CHDs	Coronary heart diseases
CLSM	Confocal laser scanning microscopy
Co-Smads	Common SMADs
CVDs	Cardiovascular diseases
CVS	Cardiovascular system
DAG	Diacylglycerol
Dll4	Delta-like ligand 4
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
ECD	Extracellular domain
ECM	Extracellular matrix
ECs	Endothelial cells
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-mesenchymal transition
EndoMT	Endothelial-to-mesenchymal transition
Eng	Endoglin
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal-regulated kinase

ET1	Endothelin-1
FFA	Free fatty acids
FGF	Fibroblast Growth Factor
FLK-1	Fetal Liver Kinase
FLT-1	FMS-like tyrosine kinase
FSP-1	Fibroblast-specific protein-1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
gCol	Glycated Collagen
GDM	Gestational Diabetes Mellitus
GRB2	Growth factor receptor-bound protein 2
HG	High glucose
HHECSs	Human cardiac microvascular endothelial cells
HS	Heparan sulfate
HSP	Hexosamine pathway
HTT	Hereditary haemorrhagic telangiectasia
I-Smads	Inhibitory SMADs
IDF	International Diabetes Federation
IF	Immunofluorescence
IGF-1	Insulin-Like Growth Factor-1
IHD	Ischaemic heart diseases
Jag1	Jagged-1
KDR	Kinase Insert Domain Receptor
LADA	Latent autoimmune diabetes of the adult
LRG1	Leucine-rich Alpha 2 Glycoprotein-1
<i>Lrg1</i> ^{-/-}	LRG1 knock-out
LTBP	Latent TGF- β binding protein
LV	Left ventricular
MAPKs	Mitogen-activated protein kinases
MCP-1	Monocyte chemoattractant protein-1
MHC	Myosin heavy chain
MI	Myocardial infarction
MMPs	Matrix metalloproteinases
mRNA	Messenger RNA
NG	Normal glucose

NO	Nitric oxide
NRARP	Notch–regulated ankyrin repeat protein
Nrp–1	Neuropilin–1
OC	Osmotic control
OxLDL	Oxidized LDL
PAH	Peripheral arterial disease
PAI–1	Plasminogen–activator inhibitor–1
PCI	Percutaneous coronary intervention
PCs	Pericytes
PDGF–B	Platelet derived growth factor subunit B
PDGFR– β	Platelet derived growth factor receptor– β
PECAM–1	Platelet endothelial cell adhesion molecule–1
PIGF	Placental growth factor
PKC	Protein kinase C
PLGF	Placental growth factor
PPAR β/δ	Peroxisome-proliferator-activated receptor β/δ
R–Smads	Receptor–regulated SMADs
RAAS	Renin–angiotensin–aldosterone system
Rac1	Rho-family G protein
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
sAGEs	Soluble AGEs
SARA	Smad anchor for receptor activation
sEng	Soluble Endoglin
SIP	Sphingosine–1–phosphate 1
SIPR	Sphingosine–1–phosphate receptor 1
siRNA	Small interference RNA
Sirt–1	Sirtuins–1
SKI	Sloan–Kettering Institute
SMCs	Smooth muscle cell
SnoN	SKI novel
SOS	Son of Sevenless
sVEGFR1	Soluble VEGFR1

T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TAC	Transverse aortic constriction
TAK	TGF- β activated kinase 1
TGF- β	Transforming growth factor- β
TGF- β 1	Transforming growth factor- β 1
TGF α	Transforming growth factor- α
Tiam1	Guanine nucleotide exchange factor
TKIs	Tyrosine kinase inhibitors
TMR	Transmyocardial laser revascularization
TNF- α	Tumor necrosis factor- α
UT	Untreated
UTR	Untranslated region
VE-Cad	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor family
VEGF-A	Vascular endothelial growth factor -A
VEGFR1	Vascular endothelial growth factor receptor-1
VEGFR2	Vascular endothelial growth factor receptor-2
VPF	Vascular-permeability factor
VSMC	Vascular smooth muscle cells
WT	Wild-type
ZO-1	Zonula occludens-1
α -SMA	α -smooth muscle actin
β -cells	Beta cells

Thesis Summary

Ischaemic heart diseases (IHDs) is the leading cause of morbidity and mortality globally, posing a major healthcare burden and enormous socio-economic impacts. Given a growing magnitude and the increasing complexity of the disease, conventional standard of care is not universally effective, particularly for patients with common major comorbidities like diabetes mellitus. Despite significant advances in the management of IHDs, there is an impending need for novel, biologically rational therapies to promote reperfusion of the ischemic myocardium and prevent decompensatory transition into terminal heart failures.

In the last two decades, therapeutic angiogenesis, a biological bypass by controlled delivery of potent pro-angiogenic growth factors to stimulate microvasculature growth at ischemic sites, emerged as a viable solution for IHDs. While the concept of therapeutic angiogenesis has been validated by a large body of preclinical evidence, the translation from bench to bedside remains largely hindered due to the highly sophisticated nature of angiogenesis. Therefore, targeting alternative or complementary angiogenic pathways may help to coordinate the formation of function vessel more effectively.

Leucine-rich alpha-2 glycoprotein 1 (LRG1) has been recently identified as a pro-angiogenic factor that facilitates pathological neovascularization in the retina. Mechanistically, LRG1 promotes vessel formation by modulating the endothelial transforming growth factor- β (TGF- β) signaling. Given the findings, this project focuses on studying LRG1's role in regulating the diabetes-related IHDs and interrogate if LRG1 could serve as a novel therapeutic angiogenesis agent. As it has been widely accepted that a single pro-angiogenic agent may not adequately establish functional blood vessel growth, hence we will study the role of LRG1 in conjunction with the known master regulator of angiogenesis, the vascular endothelial growth factor (VEGF).

Our recent findings demonstrated a pro-angiogenic effect of LRG1 on human cardiac-specific endothelial cells (ECs). In particular, we observed increased

EC viability, proliferation, migration, and tubule formation, as well as vessel outgrowth from various organ explants, under both normal and diabetic condition. Besides its established role regulating angiogenic switch through the endothelial TGF- β /activin receptor-like kinase 1 (ALK1) signaling axis, our data also suggest that LRG1 mediates a Smad4-dependent crosstalk to activate the VEGF/VEGF receptor-2 signaling cascade. LRG1 facilitates the upregulation and phosphorylation of the VEGFR2 and its downstream signaling transducer, the phosphorylated protein kinase B (PKB, also known as AKT). Furthermore, the pro-angiogenic effect of LRG1 is attenuated by the treatment with a VEGFR2 inhibitor, Linifanib suggesting LRG1 exerts its pro-angiogenic effect, at least to some extent, by mediating a signaling crosstalk through the VEGF/VEGFR2 signaling pathway.

To illustrate the critical role of LRG1 in regulating angiogenesis under IHDs, we adopted an *in vivo* model of transverse aortic constriction (TAC) induced heart failure. With that, we had successfully demonstrated that *Lrg1*-deficient suffered from enhanced adverse cardiac remodelling, and the cardiac maladaptation is exacerbated in the diabetic mice. We reported significantly lower survival rates, reduced microvessel density, and increased cardiac hypertrophy and perivascular fibrosis when compared to wild-type counterparts. Taken together, LRG1 exerts vital cardioprotective roles under both physiological and diabetic conditions.

In conclusion, we designed and validated new *in vitro*, *ex vivo* and *in vivo* models to study angiogenesis complicated with diabetic insults. The angiogenic assays had collectively highlighted that the functional importance and phenotypic difference of LRG1 in mediating physiological and diabetic cardiac angiogenesis. The molecular and signaling information extracted from cultured ECs provided new insights to LRG1-regulated TGF- β signaling, which may facilitate the development of more effective treatment strategies to treat diabetes-associated IHDs. Above all, we also uncovered novel metabolic phenotypes associated with genetic loss of LRG1 that paves way for future research efforts.

Thesis Outline

This thesis has been organized into six main chapters as follows:

- Chapter 1 includes a comprehensive literature review on the subject, the hypothesis and the specific aims of this research
- Chapter 2 provides detailed methodology and materials used for the study
- Chapter 3 presents the data supporting the key experimental findings
- Chapter 4 provides an in-depth discussion of experimental findings
- Chapter 5 highlights the limitation of current research and proposes potential future directions
- Chapter 6 draws a conclusion of the entire thesis and provides a comprehensive reflection on the past and present findings

Chapter 1: Introduction

1.1. Our Vascular System

Blood vessels line the entire circulatory system and play a central role in mediating physiology and pathogenesis. The vascular network is an elaborate transport system to supply oxygen and essential nutrients to tissues and to facilitate the removal of metabolic waste products¹. This process begins at the heart that pumps blood into the hierarchical organized vascular branches that spans across multiple order of magnitude (μm to cm)^{2,3}. The macrovasculature (including the arterial, venous, and lymphatic systems) regulates bulk oxygen delivery to accommodate systemic metabolic demands⁴⁻⁶. On the other hand, the microvasculature network (including capillaries, pre-capillary arterioles, post-capillary venules and collecting venule) regulates local perfusion and conducts effective blood–tissue exchange of macromolecules⁴⁻⁶.

1.2. Anatomy of Blood Vessels

Studies of vessels architecture reported similarities in the basic wall structure of most blood vessel (**Figure 1.1**)^{7,8}. The innermost luminal wall, also known as the tunica intima, is comprised a single concentric layer of endothelial cells (ECs) that align themselves in parallel to the blood flow⁹. This layer, also known as the endothelium, is highly metabolically active and critically regulates vessel homeostasis by governing the expression of a plethora of signaling molecules and regulate vessel permeability, vascular tone, extramural cells growth and migration and coverage, inflammatory responses and hemostasis function^{7,10,11}. Under physiological state, endothelial prostacyclin and thrombomodulin expression confers thromboresistance and maintains the blood fluidity^{12,13}. Endothelial phenotypes vary between different species, organs, vascular beds, different spatial and temporal distribution⁵. ECs demonstrate remarkable heterogeneity in cell morphology, function, gene expression, and antigen composition to serve specialized functions based on the physiological needs in the local microenvironment^{5,14}. For example, the ECs of the central nervous

system are non-fenestrated and tightly associated to form the blood-brain barrier that restricts paracellular and transcellular movement of solutes to maintain cerebral homeostasis and proper neuronal function¹⁵⁻¹⁸. On the contrary, the glomerular endothelium is highly fenestrated to regulate high flux filtration of fluid and small solutes^{6,19}.

Figure 1.1. Schematic of the Basic Structure of Blood Vessels.

Blood vessels are organised as capillaries, arterioles and venules or, arteries and veins. Capillaries are the most abundant blood vessels in our body. They consist of ECs surrounded by pericytes embedded within the EC basement membrane. Arterioles and venules are invested with VSMCs that are able to contract and regulate the blood flow. Besides the endothelium and mural cell layers, arteries and veins contain an additional layer of connective tissue with a mixture of different type of cells. Figure adapted from *Schöneberg et al., 2018*²⁰.

Ensheathing the endothelium is the tunica media layer which contains mesenchymal cells (smooth muscle cells, pericytes and fibroblast) sharing a common basement membrane with the ECs^{21,22}. Layers of spirally-arranged vascular smooth muscle cells (vSMCs) wrap around macrovascular ECs to provide mechanical support, regulates vascular tone and blood flow^{23,24}. vSMCs display remarkable level of plasticity to differentiation between the “synthetic” and “contractile” phenotype by modulating specific contractile proteins, ion channels, and cell surface receptors^{22,25}. Instead of vSMCs, a discontinuous coat of pericytes (PCs) surrounds the microvascular ECs²⁶. The PCs/ECs ratios, ranging between 1:1 and 1:10, differs across different tissue bed, often found to be enriched at the EC-EC junctions and branch points²⁷. Just like ECs, the perivascular cells also features distinct morphology, protein expression profile

and functional plasticity²⁸. For instance, mesangial cells are specialized renal pericytes that participate in ultrafiltration at the glomerulus, whereas hepatic stellate cells are specialized hepatic pericytes with additional fat-storing capacities^{24,29,30}.

Last but not least, large caliber arteries and veins are wrapped in an additional layer known as the outer tunica adventitia which contains a collagen-rich ECM and interacting cell types including quiescent fibroblasts, nerve cells, resident macrophages, T-lymphocytes, B-lymphocytes, mast cells, and dendritic cells^{26,31}. Besides providing mechanical support to the blood vessel, it serves as a biological compartment facilitating the retrieval, integration, storage, and release of key regulators that are critical for blood vessel activation and homeostasis³². Moreover, recently studies also suggested that this outermost layer of the blood vessel also functions as a niche for resident vascular progenitor cells^{31,33}.

1.3. Blood Vessels Formation

Physiological angiogenesis involves a highly organized sequence of cellular events which are tightly modulated to meet the tissue requirements³⁴. Aberrant angiogenesis, both excessive and insufficient angiogenesis, is known to modulate pathologies^{35,36}. For instance, inadequate vessel expansion or maintenance underlies many ischemic diseases including coronary artery disease, stroke, and delayed wound healing^{23,37}, whereby the poor microcirculation could result in tissue death³⁸. Conversely, excessive angiogenesis is commonly associated with malignancies and metastasis, inflammation, proliferative retinopathies, and arthritis³⁹, in which the vasculature feeds diseased tissue while impacting normal tissue homeostasis³⁸. Moreover, malformed pathological vascular networks, commonly found in tumours, readily forms but rarely mature into fully functional conduits, leads to tissue hypoxia, nutrient-deprivation, chemoresistance to therapies and fuel the

diseases progression⁴⁰. In general, new blood vessel formation follows two distinct mechanisms, vasculogenesis and angiogenesis (**Figure 1.2.**)⁴¹⁻⁴⁴.

Figure 1.2. Schematic of the Modes of Blood Vessel Formation.

De novo formation of blood vessels during embryogenesis occurs primarily through vasculogenesis, which is supported by the progenitor cells in the angioblast. Angiogenesis refers to the growth of new blood vessels from existing vasculature in development and disease. Figure adapted from Adair and Montani, 2011⁴².

1.3.1. Vasculogenesis

At the earliest stage of vertebrate embryogenesis, the embryos develop in the absence of functional vascular network with nutrients and waste products being exchanged by passive diffusion⁴⁴. From the third week of gestation, a functional vascular labyrinth starts to form to ensure an adequate supply of oxygen, nutrients and growth factor and timely evacuation of waste products in the growing embryos⁴⁵. At this stage, *de novo* blood vessel is formed in this process called vasculogenesis, which arises from the differentiation of mesenchymal cells into ECs⁴⁶. At the beginning, the blood islands, known as the angiocysts, develop in the lateral mesoderm. Subsequently, putative hemangioblasts, common precursor cells for hematopoietic stem cells and angioblasts, will be differentiated from mesodermal stem cells. Cluster of differentiation (CD)31⁺CD34⁺ VEGF receptor (VEGFR)-2-positive angioblasts are capable of differentiating into primitive endothelial cords which to give rise to the dorsal aorta, the cardinal vein and the embryonic stems of yolk sac arteries and veins^{8,47,48}. In addition, a distinct mixture of pro-angiogenic growth factors and

morphogens are reported to temporally and spatially direct the migration of angioblasts and initiation of the vasculogenesis process^{37,42}.

1.3.2. Angiogenesis

The term “angiogenesis” was first coined by John Hunter in 1787 to describe the growth of new blood vessels⁴⁹. Following the establishment of the primary vascular plexus, subsequent expansion of the vascular system is dependent on sprouting angiogenesis, a process in which ECs depart from their pre-existing vascular beds, sprout into the avascular stroma and organize into new blood vessels^{23,46,50}. Mature blood vessels are quiescent in nature and only transit into activated state during specific growth periods, or in response to tissue injury to meet the changing metabolic demands and environments²³. Physiological angiogenesis is a tightly regulated process that involves almost all components of the vascular wall in response to hypoxia⁵¹. A series of highly coordinated efforts between vascular and non-vascular cell populations, ECM breakdown and synthesis, crosstalks between multiple signaling pathways, and delicate balance between endogenous stimulators and inhibitors of angiogenesis is instrumental to the formation of stable functional blood vessels (**Figure 1.3.**)^{34,42,43,52}.

Figure 1.3. Schematic of the Sprouting Angiogenesis Process.

Sprouting angiogenesis begins with (1) ECs activation upon the exposure to an angiogenic stimulus, (2) ECs initiation mediated by basement membrane degradation and ECM remodeling, followed by (3) ECs proliferation and site-directed migration leading to vascular outgrowth and stalk extension. The nascent vessel undergoes (4) tubulogenesis (lumen formation) and anastomosis and finally undergo (5) maturation with mural cell involvement and further ECM remodeling. Figure adapted from Logsdon *et al.*, 2014⁴³.

1.3.2.1. Endothelial Activation

The onset of angiogenesis begins when the net balance of the pro-and anti-angiogenic factors is tipped in favour of angiogenesis. Hypoxia, a reduction in the physiological oxygen level, directly upregulates the hypoxia-inducible factor (HIF)-1 α and HIF-2 α , and potently activates the master regulator of angiogenesis, vascular endothelial growth factor (VEGF)^{51,53}. Experimental evidence suggest that VEGF-induced nitric oxide (NO) upregulation and increase vasodilation, one of the earliest step in angiogenesis^{44,54}. In addition, the presence of pro-inflammatory cytokines like tumour necrosis factor (TNF)- α and interleukin (IL)-1 β potently induces vascular permeability, promotes influx of inflammatory cells and upregulate the expression of pro-angiogenic cytokines and chemokines to support pro-angiogenic switch⁵⁵.

The initial vasodilation is accompanied by increased permeability, mediated by redistribution of intercellular adhesion molecules, like platelet endothelial cell adhesion molecule (PECAM)-1 and vascular endothelial (VE)-cadherin, and reorganization of cell membrane^{7,37,43}. Increased vessel permeability results to extravasation of plasma proteins, which forms a fibrin-rich provisional matrix to support vessel sprouting^{8,56}. At the same time, basement membrane wrapping around the dilated mother vessels starts to undergo structural alternations and degradation *via* a controlled proteolytic process. This is mediated by increased production of proteases, including matrix metalloproteinases (MMPs), plasminogen activators, and chymases^{23,57}. In addition to its important structural role, ECM also serves as a reservoir for growth factors and bioactive molecules, including master regulator of angiogenesis VEGF, fibroblast growth factor (FGF), transforming growth factor-beta (TGF- β), insulin-like growth factor-1 (IGF-1) and matrix-derived anti-angiogenic fragments of larger ECM molecules like endostatin and arresten^{58,59}. The breakdown releases the pool of angiogenic growth factor sequestered in the ECM which increases the local concentration angiogenic growth factor and cause ECs activation⁵⁸. Given that ECs and perivascular cells share the same basement membrane, ECM

degradation liberates ECs from the perivascular cells and enables them to freely proliferate, migrate and assemble into new vascular networks^{60,61}.

1.3.2.2. Stalk Extension

EC heterogeneity at the advancing front of angiogenic vessels is essential for a well-coordinated angiogenic process^{62,63}. ECs in the nascent sprout could be specified into the leading “tip cells” or trailing “stalk cells” by acquiring distinct morphologic and functional phenotypes⁶⁴. The highly migratory tip cell senses the VEGF gradient and leads the vascular outgrowth by dynamic filopodia extension^{23,65,66}. On the other hand, the neighbouring stalk cells adopts proliferative phenotype which supports sprout extension while establishing adherent and tight junctions to enhance the stability of the newly formed blood vessels^{63,67}. The endothelial delta-like 4 (Dll4) – Notch1 signaling pathway critically controls the extent of vascular sprouting and branching through lateral inhibition (**Figure 1.4.**)^{64,68-71}. VEGF ligand to VEGF receptor binding promotes the expression of Dll4 ligand in the tip cells⁶⁵. High Dll4 activates Notch signaling on neighbouring ECs triggers a negative regulation of VEGFR2 while promoting the expression of VEGFR1 and soluble VEGFR1, thus specifying for a stalk cell phenotype^{23,64,67,68}. Jagged1 (Jag1), a Notch ligand primarily found in the stalk cells, maintains low Notch activity in the adjacent tip cells by antagonizing the action of Dll4^{23,71}. Furthermore, signaling regulators like the notch-regulated ankyrin repeat protein (NRARP) and Sirtuins-1 (Sirt1) fine-tunes the amplitude and duration of tip and stalk transitions by negatively regulates Notch signaling^{23,63}.

1.3.2.3. Vessel Maturation and Quiescence

The stalk cells extend the nascent vessel until it meets neighbouring vascular sprouts and merge, a process known as anastomosis, to acquire a vessel lumen capable of carrying blood^{72,73}. Although the mechanisms driving circulatory anastomosis is not yet completely understood, it is been postulated that this process involves a complex series of events including EC repulsion at the cell–

cell contacts within the primitive vascular cords, junctional rearrangement and EC shape changes^{73,74}. Subsequently, the blood flow commences, and hemodynamic forces dynamically shape the apical membrane of ECs and expand the lumenized vascular tubes^{73,74}. During the final stages of capillary development, EC migration comes to a halt, while the vessel lumen and functional adherent junction re-establishes⁷³.

The rudimentary vascular cords are prone to premature regression⁷⁵. Subsequent vascular remodelling is vital to provide establishment of fully functional and quiescent vascular network to address the local homeostatic demands^{58,76}. Vessel maturation begins with the suppression of EC proliferation and sprouting facilitated by the recruitment and paracrine regulation of perivascular mural cells²³. A complex interplay involving a multitude of signaling pathway is required in this process. For instance, in response to VEGF stimulation, ECs reportedly upregulates its expression of the platelet-derived growth factor (PDGF)-B. Endothelial PDGFB binds to its cognate PDGF receptor (PDGFR)- β that is primarily expressed by mural cells and activates intracellular signaling transducers including the Ras-mitogen-activated protein kinases (MAPKs), phosphatidylinositol 3-kinase, phospholipase C γ , and extracellular-signal-regulated kinase (ERK) 1/2, all of which are the key mediators of mural cell proliferation, migration and differentiation^{23,77}. Next, TGF- β signaling pathway activation stimulates mural cell differentiation, proliferation, migration and facilitates the production of ECM for new basement membrane formation⁷⁸. Moreover, the endothelial-derived Sphingosine-1-phosphate (S1P) S1P binds to the G protein-coupled S1PRs on the mural cells and helps to stabilize the nascent vessel²³. The ligand-receptor interaction increases intracellular Ca²⁺, PI3K, guanine nucleotide exchange factor (Tiam1) and its cognate Rho-family G protein (Rac1), vascular endothelium (VE)-cadherin, β -catenin, α -actinin and Zonula occludens-1 (ZO-1) which collectively promotes adherent junction assembly and restore the endothelial barrier functions⁷⁹. Last but not least, the bidirectional paracrine signaling between mural cell-specific Angiopoietin-1 (Ang1) and its endothelium-specific receptor tyrosine kinase Tie-2 are also participating in blood vessel

stability and limit excessive plasma leakage^{37,58}. Tie-2 is highly expressed in the endothelium undergoing active angiogenesis⁸⁰. The Ang1/Tie2 signaling promotes mural cell adhesion and reinforcing the reestablishment of endothelial junctions^{23,63}. Angiopoietin-2 (Ang2) antagonizes this process by competing with Ang1 for binding to Tie2 and activates endothelial stalk cell proliferation⁸¹.

Figure 1.4. Schematic of Tip/Stalk Specification.

VEGF and Notch signaling pathways co-operatively control the tip and stalk cell specification. VEGF gradient exposes the tip cells with highest VEGF signaling, which is critical in specifying the tip cell phenotype. High VEGF signaling leads to increased expression of DLL4 in tip cells, which results in subsequent activation of Notch signaling, suppression of VEGF signaling in neighbouring ECs and specification of stalk cell phenotype. NRARP and SIRT1 control transient stalk cell elongation and facilitate the stalk stabilization. Figure adapted from *Blanco and Gerhardt, 2013*⁶⁴.

1.3.2.4. Vessel Remodelling and Pruning

To meet the nutritional requirement of the surrounding tissue, the newly formed vascular network will undergo extensive vascular remodelling and pruning to achieve optimal hierarchical organization complexity⁶³. During this process, selective pruning of specific a subset of redundant or inefficient vessel segments undergoes regression mediated by EC apoptosis following the withdrawal of key survival factors such as VEGF^{63,76}. Concurrently, driven by the mechanical influence of blood flow, modification of the branch angle of bifurcating vessels

and duplicating existing vessels occurs to optimize blood flow^{63,82-84}. In other instances, vessel retraction occurs as a result of low blood flow and subsequent recycling of ECs follows⁸⁵. ECs promptly undergo migration and redeploys themselves to new vessels in close proximity⁸⁵. High hemodynamic forces drives flow-induced enlargement of the diameter, promotes adaptive proliferation and induces provisional ECM remodelling to increase wall thickening^{86,87}. Additionally, further mural cells recruitment helps to reinforce the ECM remodelling and supports vascular basement membrane matrix assembly³⁴.

1.4. Key Regulators of Angiogenesis

Angiogenesis is regulated by a fine balance of a multitude of pro- and anti-angiogenic factors and perturbation of this balance could contribute the development and progression of pathologies³⁵. The importance of angiogenesis in health and diseases has prompted extensive investigations to identify the underlying regulators that are fundamentally important in this process. Examples of key angiogenic regulators are listed below (**Table 1.1**)^{49,88-90}. With the rapidly advancements in the cellular and molecular biology of angiogenesis, many of these angiogenic agents are now rigorously examined for their biological activities, clinical efficacies and safety to translation into therapeutic benefits⁹¹.

1.4.1. VEGF Family

The VEGF family of growth factors (VEGFs) is the most well-recognized family of pro-angiogenic regulators for their pivotal role in endothelial functions. The VEGF family members are secreted homodimeric glycoproteins of approximately 45kDa^{92,93}. The entire gene sequence of VEGF spans 16272bp and is located on chromosome 6p12⁹⁴. The family consists of five mammalian sub-types; VEGF-A, -B, -C, -D and the placental growth factor (PlGF)⁹⁵⁻⁹⁷. VEGF-A, the prototypical ligand, was originally discovered as a vascular-permeability factor (VPF) in the 1980s^{94,97,98}. In humans, VEGF-A encoded by

a single gene with eight exons and seven introns, which could be alternatively spliced into at least nine major variants including VEGF₁₂₁, VEGF₁₄₅, VEGF₁₄₈, VEGF₁₆₂, VEGF₁₆₅, VEGF₁₈₃, VEGF₁₈₉, and VEGF₂₀₆ (**Figure 1.5**)⁹⁹. These variants differ in their bioavailability, interaction with VEGF receptors leading to distinct effects in angiogenesis^{94,100}. For example, all VEGF-A isoforms, excepting VEGF₁₂₁, can bind heparin and be sequestered in the heparan sulfate (HS)-rich ECM at varying degrees¹⁰¹, while the non-heparin-binding VEGF₁₂₁ is freely diffusible upon secretion¹⁰². VEGF₁₂₁, VEGF₁₄₅ and VEGF₁₆₅ are secreted and forms dimeric proteins, whereas the VEGF₁₈₉ and VEGF₂₀₆ remained almost completely sequestered in the heparin-rich ECM^{93,99,101}. VEGF₁₆₅ is regarded as the most predominant and biological potent isoform of VEGF in promoting angiogenesis and EC permeability^{93,101}.

Activators	Inhibitors
Angiogenin	Angiostatin (plasminogen fragment)
Angiopoietin-1	Anti-angiogenic anti-thrombin III
AC133	Constatin
Chemokines*	
Del-1	Cartilage derived inhibitor (CDI)
β-estradiol	CD59 complement fragment
Ephrins	Endostatin (collagen XIII fragment)
FGF-αβ	Fibronectin fragment
FGF	Fragment of SPARC
Follistatin	Heparinases
HGF	HCG
Id1/Id3	IFN-αβγ
Integrins, αVβ3, αVβ5, α5β1	Interferon inducible protein (IP-10)
IL8	IL4, IL12, IL18
Leptin	2-methoxyestradiol
MCP-1	Maspin
MMPs	Kringle-5 (plasminogen fragment)
NOS	Osteopontin fragment
PLGF	Placental ribonuclease inhibitor
PDGF-BB	Plasminogen activator inhibitor
Pleiotrophin (PTN)	PEDF (pigment epithelium derived growth factor)
Platelet derived endothelial cell growth factor (PD-ECGF)	PF4
P1GF	
Proliferin	Prolactin 16 kDa fragment
TGF-αβ*	Retinoids
TNF-α	Tissue inhibitor of metalloproteinases (TIMPs)
VE cadherin	TSP-1
VEGF	Vasculostatin

Table 1.1. List of the Key Regulators of Angiogenesis.

The angiogenic process is regulated by a diverse array of pro- and anti-angiogenic growth factors and signaling pathways that regulates vascular homeostasis. Figure adapted from *Gupta and Zhang, 2005*⁴⁹.

Mice lacking a single VEGF-A allele (VEGF-A^{+/-}) suffer from early mid-gestational lethality between embryonic stage (ES) 11 and ES 12 owing to the development of multiple severe vascular defects^{103,104}. Specific inactivation of a single VEGF-A allele in Collagen2a1-Cre transgenic mice also resulted in severe abnormalities in heart development and premature embryonic lethality at around E10.5¹⁰⁵. Conversely, VEGF-A overexpression could also lead to

severe abnormalities in the heart and embryonic lethality at E12.5-E14¹⁰⁶. Given that, both VEGF-A excess and insufficiencies both resulted in severe pathologies during embryogenesis. Hence, a controlled, dose-dependent VEGF-A expression helps to maintain the proper functions of the vascular homeostasis⁷¹.

1.4.2. Endothelial VEGF Signaling

VEGF signals predominantly through the three structurally related VEGF receptors (VEGFR) in the consensus scheme for activation of receptor tyrosine kinases¹⁰⁷. Specifically, the VEGF receptor (VEGFR)-1 (also known as FMS-like tyrosine kinase, FLT-1) VEGFR-2 (also known as fetal liver kinase, FLK-1), and VEGFR-3 (also known as FLT4) works in conjunction with the co-receptors Neuropilin (NRP)-1 and NRP-2^{71,93,108}. The biological activities of VEGF-A are transduced mainly by the VEGFR-2 activation^{97,107}. VEGF-A binding to VEGFR-2 induces downstream receptor homodimerization, kinase activation, and autophosphorylation of specific tyrosine residues within the dimeric complex and the assembly of the membrane-proximal signaling complex^{93,107,109,110}. Subsequently, activation of specific intracellular signaling transducers eventually up-regulates target genes that facilitate EC proliferation, migration, adhesion, lumenization, and survival (**Figure 1.6**)^{93,109,111}. For example, VEGFR2 activation promotes cell survival by promoting the phosphatidylinositol 3-kinase (PI3K)-dependent activation of the anti-apoptotic protein kinase B, also referred to as the Akt kinase^{92,101}. In parallel, the VEGF-mediated Raf/MEK/ERK signaling cascade activation participates in DNA synthesis and proliferative response to achieve formation of ordered vessel networks^{112,113}. Given its important role in angiogenesis, the VEGF signaling is regulated at multiple levels, including the ligand/receptor expression, the presence of co-receptors and accessory protein, and the activation of tyrosine phosphatases¹¹⁴.

Figure 1.5. Schematic of VEGF signaling in ECs.

VEGF regulates angiogenesis primarily through VEGFR2. The ligand-receptor binding stimulates VEGFR2 dimerization and autophosphorylation of several other receptor tyrosine residues in the intracellular juxtamembrane domain leading the activation of downstream signaling transducers. Figure adapted from Cook and Figg, 2010¹¹¹.

At the ligand level, VEGF activities is governed by its own expression level, its cognate VEGFR2, as well as the presence of prominent decoy-receptor like the VEGFR1. Specifically, VEGF-A preferentially binds to VEGFR1 at 10-fold higher affinity but has relatively poor kinase activity, thus the VEGF/VEGFR1 interaction exerts a negatively impacted the VEGF signaling by limiting the bioavailability of VEGF for VEGFR2^{95,97,109,115}. Another level of the signaling regulation is mediated by the presence of co-receptors like the Nrp-1, which is enriched in endothelial tip cells, synergistically enhances VEGFR2 signaling potency and promotes VEGF-induced chemotactic and mitogenic activity^{93,95}. Consistent with the regulatory role for Nrp-1 in vascular angiogenesis, Nrp-1-deficient mice are embryonic lethal at around E12.5¹¹⁶. Along the same line, knockdown of Nrp-1 in zebrafish leads to impaired developmental angiogenesis due to the deregulated formation of the intersomitic vessels¹¹⁷.

As a master regulator of angiogenesis, a dysregulated expression of VEGF is a hallmark of many diseases¹¹⁸. Hence anti-VEGF therapy has revolutionised the treatment of multiple debilitating vaso-proliferative conditions such as proliferative diabetic retinopathy, age-related macular degeneration and various

types of cancers. For instance, commercialized VEGF-neutralising antibody, Bevacizumab, blocks the VEGF/VEGFR2 interaction, whereas aflibercept serves as a VEGF-trap by bind to a broad range of VEGF ligands including the VEGF-A, VEGF-B, and placental growth factor (PLGF)¹¹⁸⁻¹²⁰. On the other hand, tyrosine kinase inhibitors like Sorafenib, Sunitinib, Pazopanib, inhibits VEGF signaling by blocking the kinase activity of VEGFR2, thereby potentially attenuating the process of blood vessel formation¹¹⁸⁻¹²⁰.

1.4.3. Transforming Growth Factor- β (TGF- β) Family

Transforming Growth Factor (TGF)- β is a highly evolutionary conserved superfamily of growth factors that are fundamentally important in health and diseases¹²¹⁻¹²³. It comprises of more than 30 structurally-related morphogens including TGF- β 1, β 2, and β 3, Activins, Inhibins, Nodals, Growth and differentiation factors (GDF), anti- Müllerian hormone (AMH) and bone morphogenetic proteins (BMPs)¹²¹⁻¹²⁴. The TGF- β superfamily of growth factors regulates a wide variety of biological processes including proliferation, differentiation, migration, adhesion, morphogenesis, apoptosis and ECM deposition^{78,121,125}. Given that its important role, aberrant TGF- β signaling contribute to the development and progression of a variety of human diseases, ranging from embryonic development defects to autoimmune diseases, fibrosis, cardiovascular failure and malignancies^{78,125}.

Abundant evidence from genetic studies supports that the multifunctional TGF- β exerts pivotal roles in maintaining cardiovascular health^{121,125,126}. Perturbation of TGF- β signaling components has been prominently associated with impaired CVS development, cardiac malfunctions and vascular abnormalities in a variety of animal and humans studies^{78,125} (**Table 1.2**). In particular, genetic mutations at the TGF- β receptor level is associated to the development of hereditary haemorrhagic telangiectasia (HHT), Loeys-Dietz syndrome and Marfan syndrome type II, all manifesting various degree of vascular dysplasia¹²⁵. Moreover, targeted deletions of the TGF- β receptors in animal models phenocopies similar cardiovascular defects as seen in HHT, featuring fragile

leaky vessels causing spontaneous bleeding^{125,127,128}. At the ligand level, genetic polymorphism and mutations affecting TGF- β 1 are associated with varied phenotypes ranging from arteriovenous malformations, aneurysms, hypertension, atherosclerosis, autoimmune conditions etc^{125,129}. Consistently, genetic knock-out mice models also revealed variable content-dependent phenotypes^{130,131}. On the other hand, genetic variants of TGF- β 2 is strongly associated with increased susceptibility and cardiovascular complications to Kawasaki disease¹³². In addition, common TGF- β signaling mediator, Smad4, is also linked to juvenile polyposis and HHT¹³⁰. As well, in mice model, cardiomyocytes-specific deletion of Smad4 in caused cardiac hypertrophy¹³³ while EC-specific deletion of Smad4 is embryonic lethal at E10.5¹³⁴.

Gene	Human disease	Animal model
Ligands		
TGF β 1	Camurati-Engelmann disease	KO: embryonic lethal with vascular defects or postnatal lethality from autoimmune disease.
TGF β 2	Kawasaki disease	KO: aortic arch defects, cardiac septal defects, perinatal lethality.
TGF β 3	unknown	KO: cleft palate, delayed lung maturation, die shortly after birth.
Receptors		
T β RII	MFS2, LDS	KO: embryonic lethal, vascular defects
T β RI/ALK5	LDS	KO: embryonic lethal, angiogenesis defects
ALK1	HHT	KO: embryonic lethal, reduced VSMC differentiation, dilated vessels, AVMs
BMPRII	PAH	KO: pre-angiogenesis lethality Transgenic BMPRII-mutant allele: pulmonary hypertension
Accessory receptors		
ENG (endoglin)	HHT	KO: embryonic lethal due to vascular defects, reduced vSMC differentiation, heart defects. Het: vascular lesions similar to HHT.
Soluble Endoglin	Pre-eclampsia	
TGFBR3 (betaglycan)	Familial intracranial aneurysm	KO: poorly formed cardiac septa, incomplete compaction of ventricular walls
Smads		
Smad1	Unknown	KO: embryonic lethal due to defects in chorion-allantoic circulation
Smad3	Aortic aneurysm-osteoarthritis	
Smad4	JP and HHT	KO: embryonic lethal
Smad5	Unknown	KO: embryonic lethal due to angiogenesis defects
Smad6	Unknown	KO: heart abnormalities, aortic ossification and elevated blood pressure
Smad7	Unknown	KO: embryonic lethal due to cardiovascular defects
Smad8	PAH	Defective pulmonary vascular remodeling

Abbreviations: KO, knockout; Het, heterozygous; AVMs, arteriovenous malformations; LDS, Loeys-Dietz syndrome; MFS2, Marfan syndrome type 2; HHT, hereditary haemorrhagic telangiectasia; PAH, pulmonary arterial hypertension; JP, Juvenile polyposis; VSMC, vascular smooth muscle cell.

Table 1.2. List of the Pathologies Related to TGF- β signalling Dysregulation.

Given the key role of TGF- β signaling in the CVS, dysregulation of the signaling components is associated with profound changes leading to a multitude of cardiac malfunctions and vascular abnormalities. Figure adapted from *Pardali, 2012*¹²⁵.

1.4.3.1. TGF- β Signaling

TGF- β 1 is a prototypical ligand of the superfamily. It is ubiquitously expressed in all cell types in form of a latent precursor protein which¹³⁵. The latency-associated protein (LAP) component of the TGF- β 1 precursor is able to form a complex with the latent TGF- β binding protein (LTBP) via disulfide bonds leading to the formation of the large latent complex^{136,137}. LTBPs are important components of ECM, therefore, functions to maintain TGF- β 1 latency and targeting the latent form of TGF- β 1 to the ECM^{124,138}. The bioactive 25kDa TGF- β 1 dimer could be released from the latent protein by a variety of molecules, from protons to protease, in response to ECM perturbation^{122,139-141}. Following the release, TGF- β ligands evoke the multifaceted cellular effects by both canonical Smad-dependent and non-canonical signaling cascades¹⁴².

Despite of the large number of TGF- β ligands, only five TGF- β receptor II (T β RII) and seven TGF- β receptor-I (T β RI) are present in mammalian genomes¹²². The binding of the dimeric TGF- β ligand to constitutively active T β RII leads to the recruitment and phosphorylation of T β RI at the cytoplasmic serine/threonine kinases domain (**Figure 1.7**)^{122,125}. The activated T β RI regulates the downstream signal transduction *via* C-terminal phosphorylation of receptor-activated Smad (R-Smad) proteins (Smads1, -2, -3, -5, or -8)^{141,143}. Smad 2/3 mainly responds to the activation of TGF- β /Activin/Nodal mediated, whereas Smad1/5/8 mediates the cellular response induced by BMPs^{122,141,142}. The docking of R-Smads allows the phosphorylation of the last two serine residues at its carboxy terminus, which in turn, forms a transcriptional trimeric complex with a common mediator, Smad4 (Co-Smad4)^{78,135,144}. The heterotrimeric Smad complex then translocate into the nucleus, where they work with other context-dependent transcriptional regulators to activate or repress target genes^{122,141}.

TGF- β signaling could be positively-regulated by Smad anchor for receptor activation (SARA), which facilitates recruitment of Smad2 or Smad3 to T β RI for efficient phosphorylation^{122,125}. In contrast, inhibitory Smads (I-Smads), Smad6 and Smad7, mainly function to antagonize TGF- β signaling by

competing with R-Smads for binding to T β RI¹⁴³. I-Smads are also able to recruit Smurf-related ubiquitin ligases or phosphatase, leading to the receptor proteasomal degradation or dephosphorylation^{125,141,143}. Furthermore, SKI (Sloan-Kettering Institute) and SnoN (SKI novel) are Smad-interacting proteins that negatively regulate TGF- β signaling by disrupting the formation of R-Smad/Smad4 complexes^{122,145}.

Figure 1.6. Schematic of Canonical TGF- β Signaling.

TGF- β superfamily ligands signal through distinct receptor complexes. BMP ligands initiate the signaling cascade by binding and bringing together BMPRI and BMPRII receptors into a heterotetrameric complex, phosphorylating its immediately downstream substrate Smad1/5/8, and then associates with co-Smad4 and translocate into the nucleus to activate target gene transcription. On the other hand, TGF- β ligands bind and phosphorylate T β RII which, in turn, leads to the recruitment and activation of T β RI, and phosphorylation of Smad2/3. The activated Smad2/3 forms heteromeric complexes with co-Smad4. This trimeric complex subsequently translocates into the nucleus and associates with various transcription regulators leading to the activation of a distinct set of target genes. SARA facilitates the recruitment of Smad2/3 to T β RI and its phosphorylation. Additionally, the inhibitory Smad6 and 7 functions as negative regulator of TGF- β -mediated signal transduction for both the BMP and TGF- β downstream signaling cascades. Figure adapted from *Wrana, 2013*¹²².

Besides Smads, TGF- β is also able to activate non-Smad signaling transducers^{121,141}, which plays important regulatory roles in controlling cellular function and contributes to the diversity of cellular response generated by the TGF- β superfamily proteins¹⁴⁶. For instance, TGF- β receptor activation promotes the recruitment of growth factor receptor-bound protein 2 (GRB2) and Son of Sevenless (SOS) complex leading to the activation of the MAPK-ERK cascade that regulates critical cellular functions including proliferation, survival, differentiation, angiogenesis and migration¹⁴⁷⁻¹⁴⁹.

In addition to MAPK signaling transducers, TGF- β also promote the phosphorylation and activation of p38 and c-Jun N-terminal kinase (JNK) cascades, which are involved in cell apoptosis, migration and epithelial-to-mesenchymal transition (EMT)¹⁵⁰. Other examples of the TGF- β -associated non-canonical pathways includes Rho-like GTPases, PI3K-AKT and protein phosphatase 2A pathways^{78,125,151,152}. The canonical and non-canonical TGF- β signaling pathways interact with each other extensively. For example, members of the MAPK pathway, such as TGF- β activated kinase 1 (TAK-1), are reported to regulate Smad activation¹⁵³.

1.4.3.2. TGF- β and Angiogenesis

Endothelial TGF- β signaling begins with the binding of TGF- β dimers to T β RII expressed on ECs, subsequent recruitment of T β RI and the formation of a tetrameric complex¹⁵⁴. Downstream phosphorylation and conformational changes at the intracellular domain of T β RI trigger activation of the Smad transcription factors, leading to changes in ECs behaviour¹⁵⁴. TGF- β is thought to modulate EC properties by balancing angiogenic response through T β RII mediated activin receptor-like kinases (ALKs), specifically, the ALK1- and ALK5 downstream signaling¹⁵⁵.

ALK5 is ubiquitously expressed and distribution in all cell types, hence it presents as the main TGF- β receptor that results in most cellular responses to TGF- β ¹⁵⁶. At quiescent state, preferentially signals through the T β RII/ALK5

complex, in which ALK5 activation leads to phosphorylation of the Smad 2/3 and downstream target genes, such as plasminogen-activator inhibitor 1 (PAI-1), leading to the inhibition of EC migration and proliferation^{92,135,156}. In addition to ALK5, ECs also express a second type of T β RI, the activin receptor-like kinase 1 (ALK1)¹⁵⁵. ALK5 demonstrates a higher sensitivity to TGF- β 1 than ALK1, thus, a low concentration of TGF- β 1 favours the activation of the ALK5 signaling¹⁵⁵. In contrary, high concentrations of TGF- β 1 facilitates the recruitment and phosphorylation of ALK1 by T β RII, leading to the activation downstream signaling transducers Smad 1/5/8 and downstream target genes required for EC activation and angiogenesis^{78,135,144}. Hence, the TGF- β signaling exerts a biphasic effect on EC angiogenesis¹⁵⁷.

In addition, endothelial TGF- β signaling is differentially regulated by a transmembrane auxiliary TGF- β receptor, Endoglin (ENG), also known as CD105, which its expression is highly enriched in actively proliferating ECs^{158,159}. ENG is reported to interact with the TGF- β receptor complexes, including T β RII, ALK1, and ALK5, and regulates TGF- β mediated angiogenesis effect¹⁵⁹. ENG modulates a pro-angiogenic switch by promoting ALK1 kinase activity and Smad 1/5/8 signaling^{158,159}. This results in a net dominance of ALK1-mediated signaling, tipping the balance towards pro-angiogenic outcomes. Notably, membrane-bound ENG can be proteolytic cleaved and shed into the circulatory as soluble ENG (sENG). Although lesser studied, sENG is postulated to antagonize the function of membrane-bound ENG by competitive binding to TGF- β ligands, leading to an anti-angiogenic profile¹⁶⁰. Therefore, all of the research findings all substantiate that both membrane-bound ENG and sENG functions as important modulators of TGF- β signaling and controls the dynamic regulation of EC homeostasis.

1.4.4. Leucine-Rich Alpha-2-Glycoprotein-1

Leucine-rich α -2-glycoprotein 1 (LRG1) was first identified as a human serum glycosylated protein without a defined function in the late 1970s¹⁶¹. Subsequent sequence analysis mapped the LRG1 gene to chromosome 19p13.3, and

deduced that LRG1 human protein contains 347-amino acid, with a molecular mass of approximately 45kDa, and it shares 66% amino acid identity with its mouse homolog¹⁶². LRG1 is the founding member of the leucine-rich repeats (LRR) family¹⁶³. The members of the LRR family are reportedly involved in a diverse range of cellular functions including DNA transcription, DNA repair and RNA processing protein–protein interactions, signal transduction, cell adhesion, cell survival, cell apoptosis and cell metastasis¹⁶⁴. Although the biological function of LRG1 has been well-understood, it has been reported to be associated with numerous physiological events in animal and human including angiogenesis¹⁶⁴⁻¹⁶⁸, granulocyte differentiation¹⁶², fibrosis^{169,170}, inflammation^{162,171-179}, cardiac remodelling^{126,170,180-182}, autoimmune diseases^{178,183} and malignancies¹⁸⁴⁻¹⁹⁶. Apart from the speculated physiological and pathological functions, aberrantly expressed LRG1 been widely proposed as a potential plasma biomarker for inflammatory conditions^{171,197,198} and a wide range of malignancies^{184,199,200}.

Northern blot analysis of adult mouse tissues revealed that LRG1 expression is highly enriched in the liver, with a lower expression in the heart, and lesser levels in the spleen and lung¹⁶². Similarly, a quantitative transcriptomics analysis of human tissues by deep sequencing had also suggested that LRG1 is most highly expressed in the liver²⁰¹. Additionally, specific immunostainings revealed that LRG1 is predominantly found in the vasculature of various organs including the retina, breast, skin and intestine¹⁶⁴.

1.4.4.1. Endothelial LRG1 Signaling

Mechanistically, LRG1 promotes angiogenesis pathological neovascularisation in the eye by modulating the endothelial TGF- β signaling (**Figure 1.8**)¹⁶⁴. Under physiological conditions, the ECs are maintained in quiescence by the angiostatic TGF- β 1/ALK5/Smad2/3 signaling axis. However, in response to angiogenic cues, the expression levels of LRG1, TGF- β 1 and ENG becomes up-regulated. LRG1 binds to TGF- β 1, and in the presence of excessive auxiliary receptor ENG, it effectively potentiates the angiogenic switch by favoring the

angiogenic TGF- β 1/ALK1/Smad1/5/8 signaling axis, thus leading to EC activation¹⁶⁴.

Figure 1.7. Schematic of LRG1-Mediated TGF- β Signaling.

Overview of the LRG1-mediated TGF- β signaling cascade in ECs. Under the quiescent state, TGF- β signals via the T β RII-ALK5-Smad2/3 pathway. In response to angiogenic cues, there is an increased expression of LRG1, TGF- β 1 and ENG. With the presence of ENG, LRG1 is able to bind T β RII and ALK1 resulting in a switch of TGF- β 1 signaling towards the pro-angiogenic Smad1/5/8 pathway and EC activation. Figure adapted from Wang, 2013¹⁶⁴.

1.4.4.2. LRG1 and Angiogenesis

The biological function of LRG1 was first brought to light by a microarray study using fragments of pathological retinal microvasculature. LRG1 appears to be most upregulated amongst the 62 differentially expressed proteins and its role on angiogenesis was first speculated¹⁶⁴. Following the initial findings, subsequent independent research also highlighted LRG1's dominant role in mediating angiogenesis^{165,167,202-205}. For instance, LRG1 has been found to exert a neuroprotective role by promoting blood vessel formation in a mouse model of brain ischemia¹⁶⁵. On the same note, LRG1 promote *de novo* bone formation by increasing angiogenesis and mesenchymal stem cells recruiting at the osteoarthritis joints²⁰². Furthermore, in mice model of diabetic nephropathy, increase glomerular EC proliferation is accompanied with a concomitant increase in LRG1 expression²⁰³. In addition, LRG1 plays a crucial role in cancer

aggressiveness, presumably by favouring angiogenesis and epithelial-mesenchymal transition (EMT) during colorectal cancer¹⁶⁷. Additionally, LRG1 has been associated with enhanced hepatocellular carcinoma malignancies with more vascular invasion²⁰⁵. Moreover, LRG1 is also abundantly expressed in human retinoblastoma tumours sections and particularly enriched in the ischemic central tumour blood vessels²⁰⁴.

1.5. Ischaemic heart diseases (IHDs)

Ischaemic heart diseases (IHDs), including myocardial infarction (MI), stroke, and peripheral arterial disease, represent the most common form CVDs²⁰⁶. Till date, the IHDs-associated acute coronary syndrome (ACS) and sudden cardiac death remain as the leading cause of death and disability globally^{207,208}. In developed societies, advancements in IHDs treatment, population growth and increasing aging society collectively constitutes to the steep increase in the non-fatal IHDs, exerting a major healthcare and social-economical burden²⁰⁸. Similarly, in the low- and middle-income countries, a growing epidemic of IHDs is attributable to rapid economic and social transformation leading to increased lifestyle-related risk factors that outpace the development of adequate healthcare resources to manage the conditions^{209,210}.

The family diseases is characterised by restricted blood flow to the myocardium leading to inadequate blood supply to the heart muscle^{211,212}. The restricted blood supply could be caused by luminal thrombosis or constriction of blood vessels, however, the majority of such conditions is related to progressive atherosclerotic blockage in the coronary arteries^{213,214}. In general, atherosclerotic plaques develops from the deposition of cholesterol-rich lipids on the arterial wall and consequent monocyte recruitment, differentiation into macrophages and then maturation into lipid-laden foam cells²¹⁵⁻²¹⁷. These foam cells serve as major source of pro-inflammatory mediators and matrix-degrading proteases that results in the formation of the pro-thrombotic necrotic core, a characteristic feature of unstable plaque underlying atherosclerosis-related ischemias^{207,216-218}.

1.5.1. Cardiac Remodelling

Ischaemic heart diseases (IHDs) impairs normal cardiac functions and triggers a series of molecular, cellular and interstitial changes leading to structural, geometrical and physiological changes of the heart²¹⁹. Specifically, the prolonged pathophysiological stress mediated by elevated cardiac loads triggers a brief period of compensatory remodelling to restore the loss of function²²⁰. Cardiomyocytes re-expression of fetal genes like the cardiac myosin heavy chain (MHC), atrial and brain natriuretic peptides to facilitate adaptive hypertrophic expansion^{221,222}. Moreover, cardiomyocytes also undergo a series of phenotypic alternation involving excitation–contraction coupling, energy metabolism, myofibrillar content during the adaptive hypertrophy remodelling²²¹. Meanwhile, the myocardium secretes angiogenic growth factors, including VEGF, FGF, TGF- β and angiopoietins, which in turn activates coordinated angiogenesis to sustain the increase myocardial mass and performance²²³. In addition, ECs increase production of ECM proteins, pro-fibrotic factors in response to pressure overload to facilitate the adaptive remodelling process²²⁴. Above all, development of significant coronary collateral circulation is also critical to sustain cardiac health and prevent transition into HF²²⁵.

Prolonged elevated cardiac loads facilitate the transition into a pathologic maladaptive cardiac remodelling, leading to ventricular dilation and defective contractile properties. Foremost, the compromised cardiomyocytes undergo cell death^{220,221}. Given that cardiomyocytes are terminally differentiated cells with limited regenerative potential, the irreversible loss of cardiomyocytes results in increased ECM synthesis and accumulation of both interstitial and perivascular fibrotic depositions to provide the structural support in order to preserve residual cardiac function and output^{221,222,226}. This process also relies on the activation of the innate immune response, infiltration of leukocytes, cardiac fibroblasts activation and/or transition of mesenchymal cells into myofibroblasts²²². Notably, the adult epicardial cells may also undergo endothelial-to-mesenchymal transition (EndoMT) to drive this process^{225,227}. Moreover, reduced angiogenesis, vascular rarefaction and endothelial

dysfunction accompanies the maladaptive remodelling process^{221,223}. It has been postulated that NO reduction in the failing hearts impairing endothelial reparative and regenerative potentials by increasing VEGFR1 expression, which competitively binds and sequesters the VEGF bioavailability, which causally downregulates angiogenesis and reduces the cardiac microvessel density²²³. With declined angiogenesis, increased myocardial dysfunction and myocardial fibrosis, prolonged pathologic remodelling progressively transits into terminal heart failure²²⁸.

1.5.1.1. Role of TGF- β in Cardiac Remodelling

Several lines of evidence suggest that TGF- β is a key mediator of the cardiac remodelling process given its pleiotropic and multifunctional actions^{229,230}. Myocardial TGF- β synthesis is markedly and consistently upregulated in heart failures and this induction is associated with cardiac hypertrophy and fibrosis^{231,232}. An important role for TGF- β in regulating cardiac hypertrophic remodelling through promoting TAK1 activation, which leads to brief cardiomyocyte regeneration and compensatory hypertrophic expansion that improves cardiac functions^{229,233,234}. During this process, TGF- β also facilitates fibrotic remodelling by stimulating cardiac fibroblasts activation, synthesis of ECM components including fibrillar collagen, fibronectin and proteoglycans, matrix deposition and preservation to provide enhanced mechanical support^{229,230,235}. Additionally, TGF- β also induces trans-differentiation of fibroblast to contractile myofibroblast by altering gene expression and promoting synthesis of fetal contractile proteins²²⁹. Collectively, the TGF- β facilitates a brief reparative, compensatory improvement in cardiac functions. In response to prolonged stressful stimuli, the balance between ECM synthesis and degradation becomes disrupted, consequently resulting in a net excess of collagen deposition. The degradation of mature collagen fibrils is prevented by tissue inhibitors of metalloproteinases (TIMPs)²³². Excessive cardiac fibrosis results in exaggerated mechanical stiffness, loss of contractility and exacerbated tissue hypoxia²³². For that, TGF- β mediated cardiac hypertrophy and fibrosis remains as a promising target for therapeutic application²³⁶. Late,

but not early, TGF- β blockade has successfully attenuated adverse cardiac remodelling in experimental models of myocardial infarction²³¹.

1.5.1.2. Role of LRG1 in Cardiac Remodelling

Given its apparent role in angiogenesis, LRG1 has been widely implicated in regulating neovascular growth following ischemic events. For example, genetic loss of LRG1 resulted in the suppression of the pro-angiogenic Smad 1/5/8 signalling pathway, thereby resulting in reduced myocardial vessel density following the transverse aortic constriction (TAC)¹⁷⁰. On the same note, in ischemic rat brain following middle cerebral artery occlusion (MCAO), researchers speculated that LRG1 exerts neuroprotective roles by favouring angiogenesis and restoration of blood perfusion following ischemic stroke¹⁶⁵.

In addition to angiogenesis, recent data also revealed that LRG1 exerts a cardio-protective role in cardiac remodelling through regulating cardiac fibrosis^{126,169}. In particular, LRG1 gene ablation resulted in aggravated myocardial fibrosis and exacerbated cardiomyocyte apoptosis following mouse model of MI¹⁷⁰. It has been hypothesized that LRG1-producing myeloid cells facilitates the cardio-protection by reversing the adverse cardiac remodelling events¹⁷⁰. Consistent with this observation, downregulated LRG1 expression has been reported in animal models of pathological cardiac hypertrophy²³⁷. Taken together, it is evident that LRG1 is an important mediator of angiogenesis and cardiovascular health. Given that LRG1 regulates the ubiquitous TGF- β signaling pathway, it emerges as a promising potential therapeutic candidate for mitigating the multiple pathophysiological processes in the entire course of cardiac remodelling.

1.5.2. Conventional Therapeutic Strategies

With the recent advancements in treatment strategy, the morbidity and mortality rates of IHDs patients has declined substantially²³⁸. At present, primary management have focused on lifestyles modification including dietary controls,

increasing physical activities, weight management, cessation of smoking and alcohol intake to target key modifiable risk factors like hyperglycaemia, hypertension and hyperlipidaemia^{239,240}. Concurrently, additional pharmacological treatments including anti-platelet drugs (e.g. aspirins and warfarin), lipid-lowering medications (e.g. statins), blood pressure lowering agents (e.g. beta-blockers, angiotensin-converting enzyme inhibitors, angiotensin receptor blockers and calcium channel blockers), are prescribed to these IHDs patients to more effectively control their cardiometabolic risk status^{241,242}.

Despite the efforts, many patients proceed to develop advanced IHDs that requires surgical revascularization procedures to address the root of the problem. Since the first introduction in 1960s, coronary artery bypass graft (CABG) remained as the mainstream “gold standard” treatment for multi-vessel coronary artery disease²⁴³. CABG involves the engraftment of a vessel upstream of the blockage site to restore blood flow to the myocardium. CABG surgery significantly reduced the IHDs-related mortality rate, incidence of myocardial infarction, and recurrent angina^{244,245}. A notable alternative to CABG is the percutaneous coronary intervention (PCI) procedure²⁴⁶. This method, known as coronary angioplasty, uses a catheter to mechanically relief coronary arteries that are narrowed or blocked by accumulation of atherosclerotic plaque. The clinical efficacies of early coronary angiographic bare metal stents were limited due to neointimal hyperplasia and high in-stent restenosis rates²⁴⁷. In the recent years, PCI using modern drug-eluting stents (DES) that releases anti-proliferative compounds (e.g. Sirolimus and Paclitaxel) to inhibits intimal thickening and restenosis had drastically improved the long-term efficacies of PCI^{247,248}. With that, PCI and CABG surgery continues to be the mainstay treatment options for coronary artery disease. Patients with advanced, non-reparative coronary blockages progressively develops severe left ventricular dysfunction and end-stage heart failure, eventually requiring terminal heart transplantation as last resort treatment²⁴⁹.

1.6. Diabetes Mellitus

Broadly, diabetes mellitus (DM) is a class of metabolic diseases characterised by chronic hyperglycaemia²⁵⁰. Epidemiologically, DM exist as a major public health problem that has reached an epidemic globally^{251,252}. It has been projected that the number of patients with DM is estimated to increase from 382 million in 2013 to 592 million by 2035²⁵³. In addition, the International Diabetes Federation (IDF) *Diabetes Atlas*, highlighted Asia as the new “hotspot” for the diabetes epidemic due to the rapid economic development, urbanization, and lifestyle changes²⁵⁴. Indeed, there is a substantial increase in the prevalence of diabetes in Singapore since 1992 and 8.6 % of Singaporean were diagnosed with diabetes in 2017^{255,256}. In addition to the alarming magnitude of DM, around 50% of patients with diabetes are undiagnosed²⁵⁴ and these patients are likely to have a faster disease progression because of the delay in diagnosis and treatment, leading to the increased risks of diabetes-associated morbidity and mortality²⁵⁷.

The classical stratification strategy recommended by the American Diabetes Association (ADA) remains as the most accepted classification of DM²⁵⁰. According to ADA, the vast majority of DM can be divided into four categories, namely the type I DM (T1DM), type II DM (T2DM), gestational DM (GDM) and others^{250,258}. T1DM is an autoimmune disorder caused by the destruction of insulin-secreting pancreatic beta (β) cells leading absolute deficiency in insulin-production^{250,258,259}. On the other hand, T2DM represent the most prevalent subtype, is characterised by compensatory hyperinsulinaemia due to impaired sensitivity to insulin²⁶⁰⁻²⁶². Insulin resistance is not just a consequence of T2DM, but it also facilitates a vicious cycle of metabolic dysfunctions in the diabetic milieu involving development of obesity, essential hypertension^{263,264}. Pregnant women with maladaptive compensatory insulin secretion to maintain glucose homeostasis could develop GDM²⁶⁵. Unresolved GDM presents as an important risk factor related to the development of T2DM post-partum^{265,267,178}. Last but not least, other rare forms of DM are associated with genetic etiology, diseases of the exocrine pancreas, changes in hormones level and the use of drugs²⁵⁰.

DM is a highly prevalent multifaceted clinical entity, increasingly recognised as a complex disease with heterogeneous pathophysiology, whereby genetic predisposition and environmental exposure contribute to disease development and progression as well as the response to therapeutic interventions^{266,267}. Moreover, a recent large data-driven study had reported that DM family should be further stratified into five distinct clinically reproducible clusters¹⁶. The new sub-stratifications paved new ways for more improved prediction, management and treatment of the DM patient using more effective early therapeutic strategies¹⁶.

1.6.1. Vascular Dysfunction-Associated with Diabetes

Blood vessels are the basic functional unit of the cardiovascular system. Under normal physiology, circulating blood glucose is tightly regulated and stays in the range between 3.6 to 5.8 mmol/L²⁶⁸. Quiescent ECs produce factors that are important for regulating vascular tone, cell adhesion, and the homeostasis of clotting, and fibrinolysis²⁶⁸. However, circulating blood glucose could reach ≥ 7.0 mmol/L following overnight fasting in diabetic patients²⁶⁹. Notably, hyperglycemia majorly perturbs vascular homeostasis and leads to endothelium dysfunctions^{270,271}. As such, patients with DM are frequently associated with dysfunctions in both micro- and micro-vasculature^{272,273}. DM has long been considered as an independent risk factor for cardiovascular events, including myocardial infarction (MI), cardiac arrest, and stroke, which represent the major cause of morbidity and mortality in DM patients^{274,275}. Importantly, the onset of detrimental vascular damages precedes long before the presence of overt hyperglycemia^{276,277}. Recent estimates suggest that DM remains underdiagnosed in 50% of investigations²⁷⁸. Hence, many silent vascular pathologies progress and remain undetected for many years. Moreover, the concept of “hyperglycemic memory” is increasingly recognised as an emerging challenge in DM. Drawing data from large randomized studies, diabetic vascular stresses had been reported to persist after effective normalization of hyperglycemia²⁷⁹.

1.6.1.1. Macrovascular Complications

The umbrella of diabetic macrovascular diseases includes coronary artery disease (CAD), peripheral arterial disease (PAH) and cerebrovascular diseases²⁸⁰. Strikingly, the landmark Framingham study reported that DM patients are considered as risk equivalents, whereby they pose the same risk of developing of future incidence of myocardial infarction (MI) as the patients suffering from a previous episode of MI^{281,282}. In addition to the increased incidence, the consequences of MI are much greater in patients with DM than those without DM²⁷³. Even with surgical revascularization interventions, the incidence of heart failure (HF) remains substantially higher in the population impacted with DM²⁸³. This is because that the diabetic milieu accelerates the development of coronary atherosclerosis, a major risk factor leading to HF^{280,284}. Secondly, DM is also commonly associated with other comorbidities like hypertension, obesity, dyslipidaemia, inflammatory activation and hypercoagulability, which in turn partly contributes to the deterioration of cardiac health^{284,285}. Thirdly, DM is an important risk factor that predispose individuals to develop HF through specific diabetic-linked cardiomyopathies^{284,286}. Although the specific pathological mechanisms remain to be addressed, several hypotheses have been advanced. For example, it was proposed that DM is associated with an increased incidence of intramyocardial microangiopathies related to endothelial dysfunction²⁸⁴. Furthermore, chronic DM perturbs physiological metabolic balance, resulting in progressive insulin resistance and an increased release of free fatty acids (FFA)^{284,285}. The resultant changes in metabolic substrates induce the reduction in myocardial glucose transporter expression and impaired glucose uptake, thus progressively leads to maladaptive myocardial dysfunction^{284,285}. In addition, DM is often reported to be associated with the development of myocardial fibrosis, thus facilitating the progressive intramyocardial and perivascular accumulation of collagen that potentiates diabetic cardiomyopathy^{284,287,288}.

1.6.1.2. Microvascular Complications

Besides its poignant role in atherosclerosis, DM also negatively affects the microvasculature and promote the development of diabetic microvascular complications including diabetic retinopathy, nephropathy and neuropathy²⁸⁵. Diabetic retinopathy presents the leading cause of blindness in working-age population^{289,290}. The characteristic features of DM-associated microvascular injury in the eye involves the thickening of basement membrane shared between ECs and pericytes, pericytes loss, EC hyperpermeability, microaneurysm, and local tissue ischemia which triggers the abnormal blood vessel formation in the eye and ultimately leads to visual impairment and blindness^{273,291}. At present, pharmacological blockades of VEGF and laser photocoagulation remains as the conventional standard of care for managing diabetic retinopathy²⁹².

Nephropathy is the leading cause of chronic kidney disease in DM patients²⁹³. Similar to the diabetic retinopathy, diabetic nephropathy features basement membrane thickening, microaneurysm formation, excessive angiogenesis as well as glomerular hyperfiltration associated with mesangial expansion and tubular and glomerular sclerosis^{273,293}. Effective management of hypertension using angiotensin-converting-enzyme (ACE) inhibitors to block the renin-angiotensin system and/or angiotensin receptor blocker (ARBs) remains the first-line treatment of diabetic nephropathy^{293,294}. Mono- or simultaneous pancreas and/or kidney transplantation serves the last option for patients with end-stage kidney failure²⁹⁴.

Diabetic neuropathies are a heterogeneous group of disorders caused by impaired microcirculation supplying nerves²⁹⁵. It has been projected that 50% of patients with diabetes likely develop diabetic neuropathies during their lifetime²⁹⁵. Diabetic neuropathies are characterized by axonal thickening associated with the eventual the loss of distal median nerve function^{273,296}. During the course of chronic DM, neuropathic dystrophies and ineffective angiogenesis collectively increase susceptibility to infections and injuries, thus

contributing to the development of diabetic foot ulcerations²⁹⁷. While the majority (60–80%) of foot ulcers resolves over time, approximately 10–15% of them will remain as delay non-healing wounds, while another 5–24% of them results in limb amputation between 6–18 months following the initial evaluation²⁹⁸. In DM, the wound healing deficiencies is attributed to a multitude of compound factors including impaired angiogenesis, dysregulated inflammatory responses, defective accumulation of ECM components, down-regulated keratinocyte and fibroblast mobilization²⁹⁹. Unfortunately, to date, there is no effective treatment for diabetic neuropathies and associated diabetic foot ulcers. Symptomatic treatment of pain, offloading vascular management, infection management and prevention, and pressure relief are mainstay therapy^{295,298}.

1.6.2. Pathophysiology Endothelial Dysfunction in DM

Despite of the heterogeneity in the response to diabetic insults, diabetic vascular complications share similar characteristics³⁰⁰. In particular, the diabetic milieu induces EC phenotypic changes resulting in the acquisition of a proliferative, migratory, pro-inflammatory, pro-adhesion, pro-atherogenic, and pro-coagulant properties, which systemically changes the blood flow and enhanced EC permeability²⁶⁸. Furthermore, the mural cells also respond to DM insults by attaining a de-differentiated and hyperproliferative phenotype³⁰¹.

A large body of evidence suggests from both *in vitro* and *in vivo* studies had robustly demonstrate that hyperglycemia is the key determinant of the chronic vascular injuries^{302,303}. Specifically, hyperglycemia promotes retinal pericyte apoptosis and dropout in the microvasculature, which results in later loss of EC in diabetic retinopathy²⁹¹. In normal physiological condition, mature macrovessels, VSMCs maintains a contractile/differentiated phenotype, characterised by expression of contractile markers including smooth muscle myosin, α -smooth muscle actin (α -SMA), h-caldesmon, and calponin³⁰⁴. DM insults drive VSMCs into dedifferentiation by featuring a proliferative

phenotype with increased ECM accumulation, plaque formation and atherosclerosis³⁰⁴.

Mechanistically, it has been postulated that hyperglycemia promotes vascular dysfunction through a several well-recognized complication including the upregulation of the polyol pathway, the *de novo* synthesis of diacylglycerol (DAG) by protein kinase C (PKC) pathway, increased modification of proteins through the hexosamine pathway (HSP) flux and the formation and accumulation of AGEs^{272,305}. Collectively, these inter-related cascades constitutes to reactive oxygen species (ROS) overproduction and redox unbalance, leading to accumulation of oxidative stress and debilitating diabetes-related vascular dysfunction^{306,307}. The key mechanisms implicated in the pathogenic transition will be explained in detail in the following sub-sections.

1.6.2.1. Polyol Activation

Hyperglycemia-induced hyperactivation of glucose metabolism leading to chronic glucotoxicity is considered as the key mechanism of underlying diabetic pathogenesis³⁰⁸. Persistent hyperglycemia facilitates an increase flux of glucose through polyol pathway consumes the nicotinamide adenine dinucleotide phosphate (NADPH) and resulting in NADH/NAD⁺ redox imbalance and accumulation of oxidative stress^{271,305}. On the other hand, accumulation of sorbitol occurs through excessive glucose reduction and contributes to osmotic stress³⁰⁸. The altered biological metabolome also results in overproduction of fructose and accentuate diabetic vascular complications by promoting the formation of potent nonenzymatic glycation precursors, leading to increased protein structural alternations and functional impairments^{271,305}.

1.6.2.2. Protein kinase C Activation

Chronic hyperglycemia results in the accumulation of glycolytic intermediate dihydroxyacetone phosphate, that is subsequently reduced to glycerol-3-phosphate, and facilitates the *de novo* synthesis of a lipid second messenger

DAG^{271,305}. In ECs, DAG predominately regulates activation of different PKC isoforms which drives different downstream signaling transduction pathways that blunt angiogenesis^{271,309,310}. PKC activation is associated with enhanced VEGF expression and increased VEGF-induced vascular permeability^{271,309}. Additionally, PKC activation also suppresses of NO bioavailability, hence favouring endothelium-dependent vasodilator dysfunction³⁰⁹. Furthermore, PKC results in reduced blood flow and impaired vascular tone due to aberrant decrease production of prostacyclin, and induced production of thromboxane, other vasoconstrictors³¹¹. Above all, it has been reported that PKC also stimulates expression of a variety of cytokines that promotes abnormal production and accumulation of ECM³¹². Several lines of loss-of-function analysis also supported this consensus and suggested that inhibitors of PKC signaling can adequately reverse the high glucose-mediated endothelial dysfunction^{313,314}.

1.6.2.3. Hexosamine Pathway Activation

Sustained hyperglycemia results in shutting of excess glucose to a minor branch of the glycolytic pathway, the HSP pathway, which drives the many of the toxic effects of high glucose³¹⁵. Glucose is metabolized into fructose-6-phosphate, then glucosamine-6 phosphate and eventually converts to UDP (uridine diphosphate) β -D-N -acetyl glucosamine (UDP-GlcNAc)³¹⁵. In turn, UDP-GlcNAc facilitates pathologic glycosylation of other cellular protein^{311,316}. For instance, DM-induces the glycosylation of transcription factor Sp1 and contributes to increased gene transcription of TGF- β and PAI-1³¹⁷. Moreover, in the cultured heart ECs, hyperglycemia results in reduce of eNOS activity, in which the hexosamine pathway upsets the PI3-K/Akt signaling cascade³¹⁶. On the other hand, high glucose exposure increases O-GlcNAcylation of nuclear proteins, and this is associated with a reduced sarcoendoplasmic reticulum Ca²⁺-ATPase 2a (SERCA2a) mRNA expression, protein expression, and promoter activity, possibly contributing to diabetic cardiomyocyte malfunction³¹⁸.

1.6.2.4. Advanced glycation end products (AGEs)

Advanced glycation end products (AGEs) also play a dominant role in the onset of vascular complications associated with diabetes³¹⁹. Following the prolonged hyperglycaemia, glucose forms covalent adducts with proteins through a non-enzymatic Maillard reaction³²⁰. The AGEs and their precursors are capable of modifying intracellular, extracellular and circulating proteins, thus altering their expression levels at transcriptional level³²¹, and also modulates the fundamental structural and functional characteristics of these proteins³²¹. Notably, the ECM proteins like collagen and fibronectin have a relatively low turnover rate, therefore, are susceptible to non-enzymatic glycation exerted by AGEs, which resulted in increased ECM stiffness and reduced vascular compliance³²⁰⁻³²².

In addition, receptors of AGE (RAGE) has also been reported to be induced in ECs, VSMCs, and mononuclear phagocytes of the diabetic vasculature³²². The AGE-mediated activation RAGE leads to increased oxidative stress and initiation of inflammation cascade through regulating the activation of MAPK pathway, NF- κ B, IL-6, TNF- α , major contributors of diabetic vascular complications³²⁰. The soluble form of AGEs (sAGEs) also participate in vascular dysfunction by inducing monocyte migration and activation, and the expression of macrophage scavenger receptor (MSR) class A receptors, leading to upregulation of oxidized LDL (OxLDL) uptake and enhanced foam cell formation³²².

Recently, AGE has been reported to mediate the endothelial TGF- β signaling up-regulation and induce endothelial-to-mesenchymal transition (EndoMT)³²³. EndoMT is a complex biological process in which ECs lose their EC-specific cell markers, like Cluster of differentiation 31 (CD 31) and E-cadherin, and subsequently adopt a mesenchymal phenotype by expression mesenchymal cell markers including fibroblast-specific protein-1 (FSP-1), α -SMA, N-cadherin, collagen I and fibronectin^{323,324}. Notably, EndoMT triggers pro-inflammatory responses, which in turn upsets the normal vascular homeostasis by promoting EC dysfunctions³²³ and local tissue fibrosis³²⁴.

1.6.2.5. Enhanced Oxidative Stress

Regardless of the preceding mechanism, DM-associated endothelial dysfunction is associated with enhanced production of reactive oxygen species (ROS), either due to a reduced level of endothelial NO synthase (eNOS) and/or a reduced nitric oxide (NO) bioactivity^{270,325}. Under the physiological state, ECs and mural cells like VSMCs both express NADPH oxidase, which is postulated as the major source of superoxide³⁰⁵. In DM conditions, increased intracellular glucose concentration yields excessive production of superoxide in the mitochondria³⁰⁵. The increased ROS accumulation notoriously drives oxidative stress contributing to DNA and protein breakage, enhanced apoptosis and immune activation contributing to EC function impairment and vascular injuries^{305,326}. DM-associated reduction in luminal NO bioavailability also results in impaired endogenous platelet inhibition and contributes to enhanced platelet activation³²⁵. Activated platelets subsequently induce the secretion of the monocyte chemoattractant protein (MCP)-1 and alter the adhesive properties of the endothelium leading to increased leukocytes activation, recruitment and transmigration, which further promotes the vascular inflammatory reaction and atherogenesis^{270,327}.

1.7. Diabetes-associated IHDs

Diabetes-related IHDs is an important sizable subset of IHDs that is often associated with a more aggressive clinical presentation³²⁸. DM itself is a powerful independent risk factor for cardiovascular events³²⁹. Characteristically, DM patients feature smaller vessel sizes, longer lesion length, greater plaque burden, and higher incidence of mortality and restenosis than their non-diabetic counterparts^{328,330}. The processes leading to the adverse prognosis is multifactorial. By and large the diabetic milieu favours a vascular dysfunction, promotes a pro-inflammatory and pro-thrombotic state that contributes to early onset and accelerated the progression of atherosclerosis²⁷⁰. In particular, diabetes is associated with increased circulating fibrinogen, thrombin, and factor VII, and reduced fibrinolytic and antithrombin III activity, thus

facilitating hypercoagulability³³⁰. In addition, the platelets in diabetic patients are larger in size, more mitogenic and prone to activation³³⁰. Moreover, a substantial number of DM patients develop other metabolic co-morbidities such as dyslipidemia and hypertension which promotes pro-atherogenic activities³⁰⁰. Insulin resistance was reported to induce multiple metabolic alterations and exacerbates widespread atheroma formation by promoting platelet activation, adhesion and hypercoagulability^{331,332}. There is also evidence suggesting that the reversal of hyperglycaemia does not significantly eradicate IHDs burden, which is possibly due to the irreversible changes to macromolecules (e.g., ROS and AGEs accumulation) and epigenetic signatures (DNA methylations and histone modifications) induced by hyperglycaemia that continue to drive the vascular dysfunction in diabetes even after the glucose normalisation^{333,334}.

1.7.1. Challenges to Optimal Revascularization in DM

A sizable amount of patient remains refractory to conventional therapies as the lifestyle interventions, pharmacological management and current surgical revascularization strategies are not universally effective. In particular, the commonly administered surgical revascularization strategies is widely associated with complications including restenosis, stent thrombosis and recurrence of symptoms in a relatively short time, despite of maximal treatment efforts³³⁵. In patients with DM, around 12-22% of patients suffer from incomplete revascularisation and remains symptomatic after the treatment³³⁶. Typically, these patients present diffused coronary disease making conventional treatment not beneficial^{241,336}. Moreover, a substantial portion of individuals presented with CVDs are not suitable for these procedures due to poor overall health status or the presence of debilitating comorbidities^{241,336}. Also, the underlying vascular dysfunction in DM greatly enhanced IHDs progression and future complications^{337,338}. Hence, taken together, effective management of diabetes-related IHDs, especially in conditions that are non-amenable to standard revascularization, still represents a significant medical challenge³³⁹.

1.7.2. Therapeutic Angiogenesis

Angiogenesis is a natural healing process to restore perfusion and function the affected tissue³⁴⁰. However, the ischemia-induced angiogenic response is insufficient or compromised under the diabetic condition³⁴¹. In the search for novel therapeutic options for conditions that are non-amenable to standard revascularization options, many groups had turned their attention to promoting coronary collateral circulation to effectively re-establish microcirculations³⁴². In fact, a well-developed coronary collateral circulation has a favourable impact on improving overall survival rates by limiting ischemic damages and preventing terminal heart failures^{343,344}. Given the considerable interest in the physiological mechanisms that regulate new vessel over the past decade, a multitude of pro-angiogenic agents has been proven to be beneficial in improving diseases outcomes³⁴⁵. Moreover, recent advances in the field led to the identification of key mediators of this multistep process, such as VEGF, FGF-2 and PDGF. Accelerating blood vessel formation by pro-angiogenic factors is considered as an attractive therapeutic option to bypass occluded vessels, re-vascularize ischemic tissues and restore tissue function^{345,346}. Therapeutic angiogenesis approach, in forms of targeted delivery of angiogenic growth factors, gene transfer technology, and site-specific autologous delivery of endothelial progenitor cells, is supported by an impressive body of preclinical evidence^{252,25}.

For many years, VEGF remains the main angiogenic factor that has been targeted and has been shown to improve functional outcomes in patients with ischemic limb and cardiac disease^{96,347-349}. However, extensive investigations demonstrated that VEGF levels are markedly elevated in ischemic tissues of animal and patients with cardiovascular complications, which makes it inappropriate to target VEGF under these circumstances^{350,351}. Furthermore, monotherapies using VEGF or FGF have been reported to cause the formation of leaky or unstable vessels that resembles the chaotic vascular network as occurs in tumours³⁵². As the blood vessel formation is a complex and highly-coordinated process involving extensive interactions between different

cytokines, cells and ECM molecules. Administration of a single pro-angiogenic factor may not be sufficient to induce the formation of functional vasculature³⁵². In addition, there is evidence that VEGF-A has detrimental pro-atherogenic effects by influencing endothelial and immune cell function^{353,354}. Specifically, VEGF-A has been reported to promote EC adhesion molecules expression, namely VCAM-1 and PECAM-1³⁵⁴, and drives the monocyte activation and migration³⁵⁵, thus contributing to plaque expansion. The fact that VEGF is a potent vasodilator also causes concern as it may induce oedema in ischemic tissues by increasing vascular permeability³⁵⁶.

1.8. Scope of Problem

Despite the strong evidence obtained in preclinical studies, the translation of therapeutic angiogenesis into clinical trials has encountered many challenges. After almost twenty years of clinical evaluation with over 2,500 patients being recruited, none of the tested pro-angiogenic factors fulfilled the promise of clinical benefits^{345,352}. The factors that influence the outcomes of the clinical studies include the choice of angiogenic factor, the pharmacokinetics of tested drugs, and the pathophysiological differences between experimental animal models and human patients³⁵⁶⁻³⁵⁸. The existence of co-morbidity complicates the situation even further especially with endothelial dysfunction secondary to the diseases such as diabetes²¹⁴. For these reasons, novel therapeutic targets with alternative or complementary mechanisms are constantly being sought to minimize resistance to current treatments.

1.9. Proposed Project

LRG1, a novel angiogenic factor, has been shown to regulate vessel formation through endothelial TGF- β signaling pathway in aberrant choroidal neovascularization. Mechanistically, LRG1 favours a context-dependent ALK1-Smad1/5/8 downstream cascade, leading to transcriptional activation of pro-angiogenic responses including activation of EC proliferation, tube formation and *ex vivo* vessel outgrowth. Given that, we hypothesize that LRG1

could be a new therapeutic angiogenic candidate to promote functional cardiac reperfusion, restore cardiac function and limit progression to terminal heart failure. As co-morbidities often affects treatment outcome, we will study the angiogenic effects of LRG1 in physiology and under diabetic conditions. Whilst the biological regulation of LRG1 remains poorly understood, the information extracted from this study would majorly improve our understanding of the role of LRG1 in fine-tuning of cardiac-specific angiogenesis in an attempt to development more effective treatment strategies to mitigate against the ever-growing IHDs burden.

1.10. Specific Aims

To test the proposed hypothesis, we will:

- Investigate the functional role of LRG1 in cardiac-specific ECs
- Resolve the molecular and signaling mechanism leading to LRG1's function
- Study the influence of diabetic insults (hyperglycemia and AGEs) on LRG1-regulated angiogenesis
- Evaluate the potential of LRG1 as the target of therapeutic angiogenesis in diabetic mice with induced-HF

Chapter 2: Materials and Methods

2.1. *In Vitro* Assays

2.1.1. Cell Culture

All culture plates and flask for the ECs were pre-coated with a Collagen-I substrate to facilitate ECs adhesion. Culture plates and flasks were first incubated with the specified ECM coating solution for 16 hours at 37°C. following the overnight coating, the spent coating solution was discarded, and the culture vessels were rinsed with twice with 1X Phosphate Buffered Saline (PBS). All of the culture vessels were air-dried for 30 minutes prior using. (Refer to **Table 2.1.** for the components of the ECM coating solution).

Table 2.1. List of the Components in ECM Coating Solution.

Name	Composition	Source, Reference
Collagen-I Solution	<ul style="list-style-type: none">• 0.06µg/µL Rat Tail Collagen-I• 0.6% Glacial acetic acid• Hanks' Balanced Salt Solution (HBSS)	<ul style="list-style-type: none">• Corning, #354236; USA• Merck, #100063; Germany• Life Technologies, #24020-117; USA

Commercially available microvascular human cardiac ECs (referred to as HHECSs) (ScienCell, #6000; USA) were seeded onto the pre-coated culture flasks maintained in a specialized EC basal growth medium-2 (EBM-2, B3156; Lonza, USA) supplemented with microvascular EC growth supplements (Lonza, CC-3202; USA) and 1% Penicillin/Streptomycin (Nacalai Tesque, #09367-34; Japan) to make up the microvascular EC growth medium (EGM-2-MV). HHECSs were cultured in accordance to manufacturer's guide at 37°C, in a humidified incubator containing 5% CO₂ until reaching 90% confluence.

To sub-passages the cells, the spent medium was discarded, ECs were washed twice with 1X PBS, before adding a pre-warmed 0.025% Trypsin-Ethylenediaminetetraacetic acid (Trypsin-EDTA) solution (Lonza, CC-5012;

USA) and incubated for 5 minutes at 37°C. The trypsinization reaction was stopped by addition of 10mL EGM–2–MV. The resultant cell suspension would be pelleted by centrifugation at 1300 rpm for 5 minutes. The cell density was determined using standard Trypan Blue Exclusion (Gibco, 15250061; USA) method before being re-plated onto the pre-coated cell culture vessels. For prolonged culture, the culture medium would be routinely replaced every other day unless specified otherwise.

2.1.2. Cell Transfection

HHECSs were sub-cultured on 6–well culture dishes (Thermo Fischer Scientific, #140675; USA) to 70% confluence before transfections. Upon reaching the pre-defined cell density, the culture medium was aspirated, cells were washed twice with 1X PBS and maintained in 2mL of Opti–MEM® I Reduced Serum Medium (Thermo Fisher Scientific, #31985088; USA). Next, 1.5µL of Lipofectamine® 2000 (Thermo Fischer Scientific, #52887; USA) transfection reagent was mixed into 150µL of Opti–MEM® I Reduced Serum Medium in one tube while 1.5µg of plasmid DNA or 10nM small interfering RNA (siRNA) (*refer to Table 2.2. for the specification of plasmid DNA and siRNA used*) was diluted in 150µL of Opti–MEM in another tube. The contents in both tubes were mixed, incubated for 10 minutes to allow liposome–DNA generation, dropwise added into the prepared cells and left to transfect for 6 hours. At the end of the transfection, the culture medium was aspirated, cells were washed twice with 1X PBS, supplemented with another 2mL of fresh EGM–2–MV and maintained in culture for another 24 to 48 hours.

Table 2.2. List of Plasmids DNA and siRNA

Name	Genebank Accession ID	Specification	Source, Reference
Gene Overexpression Plasmids			
pcDNA3.1	–	• pcDNA3.1(+) vector	• Invitrogen, V79020; UK

pLRG1–His	Human LRG1 (NM_052972.2)	<ul style="list-style-type: none"> • Kozak sequence at 5' end • 6× His tag at 3' end • pcDNA3.1(+) vector • HindIII/XhoI cut sites 	<ul style="list-style-type: none"> • In-house preparation
pVEGFR2 _{ECD} –V5	Human VEGFR2 (NM_002253.2)	<ul style="list-style-type: none"> • VEGFR2 signaling peptide + ECD (303 to 2594 bp) • Kozak sequence at 5' end • V5 tag at 3' end • pcDNA3.1(+) vector • BamHI/EcoRV cut sites 	<ul style="list-style-type: none"> • Sino Biological Inc., Custom design, China
siRNA			
siControl	–	<ul style="list-style-type: none"> • ON–TARGETplus Non–targeting Pool 	<ul style="list-style-type: none"> • Dharmacon Inc, D–001810–10–05; USA
siLRG1	Human LRG1 (NM_052972.2)	<ul style="list-style-type: none"> • ON–TARGETplus LRG1 siRNA 	<ul style="list-style-type: none"> • Dharmacon Inc, L–015179–01–0005; USA
siSmad4	Human Smad4 (NM_005359.5)	<ul style="list-style-type: none"> • ON–TARGETplus SMAD4 siRNA 	<ul style="list-style-type: none"> • Dharmacon Inc, L–003902–00–0005; USA

2.1.3. Generation of Recombinant Human LRG1 Protein

The coding sequence of full-length human LRG1 (NM_052972) carrying a Kozak consensus sequence at the 5' and a 6X His tag at the 3' end was cloned into pcDNA3.1 (+) (Invitrogen, V79020; UK) mammalian expression vector at the HindIII/XhoI cleavage site to form the pCDNA3.1–LRG1–His. To transfect the plasmid into FreeStyle™ HEK 293F cell line (Life Technologies, R79007; UK), the cell density was adjusted to $1.2 - 1.5 \times 10^6$ cells/mL in 30mL of FreeStyle™ 293 Expression Medium (Life Technologies, #12338018; UK). Liposome–DNA mixture was prepared by adding 37.5μL of Lipofectamine® 2000 to 150μL of Opti–MEM® in one tube and 37.5μg of plasmid DNA to in 150μL Opti–MEM® in another tube. The two mixture was combined and left for 20 minutes to allow the formation of the liposome–DNA complexes. The mixture was added dropwise into FreeStyle™ HEK 293F cell suspension and incubated for 48 hours on an orbital shaker (130rpm) in a humidified incubator

which was maintained at 37°C, 8% CO₂ condition. Given that the LRG1 plasmid vector carries the Geneticin® (G418) selectable marker, generation of stable LRG1–overexpressing FreeStyle™ cell line after the initial transient transfection was initiated with 1000µg/mL G418 (Thermo Fischer Scientific, #10131027; USA), and cells were then maintained in low concentration (500µg/mL) of G418 in the following cultures. The cells were inspected every 48 hours, where the LRG1-containing conditioned media were collected and cells were sub-passaged to a density of 1.2 – 1.5 x 10⁶ cells/mL.

The conditioned medium was pooled and concentrated using Amicon® Plus centrifugal filter device (Merck, UFC903096; Germany). Ni–Sepharose 6 Fast Flow beads (GE Healthcare, #10264827; UK) was added to the concentrated LRG1-His containing conditioned medium, incubated overnight at 4°C and subsequently purified in accordance to the manufacturer’s guide. Following the overnight binding, the Ni–Sepharose beads were pelleted by centrifugation at 300g, washed twice in 1X binding buffer (*refer to **Table 2.3.** for the components of the buffer required for LRG1 production*). The LRG1-bounded beads were resuspended in 10mL of 1X elution buffer, centrifuged at 300g, and the LRG1-containing eluent was collected while leaving the Ni–Sepharose beads behind for another cycle of elution. The elution step will be repeated up to seven times for maximum recovery. Finally, the LRG1-His eluent was dialyzed in 2L of sterile 1X PBS through SnakeSkin® Dialysis Tubing (Thermo Fischer Scientific, #68100, USA) and the concentration of the resultant purified LRG1 protein was determined by Bradford protein quantification assay (Bio–Rad, #500–0006; USA).

Table 2.3. List of the Buffers Required for LRG1 Production

Name	Composition	Source, Reference
Recombinant Protein Purification Buffers		
200mM Sodium Phosphate Buffer (pH 7.4, 10X stock)	<ul style="list-style-type: none"> • 5.93g Sodium mono–phosphate (NaH₂PO₄) • 23.0g Sodium diphosphate (Na₂HPO₄) 	<ul style="list-style-type: none"> • Sigma–Aldrich, #3139; USA • Sigma–Aldrich, #71649; USA

	<ul style="list-style-type: none"> • 1L DDH₂O 	<ul style="list-style-type: none"> • Sartorius, Arium® Type 3, USA
Binding Buffer (pH 7.4)	<ul style="list-style-type: none"> • 20mM Sodium Phosphate Buffer ((pH 7.4) • 0.5M Sodium Chloride (NaCl) • 20mM Imidazole • DDH₂O 	<ul style="list-style-type: none"> • In-house preparation • Sigma–Aldrich, S9888; USA • Sigma–Aldrich, I5513; USA • Sartorius, Arium® Type 3, USA
Elution Buffer (pH 7.4)	<ul style="list-style-type: none"> • 20mM Sodium Phosphate Buffer ((pH 7.4) • 0.5 M Sodium Chloride (NaCl) • 500mM Imidazole • DDH₂O 	<ul style="list-style-type: none"> • In-house preparation • Sigma–Aldrich, S9888; USA • Sigma–Aldrich, I5513; USA • Sartorius, Arium® Type 3, USA
Dialysis Buffer		
1X PBS	<ul style="list-style-type: none"> • Dilute 10X PBS stock to 1X • DDH₂O 	<ul style="list-style-type: none"> • 1st Base, BUF–2040; Singapore • Sartorius, Arium® Type 3, USA

2.1.4. Diabetic Insults

2.1.4.1. High Glucose

To study the influence of continuous hyperglycemia on HHECSs, we subjected the cells to 22mM D–glucose (Sigma–Aldrich, A2494001; USA). This dose is widely used in endothelial functional studies and continuous 22mM high glucose stimulation had resulted in prominent detrimental changes in endothelial functions³⁵⁹⁻³⁶¹. The endothelial culture medium containing 5.6mM D–glucose serve as low glucose (LG) control. EGM–2–MV containing 22mM L–glucose (Sigma–Aldrich, G5500; USA), the metabolically inert stereoisomer of D–glucose, was used as an osmotic control (OC) to eliminate any influence due to changes in medium osmolarity.

2.1.4.2. Glycated Collagen

In DM, glucose-mediated non-enzymatic glycation of collagen, the most abundant ECM component, results in the formation of intermolecular cross-links of the collagen fibre, which causes changes in collagen solubility, charge, polymerization, and intermolecular interactions, and ultimately contributes to EC dysfunction^{321,362}. Methylglyoxal (MGO), a naturally-occurring glucose metabolite that functions as a potent precursor for AGE in our body^{320,363}, was used to chemically induce the formation of glycated Collagen I *in vitro*³⁶⁴. Specifically, 3mg/mL of Collagen I (Corning, #354236; USA) was incubated with 257mM of 40% aqueous solution of MGO (Sigma-Aldrich, M0252; USA) in 100mM Sodium Phosphate Buffer, pH 7.4 at room temperature for 0 hours or 3 hours to generate 0HgCol (process control) or 3HgCol (glycated collagen) respectively. The crude glycated samples were kept on ice immediately and dialyzed against 0.02M Glacial Acetic Acid (Merck, #100063; Germany) at 4°C to stop the glycation reaction and remove excess MGO. Successful glycation was confirmed by collagen band-shift in SDS-PAGE gel stained with Coomassie Blue (Bio-Rad, #161-0786; USA) and immunoblotting using mouse monoclonal antibody specific for MGO (Biologo, #AGE06B; Germany). (Refer to **Table 2.4.** for the list of components of the buffer used for the glycated collagen production.)

Table 2.4. List of the Buffers Required for gCol Production

Name	Composition	Source, Reference
Glycation Buffers		
100mM Sodium Phosphate Buffer (pH 7.4, 1L)	<ul style="list-style-type: none"> • 2.97 g Sodium mono-phosphate (NaH₂PO₄) • 11.5g Sodium di-phosphate (Na₂HPO₄) • 1L DDH₂O 	<ul style="list-style-type: none"> • Sigma-Aldrich, #3139; USA • Sigma-Aldrich, #71649; USA • Sartorius, Arium® Type 3, USA
Dialysis Buffer	<ul style="list-style-type: none"> • 0.02M Glacial acetic acid • DDH₂O 	<ul style="list-style-type: none"> • Merck, #100063; Germany • Sartorius, Arium® Type 3, USA

2.1.5. Endothelial Functional Assays

2.1.5.1. Cell Viability

EC viability was determined using tetrazolium compound reduction and measurement of colorimetric formazan product formation. Briefly, 1.5×10^3 HHECSs was resuspended in EBM-2 basal medium supplemented with 2% FBS (Thermo Fischer Scientific, #10082147; USA) and co-treated with additional angiogenic growth factors or glycemic insults (*refer to Supplementary Table 1. for the specification of growth factor treatments*). The cells were left to attach for 4 hours before the addition of 10 μ L of CellTiter[®] 96 AQueous One Solution reagent (Promega, G3582; USA). Finally, cell viability was determined after 3 hours by spectrophotometric measurement (Biotek, Synergy H1; USA) at the 490nm. Each of the treatment was performed in triplicates and the absorbance readings were first normalized against the blank media controls.

2.1.5.2. Cell Proliferation

EC proliferation was quantified using Ki-67 immunofluorescence (IF) staining, a marker that is constitutively expressed in the nucleus during all stages of active cell cycle except for the G₀ quiescent phase³⁶⁵. In particular, 4×10^3 cells was seeded in each well of a 48-well plate (Corning, #150687; USA) and maintained in EGM-2-MV for the first 24 hours. Subsequently, the spent medium was removed, cells were washed and starved for 16 hours in EBM-2 basal medium supplemented with 0.2% FBS (Thermo Fischer Scientific, #10082147; USA). Following the starvation, cells were subjected to additional angiogenic growth factors or glycemic treatments in 2% FBS supplemented EBM-2 for another 48 hours (*refer to Supplementary Table 1. for the specification of growth factor treatments*).

At the endpoint, cells were washed and fixed in 4% paraformaldehyde (PFA) (Sigma–Aldrich, #158127; USA) for 10 minutes at room temperature. Cells were washed and subsequently blocked in blocking buffer containing 0.5% bovine serum albumin (BSA) for 1 hour. Next, the cells were then incubated in blocking buffer containing 1:500 dilution of rabbit polyclonal anti–Ki–67 antibody (Abcam, Ab15580; UK) at 4°C, overnight. The next day, the cell were washed twice and incubated with blocking buffer containing 1:1000 dilution of Alexa Fluo® 594 conjugated goat anti–rabbit IgG secondary antibody (Thermo Fischer Scientific, A11008; USA) and Hoechst 33258 (Sigma–Aldrich, #H6024; USA) for another hour at room temperature. Finally, the cells were washed thoroughly in 1X PBS before being visualized under the fluorescence inverted microscope (Nikon, Eclipse Ti–E; USA).

2.1.5.3. Cell Migration

The 8.0–µm pore polycarbonate Transwell® (Corning, #3422; USA) were used to assess cell migration. The inserts were pre–coated with the 200µl and 500µl of collagen coating solution on the upper and lower chamber respectively for 16 hours at 37°C. Subsequently, the coating solution was removed and the Transwell® inserts were washed in thoroughly in 1X PBS and fully air–dried before use. 3.0×10^4 HHECSs was resuspended in 200µl of EBM2 supplemented with 0.2% FBS and added to the upper chambers and left to settle for 10 minutes. Next, 600µl of EBM2 containing 2% FBS was added to the lower chambers. When specified, equivalent concentrations of desired chemo–attractant was added to the both the upper and lower chambers (*refer to Supplementary Table 1. for the specification of growth factor treatments*). The cells were allowed to migrate for 5 hours at 37°C. At endpoint, the trans–migrated cells were fixed in 4% PFA for 10 minutes and counterstained with Hoechst 33258 (Sigma–Aldrich, #H6024; USA). Non–migrated cells left in the upper chamber were removed using a cotton–tipped applicator and thoroughly rinsed off with 1X PBS. Finally, the cells were visualized under the fluorescence inverted microscope (Nikon, Eclipse Ti–E; USA).

2.1.5.4. Tube Formation

Growth factor reduced Matrigel[®] basement membrane matrix (Corning, #354230; USA) has been routinely employed to demonstrate the angiogenic activity of vascular EC *in vitro*³⁶⁶. To perform the tube formation assay, Matrigel[®] was thawed overnight on ice. 80µL of the freshly thawed Matrigel[®] was added to each well of a pre-cooled 96-well plate and incubated at 37°C for at least 30 minutes to allow Matrigel polymerisation. At the same time, HHECS were trypsinized and resuspended in EBM2 supplemented with 0.2% FBS to achieve the density of 1.2×10^5 cells/mL. At this point, additional angiogenic growth factors or glycemic treatments was added to the cell suspension as specified (*refer to **Supplementary Table 1** for the specification of growth factor treatments*). 100µL of HHECS suspension was carefully seeded on top of the polymerized Matrigel[®] and incubated at 37°C for 6 hours in a humidified cell culture incubator. At the conclusion of this assay, HHECS tubules were imaged using the phase contrast mode of an inverted microscope (Nikon, Eclipse Ti-E; USA) and analysed by Image J Angiogenesis Analyzer plugin (National Institutes of Health, USA).

2.1.5.5. Cell Adhesion

Non-enzymatic glycation of collagen has been reported to interfere with cell adhesion³⁶². To evaluate EC adhesion properties, 24-well tissue culture plates (Corning, #150687; USA) were pre-coated with 0.06µg/µL native or glycated Collagen solution at 37°C in a 5% CO₂ humidified incubator for 16 hours. The plates were briefly washed twice with PBS and pre-blocked with EBM2 medium containing 10% BSA (Sigma-Aldrich, A2153; USA) to prevent non-specific binding for 2 hours at RT. Following that, the blocking solution was aspirated, and the plates were rinsed twice with PBS and air-dried before use.

2.0×10^4 HHECSs in 300µl of EBM2 medium was added to each well of the 24-well plate. After 90 minutes incubation, the non-adherent cells were washed away while the attached cells were fixed in PBS containing 10% Acetic Acid

and 10% Methanol for 15 minutes. Fixed cells were rinsed in PBS twice and stained with 5mg/ml Crystal Violet (Sigma–Aldrich, C0775; USA) solution containing 2% Ethanol (Merck, #107017; Germany) for 10 minutes at RT. The plate was then submerged into a large tank of water and rinsed thoroughly before being air–dried completely. Finally, the Crystal Violet stain were allowed to dissolve in 100ul of Acetic Acid for 15 minutes and quantified by measuring absorbance at 550nm using a spectrophotometer (Biotek, Synergy H1; USA). The experiment was repeated in duplicates and the average readout was normalized to the readings of that in non–glycated collagen control.

2.1.6. Western Blotting

2.1.6.1. HHECS treatments

Typically, the ECs were seeded onto pre-coated 6–well culture dishes (Thermo Fischer Scientific, #140675; USA) and cultured in the standard EGM–2–MV culture medium till reaching 70% confluency. When specified, cells allocated for transfection experiments would be appropriately handled as *specified (refer to Section 2.2. for detailed EC transfection protocol)*. Following the transfection, the culture medium will be removed, cells will be washed thoroughly and recovered in 2mL of EGM–2–MV for 16 hours.

For cytokines treatments, both the pre-transfected and the non-transfected cells would be first starved overnight for 16 hours in EBM-2 supplemented with 0.2% FBS. Subsequently, the cells would be treated with the appropriate growth factors 30 minutes. Some experiment plans also includes inhibitors treatment and the appropriate inhibitors will be added to the culture for 30 minutes prior to growth factors treatment (*refer to **Supplementary Table 1.** for a detailed list of growth factor and inhibitors used in this project*).

2.1.6.2. Sample Preparation

Total protein was extracted from the HHECS using 20µL of ice-cold RIPA lysis buffer containing protease and phosphatase inhibitors cocktail and the adherent cells were removed using a cell scraper (Biolab, #90020; Singapore). The crude protein lysates were snap-frozen and rapidly thawed to permeabilize the cell membrane and release intracellular proteins. This step was repeated twice before the mixture was pelleted by centrifugation at 1.3×10^4 g for 10 minutes. The resultant cell supernatant was quantified by Bradford assay (Bio-Rad, #500-0006; USA). In particular, 200µL of Bradford solution was diluted in 800µL of deionized distilled water (ddH₂O). Thereafter, 1µL of protein sample was resuspended into the mixture and incubated for 5 minutes before transferring into a cuvette for absorbance reading (Biotek, Synergy H1; USA) at 595nm. Protein standards were prepared using known serial dilutions of BSA (Sigma-Aldrich, A2153; USA) dissolved in the RIPA lysis buffer containing protease and phosphatase inhibitors cocktail. The final total protein yielded was estimated by relative comparison to a known BSA standard. (*Refer to **Table 2.5.** for the list of components in the buffers used for western blotting*)

Table 2.5. List of the Buffers Used for Western Blotting

Name	Composition	Source, Reference
RIPA Cell Lysis Buffer (pH 8)	<ul style="list-style-type: none"> • 20mM Tris-base • 420mM NaCl • 0.1mM EDTA • 10% Glycerol • 0.5% NP-40 • DDH₂O 	<ul style="list-style-type: none"> • 1st Base, BIO-1401; Singapore • Sigma-Aldrich, S9888; USA • Sigma-Aldrich, E9884; USA • Sigma-Aldrich, #49781; USA • Sigma-Aldrich, I8896; USA • Sartorius, Arium® Type 3, USA
Inhibitor cocktail (*Freshly added into RIPA buffer just before use)	<ul style="list-style-type: none"> • 1X Protease inhibitor* • 1X Phosphatase inhibitor* • 0.2M Dithiothreitol (DTT)* • 0.1M Phenyl-methylsulfonyl fluoride (PMSF)* 	<ul style="list-style-type: none"> • Nacalai Tesque, #03696-34; Japan • Nacalai Tesque, #07575-51; Japan • Sigma-Aldrich, DTT-RO; USA • Thermo Fisher Scientific, #36978; USA
4X Laemmli Buffer (pH 6.8)	<ul style="list-style-type: none"> • 8% SDS • 0.25 M Tris base • 20% 2-Mercaptoethanol 	<ul style="list-style-type: none"> • 1st Base, BUF-2052; Singapore • 1st Base, BIO-1401; Singapore • Sigma-Aldrich, M6250; USA

	<ul style="list-style-type: none"> • 40% Glycerol • 0.008% Bromophenol blue • DDH₂O 	<ul style="list-style-type: none"> • Sigma–Aldrich, #49781; USA • Sigma–Aldrich, #130915; USA • Sartorius, Arium® Type 3, USA
Running Buffer (pH 6.8)	<ul style="list-style-type: none"> • 25mM Trise base • 190mM Glycine • 0.1% SDS • DDH₂O 	<ul style="list-style-type: none"> • 1st Base, BIO–1401; Singapore • 1st Base, BIO–2080; Singapore • 1st Base, BUF–2052; Singapore • Sartorius, Arium® Type 3, USA
Transfer Buffer (pH 6.8)	<ul style="list-style-type: none"> • 25mM Trise base • 190mM Glycine • 20% Methanol • DDH₂O 	<ul style="list-style-type: none"> • 1st Base, BIO–1401; Singapore • 1st Base, BIO–2080; Singapore • Merck, #106035; Germany • Sartorius, Arium® Type 3, USA
1X Tris–Base Buffered Saline with Tween (TBST)	<ul style="list-style-type: none"> • 12.5mM Trise base • 137mM NaCL • 0.01% Tween–20 • DDH₂O 	<ul style="list-style-type: none"> • 1st Base, BIO–1401; Singapore • 1st Base, BIO–2080; Singapore • Promega, H5151; USA • Sartorius, Arium® Type 3, USA

2.1.6.3. Polyacrylamide Gel Electrophoresis

20–50 µg of each of the cell lysates was mixed with 4X Laemmli buffer and denatured by boiling at 100°C for 5 minutes. The resultant protein samples and a molecular weight reference (Thermo Fisher Scientific, #26616 or #26619; USA) were loaded and resolved by the 8%, 10% or 15% SDS–PAGE mini gels (Invitrogen, HC1000SR; USA) depending on the molecular weight of the target protein. A standard gel electrophoresis program was used for all western blots whereby all samples were first stacked at 80 volts for 30 minutes and subsequently resolved at 120 volts for another 90 minutes (*Refer to **Table 2.5.** for the list of components in the buffers used for western blotting*).

2.1.6.4. Wet Transfer

Following the electrophoresis, the resolved proteins were transferred onto an Immobilon–P[®] PVDF membrane (Merck, ISEQ00005; USA) at 80 volts for 1 hour using Mini Trans–Blot[®] Electrophoretic Wet Transfer System (Bio–Rad,

#1703930; USA). The resultant PVDF blots were then blocked in Tris–Buffered Saline containing 0.01% Tween–20 (TBST) containing 5% skim milk for 1 hour before being incubated with respective primary antibodies at 4°C overnight. Thereafter, the membranes were washed in 15mL of TBST three times and each for 15 minutes to remove any unbound primary antibody. Next, the protein blots were incubated in blocking buffer containing horseradish peroxidase–conjugated secondary antibodies. After the incubation, the blots were washed at least three times in 15mL TBST and each for 15 minutes. (*Refer to **Table 2.5.** for the list of components in the buffers used for western blotting*). (*Refer to **Table 2.6.** for the list of components in the buffers used for western blotting*).

Table 2.6. List of Antibodies Used in Western Blotting

Name	Dilution	Company
Primary antibodies (diluted in 5% BSA TBST)		
MG–AGE mouse monoclonal antibody	1:1000	Biologo, #AGE06B; Germany
6X His tag® mouse monoclonal antibody	1:1000	Abcam, Ab18184; UK
Cyclin D1 rabbit monoclonal antibody	1:1000	Cell Signaling Technology, #2978; USA
PARP rabbit monoclonal antibody	1:1000	Cell Signaling Technology, #9532; USA
Phospho–Smad1/5 rabbit monoclonal antibody	1:1000	Cell Signaling Technology, #9516S; USA
Smad 1/5 mouse monoclonal antibody	1:1000	Abcam, Ab75273; UK
Phospho–Smad2/3 rabbit monoclonal antibody,	1:2000	Cell Signaling Technology, #8828S; USA
Smad2/3 rabbit monoclonal antibody	1:2000	Cell Signaling Technology, #3102S; USA
VEGF rabbit polyclonal antibody	1:2000	Abcam, Ab46254; UK
Phospho–VEGF Receptor 2 rabbit monoclonal antibody	1:1000	Cell Signaling Technology, #19A10; USA
VEGF Receptor 2 rabbit monoclonal antibody	1:1000	Cell Signaling Technology, #9698; USA
GAPDH Rabbit Polyclonal Antibody	1:5000	Santa Cruz Biotechnology, #sc–25778; USA

Secondary antibodies (diluted in 5% Skim milk TBST)		
Goat anti-rabbit IgG HRP conjugated secondary antibody (Heavy + Light Chain)	1:5000	Bethyl Laboratories, A120-101P; USA
Goat anti-mouse IgG HRP conjugated secondary antibody (Heavy + Light Chain)	1:5000	Bethyl Laboratories, A90-116P; USA

2.1.6.5. Protein Detection

The protein of interest was detected by chemiluminescent substrates, including Clarity[™] ECL Substrate (Bio-Rad, #1705061; USA) or the SuperSignal[™] Femto Maximum Sensitivity Substrate (Thermo Fischer Scientific, #37075; USA). When needed, the blots were stripped using Restore[™] PLUS Stripping buffer (Thermo Fischer Scientific, 46430; USA) for 10 minutes and re-blocked and re-probed with another primary antibody. (*Refer to **Table 2.6.** for the list of components in the buffers used for western blotting*). All of the western blot data was convert into densitometry using ImageJ and normalized to loading control.

2.1.7. Molecular Profiling

2.1.7.1. RNA Extraction and cDNA Conversion

Total RNA was extracted from the treated cells using RNeasy[®] RT (Molecular Research Center Inc., RN 190; USA) according to manufacturer's instruction. Thereafter, cellular RNA was precipitated using 100% Isopropanol (Thermo Fischer Scientific, BP2618500; USA) and the resultant RNA concentrations were determined NanoDrop[™] spectrophotometer (Thermo Fischer Scientific, Nanodrop 2000C; USA). 1µg of RNA was reverse transcribed into cDNA using qScript[™] cDNA Supermix (Quanta Biosciences, #101414-102; USA) in a standard thermal cycler machine (BioRad, C1000; USA).

2.1.7.2. Polymerase Chain Reaction (PCR)

To carry out qualifying real-time (RT)–PCR (qRT–PCR), a master mix was prepared for every target gene and each sample was analysed in triplicates. (Refer to **Table 2.7.** for the list of components in the aRT-PCR mix). qRT–PCR was performed in the Applied Biosystems™ Real–Time PCR System (Thermo Fisher Scientific, QuantStudio™ 6; USA) using the comparative Ct protocol. The Ct value denotes the number of amplification cycles to reach the threshold fluorescence that is determined automatically by the software of the instrument³⁶⁷. Results were presented as fold change and calculated using the formula $2^{-(\Delta\Delta Ct)}$, where $\Delta\Delta Ct = \Delta Ct (\text{Test gene}) - \Delta Ct (\text{GAPDH})$, and $\Delta Ct = Ct (\text{Target gene}) - Ct (\text{Reference gene})$. (Refer to **Table 2.8.** for the list of primers for aRT-PCR).

Table 2.7. List of the Components in qRT–PCR Mix

Name	Volume	Source
All targets (except for TGF-β)		
Forward Primer	0.8μl	Integrated DNA Technologies, Inc, Custom Design; Singapore
Reverse Primer	0.8μl	Integrated DNA Technologies, Inc, Custom Design; Singapore
SYBR® Green Supermix	10μl	Bio–Rad, #1725122; USA
Template cDNA	1μl	In–house prepared
Nuclease–free Water	7.2μl	Thermo Fisher Scientific, #10977015; USA
TGF-β		
Forward Primer	1.2μl	Integrated DNA Technologies, Inc, Custom Design; Singapore
Reverse Primer	3.6μl	Integrated DNA Technologies, Inc, Custom Design; Singapore
SYBR® Green Supermix	10μl	Bio–Rad, #1725122; USA
Template cDNA	1μl	In–house prepared

Nuclease-free Water	4.2µl	Thermo Fisher Scientific, #10977015; USA
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Table 2.8. List of the Human Primers for qRT-PCR

Name	Target (Accession number)	Sequence of primer (5' to 3')
Human-specific Primers		
h-GAPDH	NM_002046 Homo sapiens glyceraldehyde-3-phosphate dehydrogenase (GAPDH), mRNA	For: GGTCTCCTCTGACTTCAACA Rev: AGCCAAATTCGTTGTCATAC
h-LRG1	NM_052972 Homo sapiens leucine-rich α -2-glycoprotein 1 (LRG1), mRNA	For: GTTGGAGACCTTGCCACCT Rev: GCTTGTTGCCGTTTCAGGA
h-TGF β	NM_000660 Homo sapiens transforming growth factor beta 1 (TGF β 1), mRNA	For: CACGTGGAGCTGTACCAGAA Rev: GAACCCGTTGATGTCCACTT
h-Smad 4	NM_005359 Homo sapiens SMAD family member 4 (SMAD), mRNA	For: CAGGATCAGTAGGTGGAATAGC Rev: TCTTTCATGCTCTGTCTTGGG
h-VEGFA	NM_001025366 Homo sapiens vascular endothelial growth factor A (VEGFA), mRNA	For: AATGTGAATGCAGACCAAAG Rev: GACTTATACCGGGATTTCTTG
h-VEGFR2	NM_002253 Homo sapiens kinase insert domain receptor (KDR), mRNA	For: GTGACCAACATGGAGTCGTG Rev: CCAGAGATTCCATGCCACTT

2.2. Ex Vivo Angiogenesis Assays

2.2.1. Ethics Statement

Lrg^{-/-} mice and corresponding wild-type controls were originally generated by Knockout Mouse Project Repository C57BL/6 used for *ex vivo* studies were purchased from the InVivos Pte Ltd., Singapore. All mice were housed in animal biosafety level 2 (ABSL-2) labs and subjected to 12-hours light/dark cycle with regulated humidity at 55%. Mice had *ad libitum* access to sterile food

and water. Animal experiments were performed in compliance with the guidelines of the Institutional Animal Care and Use Committee (ARF–SBS/NIE–A0251, A0349 and A0394) of Nanyang Technological University, Singapore and the Guide for Care and Use of Laboratory Animals published by US National Institutes of Health.

2.2.2. Fetal Metatarsal Sprouting Assay

Fetal metatarsal sprouting assay was carried out as described³⁶⁸. Inbred mice strain of C57BL/6 background was used for this assay. Time–mating was performed to achieve an accurate prediction of the embryonic stages of the fetuses. In brief, a male and two other females were placed in the same cage. Thereafter, the females were examined for copulatory plug every morning afterwards. The plugged female was transferred into a new cage and the gestation stage was considered to be E0.5. Embryos between E16.5 to E18 were sacrificed and metatarsal bones were dissected under a stereomicroscope (Leica, M165FC; Germany) and maintained in ice–cold dissection medium. Individual metatarsals were mounted onto a well of 0.1% Gelatin (Merck, ES–006B; USA)–coated 24–well culture dish (Corning, #150687; USA) and left to adhere for 5 minutes. Next, 200µl of metatarsal growth medium was added to each mounted metatarsal and incubated 48 hours to allow fibroblasts sprouting. The non-viable metatarsals without visible fibroblast were discarded and the healthy metatarsal with outgrowth were subjected to further treatments. The explants were treated with 300µl of fresh metatarsal growth medium containing the desired dose of growth factors and/or inhibitors every other day, up till day 10 to 12. (*Refer to **Table 2.9.** for the components of metatarsal assay medium*).

At the conclusion of the culture period, metatarsal explants were fixed with 4% PFA, stained with antibody specific for endothelial cell adhesion molecule–1, also known as CD31 (Abcam, Ab28364; UK) and visualized under an inverted fluorescence microscope (Nikon, Eclipse Ti–E; USA). The raw images were processed in Adobe Photoshop CS4 (Adobe, Version 13.0.6; USA) to mask

cartilage and CD31–positive sprouting area was quantified normalized to the total metatarsal size.

Table 2.9. List of the Buffers for Metatarsal Assay

Name	Components	Source
<i>Ex vivo</i> Metatarsal Assay		
Dissection Medium	<ul style="list-style-type: none"> • 10% FBS • 1X Penicillin/Streptomycin • 1X PBS 	<ul style="list-style-type: none"> • GE Healthcare, A15–101; UK • Nacalai Tesque, #09367–34; Japan • 1st Base, BUF–2040; Singapore
Metatarsal Growth Medium	<ul style="list-style-type: none"> • 10% FBS • 1X Penicillin/Streptomycin • MEM Alpha + GlutaMAX™–1 	<ul style="list-style-type: none"> • GE Healthcare, A15–101; UK • Nacalai Tesque, #09367–34; Japan • Gibco, #32561–037; USA

2.2.3. Spontaneous Cardiac Tubes Assembly Assay

In general, either 3–4 months old C57BL/6 mice or 3–4 months old wildtype and *Lrg*^{−/−} mice were used for the explant culture. When specified, 6-months old diabetic mice (with 4 months of stable hyperglycemia) and the matched buffer-injected mice were used for the explant culture. The mice were anesthetized and retrograde-perfused with 50mL of sterile ice–cold PBS before its heart was excised at the aorta arch. The dissected hearts were manually pumped to remove blood clots and then kept in ice–cold dissection medium until further use. (*Refer to Table 2.10. for the components of cardiac explants assay medium*).

Excised hearts were cut into pieces <1mm and washed twice in 15mL dissection medium to remove more blood cells and tissue debris. After which, the minced hearts were resuspended in 2mL of pre-warmed digestion medium and left in a 37°C water bath with gentle rocking. After every 15 minutes, the remaining heart tissues were disassociated by pipetting and the supernatants containing the cardiac cells were collected and neutralized with 10X volume of ice–cold neutralizing medium. A fresh aliquot of pre-warmed digestion medium will be

added back to the remaining tissues and the digestion step would be repeated 5-7 times until most of the heart tissues was digested. Meanwhile the cardiac cells-containing supernatants would be pelleted by centrifugation at 500g for 10 minutes and the cells from the same heart would be pooled together and left on ice. The final cell suspension after the series of digestion would pass through a 70µm pore size cell strainer (Falcon®, #352350; USA) and pelleted again by centrifugation at 500g for 10 minutes. Then, the isolated cells were washed thrice, each time in 15mL of neutralizing medium. The isolated cells were plated by ratio, in which cells from one heart would be seeded into 4 wells of 0.1% Gelatin (Merck, ES-006B; USA)-coated 48-well plate (Thermo Fischer Scientific, #152640; USA), with each well containing 300µl EGM2-MV. The cells were left to adhere overnight. The next day, the wells were washed thrice with 1X PBS to remove dead cell and debris and cultured in 300µl EGM2-MV for another 2-5 days before treatment of growth factors and inhibitors in EBM-2. (Refer to **Table 2.10.** for the components of cardiac explants assay medium).

Table 2.10. List of the Buffers for Metatarsal Assay

Name	Components	Source
Mouse Spontaneous Cardiac Tubes Assembly Assay		
Dissection/ Neutralizing Medium	<ul style="list-style-type: none"> • 10% FBS • 1X Penicillin/Streptomycin • 1X DMEM 	<ul style="list-style-type: none"> • GE Healthcare, A15-101; UK • Nacalai Tesque, #09367-34; Japan • Gibco, #12800-058; USA
Isolation Medium	<ul style="list-style-type: none"> • 10% FBS • 1X Penicillin/Streptomycin • 1X DMEM • 1mg/ml Collagenase / Dispase 	<ul style="list-style-type: none"> • GE Healthcare, A15-101; UK • Nacalai Tesque, #09367-34; Japan • Gibco, #12800-058; USA • Roche, #11097113001; Switzerland

The isolated cardiac cells include cardiac fibroblast which is the first cell type that adheres to the culture plates. Cardiac fibroblasts expand rapidly to form a monolayer and served as feeder cells for ECs to grow onto. Following a 5-7 days treatment periods, the explants were fixed in 4% PFA and co-stained with

CD31 (Abcam, ab28364; UK) and Hoechst 33258 (Sigma–Aldrich, #H6024; USA). Images of the IF staining was captured using an inverted fluorescence microscope (Nikon, Eclipse Ti–E; USA). Raw images were processed in Adobe Photoshop CS4 (Adobe, Version 13.0.6; USA) to quantify for CD31–positive sprouting area and the values were normalized to Hoechst 33258–positive cells across five fields.

2.3. *In Vivo* Angiogenesis Assays

2.3.1. Genotyping Protocol

PCR-based genotyping of the progeny of homozygous breeders was done using a modified protocol that has been previously published¹⁶⁴. In detail, the mice were tail-clipped upon weaning. Mouse genomic DNA was extracted using the standard HotSHOT DNA extraction method. A 1cm tail clip sample was added to a 180µL of 50mM NaOH and boiled at 95°C for 20 minutes. The lysates were then vortexed and neutralized with 20µL of 1M Tris-HCl (pH 8.0). The crude extracts were spun at 12 x 10⁴ rpm for 5 minutes and 2µL of the sample was used for subsequent PCR. PCR was performed using the manufacturer’s protocol for DreamTaq DNA Polymerase (Thermo Fischer Scientific, #EP0703; USA). (*Refer to **Table 2.11.** for further details on the genotyping primers*). A sample of the expected PCR products for both the wildtype and *Lrg*^{−/−} mice is provided (*refer to **Supplementary Figure 2.1***).

Table 2.11. List of the Genotyping Primers

Name	Target (Accession number)	Sequence of primer (5' to 3')
Genotyping Primers		
WT Primers	NM_029796 Mus musculus leucine-rich α-2-glycoprotein 1 (LRG1), mRNA	For (TUR): GATCTGACCCGCAATGCTCT Rev (TDR): CGCGTGTCGTTCTGTGAGAAC

KO Primers	NM_029796 Mus musculus leucine-rich α -2-glycoprotein 1 (LRG1), mRNA	For (NeoFwd): TCATTCTCAGTATTGTTTTGCC Rev (SD): GACCCCTGAAACAGACGTG
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2.3.2. Mice Selection Criteria

Mice were subjected to pre-injection screening at 6 weeks of age. To eliminate potential gender differences, only the male mice were used in this study. Mice were weighed and subjected to 16 hours overnight fast (from 6 pm to 10 am) to determine their basal fasting blood glucose (FBG) levels. The next day, mice FBG was tested using a point-of-care glucometer (Roche, Accu-Chek Performa; Switzerland). Male mice with body weight >20g, FBG level >100mg/dl (5.6 mmol/L) and <200mg/dl (11.1 mmol/L) were recruited for subsequent studies.

2.3.3. Diabetes Induction

Mice were subjected to 50mg/kg low-dose Streptozotocin (STZ) (Sigma-Aldrich, S0130; USA) injection for 5 consecutive days as previously described³⁶⁹. Following a brief 6-hour starvation, mice were subjected to intraperitoneal (IP) injection bolus of freshly prepared STZ solution using a 30G needle (BD, #305107; USA). Mice that do not development hyperglycemia 4 weeks after the initial induction were subjected to the second cycle of STZ-injection. Only those mice which have FBG level ≥ 250 mg/dL at 27 weeks of age were included for further studies. (Refer to **Table 2.12.** for the components of mice STZ injection).

Table 2.12. List of the Buffers for STZ Injection

Name	Components	Source
STZ injection		
0.1M Sodium Citrate Solution	<ul style="list-style-type: none">• 1.47g Sodium Citrate Tribasic Dihydrate• 500mL DDH₂O	<ul style="list-style-type: none">• Sigma–Aldrich, C0909; USA• Sartorius, Arium® Type 3, USA
0.1M Citrate Acid Solution	<ul style="list-style-type: none">• 1.05g Citrate Acid Monohydrate• 500mL DDH₂O	<ul style="list-style-type: none">• Sigma–Aldrich, C1909; USA• Sartorius, Arium® Type 3, USA
0.1M Sodium Citrate Buffer, pH 4.5	<ul style="list-style-type: none">• 2 parts of 0.1M Sodium Citrate Solution• 3 parts of 0.1M Citrate Acid Solution	<ul style="list-style-type: none">• As above• As above
STZ solution (for injection)	<ul style="list-style-type: none">• 0.02g STZ Powder• 1mL of 0.1M Sodium Citrate Buffer	<ul style="list-style-type: none">• Sigma–Aldrich, S0130; USA• As above

2.3.4. Transverse Aortic Constriction (TAC)

Chronic heart failure was induced in 32 weeks old WT and *Lrg*^{−/−} via TAC. In brief, anaesthesia was induced with 5% Isoflurane (Piramal Healthcare, #4985230; India), maintained with 3% Isoflurane throughout the procedure. The mice were placed supine on a thermoregulated surgical table at 37°C ± 0.5°C and ventilated using a volume–cycled rodent ventilator (Hugo Sachs Elektronik, MiniVent Model 845 Ventilator; Germany) with a tidal volume set according to $V(\text{ml}) = 6.2 \times \text{BW}(\text{kg})^{1.01}$ and a respiratory rate set according to $\text{rate}(\text{min}^{-1}) = 53.3 \times \text{BW}^{-0.26}$. Under the surgical microscope (Leica, M165FC; Germany), partial thoracotomy to the ribcage was performed and the sternum was retracted. The thymus and fat tissue were gently separated to locate the aorta. The aorta was ligated between the innominate and left common carotid arteries by placing a Prolene 8–0 suture (Ethicon, #2775G; USA) against a blunted 27–gauge needle (BD, #301805; USA), which functions as a spacer to determine the degree of constriction. The needle was immediately removed

following the ligation. Sham-operated mice went through the same anaesthesia and surgical procedures without aortic constriction. The ribcage and skin were sutured to close the chest. Post-operative analgesia (Troy Pharmaceutical, Ilium Meloxicam 30; Australia), was administrated intraperitoneally, at the dosage of 5mg/kg during the first three days post-surgery. The animal was closely monitored for any sign of distress and post-intervention deaths. (*Refer to Supplementary Figure 2.2 for the schematic overview of the mice model of diabetic-related heart failure*).

2.3.5. Metabolic Phenotyping

To investigate the underlying metabolic difference between the WT and *Lrg*^{-/-} mice, a comprehensive metabolic phenotyping was conducted on a cohort of age-matched (15-months old) mice. The mice were habituated for 3 days in their testing room before being relocated to climatic chambers at constant temperature of 23°C, relative humidity at 55%. Mice were subjected to a 12-hours light/dark cycle. Oxygen consumption and carbon dioxide production in cages were measured by the calorimetric chamber. Oxygen and carbon dioxide sensors were calibrated with calibration gas mixtures. The sample air flow was adjusted to 0.37 L/minute. High precision weighing stations in combination with leak- and spill-proof containers were used to record food and water intake (in grams) accurately. Spontaneous activity was recorded with two levels of infrared light beam frames surrounding each cage. Measures are considered only from the second day in the chamber and readings were taken every 15 minutes and are presented hourly or as the mean of four measurement. All of the data extracted from a total of 16 age-matched animals which included eight WT mice and eight *Lrg*^{-/-} mice.

2.3.6. Endpoint Harvesting

At the endpoint of the animal study, mice were deeply anaesthetized with 5% Isoflurane (Piramal Healthcare, #4985230; India) and blood was collected by terminal cardiac puncture using a 23G needle (BD, #302008; USA). The

collected blood was transferred into heparin-coated capillary tubes (Fisher Scientific, 22-362566; USA), sealed with Critoseal® (Leica Biosystems, #18000-296; Germany), and stored upright at RT for 30 minutes for anticoagulation to occur. Finally, plasma was collected by spinning the tube down at 3000 rpm for 10 minutes and samples were stored at -80°C until further analysis. Next, the mouse was placed in a supine position and its heart was retrograde-perfused with 50mL of 1X PBS. The heart was then excised at the aorta arch and processed for downstream applications.

2.3.7. Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme-Linked Immunosorbent Assay (ELISA) kits was used for quantitative analysis of mouse LRG1 (Immuno-Biological, #27785; Japan), Insulin (Crystal Chem Inc, #90080; USA) and VEGF (Immuno-Biological, #27102; Japan). Heparinized plasma was used for all of the ELISA assays. Each sample was tested in duplicated and all of the assays were conducted according to the manufacturers' guide.

2.3.8. Cardiac Tissues Western Blotting

Snapped-frozen hearts were crushed into fine powder and added to 500µL of RIPA lysis buffer containing protease and phosphatase inhibitors cocktail. Next, the samples were subjected to an ultrasonic processing (Sonics®, VCX 130; USA) at 60% intensity on ice, for 3 cycles of 10 seconds, with 20 seconds pause in between each pulse to prevent overheating. Crude protein lysates were snap-frozen and thawed quickly to permeabilize the cell membrane and release intracellular proteins. This step was repeated twice before the mixture was pelleted by centrifugation at 1.3×10^4 g for 10 minutes. 80–120 µg of the resultant tissue homogenates was mixed with 4X Laemmli buffer and processed for western blotting. (*Refer to **Methods and Material 2.1.6.3. to 2.1.6.5.** for the detail protocol on protein lysis, protein quantification, SDS-PAGE, western blotting and protein bands detection procedures.*)

2.3.9. Cardiac Tissues qRT-PCR

Snapped-frozen hearts were crushed into fine powder and subjected to RNA extraction, cDNA conversion and qRT-PCR as previously specified. (*Refer to **Methods and Material 2.1.7.1. to 2.1.7.2.** for the detail protocol on RNA extraction, cDNA conversion and qRT-PCR procedures*). (*Refer to **Table 2.13.** for the list of mouse primers for qRT-PCR*).

Table 2.13. List of the Mouse Primers for qRT-PCR

Name	Target (Accession number)	Sequence of primer (5' to 3')
Mouse-specific Primers		
m-GAPDH	NM_001289726 Mus musculus glyceraldehyde-3-phosphate dehydrogenase (GAPDH), mRNA	For: TCCTGCACCACCAACTGCTTAG Rev: GATGACCTTGCCCACAGCCTTTG
m-ANP	NM_008725 Mus musculus Natriuretic peptide type A (ANP), mRNA	For: GGACTAGGCTGCAACAGCTTC Rev: GTGACACACCACAAGGGCTTA
m-VEGFA	NM_001025250 Mus musculus vascular endothelial growth factor A (VEGFA), mRNA	For: GCGGATCAAACCTCACCAAA Rev: TTCACATCGGCTGTGCTGTAGGA
m-LRG1	NM_029796 Mus musculus leucine-rich α -2-glycoprotein 1 (LRG1), mRNA	For: CCATGTCAGTGTGCAGATTC Rev: AAGAGTGAGAGGTGGAAGAG

2.3.10. Tissue Processing

The apex section of the heart was reserved for histological analysis. Upon harvesting, the hearts were immediately submerged in 15mL of 4% PFA and fixed overnight at 4°C to preserve the morphologic details of the heart. Following fixation, the fixed heart tissues were transferred to 70% ethanol before being processed in automated tissue processor (Leica, ASP6025; Germany). Firstly, the tissue was fully dehydrated through a series of ethanol solutions of increasing concentration (70%, 90%, 100%, 100%), each for 15 minutes. The tissue was then washed in 100% ethanol for two times and each

for 45 minutes). Following dehydration, tissues were cleared by 3 changes in Xylene baths, for 20 minutes, 20 minutes and 45 minutes respectively). Next, the cardiac tissue was completely infiltrated and embedded in histological paraffin wax with 3 changes of the paraffin wax and each for 45 minutes before being embedded in paraffin wax blocks. 5 µm-thick cross-sectional tissue slices spanning from 1000 µm from the distal apex of the heart were cut using a tissue-sectioning microtome (Leica, RM2255; Germany) and mounted onto poly-L-lysine coated glass slides (Fisher Scientific, #12-550-15; USA). The mounted heart sections were kept at 4°C until further use.

2.3.11. Antigen Retrieval and Immunostaining

Firstly, the histological sections were deparaffinization in Xylene baths twice, each for 5 minutes before being washed in 100% ethanol twice, each for 5 minutes. Afterwards, the slides were washed in deionized water containing decreasing concentrations of ethanol (95%, 70%, 50% and 0%) for 3 minutes and kept in distilled water for later use. Antigen retrieval was performed to unmask antigenic epitopes as a result of formalin fixation or paraffin embedding. Slides were fully submerged in a container filled with 10mM Sodium Citrate Buffer at pH 9.0 and boiled at 100 °C for 10 minutes.

Slides were then allowed to cool down to room temperature before being flooded with 2% histological blocking buffer and incubated for 30 minutes. Next, the blocking buffer was gently removed by tapping and the sections were flooded with a vascular marker, FITC-conjugated Isolectin B4 (Vector Laboratories, FL1201; Canada) and Alexa Fluor® 594-conjugated Wheat Germ Agglutinin (WGA) (Thermo Fisher Scientific, W11262; USA) to delineate cell boundaries. Both of the above-mentioned antibodies were diluted 1:500 in the histology blocking buffer and incubated overnight in a dark humidified chamber. The next day, the antibody solution were aspirated, slides were rinsed thoroughly with 1X PBS and counterstained with Hoechst 33258 (Sigma-Aldrich, #H6024; USA) diluted 1:1000 in 1X PBS and stained for 20 minutes in a dark humidified chamber. Finally, the antibody solution were aspirated, slides were rinsed thoroughly with 1X PBS, lightly blotted to drain excess water

and then mounted in Mowiol® aqueous mounting media. (Refer to **Table 2.14.** for the list of buffers used in the above-mentioned histological immunostaining procedure). An inverted confocal laser scanning microscope (CLSM) with airy-scan function (Zeiss, LSM800; Germany) was employed to visualise and image the whole heart by tile-scanning. The cardiomyocytes cross-sectional area (CSA) and vessel density were manually quantified using Adobe Photoshop CS4 (Adobe, Version 13.0.6; USA).

Table 2.14. List of the Buffers Used for Histological Immunostaining

Name	Composition	Source, Reference
Histological Buffers		
Histology IF Blocking Buffer	<ul style="list-style-type: none"> • 2% BSA • 3% Triton-X • 1% Tween-20 • 0.5% Horse serum • 1X PBS 	<ul style="list-style-type: none"> • Sigma-Aldrich, A2153; USA • Sigma-Aldrich, X100; USA • Bio-Rad, #1610781; USA • Invitrogen, #31874; USA • In-house
10mM Sodium Citrate Buffer (pH 9.0, 1X stock)	<ul style="list-style-type: none"> • 2.94g Trisodium citrate dihydrate • 0.5mL Tween-20 • 1L DDH₂O 	<ul style="list-style-type: none"> • Sigma-Aldrich, S1804; USA • Bio-Rad, #1610781; USA • Sartorius, Arium® Type 3, USA
Mowiol Mounting Medium	<ul style="list-style-type: none"> • 2.4g Mowiol® 4-88 • 12mL 0.2M Tris (pH 8.5) • Sodium Azide (NaN₃) • 6mL DDH₂O 	<ul style="list-style-type: none"> • Sigma-Aldrich, #81381; USA • In-house preparation • Sigma-Aldrich, S2002; USA • Sartorius, Arium® Type 3, USA

2.3.12. Masson's Trichrome Staining

Masson's Trichrome Staining was carried out in accordance with the manufacturer's protocol (Abcam, Ab150686; UK) with some minor amendments. After antigen retrieval as described in the previous section, the slides were fully submerged in the staining vessel filled with pre-heated Bouin's Fluid (60 °C) for 60 minutes. The slides were then rinsed under the running water for 3 minutes followed by 15 minutes incubation with Biebrich Scarlet/Acid Fuchsin solution. The slides were rinsed under the running water

again for 3 minutes. Next, the tissue sections were incubated with Phosphomolybdic/Phosphotungstic acid solution for 1015 minutes with periodical checking of the level of staining under the light microscope. Subsequently, the slides were counterstained with Aniline Blue solution for 5–10 minutes followed by rinsing under the running water for another 3 minutes. Finally, the slides were incubated in Acetic acid solution for 3–5 minutes and then dehydrated by 100% ethanol before being cleared with Xylene. Finally, the slides were lightly blot dried and mounted in 1–2 drops of CV Ultra Mounting Media (Leica, #14070936261; Germany).

The level of tissue fibrosis as demonstrated by Masson's Trichrome Staining was visualised under the upright wide-field light microscope (Leica, DM5500; Germany) and image of the whole heart was captured by tile-scanning. The perivascular fibrotic area was manually quantified using Adobe Photoshop CS4 (Adobe, Version 13.0.6; USA) and normalised to the lumen area of the vessels.

2.4. Human Patient Study

2.4.1. Ethics Statement

The study followed the ethical guidelines of the Declaration of Helsinki. Human Research Ethics Committee (HREC) Approval for collection and storage of the failing hearts was provided by St Vincent's Hospital, Sydney HREC: (H03/118), and by the University of Sydney HREC 2016/923). Nanyang Technological University Institutional Review Boards granted approval for biochemical analysis of specimens (IRB-2014-11-016).

2.4.2. Sample Processing

Left ventricle (LV) samples were collected from patients with clinically overt IHDs. They were snap-frozen in liquid nitrogen within 10 minutes of the loss of coronary circulation and maintained at this temperature (-196°C) until required including their transportation from Sydney to Singapore. Donors hearts

were harvested by surgeons under the coordination by the Australian Red Cross Blood Service (ARCBS) Healthy. These hearts were acquired once brain death was certified by the ARCBS coordinators. The donor's hearts were perfused with ice-cold cardioplegia and transported by air on ice to the Sydney Heart Bank. Donors were aged 51.38 ± 2.456 years and IHDs patients were aged 53.67 ± 2.528 years. Donor and IHDs LV samples from Sydney Heart Bank were previously analyzed and published demonstrating well-preserved nucleic acids, proteins, and morphological details³⁷⁰⁻³⁷⁵.

2.4.3. Western Blotting

The same protocol used for mouse cardiac tissue western blotting was adopted. (*Refer to **Methods and Material 2.3.8 and 2.1.6.3. to 2.1.6.5.** for the detail protocol on protein lysis, protein quantification, SDS-PAGE, western blotting and protein bands detection procedures*).

Chapter 3: Results

3.1. LRG1 promotes cardiac EC activation *in vitro*

Foremost, we attempted to demonstrate the pro-angiogenic effects of LRG1 using standard cell viability and proliferation assays, on an organ-specific primary EC line, HHECSs. We starved the cells overnight to synchronize their cell cycle and increase their sensitivities to the growth factor treatments. Next, we treated the cells with the proposed pro-angiogenic factors continuously for 48 hours. Thereafter, we compared the effects of LRG1 against VEGF₁₆₅, the known master regulator of angiogenesis. The pre-determined dose of LRG1 at 20µg/mL has been proven to be pro-angiogenic in other treatment conditions¹⁶⁴, and it simulates the circulatory level under physiology³⁷⁶. On the other hand, 5ng/mL of VEGF₁₆₅ has been routinely used as a positive pro-angiogenic factor.

MTS assay indirectly measures the capacity of metabolically active healthy cells to convert the tetrazolium reagent into a coloured formazan end-product. The assay is a surrogate means to estimate the number of metabolically-viable ECs upon LRG1 and/or VEGF₁₆₅ treatments. Given our treatment conditions (**Figure 3.1.1a.**), VEGF₁₆₅ worked well as a positive control and significantly induced HHECSs viability ($***P<0.001$). Next, LRG1, our protein of interest, significantly promoted the HHECSs viability ($**P<0.01$), thus establishing itself as a pro-angiogenic factor. Interestingly, when compared to the master regulator, the extent of LRG1-induced viability is slightly lesser than the VEGF₁₆₅ treated cells ($*P<0.05$). Interestingly, the LRG1 and VEGF₁₆₅ co-treatment did not elicit further additive increments in cell viability ($P=ns$).

Notably, the overall cell viability is regulated by the fine balance between the rate of cell proliferation and cell death. Hence, to improve our understand in the LRG1-regulated cell viability, we proceeded to examine its impact on cell proliferation by staining the LRG1 treated cells with a common cell proliferation marker, Ki-67 (**Figure 3.1.1b** and **Figure 3.1.1c**). We captured the images across across five fields and manually quantify the number of Ki-67+ nuclei. Consistent with our observation in HHECSs viability, continuous

VEGF₁₆₅ treatment resulted in increased cell proliferation ($***P < 0.001$). Also similar to our previous finding, LRG1 too, has effectively promoted HHECS proliferation ($**P < 0.01$). In addition, the combinatory treatment of LRG1 and VEGF₁₆₅ did not facilitate synergistic enhancements in the cell proliferation ($P=ns$).

Figure 3.1.1. LRG1 Promotes HHECS Viability and Proliferation

Primary human cardiac microvascular ECs (HHECSs) were starved in 0.2% FBS-supplemented basal medium (EBM-2) and treated with either 50ng/mL VEGF₁₆₅ and/or 20μg/mL LRG1. The overall functional changes of HHECS was measured as (a) quantitative changes in EC viability (n=3). Changes in the HHECS proliferation was demonstrated using (b) Ki-67 proliferation marker (red) staining localized in the cell nucleus (blue), scale bar = 500μm; and quantitatively reported as (c) percentage of Ki67+ nucleus of the growth factor treated cells normalized to UT (n=3). All results were presented as percentage change normalized to the untreated (UT) and reported as mean ± SEM. The statistical significance was determined using one-way ANOVA followed by post-hoc Tukey test where $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

As mention in the introduction, sprouting angiogenesis is a highly dynamic process. In response to local angiogenic cues, ECs will undergo a rapid proliferation and directional migration in order to assemble into primitive

endothelial tubules. To examine LRG1's role in regulating EC's migration and endothelial tubule organization, we adopted other functional assays to address each of these steps. Given that the assays require a relatively short treatment period, we could therefore study the immediate effects of LRG1 in modulating direct functional changes in the HHECSs using the same pro-angiogenic dose as specified in the earlier experiments.

Transwell® migration assay, also known as the modified Boyden chamber assay, was chosen to evaluate LRG1's impact on HHECSs migration (**Figure 3.1.2a** and **Figure 3.1.2b**). Give our experimental set-up, both the upper and the lower chambers contain the same concentration of angiogenic inducers. Thus, directional migration was induced by the FBS concentration gradient, in which the cells migration from the upper well (containing 0.2% FBS) to the bottom side of the well (containing 2% FBS), across the 8.0µm porous filter membrane. Following a 5-hour incubation period, we quantify the transmigrated cells by counting the Hoechst 33258–positive nuclei across five fields. Our data revealed that VEGF₁₆₅ treatment significantly promoted HHECSs transmigration (* $P < 0.05$). Interestingly, LRG1 treatment also significantly enhanced HHECSs transmigration (* $P < 0.05$), to a comparable extent when compared to the positive control ($P = ns$). Once again, there were no combinational effect in the LRG1 and VEGF₁₆₅ co-treated cells ($P = ns$).

The innate ability of ECs to spontaneously assemble into tubular structures is a critical step during sprouting angiogenesis. Growth factor-reduced (GFR) Matrigel® is a specially formulated ECM that supports cells to form two-dimensional vascular cords. HHECSs were seeded onto solidified GFR Matrigel®, stimulated with pro-angiogenic factors and incubated for 6 hours to form stable tubule-like structures (**Figure 3.1.3**). The total tube length was quantified, and our findings revealed that VEGF₁₆₅ expectedly promoted tube formation (* $P < 0.05$), and in the same direction, LRG1 also induced tube formation (* $P < 0.05$). Similar to previous observations, the induction was comparable across the two groups ($P = ns$) and were no combinational effect in the LRG1 and VEGF₁₆₅ co-treated cell ($P = ns$).

Figure 3.1.2. LRG1 Promotes HHECSs Migration

HHECSs were resuspended in 0.2% FBS-supplemented basal medium (EBM-2) and treated with either 50ng/mL VEGF₁₆₅ and/or 20μg/mL LRG1. The immediate functional changes of HHECS was featured as changes in EC migration. (a) Representative Hoechst 33258 staining (white) of transmigrated HHECS (scale bar = 500μm), quantitatively reported as (b) percentage of migrated cells (n=3). All results were presented as percentage change normalized to the untreated (UT) and reported as mean ± SEM. The statistical significance was determined using one-way ANOVA followed by post-hoc Tukey test where * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 3.81.3. LRG1 Promotes HHECSs Tube Formation

HHECSs were resuspended in 0.2% FBS-supplemented basal medium (EBM-2) and treated with either 50ng/mL VEGF₁₆₅ and/or 20μg/mL LRG1. The immediate functional changes of HHECS was featured as changes in EC tube formation. (a) Representative phase contrast images of HHECS tubules (scale bar = 500μm), quantitatively reported as (b) percentage change in total branching length (n=3). All results were presented as percentage change normalized to the untreated (UT) and reported as mean ± SEM. The statistical significance was determined using one-way ANOVA followed by post-hoc Tukey test where * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

3.2. LRG1 promotes angiogenesis in *ex vivo* models

Apart from the ECs, perivascular cells, non-vascular cells, inflammatory cells and ECM components also actively participate in the angiogenesis process^{56,377}. Hence, the *ex vivo* angiogenesis assays provide unique opportunity to study the interplay of the multiple cell types subjected to the same microenvironment. Given that ECs are highly heterogeneous and the angiogenic process is highly organ-specific we develop and adapted two different *ex vivo* angiogenesis models to study the role of LRG1 in a robust and context-dependent manner.

Spontaneous tube formation assay uses a cell suspension prepared from a single mouse heart allowed us to study the innate cardiac neovascularization potential in the genetic knockout mice. Following 7 days of culture in microvascular EC culture medium (EGM-2-MV), we fixed and stained the explant cultures for vascular marker CD31 (**Figure 3.2.1a** and **Figure 3.2.1b**). Five fields of view were acquired for each treatment and the images were analyzed using the open-source software AngioTool³⁷⁸. The total vessel densities were normalized to the total nuclei count to account for difference in cell densities. The results tied well to the previous findings and demonstrated substantially lower vessel outgrowth in the cardiac explants yielded from the *Lrg1*^{-/-} mice in comparison to their age-matched WT counterparts (**P* < 0.05).

Figure 3.2.1 Loss of LRG1 Reduces Cardiac Vessel Outgrowth

Cardiac explants of the age-matched WT and *Lrg1*^{-/-} mice were cultured in standard microvascular ECs' growth medium (EBM-2-MV) for 7 days. The resultant vascular network was stained with CD31 and quantified using AngioTools. (a) Representative images of CD31 staining (green) of cardiac vessel outgrowth from the WT and *Lrg1*^{-/-} mice (Scale bar = 500μm). Total vessel density was tabulated and normalised to the total nuclei count indicated by Hoechst 33258 (blue) staining. Results were presented as (b) relative vessel densities (n=3) and reported as mean ± SEM. The statistical significance was determined using two-tailed, unpaired student's T test where **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Using a similar *ex vivo* experimental protocol, we isolated cardiac cells from the WT C57BL/6 mice and culture the explanted cells in standard microvascular ECs' growth medium (EBM-2-MV) for 4 days. Next, we switched the culture into the 2% FBS supplemented EC basal medium (EBM-2) to reduce the background stimulus in order to evaluate the efficacy of exogenously supplemented angiogenic growth factors, including 20 μ g/mL LRG1 and/or 50ng/mL VEGF₁₆₅ on cardiac vessels outgrowth (**Figure 3.2.2a** and **Figure3.2.2b**). Thereafter, we cultured the cells for another 3 days and proceeded with the same downstream staining protocol. The findings were largely in line with our *in vitro* data, revealing that continuous VEGF₁₆₅ (** P <0.01) and LRG1 (** P <0.01) treatments strongly promoted the cardiac vessel outgrowth. As reported, the extent of induction was similar using either of the angiogenic growth factors ($P=ns$), and co-treatment of both growth factors did not provide any further additive outcomes ($P=ns$).

Figure 3.2.2. Exogenous LRG1 Induces Cardiac Vessel Outgrowth

Cardiac explants of the WT C57BL/6 mice were first cultured in standard microvascular ECs' growth medium (EBM-2-MV) for 4 days before switching into 2% FBS-supplemented EC basal medium (EBM-2) plus additional angiogenic growth factors for another 3 days. The resultant vascular network was stained with CD31 and quantified using AngioTools. (a) Representative images of CD31 staining (green) of cardiac vessel outgrowth cultured in different conditions (Scale bar = 500 μ m). Total vessel density was tabulated and normalised to the total nuclei count indicated by Hoechst 33258 (blue) staining. Results were presented as (b) relative vessel densities (n=3) and reported as mean $\pm$ SEM. The statistical significance was determined using one-way ANOVA followed by post-hoc Tukey test where * P <0.05, ** P <0.01, *** P <0.001.

In addition to the *ex vivo* angiogenesis model using anatomically-relevant whole heart tissues, we also performed the fetal metatarsal *ex vivo* angiogenesis assay to confirm the role of pro-angiogenic effects of LRG1 (**Figure 3.2.3a** and **Figure 3.2.3b**). This assay was specifically chosen to evaluate microvascular outgrowth, a phenomenon that models the physiological angiogenesis process in a more accurate way³⁶⁸. The dissected metatarsal bones were embedded onto pre-coated plates and cultured in metatarsal growth medium containing 20µg/mL LRG1 and/or 50ng/mL VEGF₁₆₅ for an extended period, up till 12 days. Our data showed a potent promoting effect of VEGF₁₆₅ (***P* < 0.001) on microvessels outgrowth from the metatarsals. Consistent with previous findings, we observed that LRG1 treatment had significantly promoted the microvascular outgrowth (**P* < 0.05), whereas the combinational treatment containing both LRG1 and VEGF₁₆₅ did not cause further potentiates the microvessels outgrowth from metatarsal bones (*P* = *ns*). Taken together, our data robustly confirmed the pro-angiogenic role of LRG1 using both *in vitro* and *ex vivo* models of angiogenesis.

Figure 3.2.3. LRG1 Induces Vessel Outgrowth from Metatarsals

Metatarsal explants of the E17.5 mice were trimmed, mounted onto culture dishes and cultured in metatarsal growth medium supplemented with additional angiogenic growth factors for up till 12 days. The resultant vascular network was stained with CD31 and quantified using the Adobe Photoshop CS6 software. (a) Representative images of CD31 staining (white) of metatarsal microvessel outgrowth cultured in different conditions (Scale bar = 500µm). Total vessel density was tabulated and normalised to the metatarsal surface area. Results were presented as (b) relative vessel densities (n=6) and reported as mean ± SEM. The statistical significance was determined using one-way ANOVA followed by post-hoc Tukey test where **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

At present, VEGF is widely regarded as the most important angiogenic effector³⁷⁹. Interestingly, a reduced VEGF expression has been previously observed in *Lrg1*^{-/-} retina, there may be a potentially overlapping roles governing the VEGF and LRG1-mediated angiogenesis¹⁶⁴. Given that a direct evidence linking LRG1 and VEGF has yet to be identified, we went ahead to investigate if the ablation of VEGF signaling would result in a functional consequence on LRG1-induced angiogenesis.

To address our query, Linifanib, also known as ABT-869, is a potent inhibitor of the receptor tyrosine kinase (RTK) activities of VEGFR2 was included into the treatment plan³⁸⁰. HHECSs were treated with sub-optimal dosage of 5nM Linifanib (treatment dose confirmed by initial pilot studies; data not shown) in conjunction with 20μg/mL LRG1. Functional changes were assessed using the *in vitro* Matrigel® tube formation assay (**Figure 3.2.4a** and **Figure 3.2.4b**) and *ex vivo* metatarsal sprouting angiogenesis assay (**Figure 3.2.4c** and **Figure 3.2.4d**). As anticipated, 5nM Linifanib strongly inhibited the vascular tubule formation (***P* < 0.01) and reduces microvascular sprouts from metatarsal bones (***P* < 0.01) when compared to the UT control. Remarkably, the findings revealed that the promoting effect of LRG1 (**P* < 0.05) on HHECS tube formation was significantly attenuated by 5nM Linifanib (***P* < 0.01). Similarly, Linifanib also significantly reduces the LRG1-induced vessel outgrowth from metatarsal explants.

Figure 3.2.4. Linifanib Attenuates LRG1-Mediated Angiogenesis

(a-b) HHECSs were resuspended in 0.2% FBS-supplemented basal medium (EBM-2) and treated with either LRG1 or/and Linifanib at the same time. (a) Representative images of tube formation in different treatment conditions (Scale bar = 500µm) and (b) quantitative analysis of the total tube length under different treatment conditions (n=3). (c-d) Metatarsal explants of the E17.5 mice were trimmed, mounted onto culture dishes and cultured in metatarsal growth medium supplemented with LRG1 and/or Linifanib cultured up till 12 days. The resultant vascular network was stained with CD31 and quantified using the Adobe Photoshop CS6 software. (c) Representative images of CD31 staining (white) of metatarsal vessel outgrowth cultured in different conditions (Scale bar = 500µm). Total vessel density was tabulated and normalised to the metatarsal surface area. Results were presented as (d) relative vessel densities (n=6) and reported as mean $\pm$ SEM. The statistical significance was determined using one-way ANOVA followed by post-hoc Tukey test where * P <0.05, ** P <0.01, *** P <0.001.

3.3. LRG1-Mediated TGF- β signaling in HHECSs

Even though LRG1 has no impact on endogenous TGF- β 1 signaling in macrovascular HUVECs, it regulates the pathway by promoting the TGF- β 1 induced Smad1/5/8 phosphorylation¹⁶⁴. Given the highly context-dependent nature of angiogenesis and the lack of direct evidence demonstrating that LRG1 regulates TGF- β 1 signaling cascade in microvascular HHECS, we speculate that LRG1 possibly exert its pro-angiogenic effects through the previously identified signaling mechanism.

To validate the findings, we performed western blotting to examine the activation of R-Smads involved in the canonical TGF- β 1 signaling pathway (**Figure 3.3.1a-c**). Similar to the reported observations in HUVECs, the LRG1-overexpressing HHECS showed no significant difference in its basal levels of phosphorylated Smad 1/5 (pSmad1/5) (P =ns) or phosphorylated Smad 2/3 (pSmad2/3) (P =ns), in the absence of exogenous TGF- β 1. On the contrary, the

level of pSmad1/5 was significantly enhanced in the LRG1-overexpressing HHECSs, after the treatment of TGF- β 1 (**** $P < 0.0001$) in comparison to the pcDNA3.1 transfected control (**Figure 3.3.1a-c**). This finding is indicative that the HHECS demonstrated an absolute requirement of exogenous TGF- β 1 to support LRG1's action.

Figure 3.3.1. Effects on Canonical TGF- β 1 Transducers in *Lrg1*-Overexpressing HHECSs.

(a-b) Sub-confluent HHECSs were subjected to transfection and left to recover overnight in the standard EC growth medium (EGM-2-MV). Thereafter, the cells were starved overnight in 0.2% FBS-supplemented basal medium (EBM-2) in preparation for TGF- β 1 treatment. The next day, HHECSs were stimulated with 5ng/ml TGF- β 1 for 30 minutes and cell were then harvested for western blotting. (a) Representative immunoblots and densitometry quantification of (b) p-Smad1/5 normalized to t-Smad 1/5 (n=3) and (c) pSmad2/3 normalized to t-Smad 2/3 (n=3). GAPDH was used as loading control. The results were presented as mean $\pm$ SEM. The statistical significance was determined using one-way ANOVA followed by post-hoc Tukey test where * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

In order to gain understanding of the underlying molecular mechanism regulating the LRG1-mediated Smad1/5 activation, specifically in the presence of TGF- β 1, HHECSs were co-treated with TGF- β 1 and small molecule inhibitor for ALK1, LDN193189 (**Figure 3.3.2a** and **Figure 3.3.2b**). Our data showed that the promoting effect of LRG1 on TGF- β 1-mediated Smad1/5 phosphorylation was significantly reduced in the presence of LDN193189 (**** $P < 0.0001$). Hence, collectively, our findings confirmed that LRG1 regulates the TGF- β 1-mediated activation of Smad1/5 in an ALK1-dependent manner.

Figure 3.3.2. Inhibition of ALK1 Affects Activation of Smad1/5 in HHECSs

(a-b) Sub-confluent HHECSs were subjected to transfection and left to recover overnight in the standard EC growth medium (EGM-2-MV). Thereafter, the cells were starved overnight in 0.2% FBS-supplemented basal medium (EBM-2) in preparation for TGF-β1 treatment. The next day, HHECSs were treated with 100nM of small molecule inhibitor for ALK1, LDN193189 for 30 minutes and then stimulated with 5ng/ml TGF-β1 for another 30 minutes. The HHECSs were then harvested for western blotting. (a) Representative immunoblots and densitometry quantification of (b) p-Smad1/5 normalized to t-Smad 1/5 (n=3). GAPDH was used as loading control. The results were presented as mean ± SEM. The statistical significance was determined using one-way ANOVA followed by post-hoc Tukey test where * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Besides its direct role in angiogenesis, TGF-β1 was reported to regulate the expression of VEGF *via* ALK1-mediated signaling in other mammalian ECs^{381,382}. Given that LRG1 regulates the TGF-β1-mediated ALK1-Smad1/5 signaling and that there is a reduced VEGF expression in *Lrg1*^{-/-} mouse retina¹⁶⁴, we proceed to query whether LRG1 regulates VEGF expression through a TGF-β dependent manner in HHECSs. We performed qRT-PCR to identify the changes in gene transcripts associated with the gain-of-function or the loss-of-function of *Lrg1* (**Figure 3.3.3a-k**).

Lrg1-overexpression in HHECSs was first confirmed (* $P < 0.05$) (**Figure 3.3.3a**). Next, along with the up-regulated *Lrg1*, we also identified significant increments in the transcript levels of both VEGF-A (** $P < 0.01$), and its primary receptor in ECs, the VEGFR2 (** $P < 0.01$) (**Figure 3.3.3b** and **Figure 3.3.3c**). Conversely, siRNA-mediated *Lrg1*-silencing in HHECSs was first validated using qRT-PCR (*** $P < 0.001$) (**Figure 3.3.3d**). In line with previous observations, the expression levels of both VEGF-A (*** $P < 0.001$) and VEGFR2 (* $P < 0.05$) were significantly down-regulated in response to the *Lrg1*-silencing (**Figure 3.3.3e and f**). We further demonstrated that 24 hours LRG1 treatment resulted in a positive feedback loop and regulated its own mRNA

expression in HHECSs (**Figure 3.3.3g**) ($*P < 0.05$). Similar to the observations in *Lrg1*-overexpressing HHECSs, LRG1 treatment also lead to a concomitant up-regulation of both VEGF-A ($*P < 0.05$) and VEGFR2 ($*P < 0.05$) transcripts (**Figure 3.3.3h-i**). Remarkably, ALK1 inhibitor, LDN193189 significantly suppressed the promoting effect of LRG1 on VEGF-A ($**P < 0.01$) and VEGFR2 ($**P < 0.01$) expression at the mRNA level (**Figure 3.3.3j-k**).

Figure 3.3.3. LRG1 Regulates VEGF-A and VEGFR2 mRNA Expression In ALK1-dependent manner

(a-f) Sub-confluent HHECSs were subjected to transfection and left to recover in the standard EC growth medium (EGM-2-MV) for 48 hours. (g-k) Alternatively, HHECSs were starved overnight in 0.2% FBS-supplemented basal medium (EBM-2) and subsequently treated with 20μg/ml LRG1 or/and 100nM of small molecule inhibitor for ALK1, LDN193189 for 24 hours. The mRNA transcripts of (a) LRG1, (b) VEGF-A, (c) VEGFR2 in *Lrg1*-overexpressing HHECSs (n=3). The mRNA transcripts of (d) LRG1, (e) VEGF-A, (f) VEGFR2 in *Lrg1*-knockdown HHECSs (n=3). The expression of (g) LRG1, (h) VEGF-A, (i) VEGFR2 in LRG1-

treated HHECSs (n=3). The of the mRNA transcripts of (j) VEGF-A, (k) VEGFR2 in LRG1 and/or LDN193189 treated HHECSs (n=3). The changes in gene expression were calculated using fold change ($-2(\Delta\Delta Ct)$). Results were presented as mean $\pm$ SEM. The results were presented as mean $\pm$ SEM. The statistical significance was determined using student's T test or one-way ANOVA followed by post-hoc Tukey test where * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Next, we went on investigating the impact of LRG1 on the activation of VEGF signaling pathway (**Figure 3.3.4a-c**). Interestingly, a brief 30 minutes LRG1 treatment was sufficient to induce the phosphorylation of the VEGFR2 (** $P<0.001$), which subsequently led to the increased phosphorylation of its downstream signaling transducer, the pAKT (** $P<0.001$). Taken together, our data pointed out that the pro-angiogenic role of LRG1 was TGF- β 1 dependent, at least partial through TGF- β 1-mediated induction of VEGF-A and VEGFR2.

Figure 3.3.4. Inhibition of ALK1 Affects LRG1-Mediated Activation of VEGFR2

(a-c) Sub-confluent HHECSs were subjected to transfection and left to recover overnight in the standard EC growth medium (EGM-2-MV). Next, HHECSs were starved overnight in 0.2% FBS-supplemented basal medium (EBM-2) and subsequently treated with 100nM of small molecule inhibitor for ALK1, LDN193189 for 30 minutes. (a) Representative immunoblots and densitometry quantification of TGF- β 1 signaling downstream effectors, p-VEGFR2 normalized to t-VEGFR2 and p-AKT normalized to t-AKT (n=3). GAPDH was used as loading control. The results were presented as mean $\pm$ SEM. The statistical significance was determined using one-way ANOVA followed by post-hoc Tukey test where * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Following the activation of ALK1 and R-Smads, specifically Smad1/5/8 in ECs, co-Smads, also known as Smad4, will form a heterotetrameric complex with pSmad1/5/8 and translocated into the nuclei^{383,384}. Given the pivotal role of Smad4 in mediating the TGF- β 's effect, the LRG1-mediated induction of VEGF-A and VEGFR2 was assessed in HHECSs subjected to siRNA-mediated

Smad4 knock down. qRT-PCR analysis demonstrated a successful knockdown of Smad4 in Smad4 siRNA transfected HHECSs (**Figure 3.3.5a**). As expected, the LRG1-induced VEGF-A ($****P<0.0001$) and VEGFR2 ($**P<0.01$) expressions were significantly attenuated in Smad4-knockdown HHECSs (**Figure 3.3.5b-c**). In line with this observation, the promoting role LRG1 in HHECS tube formation was lost in Smad4-knockdown cells ($***P<0.001$) (**Figure 3.3.5d-e**). Taken together, these findings indicate that the LRG1 mediates angiogenesis is, at least partially regulated through VEGF-A and VEGFR2, in a TGF- β dependent manner.

Figure 3.3.5. Smad4 Regulates the LRG1-Mediated Angiogenesis

(a-c) Sub-confluent HHECSs were subjected to transfection and left to recover overnight in the standard EC growth medium (EGM-2-MV). Next, HHECSs were starved overnight in 0.2% FBS-supplemented basal medium (EBM-2) and subsequently treated with 20 μ g/ml LRG1 for 24 hours. The expression of (a) Smad4, (b) VEGF A, (c) VEGFR2 in HHECSs subjected to siRNA knockdown of Smad 4 with the presence or absence of LRG1. The changes in the gene expression were calculated using fold change ($-2(\Delta\Delta Ct)$). (d-e) Transfected HHECSs was left to recover overnight and trypsinized for tube formation assay the next day (d) Representative images and (e) quantitative analysis (right) of total tube length of formed by HHECSs subjected to siRNA knockdown of Smad 4 with the presence or absence of 20 μ g/ml LRG1. The results were presented as mean $\pm$ SEM. The statistical significance was determined using one-way ANOVA followed by post-hoc Tukey test where $*P<0.05$, $**P<0.01$, $***P<0.001$.

Moving on, to further investigate if the LRG1-regulated activation of VEGF signaling pathway is dependent on the bioavailability of VEGF, HHECSs were treated with LRG1 and/or 10 μ g/mL of soluble VEGF-trap, Aflibercept, and VEGF₁₆₅ was used as a positive control (**Figure 3.3.6a** and **Figure 3.3.6b**). As expected, VEGF₁₆₅ -induced VEGFR2 phosphorylation was completely abolished by the addition of Aflibercept. Similar observation was made in LRG1 treated HHECSs. Although LRG1 was able to promote VEGFR2 phosphorylation ($***P<0.001$), the promoting effect of LRG1 was completely abolished by Aflibercept ($****P<0.0001$). Together, these data suggested that the promoting effect of LRG1 on VEGFR2 phosphorylation is dependent on the presence of VEGF-A.

Figure 3.3.6. LRG1-Mediated VEGFR2 Phosphorylation is VEGF-A Dependent

(a-b) HHECSs were starved overnight in 0.2% FBS-supplemented basal medium (EBM-2) and subsequently treated with 20 μ g/ml LRG1, 50ng/ml VEGF₁₆₅, and/or 10 μ g/ml Aflibercept for 30 minutes. Thereafter, the HHECSs were harvested for western blotting. (a) Representative immunoblots and (b) densitometry quantification of p-VEGFR2 and t-VEGFR2 in the presence of LRG1, VEGFR and/or Aflibercept. GAPDH was used as loading control. The results were presented as mean $\pm$ SEM. The statistical significance was determined using one-way ANOVA followed by post-hoc Tukey test where $*P<0.05$, $**P<0.01$, $***P<0.001$.

In summary, our findings could be integrated in this working model (**Figure 3.3.7**). In particular, LRG1 promotes angiogenesis through mediating multiple signaling pathways in HHECSs. Besides its regulatory role in endothelial TGF- β signaling, LRG1 is also able to induce the expression of VEGF-A and VEGFR2 in a Smad4-dependent manner. Furthermore, LRG1 is able to regulate the phosphorylation of VEGFR2 in the presence of VEGF-A. Blocking VEGF signaling either by tyrosine kinase inhibitor or VEGF-trap significantly attenuated the pro-angiogenic effect of LRG1.

Figure 3.3.7. Working Model of the LRG1-Mediated TGF- β Signaling in HHECSs.

During active angiogenesis, LRG1 promotes the TGF- β /ALK1/Smad1,5,8 signaling in EC. Besides its direct impact on EC activation and angiogenesis, TGF- β promotes angiogenesis indirectly by inducing the expression of VEGF-A and VEGFR2. Similarly, LRG1 is able to induce the expression of VEGF-A and VEGFR2 in a Smad-dependent manner. Furthermore, LRG1 is able to regulate VEGFR2 phosphorylation, possibly by mediating the availability of VEGF to its receptor.

3.4. Angiogenesis in Diabetic Conditions

3.4.1. High Glucose (HG) Affects ECs Functions

The impacts of diabetes on vascular injury and collateral blood vessel formation in response to tissue ischemia are well-documented^{214,337,338}, which are partially responsible for the unsuccessful translation of therapeutic angiogenesis in human patients. In order to understand the pro-angiogenic effect of LRG1 under diabetic condition, we first established various *in vitro* diabetic models and validated the vascular dysfunction under diabetic conditions. 22mM D-glucose was used to simulate the hyperglycaemic condition and 22mM metabolically inert L-glucose was used as an osmotic control to eliminate the osmotic effect.

MTS assay had demonstrated a significant reduction in HHECSs survival (** $P < 0.01$) after 48-hour treatment with high glucose (HG) (**Figure 3.4.1.1a**). A significant decreased in HHECSs proliferation (** $P < 0.01$) as represented by the presentation of Ki67-positive cells was also observed in 22mM D-glucose

treated HHECSs (**Figure 3.4.1.1b-c**). In addition, there is a significant increase in apoptosis in 22mM D-glucose treated HHECSs (** $P < 0.01$) as indicated by the expression of poly-ADP-ribose polymerase cleavage (PARP), a signature feature of cell-death related proteases in action (**Figure 3.4.1.1d**). Taken together, there data provided compelling evidence of successful establishment of hyperglycemic condition *in vitro*.

Figure 3.4.1.1. Continuous HG Treatment Reduces HHECSs Survival.

(a-e) HHECSs were subjected to 48 hours of 5mM glucose (LG), 22mM D-glucose (HG) and 22mM L-glucose (OC) treatment in standard EC growth medium (EGM-2-MV). (a) HHECS viability, (b and c) proliferation; scale bar = 500μm, and (d and e) the expression of cleaved PARP normalized to the non-cleaved PARP, in which GAPDH was used as loading control. The results were presented as mean $\pm$ SEM. The statistical significance was determined using one-way ANOVA followed by post-hoc Tukey test where * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Besides cell proliferation, the ability of ECs to migration and spontaneous tubule assembly capacities plays critical roles in the regulation of sprouting angiogenesis. Remarkably, a brief 5-hour exposure to 22mM D-glucose resulted

in a significant reduction in HHECSs migration ($***P<0.001$) in comparison to the OC (**Figure 3.4.1.2a** and **Figure 3.4.1.b**). In addition, of the ability of HHECSs to form tube like structure was significantly compromised under the influence of HG ($**P<0.01$) (**Figure 3.4.1.2c** and **Figure 3.4.1.d**). Taken together, the angiogenic response of HHECS was found to be largely attenuated in the presence of acute HG insults.

Figure 3.4.1.2. Acute HG Decreases HHECSs Migration and Tube Formation.

(a-b) HHECSs were trypsinized and seeded onto the upper chamber of 8.0µm Transwell®. Both the upper and the lower chambers contained the same glucose concentrations and directional migration was induced by FBS concentration gradient (from 0.2% FBS on the upper well to 2% FBS in the lower wells) during a 5-hour incubation period. (a) Representative images of HHECSs migrated across the Transwell® insert as indicated by DAPI staining (Scale bar = 500µm) and (b) quantitative analysis of percentage change in HHECS migration (n=3). (c) Representative images (of tube formation in 22mM D-glucose (HG) and 22mM L-glucose (OC) (Scale bar = 500µm), and (d) quantitative analysis of total tube length of formed by HHECSs following a 6 hours incubation period (n=3). The results were analysed in ImageJ and were presented as mean $\pm$ SEM. The statistical significance was determined by two-tailed, unpaired, student's test where $*P<0.05$, $**P<0.01$, $***P<0.001$.

3.4.2. HG Affects Angiogenic Gene Expression

To confirm HG-mediated EC dysfunction, we investigated the key angiogenic genes that has been widely associated with hyperglycaemic insults. We simply subjected the HHECSs to 48-hours of continuous HG treatment and harvest the cells for qRT-PCR profiling. Given that many literature has provided strong experimental data claiming that the molecular basis of diabetes-related endothelial dysfunction is attributable to the increased oxidative stress and reactive oxygen species (ROS) associated with the down-regulated expression

of nitric oxide synthase (eNOS) and the co-factors for its synthesis^{257,385,386}, we went ahead to quantify for changes in the eNOS transcript levels in HHECSs following 48 hours of continuous HG treatment. As expected, we observed a significant reduction of the eNOS transcript levels ($*P<0.05$) (**Figure 3.4.2.1a**) when compared to its OC. Given that, we could confirm a successful establishment of *in vitro* diabetic conditions on HHECSs. Interestingly, in the same treatment conditions, we observed a concomitant reduction in LRG1 ($***P<0.001$) and VEGFR2 ($***P<0.001$) expression (**Figure 3.4.2.1b-c**). Moreover, we also observed an up-regulated expression of VEGF-A ($*P<0.05$) (**Figure 3.4.2.1d**), which is likely through a non-LRG1-dependent pathway in an attempt to improve EC survival and function. Taken together, our data demonstrated a successful establishment of HG-induced EC dysfunction and impairment of angiogenic potential *in vitro*.

Figure 3.4.2. Molecular Changes Associated with Prolonged HG Treatment.

HHECSs were subject to continuous low glucose (LG, 5.5mM), high glucose (HG, 22mM D-glucose) and osmotic control (22mM, L-glucose) for 48 hours. qRT-PCR analysis of the mRNA transcript levels of (a) eNOS, (b) LRG1, (c) VEGFR2, and (d) VEGF-A were tested (n=3 for all). The changes in gene expression were calculated using fold change ($-2(\Delta\Delta Ct)$) and the tabulated results were presented as mean $\pm$ SEM. The statistical significance was determined using one-way ANOVA followed by post-hoc Tukey test where $*P<0.05$, $**P<0.01$, $***P<0.001$.

3.4.3. Advanced Glycated End-products (AGE) Affects Endothelial Function

Following the prolonged hyperglycaemia, glycation of plasma and extracellular protein ultimately contributed to the pathophysiological sequelae of vascular dysfunction associated with late-stage DM^{387,388}. Collagen, the most abundantly-present ECM component in the vasculature has a relatively long turnover rate^{387,389}. Methylglyoxal (MGO) are highly reactive α -oxalaldehyde intermediates of glucose metabolism that could rapidly induce intramolecular or intermolecular crosslinking of target proteins^{320,390}. In addition to the direct hyperglycaemic effects brought upon by the high level of circulatory glucose, we are also interested in the long-term biological events associated with chronic hyperglycaemic status. We began by studying the role of advanced glycated end-products (AGEs) in the regulation of HHECSs function.

We adapted a previously published protocol to chemically induce the formation of glycated Collagen I *in vitro*³⁶⁴. Our protocol demonstrated that 257mM MGO led to the formation of glycated Collagen I as indicated by a prominent shift of Collagen I protein bands in the SDS-PAGE which were stained with Coomassie Brilliant Blue (**Supplementary Figure 3a**). The shift in protein molecular weights was also confirmed by western blot analysis using a monoclonal antibody that is specifically raised against MGO (**Supplementary Figure 3b**).

Following the establishment of the glycation protocol, we examined the effects of the glycated Collagen (gCol) on HHECSs' function. HHECSs were seeded on control Collagen and gCol-coated cell culture plates and cultured for 72 hours. Firstly, changes in cell viability was investigated using the MTS assay (**Figure 3.4.3.1a**). The experimental findings demonstrated prominent inhibition of HHECS viability when cultured on gCol ECM ($*P < 0.05$). In addition, we were able to demonstrate that changes in HHECS viability was not due to the reduced ability of cells to adhere to the to the gCol substrates ($P = ns$) as our gCol products did not significantly changed HHECSs adhesion properties (**Figure 3.4.3.1b**). Besides its impact on HHECS viability, the gCol product had also negatively-impeded with the motility of HHECSs using the Transwell[®] migration assay ($**P < 0.01$) as compared to those seeded on non-glycated control substrate (**Figure 3.4.3.1c** and **Figure 3.4.3.1d**). Moreover, we adapted the standard tube formation assay to examine if the ability of HHECSs to form

tube-like structure would be affected in the gCol-containing versus control Col-containing Matrigel® by mixing the ECM substrates in 1:1 ratio (**Figure 3.4.3.1e** and **Figure 3.4.3.1f**). Our findings revealed that the tube formation capacities were compromised in HHECSs cultured on the gCol-containing Matrigel® as compared to those cultured on the Matrigel® containing native collagen ($***P < 0.001$). In summary, taken together, our study confirmed the detrimental effect of gCol on HHECSs' function and angiogenesis.

Figure 3.4.3. Effects of gCol on HHECS Functions and Angiogenesis

HHECSs were cultured on culture surfaces coated with normal Collagen I and 3HgCol for different durations to examine the changes in EC functional properties. HHECS demonstrated (a) significant reduction in cell viability ($n=3$), but (b) no obvious change in initial cell adhesion ($n=3$). (c) Representative nuclei staining (white) denoting HHECSs transmigration facilitated by normal collagen and gCol substrates (Scale bar = 500µm), (d) tabulated HHECS transmigration suggesting 3HgCol reduces migratory properties ($n=3$). (e-f) Matrigel® was mixed with either normal Collagen I and 3HgCol at 1:1 ratio and HHECSs tube formation capacities were examined. (e) Representative figure denoting reduced ability to form tube-like structure in Matrigel® mixed with gCol in comparison to the normal Collagen (Scale bar = 500µm), (f) quantification of the total branching length ($n=3$). All results were presented as mean $\pm$ SEM. Statistical significance was determined by two-tailed, unpaired, student's test where $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

3.4.4. AGE Affects Angiogenic Gene Expression

Having established the detrimental role of gCol on HHECS function and angiogenesis, we went on examining the expression levels of key molecular mediators of EC function in associated with the functional changes (**Figure 3.4.4.a-e**). To do so, we pre-coated the culture dishes with native collagen and 3HgCol substrates and cultured HHECSs on these surfaces for 48 hours, in normal EC growth medium (EGM-2-MV). At endpoint, we harvested the HHECSs for angiogenic gene profiling using qRT-PCR.

Foremost, we examined the mRNA transcripts of multi-ligand receptor of AGE (RAGE), which is expected to be up-regulated upon AGE treatments (**Figure 3.4.4.a**). Indeed, RAGE was significantly up-regulated in HHECSs that was cultured on gCol substrate ($*P<0.05$) in comparison to the HHECSs that was on native collagen substrate. Next, given that ROS accumulation due to reduced eNOS bioavailability is a widely appreciated hallmark of EC dysfunction in diabetic conditions^{307,391}, we went ahead to validate if this notion holds true in gCol treatments (**Figure 3.4.4.b**). Concomitant with the RAGE up-regulation, the eNOS transcript level was also reduced ($**P<0.01$). In addition, similar to our observation in the HG-treated HHECSs, HHECSs cultured on the gCol substrate demonstrated a markedly reduced expression of LRG1 ($***P<0.001$) (**Figure 3.4.4.c**) and VEGFR2 ($***P<0.001$) (**Figure 3.4.4.d**), but an up-regulated expression of VEGF-A ($***P<0.001$) (**Figure 3.4.4.e**). Together, our results demonstrated an impaired EC function and angiogenic potential in the presence of AGE.

Figure 3.4.4. Molecular Changes Associated with Native and gCol Substrates

HHECSs were cultured on culture surfaces coated with native Collagen I and 3HgCol for 48 hours in the standard EC growth medium (EGM-2-MV). The cells were harvested for qRT-PCR at the endpoint. qRT-PCR analysis of the transcript levels of (a) AGER, (b) eNOS, (c) LRG1, (d) VEGFR2, and (e) VEGF-A in HHECSs subjected to native or gCol substrates. The changes in gene expression were calculated using fold change ($-2(\Delta\Delta Ct)$). The assay was repeated in at least three independent settings ($n \geq 3$). Results were presented as mean $\pm$ SEM; $n=3$ for all gene targets. Statistical significance was determined by two-tailed, unpaired, student's test where * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

3.5. LRG1 Promoted Cardiac Angiogenesis in Diabetic Conditions

Following the successful establishment of the diabetic condition *in vitro* and confirming the reduced production of pro-angiogenic growth factors, including the reduced LRG1 gene expression in the HHECSs, we proceeded to assess if exogenous LRG1 could promote HHECS function and angiogenesis under the diabetic condition (**Figure 3.5.1a-d**). As demonstrated in Matrigel[®] tube

formation assay (**Figure 3.5.1a**), although HG led to a significant reduction in the ability of HHECSs to form tube-like structure, the pro-angiogenic effect of LRG1 has been well-preserved under the HG condition (**Figure 3.5.1b**). Similarly, results from the modified tube formation assay (**Figure 3.5.1c**) revealed that exogenous LRG1 treatment was still effective in promoting the HHECS tube formation in HHECSs cultured on gCol-containing Matrigel® (** $P<0.01$) (**Figure 3.5.1d**).

Figure 3.5.1. LRG1 Promotes HHECS Tube Formation in Diabetic Conditions

(a) Representative images of HHECS tube formation in Matrigel® in the presence of LRG1 under normal glucose and HG condition (Scale bar = 500µm), (b) quantification of the total branching length (n=4). (c) Representative images of HHECS tube formation in Matrigel in the presence of LRG1 with the presence of native collagen I or gCol (Scale bar = 500µm), and the (d) quantification HHECS branching length (n=3). The tube formation was analysed in ImageJ using Angiogenesis analyser plugin. All results were presented as mean ± SEM. The statistical significance was determined by one-way ANOVA followed by post-hoc Tukey test where * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Subsequently, we assessed the pro-angiogenic effect of exogenous LRG1 under the diabetic condition in several *ex vivo* model of angiogenesis (**Figure 3.5.2a-**

d). Firstly, the fetal metatarsal explants cultured in 22mM D-glucose supplemented growth medium exhibited a significant reduction in vascular sprouting (**** $P < 0.0001$), which could be rescued by the treatment with exogenous LRG1 (**** $P < 0.0001$) (**Figure 3.5.2a** and **Figure 3.5.2b**). Similarly, the vascular outgrowth from metatarsal explants cultured on gCol-coated culture surfaces was markedly reduced as compared to those grown on native collagen (* $P < 0.05$), whereas LRG1 significantly promoted the vessels outgrowth from metatarsal explants grown on both native and gCol (** $P < 0.01$) (**Figure 3.5.2c** and **Figure 3.5.2d**). Taken together, our study showed a potent pro-angiogenic effect of LRG1 under both normal and diabetic conditions in *ex vivo* organ cultures.

Figure 3.5.2. LRG1 Promotes Vessels Outgrowth from Metatarsals in Diabetic Conditions

(a) Representative images (Scale bar = 500 μ m) and (b) quantification of vessel outgrowth from metatarsal explants subjected to treatment with exogenous LRG1 under normal glucose and HG condition (n=6). (c) Representative images (Scale bar = 500 μ m) and (d) quantification of vessel outgrowth from metatarsal

explants cultured on native or gCol-coated plate and subjected to additional LRG1 treatment (n=5). Metatarsal sprouting area were quantified using Adobe Photoshop CS6 (Version 13.0.6, USA) and normalised to total explant area. All results were presented as mean $\pm$ SEM. The statistical significance was determined by one-way ANOVA followed by post-hoc Tukey test where * P <0.05, ** P <0.01, *** P <0.001.

Having established the pro-angiogenic effect of LRG1 under the diabetic condition in the multi-cellular metatarsal assay, we went on evaluating the pro-angiogenic effect of LRG1 in cardiac tissue as angiogenesis is a highly-context dependent process. Firstly, we subjected the WT mice to STZ-induced diabetes. After 20-weeks following the initial induction of diabetes, the mouse hearts were harvested for explant cultures (**Figure 3.5.3a** and **Figure 3.5.3b**). Our study revealed a significant increase in vessel densities upon treatment with LRG1 (**** P <0.0001) (**Figure 3.5.3a**) and VEGF₁₆₅ (**** P <0.0001) (**Figure 3.5.3b**). As mentioned in several *in vitro* and *ex vivo* angiogenesis, the extent of pro-angiogenesis induction from LRG1 and VEGF₁₆₅ was comparable. This assay had promptly recapitulated our previous observations and demonstrated no significant difference between the vessel densities of LRG1 and VEGF₁₆₅ explants ($P=ns$). Interestingly, using this model, we observed a prominent additive synergistic angiogenesis effect in the LRG1 and VEGF₁₆₅ co-treated explants (**** P <0.0001) when compared the explants treated with either of the angiogenic factor.

Figure 3.5.3. LRG1 Promotes Cardiac Vessels Outgrowth in Diabetic Conditions

(a) Representative immunostaining of vessel formation as demonstrated by CD31 staining (green) from the signal cell suspension from the heart of WT mice-subjected to STZ-induced diabetes (Scale bar = 500µm). (b) Quantitative analysis of vessel density using Adobe Photoshop CS6 (n=3) and normalised to total nuclei count stained using Hoechst 33258 (blue). The results were presented as mean $\pm$ SEM. The statistical

significance was determined by one-way ANOVA followed by post-hoc Tukey test where * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

3.6. Role of LRG1 in Mice Model of Heart Failure

The mice model of pressure-overload-induced heart failure generated by transverse aortic constriction (TAC) surgery and this model has been widely adopted to study the cardiac remodeling process *in vivo*. TAC mimics aortic stenosis, which is associated to the manifestation of the many of the features of human IHDs related to atherosclerosis³⁹²⁻³⁹⁴. To understand the role of LRG1 in pathological cardiac remodeling, WT and *Lrg1*^{-/-} mice were subjected to TAC surgery 20-weeks after the induction of diabetes induced by STZ (**Figure 3.6.1**). The Kaplan-Meier survival plot demonstrated a significant reduction in survival rate in TAC-operated WT and *Lrg1*^{-/-} mice as compared to sham-operated control mice under both normoglycemic and diabetic condition. There is no difference in terms of TAC-induced mortality between WT and *Lrg1*^{-/-} mice under normoglycemic condition. However, the STZ-induced diabetes caused a significant increase in TAC-induced mortality in *Lrg1*^{-/-} mice but not in WT controls. These data suggested a potential role of LRG1 in mediating the TAC-induced cardiac remodeling under diabetic condition.

In order to further understand the role of LRG1 in diabetes-associated cardiac remodelling, we first evaluated the development of diabetes in WT and *Lrg1*^{-/-} mice. The fasting blood glucose (FBG) levels was measured 20-weeks after the initial STZ injection. Our data showed a significant increase in FBG levels in WT and *Lrg1*^{-/-} mice 20-week following STZ-injection. However, there is no difference in FBG levels between the STZ-treated WT and *Lrg1*^{-/-} mice (**Figure 3.6.2**). This data suggested that the worse survival rate observed in *Lrg1*^{-/-} mice with diabetes was not due to the increased severity of diabetes. Hence, the evidence supported the proposition that genetic *Lrg1*-deficiency resulted in enhanced maladaptive deaths following TAC and this is not related to the underlying differences susceptibility and severity of STZ-induced diabetes.

Figure 3.6.1. LRG1 Promotes Cardiac Vessels Outgrowth in Diabetic Conditions

The plot features the eight different animal cohorts namely (1) WT buffer-injected, Sham-operated mice, WBS; (2) WT buffer-injected, TAC-operated mice, WBT; (3) WT STZ-injected, Sham-operated mice WSS; (4) WT STZ-injected, TAC-operated mice, WST; (5) *Lrg1*^{-/-} buffer-injected, Sham-operated mice, KBS; (6) *Lrg1*^{-/-} buffer-injected, TAC-operated mice, KBT; (7) *Lrg1*^{-/-} STZ-injected, Sham-operated mice KSS; and (8) *Lrg1*^{-/-} STZ-injected, TAC-operated mice, KST. Statistical significance between the different experimental groups was determined by Logrank-test (Mantel-Cox), where **P*<0.05, ***P*<0.01, ****P*<0.001. *n*≥6 mice per cohort.

Figure 3.6.2. Fasting Blood Glucose in WT and *Lrg1*-Deficient Mice

The FBG following 16 hours overnight fast period was measured in buffer or STZ-injected WT and *Lrg1*^{-/-} mice 20-week after the initial buffer or STZ injection (*n*=10 mice per cohort). All results were presented as mean ± SEM. Statistical significance was determined by one-way ANOVA followed by post-hoc Tukey test, where **P*<0.05, ***P*<0.01, ****P*<0.001.

To better understand the pathophysiological role of LRG1 in diabetes-associated cardiac remodeling, we examined the circulating levels of LRG1 in

buffer and STZ-injected WT mice at 27-weeks of age. Our study demonstrated a drastic increase in plasma LRG1 levels in diabetic mice as compared to that in buffer-injected control mice ($***P<0.001$) (**Figure 3.6.3a**). Similarly, the level of plasma VEGF-A was also significantly higher in WT mice with established diabetes as compared to that in normoglycemic control mice (**Figure 3.6.3b**). Given that a reduced VEGF-A expression was previously observed in *Lrg1*^{-/-} mouse retina¹⁶⁴, consistent with this observation, we found that the plasma VEGF-A level was markedly lower in *Lrg1*^{-/-} mice with diabetes ($*P<0.05$) as compared to their WT-counterparts ($**P<0.01$). In summary, our findings demonstrated a concomitant increase in plasma LRG1 and VEGF-A levels in diabetic mice and a significant reduction in VEGF-A level in the *Lrg1*^{-/-} mouse plasma.

Figure 3.6.3. Plasma Level of Angiogenic Factors in Diabetic Mice

Circulating levels of LRG1 in buffer and STZ-injected WT mice at 27-weeks of age. (a) tabulated plasma LRG1 levels (n=10), (b) tabulated plasma VEGF-A levels (n=10). Results were presented as mean $\pm$ SEM. Statistical significance was determined by unpaired, two tailed, student's test or one-way ANOVA followed by post-hoc Tukey test where $*P<0.05$, $**P<0.01$, $***P<0.001$.

To further investigate the underlying mechanism related to the reduced survival rate in *Lrg1*^{-/-} mice-subjected to STZ-induced diabetes, we went on analyzing the heart morphology and histology in the different groups mice. Our study revealed a significant enlargement of the heart in WT mice 6-weeks after the TAC surgery suggesting the successful induction of cardiac remodeling, which was further enlarged in *Lrg1*^{-/-} mice (**Figure 3.6.4a**). However, the induction of diabetes had no difference ($P=ns$) on heart size in both the WT and *Lrg1*^{-/-} mice.

Concomitant with this observation, there was a significant increment in TAC-induced increase in heart weight (HW) to body weight (BW) ratio (**Figure 3.6.4b**) and HW to tibia length (TL) ratio (**Figure 3.6.4c**) in *Lrg1*^{-/-} mice as compared to WT controls. However, prolonged diabetes did not result in further changes in both parameters in both WT and *Lrg1*^{-/-} mice.

Figure 3.6.4. Development of Cardiac Hypertrophy 6-weeks Post-TAC

(a) Representative images of the heart isolated from mice in different treatment groups. Heart weight ratios normalised to either (b) body weight or (c) tibia length. Each experimental group contains at least 6 animals ($n \geq 6$). All results were presented as mean $\pm$ SEM. Statistical significance was determined by the one-way ANOVA followed by post-hoc Tukey test where * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Moreover, to further validate the cardioprotective role of LRG1, we analyzed the histological abnormalities of the heart harvested 6-weeks post-TAC procedure. In particular, the hearts were co-stained with Alexa-Fluo[®] 594-

conjugated wheat-germ agglutinin (WGA) to demarcate its cell boundaries and the FITC-conjugated Isolectin B4 (IB4) to label the cardiac microvasculature. Each of the parameters was then tabulated manually by sampling at least 100 cells across the field (**Figure 3.6.5b** and **Figure 3.6.6b**). Interestingly, the results suggested a more substantial TAC-induced increase in cardiomyocyte cross-sectional area (CSA) in *Lrg1*^{-/-} mice as compared to WT counterparts (**Figure 3.6.5b**), and this holds true for both the buffer-injected control mice and STZ-injected diabetic mice. This observation also accurately reflects our previous claims in which *Lrg1*^{-/-} mice had higher HW/BW and HW/TL ratios than their WT counterparts (**Figure 3.6.3b** and **Figure 3.6.4c**). Therefore, there appears to be innate difference between the cardiac remodeling process in the WT and *Lrg1*^{-/-} mice, suggestion that LRG1 may exert a cardioprotective role in limiting maladaptive cardiac hypertrophy following TAC. Unexpectedly, the presence of diabetes did not further induce cardiomyocyte CSA in both WT and *Lrg1*^{-/-} mice.

Atrial natriuretic peptide (ANP) is an important pro-hypertrophic marker associated with hypertrophy responses during cardiac remodeling³⁹⁵. To understand the molecular mechanism underlying increased cardiomyocyte CSA in response to TAC in *Lrg1*^{-/-} mice, we investigated the mRNA expression of ANP in the heart of mice from different experimental groups (**Figure 3.6.5c**). Our results revealed significant up-regulation of ANP mRNA transcript induced by TAC surgery in both WT and *Lrg1*^{-/-} mice under normoglycemic and hyperglycemic conditions. Furthermore, diabetes caused a further increase in TAC-induced increase in ANP levels in both WT and *Lrg1*^{-/-} mice and *Lrg1*^{-/-} deficiency was also positively correlated with elevated ANP expression.

Figure 3.6.5. Characterization of Cardiomyocyte CSA 6-weeks Post-TAC

(a) Representative images of immunofluorescence staining with WGA (red) and DAPI (blue) and (b) quantitative analysis of the ventricle cross-sections of the whole heart in buffer or STZ-injected WT and *Lrg1*^{-/-} mice. Image was taken with CLSM, scale bar = 5000μm. (c) Changes in ANP in the heart of mice from different experimental groups. The changes in gene expression were calculated using fold change (-2(ΔΔCt)). Each experimental group contains at least 6 animals (n≥6). All results were presented as mean ± SEM. Statistical significance was determined by the one-way ANOVA followed by post-hoc Tukey test where **P*<0.05, ** *P*<0.01, ****P*<0.001.

Moreover, in consistent with the potent role of LRG1 in cardiac angiogenesis, there is a significant reduction in cardiac vascular density in both sham and TAC-operated *Lrg1*^{-/-} mice as compared to their WT counterparts (*****P*< 0.0001, across all groups), (**Figure 3.6.6a** and **Figure 3.6.6b**). Interestingly, STZ-induced diabetes resulted in a further induction in the TAC-induced vascular rarefaction, reflected as reduced cardiac microvessel densities in both the WT (***P*< 0.01) and *Lrg1*^{-/-} mice (**P*< 0.05). Hence, our results underline

that LRG1 may elicit critical cardio-protective mechanisms by regulating vessel remodeling in IHDs.

Figure 3.6.6. Characterization of Microvessels Densities 6-weeks Post-TAC

(a) Representative images of immunofluorescence staining with WGA (red), IB4 (green) and DAPI (blue) and (b) quantitative analysis of the ventricle cross-sections of the whole heart in buffer or STZ-injected WT and *Lrg1*^{-/-} mice. Image was taken with CLSM, scale bar = 5000µm. Each experimental group contains at least 6 animals (n≥6). All results were manually tabulated and presented as mean ± SEM. Statistical significance was determined by the one-way ANOVA followed by post-hoc Tukey test where **P*<0.05, ** *P*<0.01, ****P*<0.001.

Consistent with our previous observation, we noticed that the LRG1 mRNA transcript level (**Figure 3.6.7a**) is significantly lower in the cardiac tissues of the TAC-operated mice, in both the buffer-injected (**P*< 0.05) and STZ-injected (**P*< 0.05) groups. Notably, the in the WT STZ-injected, TAC-operated mice, we observed a further reduction in LRG1 transcripts (***P*< 0.01)

when compared to the buffer-injected group. Interestingly, the LRG1 protein expression levels (**Figure 3.6.7b**) revealed a similar observation. We found that LRG1 protein expression is reduced with TAC surgery, in both the buffer-injected (**** $P<0.0001$) and STZ-injected (* $P<0.05$) groups. Similarly, the LRG1 protein expression level is significantly lower in the cardiac tissues of the WT STZ-injected (** $P<0.01$) compared to the buffer-injected mice.

Controversially, although there is a parallel reduced VEGF-A mRNA transcript level in *Lrg1*^{-/-} mice, VEGF-A is significantly elevated in the heart following the TAC surgery across all experimental groups (**Figure 3.6.7c**). Furthermore, a relative increase in cardiac production of VEGF-A is observed in diabetic WT and *Lrg1*^{-/-} mice when being compared to their respective buffer injected normoglycemic controls (**Figure 3.6.7c**). Consistent with the observation by qRT-PCR, VEGF-A protein levels were substantially reduced in *Lrg1*^{-/-} mice in different treatment groups (**Figure 3.6.7d**). However, both the TAC surgery and the establishment of diabetes caused a significant suppression on VEGF-A protein expression (**Figure 3.6.7d**), suggesting that the VEGF-A was post-translationally regulated during diabetes-associated cardiac remodeling. The reduced protein levels of both LRG1 and VEGF-A may be collectively responsible for the reduced angiogenic potential observed in diabetic mice.

Figure 3.6.7. Characterization of LRG1 and VEGF-A Expression in the Mouse Hearts 6-weeks Post-TAC

Cardiac tissues of diabetic and non-diabetic WT and *Lrg1*^{-/-} mice were subjected to analysis of (a) LRG1 mRNA, (b) LRG1 protein, (c) VEGF-A mRNA and (d) VEGF-A protein levels at 6-weeks post-TAC induction. The changes in gene expression were calculated using fold change ($-2(\Delta\Delta Ct)$) while changes in protein expression were calculated using GAPDH as loading control, and an internal standard as a process control. All results were tabulated and presented as mean $\pm$ SEM. $n \geq 6$ animals in each experimental group. Statistical significance was determined by one-way ANOVA followed by post-hoc Tukey test where * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Besides of the changes in CSA, vascular density and associated molecular markers, the *Lrg1*^{-/-} mice also exhibited an increase in TAC-induced perivascular fibrosis under normoglycemic condition (**** $P < 0.0001$ across all groups) as demonstrated by Masson's trichrome staining (**Figure 3.5.7a** and **Figure 3.5.7b**). Moreover, the induction of perivascular fibrosis was further exacerbated (**** $P < 0.0001$) with the induction of diabetes by STZ. In summary, increased CSA, reduced vascular density and elevated perivascular fibrosis all collectively contributed to worse survival and maladaptive cardiac remodeling observed in TAC-operated *Lrg1*^{-/-} mice under diabetic condition.

Figure 3.6.8. Characterization of Perivascular Fibrosis in the Mouse Hearts 6-weeks Post-TAC

(a) Representative images and (b) quantitative analysis of perivascular fibrosis as indicated in blue by Masson's trichrome staining of the cross-sections of the whole heart in buffer or STZ-injected WT and *Lrg1*^{-/-} mice (n=6). Images were manually analysed, and data were presented as mean $\pm$ SEM. Statistical significance was determined by one-way ANOVA followed by post-hoc Tukey test where * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

3.7. Role of LRG1 in Human Heart Failure

The exaggerated maladaptive myocardial remodelling observed in *Lrg1*^{-/-} mice suggests a cardio-protective role of LRG1. Interesting, we observed a reduced LRG1 level in left ventricular (LV) samples of diabetic patients with IHDs, as compared to their age-matched donors (**Figure 3.6.8a-b**). As expected, we observed a reduced VEGF-A protein levels in diabetic IHDs patients (**Figure 3.6.8c**). In summary, our data demonstrated that reduced LRG1 and VEGF protein levels are associated with diabetes-associated IHDs in both mice and humans.

Figure 3.7.1. Characterization of LRG1 and VEGF-A Expression in LV Samples from IHDs Patients
(a) Representative immunoblot of LRG1 and VEGF-A (top) and relative quantification of (b) LRG1 and (c) VEGF-A protein expression (bottom). Each experimental group contains at least 5 human patient samples ($n \geq 5$). The changes in protein expression were calculated using GAPDH as loading control, and an internal standard as a process control. The findings were presented as mean $\pm$ SEM. All results were tabulated and presented as mean $\pm$ SEM. Statistical significance was determined by one-way ANOVA followed by post-hoc Tukey test where * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

3.8. Biological Role of LRG1 Beyond Angiogenesis

Besides changes in TAC-induced cardiac dysfunction and remodeling, we also observed substantial differences in BW at 34-weeks-of age (**Figure 3.8.1a-b**). Notably, the WT and *Lrg1*^{-/-} mice in sham-operated, non-diabetic control mice demonstrated a significant difference (*** $P > 0.001$) in their BW recorded at the experimental end-point of our TAC-induced HF model (**Figure 3.8.1a**). This observation was further confirmed in a separate study using age and gender-matched WT and *Lrg1*^{-/-} mice over a time course of 36 weeks suggesting a potential metabolic phenotype in *Lrg1*^{-/-} mice (**Figure 3.8.1b**).

Figure 3.8.1. Characterization of progressive BW changes in WT and *Lrg1*^{-/-} mice.

(a) changes in BW in 38-week old WT and *Lrg1*^{-/-} mice (b) Changes in BW of WT and *Lrg1*^{-/-} mice over a time course of 36 weeks. Data were presented as mean $\pm$ SEM; $n \geq 6$ mice per group. Statistical significance for the endpoint comparison of mice BW was determined by one-way ANOVA followed by post-hoc Turkey test, while the significance for the continuous monitoring of BW evolution was performed computed by two-tailed unpaired student's t test at every time point, where * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ are deemed as significant.

To investigate and explain for the potential differences in metabolic homeostasis between the WT and *Lrg1*^{-/-} mice, we performed continuous FBG monitoring study and the findings revealed no difference in FBG levels between WT and *Lrg1*^{-/-} mice at the 7-, 12-, 16- and 28-weeks of age (**Figure 3.8.2a**). However, the glucose tolerance test (GTT) revealed that *Lrg1*^{-/-} mice suffered from impaired GTT when compared to their age and gender-matched WT counterparts (**Figure 3.8.2b**). Interestingly, the data suggested plausible glucose metabolism defect causing in a lower glucose clearance in the *Lrg1*^{-/-} mice when compared to the WT, hence strengthen our conviction that LRG1 may elicit a role in metabolic regulation.

Subsequently, to further understand the potential metabolic phenotype in *Lrg1*^{-/-} mice, we performed a comprehensive analysis on whole body metabolism. The extrapolated data were robust and reproducible with mice displaying a typical circadian rhythm with recognizable general respiration indexes and metabolic functions features in typical light/dark cycle. Remarkable, we noted that the *Lrg1*^{-/-} mice exhibited distinctly reduced metabolic activity when compared to their age-match (8-months of age) WT counterparts (**Figure 3.8.3a-d**). Firstly, the indirect calorimetry readings revealed higher volume of inspired oxygen (**Figure 3.8.3a**) and volume of expired carbon dioxide expired in *Lrg1*^{-/-} mice (**Figure 3.8.3b**). The ratio of VCO_2/VO_2 was calculated to obtain the Respiratory Exchange Ratio (RER) (**Figure 3.8.3c**), which is later normalized to the mice BW (**Figure 3.7.3d**). The RER plot offers compelling evidence that the fuel consumptions between the *Lrg1*^{-/-} mice and WT mice were markedly different in their active periods (dark cycle) (**Figure 3.8.3c**), that the *Lrg1*^{-/-} mice preferentially relies fat oxidation rather than glycolysis. In addition, similar to earlier reports, continuous BW measurement revealed difference between WT and *Lrg1*^{-/-} mice (*** $P < 0.001$). However, there was no significant difference in the daily average RER readings (**Figure 3.8.3d**). Notably, the increased BW in *Lrg1*^{-/-}

mice is not related to increased food or water consumption (**Figure 3.8.3e-h**). Furthermore, it is crucial to highlight that *Lrg1*^{-/-} mice are significantly more active in both the light and dark cycles when compared to WT counterparts, and the increase activity level cannot counter-balance the BW gain. Therefore, taken together, it is evident that LRG1 may exert novel metabolic regulatory roles that has never been reported before.

Figure 3.8.2. Characterization of Blood Glucose Changes in WT and *Lrg1*^{-/-} mice.

(a) Continuous monitoring of FBG at 7-, 12-, 16- and 28-week old WT and *Lrg1*^{-/-} mice (b) glucose tolerance test on 28-week old WT and *Lrg1*^{-/-} mice and (c) quantitative analysis of the area under curve (AUC). Data were presented as mean $\pm$ SEM; $n \geq 8$ mice per group. Statistical significance for the endpoint comparison of mice BW was determined by one-way ANOVA followed by post-hoc Turkey test, while the significance for the continuous monitoring of BW evolution was performed computed by two-tailed unpaired student's t test at every timepoint, where * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ are deemed as significant.

Figure 3.8.3. Characterization of Systemic Metabolism in WT and *Lrg1*^{-/-} Mice.

Tabulated data for (a) volume of oxygen inspired, (b) volume of carbon dioxide expired, (c) Respiratory Exchange Ratio (RER), (d) BW/RER correlation analysis, (e, g) food consumption plots and relative quantification, (f, h) drink consumption plots and relative quantification. (i, k), horizontal activity plots and its relative quantification (i-l) vertical activity levels and its relative quantifications. Indexes were expressed as an average per day, or per phase of the day (light/dark phase). Each experimental group contains at least 8 animals (n=8). The data were presented as mean ± SEM. Statistical significance for was calculated by SPSS using determined by two-tailed unpaired student's t test and ACNOVA analysis, where **P*<0.05, ***P*<0.01, ****P*<0.001 are deemed as significant.

Chapter 4: Discussion

4.1. Role of LRG1 in Regulating Physiological Angiogenesis

Angiogenesis plays a critical role in embryonic development and physiological processes such as wound healing. The defective angiogenic response following acute myocardial infarction is responsible for worse ischemic injury and poor prognosis. Promoting functional blood vessel formation is considered as an attractive strategy to restore blood perfusion to the ischemic tissue and improve the cardiac functions³⁹⁶. New blood vessel formation is tightly regulated in a spatial and temporal manner³⁵⁸ and VEGF is a master regulator in this process¹⁰⁹. Although preclinical studies had demonstrated a great efficacy of VEGF-mediated therapeutic angiogenesis, the result in clinical trials has not been very promising, except for safety^{397,398}. In addition to angiogenesis, VEGF also confers potent vascular permeability effects³⁹⁹. Myocardium oedema has been observed in patients subjected to VEGF-A mediated therapeutic angiogenesis⁴⁰⁰. This is not surprising since angiogenesis is a tightly regulated process involving coordinated interactions between different types of cells, growth factors and ECM components^{37,345,358}. Combinational treatment with multiple angiogenic factors may be necessary to achieve optimal therapeutic outcomes^{345,401}.

LRG1 is a novel angiogenic factor that was previously demonstrated to regulate ocular angiogenesis through mediating the endothelial TGF- β signalling pathway¹⁶⁴. However, it is important to highlight that angiogenesis is a highly-context-dependent process and the response of different types of ECs to angiogenic stimuli could be highly different⁴⁰². In this study, we evaluated LRG1's effect in cardiac-specific microenvironment using a number of *in vitro* and *ex vivo* functional assays and revealed a promoting effect of LRG1 on HHECSs' survival, proliferation, migration and its ability to assemble into tubular network, which is largely consistent with previous findings on LRG1's role in the brain¹⁶⁵, kidney glomerulus⁴⁰³, eyes^{164,404} and tumorigenesis^{184,188,193,204,405}. We further confirmed that the pro-angiogenic effect of LRG1 in cardiac-specific *ex vivo* spontaneous tube formation assay and metatarsal assay.

It is worth mentioning that the angiogenic potential of LRG1 is comparable to that of VEGF₁₆₅. Given the unfavourable clinical outcome of current single angiogenic factor therapy, a combinatorial therapy regime targeting multiple angiogenic pathways may result in a better therapeutic outcome³⁴⁷. To our surprise, simultaneous treatment of LRG1 and VEGF₁₆₅ did not result in a further increase in angiogenesis under normal physiological conditions. One possibility is that LRG1 and VEGF may signal through a common pathway in HHECSs.

4.2. Role of LRG1 in Angiogenic Signal Transduction

LRG1 was previously demonstrated to regulate the switch of the quiescent TGF- β 1-ALK5-Smad2/3 pathway towards the proangiogenic TGF- β 1-ALK1-Smad1/5/8 pathway¹⁶⁴. Using combinations of gain-of-function and lost-of-function approach, this observation was confirmed in HHECSs. Our previous study in a mouse model of oxygen-induced retinopathy (OIR) demonstrated a concomitant suppression of LRG1 and VEGF-A expression in the retina subjected to hyperoxia but an upregulated expression of LRG1 and VEGF-A in the retina with excessive neovascularization¹⁶⁴. Consistently, there is a reduced VEGF-A expression in *Lrg1*^{-/-} mouse retina¹⁶⁴. More recently, histological analysis of retinoblastoma tumours revealed a co-localization between LRG1 and VEGF-A in tumour vasculature by immunofluorescence staining²⁰⁴. In a separate study, both LRG1 and VEGF-A expression were up-regulated in ischemic rat brain at 12-hours and 24-hours following middle cerebral artery occlusion⁴⁰⁶. Together these data suggest a potential link between LRG1 and VEGF signalling pathway. VEGF-A exerts its proangiogenic effect mainly through its cognate VEGFR2^{118,407}. Moreover, TGF- β -Smad4 was previously reported to regulate the expression of VEGF and its receptor in various types of cells, including ECs, under hypoxia condition^{125,408}. Our study confirmed the previous findings and validated that LRG1 is able to induce the expression of both VEGF-A and VEGFR2 in a Smad4-dependent manner.

Recent studies demonstrated that VEGF-induced angiogenesis is dependent on EC-specific type III TGF- β receptor, Eng and VEGF-induced vascular outgrowth from spheroids was significantly attenuated in Endoglin-knockdown endothelial cells and the vascular outgrowth from Eng^{+/-} fetal metatarsal explants⁴⁰⁹. Moreover, treatment of Eng neutralizing antibody also resulted in prominently decreased VEGF-induced EC sprouting⁴⁰⁹. In addition, Eng was reported to interact with VEGFR2 directly through its cytoplasmic domain *via* a VEGF-dependent manner and potentiates the VEGF-VEGFR2 signaling⁴¹⁰. Furthermore, our previous study showed that Endoglin functions as a major binding partner for LRG1 and it plays an important role in LRG1-mediated switch of TGF- β signalling in ECs¹⁶⁴. It will be interesting to know whether LRG1 physically interacts with VEGFR2 and whether this process involves Eng. It will also be interesting to see whether LRG1 is required for the interaction between Endoglin and VEGFR2 and its role in Eng-mediated VEGF signalling as well as angiogenesis. On that note, we hope to investigate whether Endoglin is involved in the LRG1-regulated expression of VEGF signalling pathway components and angiogenesis in the future follow-up studies.

Besides inducing the expression of VEGF and VEGFR2 in HHECSs, we found that LRG1 promotes the phosphorylation of VEGFR2. We further showed that the promoting effect of LRG1 on VEGFR2 phosphorylation is attenuated in the presence of Aflibercept, a biopharmaceutical VEGF-trap that contains the ECD of human VEGFR1 and VEGFR2 that are fused to the Fc portion of the human IgG1 immunoglobulin⁴¹¹⁻⁴¹³. Aflibercept has been shown to bind VEGF-A in a dose-dependent manner⁴¹⁴. Our study showed that the promoting effect of LRG1 on VEGFR2 phosphorylation is attenuated in the presence of Aflibercept suggesting the requirement of VEGF-A in LRG1-induced VEGFR2 phosphorylation. It is possible that LRG1 exerts its function by promoting the recruitment of VEGF-A to its cognate type 2 receptor. To confirm that, we will carry out the GST-pulldown assay or co-immunoprecipitation to evaluate the binding between VEGF-A and VEGFR2 in the presence or absence of LRG1. In addition, our study showed that treatment with ALK1 inhibitor, LDN193189, also attenuated the LRG1-induced phosphorylation of VEGFR2 and its downstream signalling transducer suggesting that LRG1 may regulate VEGFR2

phosphorylation indirectly through TGF- β 1 signalling and subsequent induction of VEGF-A and VEGFR2 expression. In summary, our findings provided further experimental evidence regarding the TGF- β -dependent and -independent LRG1 role in HHECSs signalling.

4.3. Dysregulated Angiogenesis in Diabetes

DM is a common comorbidity associated with IHDs and it is a key factor affecting the efficacy of therapeutic angiogenesis^{272,273}. Critically, continuous hyperglycemia in long-standing DM majorly perturbs vascular homeostasis and leads to endothelium dysfunctions^{270,271}, mainly attributable to increase accumulation of oxidation stress and advanced glycated end-products (AGEs)²⁷⁹. Moreover, the mural cells respond to DM insults by attaining a de-differentiated and hyperproliferative phenotype leading to ECM accumulation and accelerate atherosclerosis progression³⁰¹. In this study, we established in vitro models of diabetes using HG and gCol and demonstrated impaired HHECS function, such as reduced survival, proliferation, migration and tube formation. We also showed an impaired vessel formation in cardiac-specific *ex vivo* spontaneous tube formation assay and metatarsal assay. Interestingly, we consistently observed an elevated VEGF-A mRNA levels but a reduced VEGFR2 mRNA level in HHECSs subjected to HG and gCol treatment.

In support of our observation, elevated serum VEGF-A levels were elevated in the diabetic patient with a poorer prognosis, whereby the source of elevated VEGF-A is postulated to be derived from the circulating inflammatory cells instead of ECs⁴¹⁵. Under DM, high VEGF-A was reported to cause further damages to endothelium by inducing the production of reactive oxygen species (ROS) and further activation of critical proinflammatory cytokines like NF- κ B, tumour necrosis factor- α (TNF- α), Interleukin-6 (IL-6) and vascular cell adhesion protein1 (VCAM-1)^{416,417}. Diabetes was also reported to promote ligand-independent phosphorylation of VEGFR2 leading to progressive loss of VEGFR2 at the cell surface. The reduced VEGFR2 expression in challenged

HHECS may explain the failure of inducing neovascularization by exogenous VEGF in diabetic patients.

4.4. LRG1 for Therapeutic Angiogenesis in Diabetes

In this study, we evaluated the pro-angiogenic role of LRG1 under the diabetic condition, including hyperglycemia and the presence of AGEs. Despite the detrimental effect of diabetes on the vasculature, our study showed that LRG1 is still able to promote the activation of endothelial cells and leads to increased blood vessel formation under the diabetic condition in a number of *in vitro* and *ex vivo* angiogenesis assays. Once again, the pro-angiogenic effect of LRG1 is comparable to that of VEGF-A. Although, there is no synergistic angiogenic effect between LRG1 and VEGF under physiology state, the co-treatment elicited a much stronger angiogenic effect than each growth factor on its own in *ex vivo* spontaneous tube formation assay using cardiac tissue isolated from WT mice subjected to STZ-induced DM, which could be explained, at least in part, by LRG1's effect on VEGFR2. Under physiological conditions, there is a sufficient amount of VEGFR2 on ECs' membrane and the amount of VEGF-A is a limiting factor for the response of ECs to angiogenic cues. Although LRG1 is able to induce the mRNA levels of VEGF-A and VEGFR2, its impact on angiogenesis through mediating VEGF signalling will be trivial when there is sufficient amount of exogenous VEGF-A. This explains the lack of combinational angiogenic effective between LRG1 and VEGF-A under physiological condition. Diabetes, on the other hand, affects not only the expression but also the membrane association of VEGFR2⁴¹⁸. In this case, the availability of VEGFR2 on EC surface is the rate-limiting factor, which at least partially, responsible for the compromised angiogenic response despite the elevated levels of VEGF-A in diabetic patients. Hence, this may account for the limited clinical benefits of therapeutic angiogenesis mediated by VEGF-A under diabetes. Treatment with LRG1, not only promotes angiogenesis through TGF- β -dependent pathway, but also leads to increased VEGFR2 expression and phosphorylation. Hence, significantly increases the efficiency of VEGF signalling and further increases the VEGF-induced angiogenic effect.

4.5. Role of LRG1 in Heart Failure

Elevated plasma LRG1 levels were positively correlated with arterial stiffness, endothelial dysfunction, and PAD in diabetic patients³⁷⁶. Increased plasma LRG1 levels were also reported in human patients with IHDs⁴¹⁹. A recent publication claimed that the infiltrating myeloid cells serve as a major source of LRG1 following acute MI in the heart¹⁷⁰. Controversially, despite the elevated circulating level of LRG1 in diabetic mice, our data demonstrated a reduced LRG1 expression in the heart of WT mice subjected to STZ-induced diabetes, which was further suppressed following TAC surgery. On the contrary, VEGF-A expression was induced by both diabetes and TAC surgery in the wild-type mouse heart. This finding is consistent with previous observations of elevated circulating levels of VEGF in diabetes patients⁴¹⁵ and the activated angiogenic process in response to heart ischemia³⁵⁰.

To understand the role of LRG1 in diabetes-associated IHDs, TAC-induced heart failure was carried out in wild-type and *Lrg1*^{-/-} mice subjected to STZ-induced diabetes. Notably, while relatively young mice (~8-weeks old) have been routinely used in most TAC-induced heart failure studies, and these mice tend to tolerate induced-HF better and the finding might not be relevant to human IHDs, which the disease typically manifests during middle-age⁴²⁰. Given that, we purposely chose our experimental endpoint at 34-weeks of age as mice at this age is deemed as early mild-age mice, which more accurately phenocopies the human IHDs.

Interestingly, diabetic *Lrg1*^{-/-} mice demonstrated a higher TAC-induced mortality compared to diabetic wild-type mice. It is worth to note that the survival rate of diabetic *Lrg1*^{-/-} mice is only 28%. It is likely that the mice suffering from more severe phenotype didn't survive till the experimental endpoint. Furthermore, the hearts isolated from diabetic *Lrg1*^{-/-} mice following TAC surgery are larger in size, heavier and demonstrated increased cardiomyocyte hypertrophy with reduced vascular density and increased perivascular fibrosis, all of which indicate more severe maladaptive cardiac

remodelling in *Lrg1*^{-/-} mice. Moreover, consistent with the reduced vascular density, the expression level of VEGF-A was significantly reduced in *Lrg1*^{-/-} mice in different treatment groups compared to WT counterparts, further substantiating the key role of LRG1 in mediating angiogenesis following TAC-induced cardiac remodelling.

Besides its role in angiogenesis, TGF- β 1 is a potent regulator of tissue fibrosis^{229,421}. Interestingly, worse TAC-induced perivascular fibrosis was observed in *Lrg1*^{-/-} mice as compared to its WT counterparts, which was further exaggerated under diabetic condition suggesting a negative regulatory role of LRG1 in cardiac fibrosis. Perivascular fibrosis is characterized by excessive deposition of connective tissue around the blood vessels and has been demonstrated to promote cardiac dysfunction in human studies⁴²². It contributes to impaired coronary blood flow and oxygen diffusion to the surrounding myocardium. Furthermore, perivascular fibrosis is a strong prognostic indicator correlated with the mortality of HF patients⁴²³. Our previous study demonstrated a direct interaction between LRG1 and pro-fibrotic TGF- β receptor, ALK5¹⁶⁴. Unlike ECs, most cell types, including cardiac fibroblasts, response to TGF- β signalling through ALK5-Smad2/3 axis. It is possible that LRG1 and ALK5 interaction prevents the access of TGF- β to its receptors, therefore, leading to reduced activation of downstream signalling transducers and pro-fibrotic effects. To confirm that, we will carry out GST-Pulldown or co-immunoprecipitation assay to study the impact of LRG1 on the binding kinetics between TGF- β and various TGF- β receptors as well as the activation of downstream signalling transducer, cardiac fibroblast proliferation, migration and contractility. Besides its role in regulating TGF- β signalling pathway, LRG1 has been reported to be a direct target gene of the peroxisome-proliferator-activated receptor β/δ (PPAR β/δ)¹⁶⁹ and PPAR β/δ has been reported in tissue fibrosis in different animal models, including cardiac fibrosis⁴²⁴⁻⁴²⁶, it will be interesting to study whether LRG1 is required for PPAR β/δ –mediated cardiac fibrosis.

Besides perivascular fibrosis, TAC was reported to induce ECM deposition in the cardiac interstitium, which represents a major cause of increased stiffness of the ventricle, impaired transduction of cardiomyocyte contraction and

reduced tissue perfusion^{392,427}. However, we were unable to identify major changes regarding interstitial fibrosis in our mouse model of TAC-induced cardiac remodelling. As the development and progression of cardiac fibrosis may be strain-dependent, we would like to do a time-course study in the future to identify a suitable time point for investigating the role of LRG1 in interstitial fibrosis.

Despite our initial molecular and histological characterization, it is not sure whether these structural changes are sufficient to cause impaired cardiac function. Hence in the future, we would like to carry out echocardiogram and pressure-volume loop analysis to further investigate the functional impact of LRG1 on heart failure.

Together, our data demonstrated a worse diabetes-associated cardiac remodelling in *Lrg1*^{-/-} mice as compared to its WT controls, suggesting a cardiac protective role of LRG1. To confirm this notion, we would like to employ adenovirus vector-mediated *Lrg1*-overexpression in the heart and study its consequence on diabetes-associated maladaptive cardiac remodelling in the future work.

4.6. LRG1's Biological Roles Beyond Angiogenesis

It was previously reported that circulatory LRG1 level is positively associated with obesity¹⁷¹. However, the BW of *Lrg1*-deficient mice is significantly heavier than age- and gender-matched WT control mice. In addition, the *Lrg1*^{-/-} mice also exhibits a retarded glucose clearance when subjected to IPGTT thus suggesting a potential underlying metabolic impairment.

Interestingly, there is an intimate association between adipocyte and the vasculature in the adipose tissue⁴²⁸. Remarkably, both the vasculature and the adipose tissues are high plastic and can rapidly response to environmental cues, resulting in constant vascular and adipocyte expansion and/or regression throughout the entire lifetime⁴²⁹. Despite of the limited understanding in the molecular mechanism of adipose angiogenesis, the possibility of targeting the

adipose vasculature for the treatment of obesity and metabolic disease has been explored^{428,430}. Furthermore, VEGF-A has been demonstrated to play a crucial role in adipose tissue homeostasis and metabolic dysfunction. VEGF-A overexpression in white adipose tissue (WAT) has been shown to promote angiogenesis leading to the rapid appearance of beige fat cells caused by “beiging effect” in subcutaneous WAT^{428,430}. VEGF-A overexpression in the brown adipose tissue (BAT) has also been reported to facilitate vascularization and thermogenesis during chronic cold exposure and protects against systemic metabolic dysfunction induced by a high-fat diet (HFD) challenge⁴³¹. On the other hand, inhibition of VEGF-A–induced activation of VEGFR2 during the early phase of high-fat diet-induced weight gain causing systemic insulin resistance^{432,433}. Considering the important role of LRG1 in angiogenesis and the role of VEGF in LRG1-mediated angiogenic response, it is likely that LRG1 also participates in regulating adipose tissue angiogenesis, hence promotes lipolysis and browning in adipose tissue.

The propensity for a lean or obese phenotype in an individual is determined by the balance between energy uptake and energy expenditure. Chronic excessive energy uptake and reduced expenditure lead to increased energy storage in the form of lipids within adipocytes, obesity and metabolic diseases in the long run. Interestingly, *Lrg1*^{-/-} mice had high activity levels yet excessive adipose storage, which is not related to the difference in food intake. Taken together, our finding highlights a potential metabolic phenotype of *Lrg1*^{-/-} mice, which is worth further investigation as a part of our future work.

Chapter 5: Conclusion

Interest in the field of vascular angiogenesis has grown exponentially in the recent years and the vasculature is considered as a major gatekeeper governing physiological and pathological status. This project validated LRG1-mediated angiogenesis in cardiac-specific *in vitro* and *ex vivo* models. Besides its well-established role in regulating the endothelial TGF- β signalling, our study demonstrated a regulatory role of LRG1 in the expression of VEGF signaling pathway components and VEGFR2 phosphorylation. Furthermore, we have demonstrated a potential involvement of LRG1 in cardiac hypertrophy and fibrosis. Targeting LRG1 may offer a unique opportunity to control multiple pathologies leading to maladaptive cardiac remodeling. Interestingly, we also observed a remarkable metabolic syndrome of *Lrg1*^{-/-} mice. A deeper understanding of LRG1-regulated cell metabolism will facilitate the on-going development of LRG1-targeted therapeutics.

Supplementary Figures

Supplementary Figure 1. PCR Products for Mice Genotyping

To confirm the genotype of the mice colonies, endpoint PCR-based genotyping was done. The expected PCR products for the wildtype mice would be a 693bp band while the expected PCR products for the *Lrg*^{-/-} mice would be a 307bp band. When heterozygous breeders were involved, the heterozygous progenies will carry both the 693bp band and 307 bp products.

Supplementary Figure 2. Schematic Overview of the Animal Model

Mice between 6–8 weeks of age were subjected to STZ-induced diabetes. Mice will be screened 4- and 8- weeks later to confirm the development of hyperglycemia. Mice may be subjected to a repeated cycle of STZ-injection if hyperglycemia did not manifest. Diabetic status was confirmed at 28-weeks of age, prior to the TAC procedure and mice will be sacrificed at 6-weeks post-TAC induction.

Supplementary Figure 3. Validation of the Collagen I Glycation.

(a) Coomassie blue stained SDS-PAGE effectively resolve the differentially glycosylated products and demonstrated increased band shifts with prolonged incubation of Collagen I with methylglyoxal (MGO). (b) Positive glycation was further validated by western blotting using an anti-MGO antibody.

Supplementary Tables

Supplementary Table 1. List of Growth Factors and Inhibitors

Name	Reconstituted in	Treatment Concentration	Source, Reference
Growth factors			
CHO cells-derived recombinant human (TGF- β 1)	Citric acid	5ng/ml	PreproTec, #100–21, USA
Animal-free (<i>E.coli</i> -derived) recombinant VEGF-165	0.1% BSA	50ng/ml	PreproTec, #100–20, USA
Freestyle™ cells-derived Recombinant human LRG1 (LRG1)	PBS	20 μ g/ml	In-house preparation
Signalling inhibitors			
ALK1 inhibitor, LDN193189	DMSO	100nM	SML0559, Sigma–Aldrich
ALK5 inhibitor, SB431542	DMSO	10 μ M	S4317, Sigma–Aldrich
Humanised monoclonal antibody against LRG1, Magacizumab	PBS	20 μ g/ml	In-house preparation
VEGFR2 inhibitor, Linifanib, ABT–869	DMSO	5nM	Selleckchem, # S1003, USA
VEGF–Trap, Aflibercept	DMSO	10 μ g/ml	Bayer, Eylea; Germany

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