Roles of leucine-rich alpha-2-glycoprotein 1 in normal and diabetic wound healing

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**ROLES OF LEUCINE-RICH ALPHA-2-
GLYCOPROTEIN 1 IN NORMAL AND DIABETIC
WOUND HEALING**

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**SCHOOL OF CHEMICAL AND BIOMEDICAL
ENGINEERING**

2019

**Roles of Leucine-Rich alpha-2-Glycoprotein 1 in
Normal and Diabetic Wound Healing**

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**SCHOOL OF CHEMICAL AND BIOMEDICAL
ENGINEERING**

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

2019

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SUMMARY

Transforming growth factor beta (TGF β) 1 is a master-regulator of wound healing. Our study shows that Leucine-rich alpha-2-glycoprotein 1 (LRG1), a novel regulator of TGF β 1, is expressed in keratinocytes, endothelial cells and fibroblasts of normal mouse skin tissues. During wound healing, LRG1 is significantly induced at the inflammatory and proliferative phases, but downregulated at the remodeling phase. Consistent with this observation, infiltrated neutrophils, activated keratinocytes and angiogenic endothelial cells are major sources of LRG1 during the wound healing process. Importantly, *Lrg1*^{-/-} mice demonstrate a delayed wound closure in a mouse model of excisional wound healing.

Tumor necrosis factor alpha (TNF- α) is a key mediator of the inflammatory response in wound healing and it exerts its function through activating transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B). Interestingly, TNF- α is able to induce LRG1 expression at both mRNA and protein levels in different types of skin cells *in vitro*. *In silico* analysis reveals two putative NF- κ B binding sites on the *LRG1* promoter region. As demonstrated by ChIP assay, TNF- α promotes the recruitment of NF- κ B to the *LRG1* promoter.

Blood vessel permeability increases significantly to allow inflammatory cytokines and cells to reach the damaged tissue at the inflammatory phase of the wound healing. Miles assay shows an increased dermal vessel leakage in mice subjected to subcutaneous injection of recombinant LRG1 (rLRG1). Consistently, *Lrg1*^{-/-} mice are resistant to recombinant vascular endothelial growth factor (rVEGF)-induced dermal vessel leakage and demonstrated a reduced neutrophil infiltration one-day after the injury. Mechanistically, we show that rLRG1 disrupts the membrane distribution of endothelial adherens junction protein, VE-cadherin, leading to increased permeability of dermal endothelial cells via activating src family kinase (SFK) pathway.

Besides the compromised inflammatory response, epithelialization in *Lrg1*^{-/-} mice is seriously delayed, which is accompanied by a reduced number of proliferating keratinocytes in the wound edge. Consistently, LRG1 promotes cell sheet migration of keratinocytes by attenuating the anti-proliferative TGFβ1/Smad2 signalling *in vitro*.

In agreement with the important role of LRG1 in angiogenesis, we observed a reduced vessel density in granulation tissue of the wound bed. Consistently, rLRG1 significantly induces HDMEC proliferation, motility and tube formation through regulating the canonical TGFβ1/Smad1/5/8 signalling pathway.

Interestingly, there is an increased expression of fibrotic markers at the remodelling phase of wound healing in *Lrg1*^{-/-} mice. Increased fibrosis is also observed in the skin of *Lrg1*^{-/-} mice subjected to bleomycin-induced dermal fibrosis. Consistently, mouse embryonic fibroblasts (MEFs) isolated from *Lrg1*^{-/-} mice are more sensitive to TGF β1-induced expression of fibrotic markers and contractility as compared to wild-type MEFs. On the other hand, TGFβ1-induced activation of dermal fibroblasts is significantly attenuated by treatment with rLRG1.

Despite the important role of LRG1 in wound healing under the physiological condition, the speed of wound closure is much faster in *Lrg1*-deficient mice under diabetic conditions, which is, at least in part, due to the reduced inflammatory response. In addition to the reduced number of infiltrated neutrophils, *Lrg1*-deficient neutrophils are less susceptible to form neutrophil extracellular traps (NETs). Consistently, rLRG1 can induce NET activation and release (NETosis) in human neutrophils by activating extracellular signal-regulated kinases (ERK). Taken together, this study provides new insights into a complex role of LRG1 in normal and diabetic wound healing.

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1. **Chenghao Liu*** et al, Collaborative regulation of LRG1 by TGF β 1 and PPAR β/δ in pressure overload-induced cardiac fibrosis. (Submitted, *co-first author)
2. **Chenghao Liu** et al, Different role of LRG1 in normal and diabetic wound healing. (In preparation)
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7. Beiying Qiu, Alison Tan, Veluchamy Barathi, Yong Li, **Chenghao Liu**, Hannah Murray, Wei Cheng et al. Synthetic Apratoxin S4 inspired by a marine natural product, a new treatment option for ocular angiogenic diseases. (Submitted)

LIST OF ABBREVIATIONS

ALK	Activin receptor-like kinase
Ang	Angiopoietin
ANOVA	Analysis of variance
BALF	Bronchoalveolar lavage fluid
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
CD31/PECAM-1	Platelet endothelial cell adhesion molecule
ChIP	Chromatin immunoprecipitation
CTGF	Connective tissue growth factor
DAPI	4',6-diamidino-2-phenylindole
DFU	Diabetic foot ulcers
DG	D-Glucose
DM	Diabetes mellitus
DMEM	Dulbecco's modified Eagle's medium
EBM2	Endothelial cell growth basal medium-2
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor

EGM2-MV	Microvascular endothelial cell growth medium-2
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-to-mesenchymal transition
ENG	Endoglin
ERK	Extracellular-signal-regulated kinase
FBG	Fasting blood glucose
FBS	Fetal bovine serum
FGF	Fibroblast growth factors
FGM-2	Fibroblast growth medium-2
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GM-CSF	Granulocyte-macrophage colony stimulating factor
H&E	Haematoxylin and Eosin
H3Cit	Citrullinated histone H3
HB-EGF	Heparin-binding EGF-like growth factor
HBOT	Hyperbaric oxygen therapy
HCC	Hepatocellular carcinoma
HDMECs	Human dermal microvascular endothelial cells
HPF	High power field

HRP	Horseradish peroxidase
HUVECs	Human umbilical vein endothelial cells
I-Smads	Inhibitor Smads
IACUC	Institutional Animal Care and Use Committee
ICAM	Intercellular adhesion cellular molecule
IGF-1	Insulin-like growth factor-1
IgG	Immunoglobulin G
IL	Interleukin
JNK	c-Jun N-terminal kinase
KGF	Keratinocytes growth factor
KOMP	Knockout Mouse Project
LAD	Left anterior descending
LLC	Lewis lung carcinoma
LPS	Transforming growth factor-β-activated kinase 1
LRG1	Leucine-rich α-2 glycoprotein 1
<i>Lrg1</i>^{-/-}	Global <i>Lrg1</i> knockout
LRR	Leucine-rich repeat
Ly6G	Lymphocyte antigen 6 complex locus G6D
MCP	Monocyte chemoattractant protein

MEF	Mouse embryonic fibroblasts
MIP1α	Macrophage inflammatory protein 1 alpha
MIS	Mullerian inhibitory substance
MMPs	Matrix metalloproteinases
MPO	Myeloperoxidase
mTOR	mammalian target of rapamycin
MTS	5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazoly)-3-(4-sulfophenyl)tetrazolium, inner salt
NACLAR	National Advisory Committee for Laboratory Animal Research
NETs	Neutrophil extracellular traps
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NG	Normal glucose
NHDFs	Normal human dermal fibroblasts
OC	Osmotic control
ORC	Oxidized regenerated cellulose
p38 MAPK	p38 mitogen-activated protein kinase
PAD4	Peptidylarginine deiminase 4
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor

PEDFs	Pigment epithelium-derived factors
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear neutrophils
PPAR	Proliferator-activated receptor
qRT-PCR	Quantitative real time PCR
R-Smads	Regulated Smads
rLRG1	Recombinant LRG1
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
rVEGF	Recombinant vascular endothelial growth factor
SDS	Sodium dodecyl sulfate
SEM	Standard error of mean
SFK	Src family kinases
Smurfs	Smad ubiquitination regulatory factors
STZ	Streptozotocin
TAK1	Transforming growth factor-β-activated kinase
1	

TEM	Transendothelial migration
TGF	Transforming growth factor
TGFβR	Transforming growth factor beta receptor
Tie2	Angiopoietin-2 receptor-2
TIMP	Tissue inhibitor of metalloproteinase
TLRs	Toll-like microbial pattern recognition receptors
TNF	Tumor necrosis factor
TSS	Transcription initiation site
VE-Cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth factor receptor
αSMA	Alpha-smooth muscle actin

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Chapter 1 Background

1.1 Wound Healing

Wound healing is a fundamental physiological process that is important for tissue homeostasis. It consists of three consecutive phases, including inflammatory phase, proliferative phase and tissue remodeling phase (**Figure 1**) [1-4]. Many types of cells work in a highly orchestrated fashion to achieve successful tissue repair. It begins with hemostasis by temporary plugging damaged blood vessels and the barrier layer with a fibrin clot. Subsequently, innate immune cells are recruited from the circulation and adjacent tissue. This is followed by re-epithelialization to close the wound margins, and reconstitution of dermis by fibroblasts to form a temporary and highly vascularized wound granulation tissue. Realignment of disorganized matrix and removal of endothelial cells, myofibroblasts and macrophages occur in the following remodeling phase to strengthen the newly formed tissue. Defects in each of these processes will lead to impaired wound closure.

A

B

C

Figure 1. The cutaneous wound healing consists of three overlapping phases. A, Inflammatory phase. Neutrophils, macrophages, mast cells are recruited to the injured site to exert their immune functions. **B**, Proliferative phase. Fibroblasts, endothelial cells and keratinocytes commence proliferation, differentiation and migration to cover the wound and form granulation tissue. **C**, Tissue remodeling phase. Newly formed skin tissue is remodeled to restore the normal skin structure. (Adapted from Nature volume 453, pages 314–321 (15 May 2008) [4])

1.1.1 Inflammatory phase of wound healing

The inflammatory phase commences immediately after tissue injury [4]. It is initiated by blood clotting within the first few minutes after injury. The wound gap will be filled with a provisional wound matrix containing fibrin molecules, fibronectin, vitronectin and thrombospondins loaded with platelet, which serves to plug the breached vessels and stop further bleeding [5]. Growth factors and cytokines released by platelets and disrupted cells trigger vasodilation and increased vessel permeability, which facilitate the recruitment of a large number of leukocytes to the wound bed to combat pathogens and remove dead skin cells [6]. Neutrophils are the most abundant leukocytes in the blood and they lead the first wave of host defense to tissue damage. Within few hours after injury, neutrophils are recruited to the wound bed from nearby blood vessels through a sequential, multistep cascade [7]. It starts with an initial contact of floating neutrophils with the activated vascular endothelium, followed by the rolling of neutrophils along the vessel wall [8]. This process is mediated by endothelial expressed E- and P-selectin and neutrophil expressed L-selectin and E- and P-selectin ligands [8]. A delayed wound healing is observed in mice with deficient L-selectin due to compromised leukocyte recruitment [9, 10]. The firmly adhered neutrophils will then transmigrate through the endothelial cells, a process mediated by several endothelial transmembrane proteins, including intercellular adhesion cellular molecule-1, -2 (ICAM-1, -2), and vascular cell adhesion molecule 1 (VCAM-1) [11]. It also requires an increase in permeability of the endothelium, which is partly achieved by the disruption

of the membrane distribution of endothelial cell junctional proteins, such as vascular endothelial cadherin (VE-cadherin) [11, 12]. Src and Pyk2 kinases can phosphorylate tyrosine residues of VE-cadherin, leading to its disassembly, and facilitate transendothelial migration (TEM) of neutrophils [13].

Neutrophils mediate tissue damage through several strategies. A plethora of antimicrobial substances, such as myeloperoxidase (MPO), reactive oxygen species (ROS), cationic antimicrobial proteins are released by infiltrated neutrophils to kill invading microorganisms [7]. Neutrophils are also capable of engulfing and digesting microbial invaders through phagocytosis [7]. As a part of the antimicrobial defence mechanisms, neutrophils undergo programmed death and release their nuclear and granular contents to form neutrophil extracellular traps (NETs) [14], a process that is activated by pathogens through Toll-like receptors, Fc receptors or complement receptors [15]. Inflammatory cytokines and chemokines, such as IL8, TNF, and IFN- γ , have also been reported to trigger NETs formation, also known as NETosis [16]. Following the activation of respective receptors, various kinases such as mitogen-activated protein kinase (MEK), extracellular signal-regulated kinases (ERK), phosphoinositide 3-kinase (PI3K), protein kinase C (PKC) and protein kinase B (Akt) will be phosphorylated to mediate the downstream signaling transduction [17]. During degranulation, neutrophils secrete various proteases, which are critical for degrading extracellular matrix (ECM) components, and pave the way for subsequent migration of other types of skin cells into the wound bed [7]. Consistent with the fundamental role of neutrophils at the inflammatory phase of wound healing, reducing neutrophil infiltration by deleting formylpeptide receptors results in a delayed wound healing *in vivo* [18]. Similarly, aged mice with neutropenia, a condition characterised with an abnormally low level of neutrophils, show delayed wound healing [19]. Despite being indispensable to host defence against infection, free radicals and NETs released by neutrophils

can also cause collateral damage to host cells [15]. For example, the healing process is accelerated in neutrophil-depleted mice [20].

Following neutrophil infiltration, circulating monocytes will be recruited to the wound site to amplify the inflammatory response [21]. Neutrophils, platelets, and the activated surrounding non-immune cells, keratinocytes and fibroblasts, contribute to the recruitment of circulating monocytes by producing various chemokines, including macrophage inflammatory protein 1a (MIP1 α) and monocyte chemoattractant proteins (MCPs) [6, 22]. As monocytes invade tissues, they mature into macrophages that function to clear matrix and cell debris, particularly red blood cells, spent neutrophils and damaged host cells, through phagocytosis [21]. Macrophages have also been proven to be a sustained source of numerous cytokines and growth factors, including transforming growth factors (TGFs), insulin-like growth factor 1 (IGF-1), platelet-derived growth factor (PDGF), vascular endothelial growth factors (VEGFs), tumor necrosis factors (TNFs) and interleukins (ILs) [23]. They have potent paracrine impacts on the activity of other cell lineages in the following proliferative phase [21]. Consistent with the pleiotropic role of macrophages in wound healing, depletion of macrophages with antimacrophage sera [24], by neutralizing macrophage chemoattractant MIP1 α [25] or by diphtheria toxin-mediated killing of macrophages [26], results in a failure of debridement and retarded healing.

Other immune cells, including dendritic cells, mast cells, Langerhans cells, and T cells, are also required for efficient wound healing [27]. However, the mechanisms of action are less well-understood [28].

1.1.2 Proliferative phase of wound healing

Keratinocytes are the major cellular component of the epidermis, the first barrier of the skin. Re-epithelialization is one of the key steps of

cutaneous wound healing. In response to damage signals such as H₂O₂ and calcium, altered mechanical tension, invaded pathogens and serum exposure [29-34], keratinocytes are rapidly activated and undergo a phenotypical switch to a partial epithelial-to-mesenchymal transition (EMT) phenotype [35, 36]. This renders the keratinocytes at the wound-edge to become migratory, invasive and proliferative [37, 38].

In response to pro-inflammatory signals, such as IL-1 and TNF- α , the connection between neighbouring keratinocytes will be considerably loosened [39], which will provide a space for follower cells to shuffle forward [40]. Meanwhile, the interaction between keratinocytes and the unusual provisional wound ECM triggers the expression of a plethora of proteinases, including collagenase and other matrix metalloproteinases (MMPs) [41, 42]. The adoption of this degradative phenotype is critical for the advancing epidermis to pave its way between scab and granulation tissue until the wound gap is sealed. Basal keratinocytes in the epidermis adjacent to the wound edge, on the other hand, adopt a hyper-proliferative phenotype to provide a sustained supply of keratinocytes required for encasing the wound [43]. This phenotype is activated by Heparin-binding EGF-like growth factor (HB-EGF), EGF, TGFs, keratinocytes growth factor (KGF) and granulocyte-macrophage colony stimulating factor (GM-CSF) [39, 44].

Besides re-epithelialization, dermal fibroblasts recruited from the surrounding tissue are also important for regenerating lost tissue in the wound. After sensing the damage signals, reticular wound-margin dermal fibroblasts will be recruited into the wound bed and contribute to the formation of the new dermis [45]. When faced with the complex wound environment, the infiltrated fibroblasts undergo a phenotypic switch. They are able to secrete plasminogen activator to degrade fibrin clots and produce new ECM to form granulation tissue [46]. The activated fibroblasts also express increased levels of alpha-smooth muscle actin

(α -SMA) which makes the wound-associated fibroblasts contractile to facilitate wound closure [47]. This process is mainly modulated by various growth factors, including TGF β s, connective tissue growth factor (CTGF) and fibroblast growth factors (FGFs) [47].

Another characteristic feature of the proliferative phase is a considerable angiogenic response, which is triggered by the increased metabolic demands of repairing [48]. This process is regulated by angiogenic growth factors, such as VEGFs, FGFs, angiopoietins and TGF β s [49, 50]. The activated endothelial cells from adjacent unwounded skin proliferate and migrate into the ECM of the wound bed. These endothelial cells form tubes and connect to existing vascular loop before differentiating into arterial and venous vessels. Finally, perivascular cells (smooth muscle cells or pericytes) will be recruited to stabilize the newly formed blood vessels [51]. The cross talks between different cellular populations, ECM components and growth factors are critical for efficient wound healing [52-55]. For instance, fibroblasts release various growth factors, such as KGF, IL-6, GM-CSF, HGF, actin, TGF β 1 and HB-EGF, in response to injury, which functions to regulate the activation of keratinocytes, fibroblasts and endothelial cells [55].

1.1.3 Tissue remodeling phase of wound healing

After the successful closure of the wound gap, collagen will be realigned and degraded by MMPs. Excess cells such as myofibroblasts, endothelial cells, and inflammatory cells will be removed from the wound site by emigration and apoptosis [56, 57]. For instance, myofibroblasts are removed from the wound site by a prominent wave of apoptosis as a consequence of reduced local growth factors production [47], a process that is regulated by TGF β 1-mediated activation of Akt signalling and endothelin-1 [58]. With the maturation of granulation tissue, the metabolic demand of the wound bed is significantly reduced. Several angiogenic

factors, including VEGF, are downregulated [59], and endothelial cells will undergo apoptosis [60] leading to vessel regression and the restoration of the wound bed to an angiostatic state [61]. C-X-C motif chemokine 10 (CXCL10)/C-X-C motif receptor 3 (CXCR3) signaling has been reported to activate the anti-angiogenic pathways and promote vessel regression during the tissue remodeling phase of wound healing [61]. Abnormalities in the remodeling phase usually cause excessive fibrotic diseases, including keloid and hypertrophic scars.

1.2 The central role of TGF- β in wound healing

TGF- β superfamily of growth factors regulates a broad range of biological responses, including cell proliferation, differentiation, apoptosis, autophagy and the immune response [62]. Based on structural and biological similarities, they can be divided into four families, the TGF- β family, bone morphogenetic protein (BMP) family, Mullerian inhibitory substance (MIS) family and inhibin/activin family [63]. Among them, TGF β family growth factors, specifically TGF β 1, is believed to serve as a 'master regulator' in the wound healing process by critically modulating the crosstalk between different types of cells [64].

1.2.1 The canonical and non-canonical pathways of TGF β signaling

TGF β 1 is expressed as a precursor molecule containing the active TGF β 1 molecule and a latency-associated peptide (LAP) [65]. The active TGF β 1 molecule can be released from LAP by changes in pH, proteases, and thrombospondin [66-68]. TGF β 1 exerts its function through various types of cell-surface serine/threonine kinase receptors. In most cell types, the binding of TGF β 1 to constitutively active TGF β RII leads to the recruitment and trans-phosphorylation of a ubiquitously expressed type I receptor, also known as ALK5, which in turn activates the receptor regulated Smads (R-Smads), Smad 2 and 3 [69]. Apart from ALK5, TGF β 1 can also

signal through a second type I receptor, termed ALK1, and trigger the activation of R-Smads, Smad1/5/8, in endothelial cells [70]. After being phosphorylated, R-Smads will be disassociated from the type I receptor and form trimeric complexes with the Co-Smad (Smad4), which will be translocated to the cell nucleus to exert their function as transcription factors [71]. This signaling transduction is negatively regulated by Smad ubiquitination regulatory factors (Smurfs) and inhibitor Smads (I-Smads), including Smad 6 and Smad 7 [71, 72]. Besides Smad-mediated transcription, it is increasingly appreciated that TGF β 1 activates many non-canonical signaling transducers, such as ERK, c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (p38 MAPK), PI3K/Akt, and Rho-like GTPase [73]. The specificity of TGF β 1-induced transcriptional response relies on the presence of ancillary receptors, betaglycan and Endoglin (ENG) [74]. The combinatorial effects of both canonical and non-canonical TGF β signaling define the cell type and context-dependent effect of TGF β (**Figure 2**).

Figure 2. Smad-dependent and Smad-independent pathways of TGF β family signaling. TGF β ligands, including TGF β 1, bind to TGF β RI and TGF β RII, triggering canonical pathways by activating R-Smads with the presence of Smad 4, or triggering Smad-independent pathways including JNK, p38 MAPK, ERK and Rho-like GTPase (Adapted from Nature volume 425, pages 577–584 (09 October 2003) [71])

1.2.2 The role of TGF β 1 in immune cells

Platelets are a major source of TGF β 1 at the early phase of wound healing [75]. TGF β 1 is one of the most potent chemoattractants to neutrophils [76, 77] and mediates neutrophil degranulation and its anti-bacterial function [78]. TGF β 1 also plays a critical role in mediating monocyte functions. TGF β 1 derived from hemopoietic cells can induce monocyte chemotaxis and growth factor production in the early inflammatory phase [79]. TGF β 1 overexpression results in constitutive and massive immune cell infiltration, which eventually leads to delayed wound healing [80].

During the acute inflammation, recruited monocytes differentiate into macrophages which mainly undergo classical activation to become pro-

inflammatory M1 macrophages [81]. On the other hand, macrophages undergo alternative activation to become anti-inflammatory M2 macrophages, which is critical in initiating the proliferative phase by secreting numerous critical growth factors at the late stage of inflammation phase [81]. This process is mediated by TGF β 1 [82, 83].

1.2.3 The role of TGF β 1 in re-epithelialization

Besides its role in inflammation, TGF β 1 has been reported to inhibit keratinocyte proliferation [84] and TGF β 1-overexpression in the epidermis is lethal due to insufficient epidermal proliferation in the embryonic stage [85]. On the other hand, TGF β 1 is one of the most potent inducers of keratinocyte migration [85]. It functions to increase the expression of integrins, specifically alpha 3 beta 1 integrin (α 3 β 1), which is required for keratinocyte adhesion and migration [86]. TGF β 1 is reported to regulate re-epithelialization through the canonical TGF β /Smad2/3 pathway and it is negatively regulated by Smad 7 [85]. However, the requirement for both keratinocyte proliferation and migration in wound healing highlights a controversial role of TGF β 1 in re-epithelialization of wound healing.

In vitro and *ex vivo* studies have demonstrated that exogenous TGF β 1 can promote or inhibit re-epithelialization in different models. While exogenous TGF β 1 delays re-epithelialization by suppressing keratinocyte proliferation in an organotypical model [84], it stimulates the initiation of epidermal cell outgrowth from porcine skin explant cultures [87]. This contraction is further demonstrated by *in vivo* studies using transgenic mice. TGF β 1 null mice show increased rate of re-epithelialization but reduced epidermal thickness following wounding [88], whereas transgenic mice overexpressing TGF β 1 in keratinocytes show accelerated re-epithelialization in partial-thickness ear wounds and delayed re-epithelialization in full-thickness back wounds [89]. These

contradictory observations could be explained by the presence and absence of dermis, and the cross-talk between epidermis and other cell types [85]. The effects of TGF β 1 on re-epithelialization might be masked by its promoting effects on fibroplasia [90] and regulatory roles in inflammation [80]. Therefore, tackling the effects of TGF β 1 on re-epithelialization is still challenging due to the complexity of the wound healing and TGF β signaling pathway.

1.2.4 The role of TGF β 1 in angiogenesis

Similar to keratinocytes, LRG1's role in endothelial cell function and angiogenesis is equally complicated. Although TGF β 1 serves as a potent inhibitor of endothelial cell proliferation and an inducer of apoptosis, it is required for the formation of capillary-like structure *in vitro* [91-93]. It has been proven that TGF β 1 promotes migration, differentiation, and capillary formation of endothelial cells in animal wound healing models [49, 94]. The effect of TGF β 1 on endothelial cells is mechanistically different from other cell types due to the capability of signaling through a second type I receptor, ALK1 and the presence of ENG, a type 3 TGF β receptor in endothelial cells [95]. As mentioned above, while ALK5/Smad2/3 mediates the response to TGF β 1 in most cell types, TGF β 1 signals through ALK1/Smad1/5/8 in endothelial cells, and the balance between ALK1/Smad1/5/8 and ALK5/Smad2/3 is mediated by ENG, which defines the cellular response to TGF β [96, 97].

Furthermore, this pro-angiogenic effect of TGF β 1 is contributed not only by its direct impact on endothelial cells but also by its role in mediating the action of other cells. For instance, TGF β 1 is required for the chemotaxis of myeloid cells [76, 79]. Recent studies have demonstrated that monocytes/macrophages and neutrophils are major sources of angiogenic factors, including VEGF and placental growth factor (PIGF), during wound healing [98, 99]. TGF β 1 is able to induce VEGF expression

in multiple cell types, including macrophages, dendritic cells, fibroblasts, perivascular cells and keratinocytes [100-106], and promote angiogenesis in a paracrine manner.

1.2.5 The role of TGF β 1 in ECM deposition and remodeling

ECM is an important component of granulation tissue during the wound healing process and TGF β 1 is one of the most important mediators of ECM homeostasis and remodeling [107]. In dermal fibroblasts, TGF β 1 has been reported to promote cell proliferation, migration, ECM synthesis and transdifferentiation into myofibroblasts [108-110]. In addition, the pro-fibrotic effect of TGF β 1 was further potentiated by TGF β 1-induced expression of other pro-fibrotic growth factors, such as CTGF in fibroblasts [111, 112]. In addition, the TGF β 1-induced myofibroblasts transdifferentiation contributes not only to ECM deposition but also wound contraction by inducing α -SMA [47]. The deposition and remodeling of ECM are regulated by the balance between MMPs and their inhibitors, tissue inhibitors of metalloproteinase (TIMPs) [113]. TGF β 1 was reported to inhibit the expressions of MMP-1, MMP-3, and MMP-9 but promote the expression of TIMP-1 [114]. Exogenous TGF β 1 can significantly accelerate incisional wound closure in rats, which partially due to the increased collagen deposition [115]. Both canonical TGF β /Smad2/3 and non-canonical Smad-independent pathways have been implicated in the TGF β 1-regulated fibroblasts functions [116, 117].

1.3 Leucine-rich alpha-2-glycoprotein 1, a novel regulator of TGF β signaling pathway

Leucine-rich α -2 glycoprotein 1 (LRG1) is a member of the leucine-rich repeat (LRR) family of proteins, many of which are involved in protein-protein interactions, signaling and cell adhesion [118-121]. LRG1 is expressed at low levels in quiescent vasculature of many organs including

the skin, and its expression is significantly up-regulated in pathological vessels in the eye [122]. The previous study has demonstrated that LRG1 interacts with multiple TGF β receptors, including the type III TGF β receptor, ENG [122]. Together with TGF β 1, ENG promotes the binding of LRG1 to the pro-angiogenic type I TGF β receptor, ALK1, but inhibits the association between LRG1 and the angiostatic type II TGF β receptor, ALK5 (**Figure 3**) [122]. Besides its role in ocular angiogenesis, LRG1 has been reported to mediate tumor angiogenesis by inducing VEGF expression in colorectal cancer cells [123].

Figure 3. Proposed working model of LRG1 mediated endothelial TGF β signaling pathway. In quiescent blood vessels, LRG1 binds to angiostatic TGF β receptor ALK5. Under angiogenic conditions, both LRG1 and ENG are up-regulated leading to the switch of the endothelial TGF β signaling from ALK5/Smad2/3 pathway to the angiogenic ALK1/Smad1/5/8 pathway. (Adapted from Nature volume 499, pages 306–311 (18 July 2013) [122])

In addition to angiogenesis, there are evidence that LRG1 is involved in other TGF β 1-regulated processes such as inflammation, tissue fibrosis and EMT in cancer [123-125]. For example, previous studies reported an up-regulated expression of LRG1 in various inflammatory diseases, such as adult-onset Still's disease, rheumatoid arthritis, ulcerative colitis, osteoarthritis, and asthma [126-130]. The relation between LRG1 and inflammation was further strengthened by the observation of an increased LRG1 expression in human umbilical vein endothelial cells (HUVEC) treated with pro-inflammatory cytokine TNF- α [130]. Additionally, myeloid cells have been identified as a source of LRG1 in infarcted myocardium *in vivo* and during granulocytic differentiation *in vitro* [131-133]. Furthermore, LRG1 has been reported to mediate TGF β -regulated hematopoietic progenitor cell growth and myelopoiesis [132]. A recent study also demonstrated a regulatory role of LRG1 on T-helper cells function through canonical TGF β /Smad2 pathway in arthritis [124]. All these evidence indicate a critical role of LRG1 in inflammatory responses.

Besides inflammation, an altered serum LRG1 was reported in multiple types of cancers, including pancreatic cancer, colorectal cancer, glioblastoma, retinoblastoma, gastric cancer, squamous cell carcinoma, ovarian cancer and endometrial carcinoma [134-142]. Recent studies reveal the context-dependent roles of LRG1 in cancer development and progression. LRG1 was reported to promote the migration and proliferation of colon cancer cells, gastric cancer cells and glioma cells [120, 140, 142], but exert an inhibitory role in Lewis lung cancer cell and hepatocellular carcinoma cell viability and migration [118, 143]. Although the mechanism of opposite effects of LRG1 in different cancer cells is poorly understood, several studies indicate a role of LRG1 in mediating EMT in cancer progression [120, 123], which is highly similar to changes of keratinocytes in re-epithelialization [144, 145].

As LRG1 is able to bind the pro-fibrotic TGF β receptor, ALK5, LRG1's

role in fibrotic diseases has been explored recently. Our study showed that peroxisome-proliferator-activated receptor beta/delta (PPAR β/δ) antagonizes the TGF β 1-induced fibroblast activation in an LRG1-dependent manner in the skin [146]. Similarly, *Lrg1*^{-/-} mice demonstrate a reduced vessel density but an increased cardiac fibrosis in infarcted myocardium in a mouse model of left anterior descending (LAD) artery occlusion-induced myocardial infarction [131]. However, bleomycin-induced pulmonary fibrosis is substantially suppressed in *Lrg1*-deficient mice [125].

In summary, a dysregulated LRG1 expression is associated with different types of diseases (**Table 1**). It is reported to play important but controversial roles in various biological and pathological processes, including inflammation, angiogenesis, fibrosis and epithelial cells functions, all of which are critical for efficient wound healing. However, its role in cutaneous wound healing remains to be elucidated.

Table 1 Expression and functions of LRG1 in different diseases

Disease	Objectives of studies	Reported changes of LRG1 expression	Reported functions of LRG1	Reference
Proliferative diabetic retinopathy	Human vitreous, plasma and mouse tissues	Upregulation	Promotes angiogenesis	[122, 147]
Rheumatoid arthritis	Human serum, mouse model	Upregulation	Promotes Th17 differentiation and arthritis development	[124, 126]
Ulcerative colitis	Human and mouse serum	Upregulation	Unknown	[127]
Lung fibrosis	Mouse tissues, serum and bronchoalveolar lavage fluid (BALF)	Upregulation	Promotes lung fibrosis	[125]
Myocardial infarction	Mouse model	Upregulation	Suppresses adverse cardiac remodeling	[131]
Asthma	Human Sputum, Mouse BALF and tissues	Upregulation	Unknown	[129]
Osteoarthritis	Mouse model	Upregulation	Promotes angiogenesis and mesenchymal stem cell migration	[130]
Colorectal cancer	Human tissues	Upregulation	Promotes tumor cell proliferation and epithelial-mesenchymal transition, and angiogenesis	[123, 136, 137, 148]
Endometrial carcinoma	Human tissues	Upregulation	Unknown	[138]
Glioma and glioblastoma	Human cell lines	Unknown	Promotes tumor development	[120, 149]
Retinoblastoma	Human tissues	Upregulation	Unknown	[135]
hepatocellular carcinoma (HCC)	Human tissues and human HCC cell lines	Upregulation	Suppresses migration and invasion of HCC	[118, 150]
Gastric cancer	Human tissues and serum	Upregulation	Enhances tumor migration	[140]
Pancreatic cancer	Human serum/Plasma and tissues	Upregulation	Unknown	[141, 151]
Ovarian cancer	Human serum and tissues	Upregulation	Unknown	[142, 152]
Lewis lung carcinoma (LLC)	Mouse allograft model and cell line	Unknown	Suppresses LLC growth	[143]
Head and neck squamous cell carcinoma	Human tissues	Downregulation	Unknown	[134]

1.4 Diabetes mellitus and diabetic wound healing

Diabetes mellitus (DM) is a disease characterized by high blood glucose [153]. It affects 451 million people age from 18 to 99 years, and is attributed to 5 million deaths in 2017 [154]. By 2045, the number of patients with DM is projected to reach 693 million [154]. There are two major types of DM, type 1 and type 2. Most of type 1 DM is caused by β -cell destruction resulting from autoimmune dysfunctions, while a small part of type 1 diabetes is due to non-autoimmune causes including genetic deficiency [153]. Patients with type 1 diabetes, therefore, cannot produce sufficient quantities of insulin [153]. Type 1 diabetes accounts for 5-10% of patients with diabetes and it is usually diagnosed in children and young adults, although it can appear at any age [153]. Type 2 diabetes is mainly caused by insulin-resistance but relative low insulin secretion is also observed in some patients with type 2 diabetes [153]. Type 2 diabetes represents 90-95% of all diagnosed diabetes cases [155].

Diabetes is known to cause adverse effects on different body parts and organs. Since the endothelial cells are in direct contact with blood, vascular complications are the most serious manifestation of diabetes [156]. Large vessel (macrovascular) problems may lead to the development of cardiovascular disease, cerebrovascular disease, and peripheral arterial disease. When small vessel (microvascular) are affected, patients may develop retinopathy, nephropathy, or neuropathy [157].

As the most distal part of the body, the foot is subjected to a great stress of weight bearing and walking. Reduced sensitivity because of neuropathy let the mild injury go unnoticed. Impaired circulation associated with peripheral arterial diseases may cause tissue anoxia leading to compromised healing. If left untreated, it can lead to infection, amputation and even death [158]. Around 19-34% of patients with

diabetes will develop a foot ulcer during their lifetime [158]. Following a first wound, 65% of patients will suffer from a recurrent wound within 5 years and around 20% of moderate and severe diabetic foot infection lead to amputation [158]. Following an amputation, the survival rate is less than 30% after 5 years [158, 159].

Diabetic foot ulcer (DFU) is a multifactorial disease characterized by dysregulated growth factor production, decreased new blood vessel formation, disrupted inflammatory response, impaired re-epithelialization, and compromised production, assembly, and remodeling of the ECM by fibroblasts [160]. While there is a significant upregulation of TGF β 1, TGF β R1, GM-CSF and EGF in keratinocytes, and MCP-1, GM-CSF and TGF β R1 in endothelial cells, the expressions of TGF β 1, IL10, IL15 and bFGF in endothelial cells are reduced in the ulcer margin [161]. Meanwhile, the lack of upregulated expressions of IL-8, IL10 receptor, GM-CSF receptor, VEGF and VEGFR2, PDGFs and their receptors, EGF receptor and insulin-like growth factor-1(IGF-1) are observed in diabetic wound healing [161]. The abnormal growth factor signaling leads to reduced recruitment of immune cells, fibroblasts and endothelial cells to the wound bed, which creates a vicious cycle of impaired growth factor response in diabetic ulcers.

1.4.1 Inflammation in diabetic wound healing

A dysregulated inflammatory response is intimately associated with non-healing wounds in diabetic patients. An elevated expression of IL8 leads to increased recruitment of neutrophils into the wound sites [162]. Furthermore, neutrophil over-activation, including excessive NETosis, increased MPO expression and increased neutrophil infiltration, have been demonstrated in rodent models of diabetic wound healing and systemic application of neutrophil-specific inhibitor drastically improves wound healing in diabetic mice [162, 163]. Peptidylarginine deiminase 4

(PAD4), a nuclear citrullinating enzyme involved in releasing decondensed chromatin from neutrophils in the form of NETs [16], and an up-regulated expression of PAD4 is observed in neutrophils isolated from diabetic patients [164]. Consistently, global knockout of PAD4 gene leads to an accelerated wound closure in both normal and diabetic mice. Furthermore, removing NETs by DNase 1 significantly increases the speed of wound closure in diabetic and normoglycemic mice [164].

Besides increased recruitment and activation of neutrophils, there is an increased macrophage infiltration at the edge of diabetic ulcers [165, 166]. Furthermore, hyperglycemia has been shown to induce the expression of pro-inflammatory cytokines in M1 macrophages *in vitro* [167]. Consistently, increased expressions of macrophage-derived pro-inflammatory cytokines, such as TNF- α , IL-1 β and MMPs, are observed in the diabetic wound fluid [168-170], whereas the expression of anti-inflammatory cytokines, including IL-10, are downregulated in diabetic wounds [171]. Moreover, macrophages isolated from diabetic patients show impaired efferocytosis, demonstrating an impaired switch from M1 to M2 phenotype [172].

To fight the persistent inflammation, several anti-inflammatory drugs and supplements, including curcumin, protein supplementation, nutraceutical, neurotensin, photodynamic therapy, doxycycline and TNF- α antibody Inflixmab, have been used to treat diabetic foot ulcers in pre-clinical models and in human patients, and demonstrated promising treatment outcomes [173]. In addition, numerous technologies, such as transcutaneous electrical nerve stimulation, hyperbaric oxygen therapy (HBOT), silver dressing and negative pressure wound therapy, have also been used to attenuate inflammation in diabetic patients with foot ulcers [173].

1.4.2 Re-epithelialization in diabetic wound healing

Failure of re-epithelialization is also commonly observed in diabetic foot ulcers [174]. Despite the inhibitory effect of high glucose on keratinocyte proliferation *in vitro* [175], the epidermis of diabetic ulcers in the wound edge is hyperproliferative as compared to that in normal wounds [176]. Furthermore, the motility of keratinocytes is significantly compromised under the diabetic condition [177, 178], which is partly due to excess MMP9 production by keratinocytes [179]. Increased glycation of ECM proteins, such as collagen, has also been shown to impede the adhesion and migration of keratinocytes under diabetic conditions [174, 180]. Surgical debridement to remove the hyperproliferative/hyperkeratotic, non-migratory epidermis at the wound edge is one of the routine procedures for managing chronic wounds and promoting healing [181, 182].

1.4.3 Angiogenesis in diabetic wound healing

Besides dysregulated inflammatory response and re-epithelialization, there is reduced angiogenesis at the diabetic wound bed [183]. As mentioned previously, angiogenesis is critical in granulation tissue formation and VEGF is known as one of the most potent angiogenic growth factors [184, 185]. During normal wound healing, VEGF is expressed by several cell types, including keratinocytes, M2 macrophages, fibroblasts, smooth muscle cells, platelets and neutrophils [186]. However, VEGF expression is significantly reduced in diabetic wounds [106, 187], which is associated with elevated expression of anti-angiogenic factors, such as pigment epithelium-derived factors (PEDFs) [188]. In addition to reduced angiogenesis, blood vessel maturation is also affected by DM. Perivascular cells play a critical role in stabilizing newly formed blood vessels and this process is regulated by Angiopoietin-1/Angiopoietin-2/angiopoietin-2 receptor (Ang1/Ang2/Tie2) complex and

PDGF pathway [183]. Disturbed Ang1/Ang2/Tie2 complex formation and reduced PDGF expression are observed in diabetic wounds [189], which leads to destabilization of blood vessels that ultimately causes hasty vessel regression and reduced perfusion of newly formed granulation tissue in wound bed [190].

Promoting functional blood vessel formation, therefore, serves as an alternative strategy to promote the healing of diabetic foot ulcers. Unfortunately, while topical application of VEGF was reported to promote neovascularization leading to accelerated wound healing in diabetic mice [191], long term application of exogenous VEGF has been reported to induce edema caused by increased vessel leakage and malformation of vessels [183, 192]. The clinical trial of recombinant human VEGF (Telbermin) failed to demonstrate clinical benefits to patients with diabetic foot ulcers [183]. Therapeutic potential of PDGF has also been tested in mouse models and clinical trials. Despite the clear beneficial effect [193, 194], PDGF (rh-PDGF-BB) (Becaplermin) causes multiple adverse effects including rash, burning sensation, and increased risks of osteomyelitis, cellulitis and cancer [183, 192].

1.4.4 ECM formation and remodeling in diabetic wound healing

Dysregulated ECM remodeling is another defining feature of diabetic wounds [195]. Fibroblasts isolated from chronic diabetic wounds have been shown to grow at a much slower rate [196] and are less sensitive to the stimulation of mitogenic growth factors, such as EGF, IGF-1, bFGF, and PDGF-AB, as compared to those isolated from non-diabetic wound tissues [197]. Furthermore, fibroblasts isolated from *db/db* mice are less migratory as compared to their wild-type counterparts [198]. Reduced contractility has also been observed in fibroblasts isolated from diabetic ulcers compared to those isolated from non-diabetic skin [195]. In addition, both reduced ECM production and increased degradation of ECM

components have been reported in diabetic wounds [168, 199]. For example, excessive activation of MMP9 is observed in diabetic wounds, which may lead to the breakdown of important matrix proteins and growth factors [200, 201] and compromise the formation of provisional matrix and granulation tissue [202]. Decellularized collagen and ECM biological scaffolds and biomaterials derived from a variety of mammalian sources and anatomic locations have been used in treating chronic wounds including diabetic foot ulcers [203]. Diabetic foot ulcers treated by ECM products, including oxidized regenerated cellulose (ORC)/collagen with and without added silver, acellular human dermal matrix (Graftjacket), and porcine small intestine submucosa, have shown promising improvement in clinical trials [203].

TGF β 1 is the primary regulator of ECM deposition and wound contraction during wound healing [64]. Lack of TGF β 1 upregulation was observed in the wound tissue of both diabetic mice and human patients [204, 205], and there is an impaired TGF β signal transduction due to reduced TGF β R2 expression in fibroblasts derived from diabetic skin [206]. Indeed, restoration of TGF β signaling with exogenous TGF β 1 can rescue delayed wound healing by improving granulation tissue formation in diabetic rats [207]. However, Clinical trials of TGF β 1 treatment have achieved limited success due to insufficient efficacy [80].

In summary, impaired wound healing in diabetic patients is attributed to the defects in the action of various cytokines, chemokines, and cell types. A better understanding of the pathophysiological mechanisms underlying DFU may facilitate the development of new treatments.

1.5 Aims and objectives

The overarching aim of this project is to explore the role of LRG1, a novel regulator of TGF β signalling pathways, in wound healing process under normoglycemic and diabetic conditions. We hypothesize that an altered LRG1 expression plays a causative role in delayed wound closure under normal and diabetic conditions. The specific aims are i) to evaluate the expression pattern of LRG1 during wound closure under normal and diabetic conditions; ii) to understand the role of LRG1 in different biological processes involved in wound healing, including inflammation, angiogenesis, fibroplasia and re-epithelialization; iii) to assess the impact of LRG1 deletion on wound closure under normal and diabetic conditions.

Chapter 2 Materials and Methods

2.1 *In-Vitro* work

2.1.1 Cells and cell culture

All cells used in this project were cultured in a humidified incubator kept at 5% CO₂ and 37°C.

Primary human dermal microvascular endothelial cells (HDMECs, Promocell, Germany) and normal human dermal fibroblasts (NHDFs, Promocell, Germany) were maintained in Microvascular Endothelial Cell Growth Medium-2 (EGM-2MV, Lonza, USA) and Fibroblast growth medium (FGM-2, Promocell, Germany) respectively. Only cells between passages 4 to 9 were used for the proposed study.

Immortalized keratinocyte cell line, HaCaT (ATCC, USA) was maintained in high glucose Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, USA) supplemented with 10% FBS (Gibco, USA), 1% Penicillin-Streptomycin (Sigma-Aldrich, USA) and 1% L-glutamine (Life Technologies, USA).

Mouse embryonic fibroblasts (MEFs) were isolated from embryonic day 14.5 wild-type and *Lrg1*^{-/-} mice as described previously [208], and maintained in high glucose DMEM (Life Technologies, USA) supplemented with 15% FBS (Gibco, USA), 1% Penicillin-Streptomycin (Sigma-Aldrich, USA) and 1% L-glutamine (Life Technologies, USA). Briefly, heads, livers and blood clots were removed from embryos. The tissues were minced and trypsinized with 2ml of Trypsin (0.05%)-EDTA (0.53mM) at 37°C for 10 minutes. The fetal tissues were then resuspended to make a single cell suspension by vigorous pipetting, and frozen or seeded for future experiments. Only cells between passages

one to three were used for the proposed study.

All adherent cells were grown as an adherent monolayer and were trypsinized by 0.25% Trypsin-EDTA (Sigma-Aldrich, USA) before being seeded at optimal cell densities for respective assays.

Human polymorphonuclear neutrophils (PMN) were isolated from blood using Ficoll-Paque density gradient according to a previous protocol [209]. In brief, human peripheral venous blood was mixed with equal amount of 0.1% BSA in phosphate-buffered saline (PBS), layered on 1.5 x blood volume of Ficoll-Paque solution (GE healthcare, USA), before being centrifuged at 300g for 30 min at room temperature. The bottom layer containing granulocytes and erythrocytes was collected and topped up to 20 ml with PBS before mixing with an equal volume of 3% dextran (Sigma-Aldrich, USA) for 20-minutes sedimentation. PMN-rich supernatant was collected and centrifuged at 300g for 10 minutes. Remaining erythrocytes were lysed by hypotonic lysis buffer (13.6 ml 10× PBS in 600 ml water) for 30 seconds before restoring isotonicity using re-equilibration buffer (13.6 ml 10× PBS in 600 ml water). This lysing step was repeated until no significant erythrocyte contamination was observed. Cells were resuspended in 0.1% BSA in PBS for future experiments.

Mouse bone-marrow derived neutrophils were isolated from the tibia and femur of age-matched wild-type and *Lrg1*^{-/-} mice according a modified protocol [210]. Briefly, tibia and femur were dissected and kept in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% FBS and 1% L-glutamine. Bone marrow was flushed out using 0.1% BSA in PBS followed by centrifugation at 500g for five minutes. Erythrocytes were lysed by hypotonic lysis buffer for 30 seconds before restoring isotonicity using re-equilibration buffer. Tissue debris was removed by 70-µm cell strainer. Cells were resuspended in 0.1% BSA in PBS, layered on a 62.5% Percoll gradient. (GE healthcare, USA), and centrifuged at a speed of

1000g for 30 minutes. Neutrophils in the bottom layer of the gradient were harvested and validated by immunofluorescence staining with CD66b antibody (1:100 dilution, abcam, UK). Cells were resuspended in 0.1% BSA in PBS for future experiments.

2.1.2 Growth factors and inhibitors treatments

Recombinant human TGF β 1 (PeproTech, USA) was used at the concentration of 5ng/ml for HDMEC treatments and 10ng/ml for NHDFs treatments. Recombinant human VEGF₁₆₅ (rVEGF, PeproTech, USA) was used at the concentration of 50ng/ml for all *in vitro* assays. Recombinant human TNF- α (PeproTech, USA) was used at the concentration of 10ng/ml to 50ng/ml. Recombinant human LRG1 (rLRG1) was expressed in FreeStyle™ 293-F cells (Thermo Fisher Scientific, USA) and purified as described previously [122]. 100 μ g/ml of rLRG1 was used to treat human and mouse neutrophils. 20 μ g/ml of rLRG1 was used to treat HDMECs and 200ng/ml of rLRG1 was used to treat NHDFs, HaCaT cells and MEFs. 100 nM ALK1 inhibitor (LDN 193189, Axon Medchem BV, Netherlands) and 10 μ M ALK5 inhibitor (SB43152, Sigma-Aldrich, USA) were used as previously described [122]. Neutralizing mouse anti-human TGF β 1 antibody (R&D system, USA) was used at the concentration of 1ng/ml for HDMEC signaling study.

2.1.3 *In vitro* endothelial cell monolayer FITC-Dextran permeability assay

300,000 HDMECs were seeded onto Transwell inserts (0.4 μ m polycarbonate membrane, Corning, USA) and cultured for 4-5 days. Cells were pretreated with 50ng/ml rVEGF and 20 μ g/ml rLRG1 after serum starvation in endothelial cell basal medium (EBM2) containing 0.2% FBS overnight. Fluorescein isothiocyanate (FITC)–dextran 70 (Sigma-aldrich, USA) was added to the upper chamber of the Transwell inserts to reach

a final concentration of 0.1mg/ml. Two hours later, the FITC-dextran leaked through the HDMEC monolayer was quantified using Synergy H1 microplate reader at the wavelength of 495 nm/519 nm (BioTek, USA).

2.1.4 LRG1 overexpression and small interfering RNA silencing

The 6xHis-tagged coding sequence of human LRG1 (NM_052972) was cloned into the pcDNA3.1 plasmid backbone (Invitrogen, USA) at the HindIII/XhoI sites to generate LRG1 expression vector pcDNA3.1-LRG1.

80,000 NHDFs in FGM-2 medium were seeded into each well of 6-well culture plates (Corning, USA) and cultured overnight, before they were transfected with 1500ng of either pcDNA3.1 or pcDNA3.1-LRG1. HaCaT cells were seeded into 6-well plates at a cell density of 200,000 cells per well and cultured for 48 hours in complete DMEM, before being transfected with 2500ng of either pcDNA3.1 or pcDNA3.1-LRG1, and 33mM of scramble siRNA or LRG1 siRNA (Dharmacon, USA). Transfection was performed using Lipofectamine 2000 (Invitrogen, USA) and Opti-MEM® (Life Technologies, USA) according to manufacturer's protocol.

Briefly, indicated amounts of plasmid/siRNA were mixed with Lipofectamine 2000 at a ratio of 1000ng of plasmid to 2.5µl of Lipofectamine 2000 or 100pmol siRNA to 6µl of Lipofectamine 2000 in 1.5ml Opti-MEM® (Life Technologies, USA) and incubated at room temperature for 20mins to allow the formation of liposome-DNA/siRNA mixture. Next, each well of sub-confluent cells was incubated with 1.5ml of liposome-DNA/siRNA mixture for six hours. Transfected cells were recovered in complete mediums for 24 hours and subsequently used for downstream experiments.

2.1.5 Matrigel tube formation assay

60µl of Growth Factor Reduced Matrigel Basement Membrane Matrix (Corning, USA) containing vehicle control, 50 ng/ml rVEGF or 20 µg/ml rLRG1, was added to each well of a 96-well plate and incubated for 30 minutes at 37°C to allow polymerization. 15,000 HDMECs in 100µl of EBM2 medium containing vehicle control, 50 ng/ml rVEGF or 20 µg/ml rLRG1 were seeded onto the polymerized Matrigel gel. After an overnight incubation, the HDMEC tube formation was imaged using phase contrast mode on Eclipse Ti-E Inverted Research Microscope (Nikon, USA) and the complexity of the vascular network was analyzed by Image J (National Institutes of Health, USA) for total vessel per well.

2.1.6 Cell viability assay

Cell viability was evaluated by colorimetric MTS Tetrazolium Assay (Promega, USA). 2,000 HDMECs were resuspended in 100µl of EMG2-MV medium and cultured in each well of a 96-well plate for 24 hours. The cells were then rinsed once by PBS before being starved for 24 hours in EBM-2 basal medium (Lonza, USA) containing 0.2% FBS (Thermo Fisher Scientific, USA), to synchronize cell growth. HDMECs were subjected to treatments in 100µl EBM2 medium (Lonza, USA) with 50ng/ml rVEGF or 20 µg/ml rLRG1 for 2 days. 10µl of MTS reagent was directly added into each well, the absorbance of formazan product was measured after a 3-hour incubation using Synergy H1 microplate reader (BioTek, USA) at the wavelength of 490nm.

2.1.7 Glucose Treatments

A final concentration of 22 mmol/L D-Glucose solution (Thermo Fisher Scientific, USA) was supplemented to the growth medium to recapitulate

hyperglycemic conditions. Same amount of D-mannitol (Sigma-Aldrich, USA) was added to the medium as an osmotic control.

2.1.8 Trypan blue exclusion assay

HaCaT cells were seeded into 6-well culture plates at the density of 200,000 cells per well and grown for two days. Cells were transfected with pcDNA 3.1/pcDNA3.1-LRG1 or scramble siRNA/LRG1 siRNA as previously described. At 24 hours after transfection, cells were collected and stained with Trypan blue solution. Enumeration of viable cells was conducted using hemocytometer under a phase contrast microscope.

2.1.9 Cell proliferation assay

4,000 HDMECs or 3,000 NHDFs were seeded in each well of a 48-well culture plate and grown for 24 hours. Cells were then washed once in PBS before being starved for 24 hours in EBM-2 basal medium (Lonza, USA) containing 0.2% FBS (Thermo Fisher Scientific, USA) or basal FGM-2 medium (Promocell, Germany) to synchronize cell growth. HDMECs and NHDFs were subjected to their respective treatment medium for 48 hours before being fixed in 4% paraformaldehyde (PFA) for 15 minutes. Cells were then blocked, permeabilized in 1X blocking buffer containing 0.5% bovine serum albumin (Biowest, Denmark), 3% Triton-X 100 (Bio-Rad, USA) and 1% Tween-20 (Bio-rad, USA in 1X PBS) for 45 minutes before being incubated in blocking buffer containing rabbit anti-Ki67 antibody (1:500 dilution, Abcam, UK) overnight at 4°C. Cells were washed three times and incubated with Alexa Fluor® 594 conjugated goat anti-rabbit IgG secondary antibody (1:500 dilution, Thermo Fisher Scientific, USA) for two hours, and stained with DAPI dye (1:500 dilution, Thermo Fisher Scientific, USA) for ten minutes. Images were captured using Eclipse Ti-E Inverted Research Microscope (Nikon, USA). Cell proliferation rate was calculated by the percentages of Ki67 positive cells

using Image J (National Institutes of Health, USA).

2.1.10 Scratch wound healing assay

Transfected HaCaT cells were subjected to serum starvation in basal high glucose DMEM containing 0.2% FBS (Thermo Fisher Scientific, USA) for 24 hours, and cell monolayer was scratched vertically using a sterile p200 pipette tip after a one-hour incubation with 10 μ g/ml mitomycin C (Sigma-Aldrich, USA). The cells were cultured in complete DMEM and imaged at different time points using Eclipse Ti-E Inverted Research Microscope (Nikon, USA). The area covered by cells was measured using Image J (National Institutes of Health, USA).

2.1.11 Transwell migration assay

50,000 HDMECs were pre-treated by rLRG1 and rVEGF for 24 hours before being seeded onto 100 μ g/ml rat tail collagen I (Corning, USA) coated 8 μ M Transwell® 24-Well Plate Inserts (Corning, USA). EBM2 medium was placed into the upper chamber of the transwell, whereas the EBM2 medium containing 5% FBS was added into the lower chamber as a chemoattractant. After five hours of incubation, inserts were fixed in 1% PFA for 10 minutes, and permeablized with 0.2% Triton-X 100 for ten minutes. Non-migrated cells on the upper side of inserts were scraped off with cotton swabs. Migrated cells on the other side of inserts were stained with DAPI (1:500 dilution, Thermo Fisher Scientific, USA) and imaged using Eclipse Ti-E Inverted Research Microscope (Nikon, USA). Number of migrated cells were counted manually using Image J (National Institutes of Health, USA).

2.1.12 Collagen gel contraction assay

Rat Tail Collagen Type I (Corning, USA) was dissolved in 0.01% glacial

acetic acid (Thermo Fisher Scientific, USA) to achieve a working concentration of 3.96mg/ml. NHDFs and MEFs were trypsinized and diluted at the concentration of 150,000 cells/ml in basal FGM-2 (Promocell, Germany) or DMEM (Life Technologies, USA) supplemented with 1mM NaOH (Sigma-Aldrich, USA) and 1.5mg/ml Rat Tail Collagen Type I (Corning, USA). After a 20-minute incubation at room temperature, 0.5ml of fresh basal FGM-2 medium (Promocell, Germany) or DMEM medium (Life Technologies, USA) with 15% FBS and appropriate treatments were added to each well. Gel contraction was followed over a period of 2-5 days and images were captured using Fluorescent Stereo Microscope Leica M165 FC (Leica Microsystems, USA).

2.1.13 SYTOX green assay

50,000 mouse neutrophils and human polymorphonuclear neutrophils were seeded onto each well of a 96-well clear bottom black plate. Cells were treated with 5 μ M calcium ionophore A23187 or 100 μ g/ml rLRG1 for 2-4 hours, before being incubated with the DNA dye SYTOX green (Invitrogen, USA) at a final concentration of 1 μ M for 15 minutes. NETs formation was determined by measuring the fluorescence intensity using Synergy H1 microplate reader at a wavelength of 504 nm/523 nm (BioTek, USA).

2.1.14 Induction of NETosis for immunofluorescence staining

300,000 mouse neutrophils were seeded onto each well of an 8-well chamber slide and were cultured in RPMI-1640 medium containing 25mM HEPES for 20 minutes. After removing unattached cells, attached cells were incubated with 5 μ M calcium ionophore A23187 for two hours. Cells were fixed with 1% PFA. NETs were stained by H3Cit antibody (1:100 dilution, abcam, UK) and nuclei was stained by DAPI following a standard protocol. Cells undergoing NETosis were counted manually.

2.2 Molecular and biochemistry techniques

2.2.1 SDS-PAGE and Western blot

Proteins were harvested using RIPA buffer (1 X protease inhibitors (Roche, Switzerland), 1 X phosphatase inhibitors (Sigma, USA), 0.1M phenylmethylsulfonyl fluoride (Sigma, USA) and 1mM dithiothreitol (Sigma, USA)) and the amount of protein was quantified by Bradford assay (Bio-Rad, USA) according to the manufacturer's instructions. Protein samples were loaded and separated by 10% SDS-PAGE before being transferred onto a Immobilon-PSQ PVDF membrane (Merck Millipore, USA) using Pierce G2 fast blotter transfer system (Thermo Fisher Scientific, USA) or Mini Trans-Blot® Cell (Bio-Rad, USA). Clarity™ ECL western blotting substrate (Bio-Rad, USA) or the Supersignal™ Femto maximum sensitivity substrate (Thermo Fisher Scientific, USA) were used for target detection and Restore™ western blot stripping buffer was used to strip blots for re-probing purposes. The antibodies used are described in **Table 2**.

Table 2 Details of antibodies used for Western blot

Name	Dilution
CTGF Antibody; Goat, Polyclonal (sc-14939, Santa Cruz Biotechnology, USA)	1:450
α-SMA Antibody; Mouse, Monoclonal (ab32575, AbCam, UK)	1:2500
Cyclin D1 Antibody; Rabbit, Monoclonal (#2922, Cell Signaling Technology, USA)	1:1000
Phospho-Src Family Antibody; Rabbit, Monoclonal (#2101, Cell Signaling Technology, USA)	1:1000
Src Antibody; Rabbit, Monoclonal (#2108, Cell	1:1000

Signaling Technology, USA)	
LRG1 Antibody; Rabbit, Monoclonal (13224-1-AP, ProteinTech, USA)	1:2500
α-tubulin Antibody; Rabbit, Monoclonal (#2144, Cell Signaling Technology, USA)	1: 1000
Histone H3 (citulline R2 + R8 + R17) antibody; Rabbit, Polyclonal (ab5103, AbCam, UK)	1:1000
Histone H3 antibody; Rabbit, Polyclonal (ab1791, AbCam, UK)	1:1000
Phospho-Smad1/Smad5; Rabbit, Monoclonal (#9516, Cell Signaling Technology, USA)	1:2500
Smad1/Smad5, Mouse, Monoclonal (ab75273, AbCam, UK)	1:2500
Phospho-Smad3; Rabbit, Monoclonal (#9520, Cell Signaling Technology, USA)	1:2500
Smad3; Rabbit, Monoclonal (#9513, Cell Signaling Technology, USA)	1:2500
Phospho-Smad2; Rabbit, Monoclonal (#18338, Cell Signaling Technology, USA)	1:2500
Smad2; Rabbit, Monoclonal (#5339, Cell Signaling Technology, USA)	1:2500
Phospho-Akt, Monoclonal (#9271, Cell Signaling Technology, USA)	1:2500
Akt Antibody, Monoclonal (#9272, Cell Signaling Technology, USA)	1:2500
Phospho-p38 MAPK ,Rabbit, Monoclonal (#9211, Cell Signaling Technology, USA)	1:2500
p38 MAPK, Rabbit, Monoclonal (#9212, Cell Signaling Technology, USA)	1:2500
Phospho-p44/42 MAPK (ERK1/2); Rabbit, Monoclonal (#4370, Cell Signaling Technology, USA)	1:2500
p44/42 MAPK (ERK1/2); Rabbit, Monoclonal (#4695,	1:2500

Cell Signaling Technology, USA)	
GAPDH Antibody; Rabbit, Polyclonal (sc-32233, Santa Cruz Biotechnology, USA)	1:50,000
Goat anti-rabbit IgG HRP Antibody (Santa Cruz Biotechnology, USA)	1:5000
Goat anti-mouse IgG HRP Antibody (Santa Cruz Biotechnology, USA)	1:5000
Donkey anti-goat IgG HRP Antibody (Santa Cruz Biotechnology, USA)	1:5000

2.2.2 Quantitative real time polymerase chain reaction (qRT-PCR)

For qRT-PCR experiments, MEFs, NHDFs and HDMECs were seeded at the optimal density in their respective medium in 6-well culture plates (Corning, USA). Cells were starved for 24 hours in respective starvation medium once reaching 70-80% confluence. Cells were then treated with medium containing different growth factors for desired period. At the end of treatments, cells were washed in PBS twice before being subjected to RNA isolation.

RNAzol®RT (Molecular Research Center, USA) was used for mRNA isolation and purification according to the manufacturer's instructions. RNA concentrations, 260/280 ratio and 260/230 ratio were determined using Nanodrop 2000C Spectrophotometer (Thermo Fisher Scientific, USA). 800-1000ng of RNA was reverse transcribed to cDNA using qScript cDNA Supermix (Quanta Biosciences, USA) according to manufacturer's instructions. PCR master mix containing relevant primers, nuclease free water and iTaq™ Universal Synergy Brands (SYBR®) Green Supermix (Bio-Rad, USA) was prepared. qRT-PCR was performed using Applied Biosystems StepOnePlus™ Real Time PCR System (Life Technologies, USA). Primer sequences are listed in **Table 3** and **Table 4**.

Table 3 Mouse primers used for qRT-PCR

Gene (Mouse)	Forward Primer Sequence	Reverse Primer Sequence
CTGF	5'-GGGCCTCTTCTGCGATTTTC-3'	5'-ATCCAGGCAAGTGCATTGGTA-3'
α -SMA	5'-GTCCCAGACATCAGGGAGTAA-3'	5'-TCGGATACTTCAGCGTCAGGA-3'
Lrg1	5'-TGCACCTCTCGAGCAATCG-3'	5'-TGCAGGCGCTGTCTCCCGAGTT - 3'
GAPDH	5'-AGGTCGGTGTGAACGGATTTG-3'	5'-TGTAGACCATGTAGTTGAGGTCA-3'

Table 4 Human primers used for qRT-PCR

Gene (Human)	Forward Primer Sequence	Reverse Primer Sequence
LRG1	5'-GTTGGAGACCTTGCCACCT-3'	5'-GCTTGTTGCCGTTTCAGGA-3'
GAPDH	5'-GGTCTCCTCTGAC TTCAACA-3'	5'-AGCCAAATTCGTTGTCATAC-3'

2.2.3 Chromatin immunoprecipitation (ChIP) assay

HDMECs were cross-linked with 1% formaldehyde (Sigma, USA) for ten minutes before being quenched with 0.125M glycine for five minutes. Cells were then lysed in SDS lysis buffer containing 1% SDS, 5mM EDTA, 50mM Tris-HCl (pH 8.1) and 1x cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail (Roche, USA) for 10 minutes on ice. Whole-cell lysates were sonicated into 500-bp fragments with a sonicator (Sonics Vibracell). Sheared chromatin was cleared of debris and incubated overnight at 4°C with either rabbit IgG (Sigma, USA), or 2-μg rabbit polyclonal NFκB p65 antibody (Santa Cruz, USA). After washing, the protein-DNA crosslinks were reversed and DNA was eluted in 240μl elution buffer containing 1% SDS and 0.1M NaHCO₃. DNA purification was performed using the QIAquick PCR Purification Kit (Qiagen, Germany) according to

manufacturer's instructions. qRT-PCR was performed using the primers spanning the NFκB binding sites near the LRG1 TSS:

Forward primer, 5'-GGGGTTGAACAGGAAACAAT-3'

Reverse primer, 5'-GCTCACTGCAGCCTCTGAA-3'

2.2.4 LRG1 Enzyme-linked immunosorbent assay (ELISA)

The serum LRG1 levels of patients were measured by ELISA using Human LRG Assay Kit (Immuno-Biological Laboratories Co,Ltd, Japan) according to manufacturer's instruction.

2.3 *In-Vivo* work

2.3.1 Animal care

Lrg1^{-/-} mice were generated by the Knockout Mouse Project (KOMP). C57BL/6 mice were purchased from INVIVOS. The care and use of all animals were performed in compliance with the National Advisory Committee for Laboratory Animal Research (NACLAR) and the Institutional Animal Care and Use Committee (IACUC, ARF-SBS/NIE-A0268) of Nanyang Technological University.

2.3.2 Miles assay

Miles assay was conducted as previously described [211, 212] with minor modifications. Briefly, 8-12-week-old age-matched female wild-type and *Lrg1*^{-/-} mice were restrained with a restraint device. Dorsal hair was removed by shaving and hair removal cream (Veet, USA). 200μl of 0.5% Evans Blue solution (Sigma-Aldrich, USA) was then injected into the tail vein of each mouse. 50μl of PBS, PBS containing 250ng rVEGF (PeproTech, USA) or 60μg rLRG1 were injected subcutaneously into

three separate sites of the shaved skin 30 minutes later. Next, the mice were sacrificed and the skin was separated from the muscle layer and photographed 30 minutes after treatments. The tissue surrounding the injection site was excised using 6-mm Integra™ Miltex™ Standard Biopsy Punches (Thermo Fisher Scientific, USA) and dried for 48 hours at 55 °C. Evans Blue was extracted by incubating with formamide at 56 °C for 24 hours and quantified using Synergy H1 microplate reader at the absorbance of 620 nm (BioTek, USA).

2.3.3 Mouse excisional wound healing model

In brief, 8-12-week old age-matched male wild-type and *Lrg1*^{-/-} mice were anesthetized *via* intraperitoneal injection of ketamine (120 mg/kg) and xylazine (16 mg/kg) mixture dissolved in 0.9% NaCl solution. Dorsal hair was removed by shaving and hair removal cream (Veet, USA). Six full-thickness cutaneous wounds were created on the dorsal skin using 4-mm Integra™ Miltex™ Standard Biopsy Punches (Thermo Fisher Scientific, USA) and wounds were imaged and harvested at respective time points. Wound sizes were quantified using Image J (National Institutes of Health, USA).

2.3.4 Mouse diabetic excisional wound healing model

Diabetes was induced in 6-8 week old, male wild-type and *Lrg1*^{-/-} mice by streptozotocin (STZ) according to previous study [213]. Briefly, mice were fasted for six hours prior to an intraperitoneal injection of 40mg/kg STZ in 50mM sodium citrate buffer (pH 4.5) for five continuous days. Mice were allowed to access food and water freely afterward. Fasting blood glucose (FBG) was measured using glucometer 28 days after the last injection. Mice with FBG higher than 200mg/dl were proceeded with wound healing study. Two 4-mm wounds were created symmetrically on the dorsal skin of each mouse. The experimental design of diabetic wound healing model

is illustrated in the following figure (Figure 4).

Figure 4. Experimental design of mouse diabetic excisional wound healing model. 6-8 week old male wild-type and *Lrg1^{-/-}* mice were subjected to 5 continuous days of injection of 40mg/kg STZ. On day 33, FBG was measured, and non-diabetic mice (FBG $\leq$ 200mg/dl) were eliminated from the study. On day 47, two wounds were created on the dorsal skin of each mouse, and digit photos were taken daily for nine days after injury.

2.3.5 Bleomycin-induced skin fibrosis model

Skin fibrosis was induced as previously described [214] in 8-week-old, female wild-type and *Lrg1^{-/-}* mice. Briefly, mice received a subcutaneous injection of 100 μ l of 1.5mg/ml bleomycin (Sigma-Aldrich, USA) in 0.9% NaCl at the predefined area of the upper dorsal skin every other day. Mice injected with same amount of 0.9% NaCl served as vehicle controls. Mice were sacrificed using CO₂ chamber 28 days after injection. Skin was harvested for histological and molecular analysis.

2.4 Histology and immunostaining

2.4.1 Haematoxylin and Eosin (H&E) staining

Skin tissues were fixed in 4% PFA and embedded in paraffin. 5µm skin paraffin sections were deparaffinized in two changes of xylene for five minutes each, and rehydrated with a graded series of ethanol (95%, 70%, 50% and 0%) for two minutes each. The sections were stained with Hematoxylin 560 solution (Leica, Germany) for two minutes, before differentiating in 0.1% acid alcohol. Next, the sections were stained with Alcoholic Eosin Y 515 solution (Leica, Germany) for one minute, followed by quick dehydration in a graded series of ethanol (0%, 50%, 75%, 95% and 100%). Dehydrated sections were cleared in two changes of xylene for five minutes each, and mounted with CV Ultra Mounting Media (Leica, Germany). Images were captured using Leica DM5500 Microscope (Leica Microsystems, USA), and analyzed using Image J (National Institutes of Health, USA).

2.4.2 Immunohistochemistry Staining

The slides were deparaffinized in two changes of xylene and rehydrated with a graded series of ethanol (95%, 70%, 50% and 0%). Antigen retrieval was performed by boiling the sections in Tris-EDTA Buffer (10mM Tris Base, 1mM EDTA Solution, 0.05% Tween 20, pH 9.0) for 10 minutes. Next, endogenous peroxidase was blocked using Peroxidase-Blocking Solution for 30 minutes (Dako, USA). The sections were rinsed seven times with TBS buffer (500ml of 1M Tris, 90g of NaCl, 10 litre of deionized water) before a 40-minute incubation with 10% horse serum (Sigma-Aldrich, USA) in TBS diluent (0.1% of BSA and 0.01% of Sodium azide in TBS buffer). Next, the sections then were incubated with LRG1 antibody in TBS diluent overnight at room temperature (1:200 dilution, Proteintech, USA). Excess primary antibody was removed by rinsing the sections seven times with TBS buffer, before incubating with Goat anti-rabbit IgG HRP Antibody (1:200, Santa Cruz Biotechnology, USA) for two hours at

room temperature. Excess secondary antibody was removed by seven washings of TBS, and followed by visualization with 1:50 diaminobenzidine (DAB) peroxidase substrate solution for five minutes. The sections were counterstained with Hematoxylin 560 solution (Leica, Germany) before being dehydrated in a graded series of ethanol (0%, 50%, 75%, 95% and 100%). Dehydrated sections were cleared in two changes of xylene for five minutes and subsequently mounted with CV Ultra Mounting Media (Leica, Germany). Images were captured using Leica DM5500 Microscope (Leica Microsystems, USA).

2.4.3 Immunofluorescence staining

PFA-fixed cells were blocked using blocking buffer containing 0.5% bovine serum albumin (Biowest, Denmark), 3% Triton-X 100 (Bio-Rad, USA) and 1% Tween-20 (Bio-rad, USA in 1X PBS) for 45 minutes before being incubated with primary antibodies overnight at 4°C. Cells were washed three times in 1X PBS before being incubated with secondary antibody at room temperature for two hours followed by DAPI staining (1:500 dilution, Thermo Fisher Scientific, USA) at room temperature for ten minutes. The sections were washed in PBS three times before being mounted with Mowiol® aqueous mounting medium. Images were captured using Eclipse Ti-E Inverted Research Microscope (Nikon, USA), and quantified using Image J (National Institutes of Health, USA). The details of primary antibodies used are indicated in **Table 5**.

Table 5 Details of primary antibodies used in cell-based staining

Primary antibody for cell-based staining			
Antibody	Origin	Dilution	Source
VE-cadherin	Rabbit	1:100	Cell Signaling Technology, USA
Ki67	Rabbit	1:500	Abcam, USA
CD31	Rat	1:250	BD Bioscience, USA
CD66b	Rabbit	1:100	Abcam, USA
H3Cit	Rabbit	1:100	Abcam, USA

For tissues, PFA-fixed skin tissues were cryoprotected in 30% sucrose before being embedded in Tissue-Plus™ O.C.T. Compound (Fisher Scientific, USA). Skin tissues were cryosectioned at a thickness of 5µm, and blocked in histological blocking buffer containing 2% BSA, 1% Tween 20, 3% Triton-X and 0.4% horse serum (Sigma-Aldrich, USA) for one hour. The slides were then incubated overnight with respective primary antibodies at the desired concentrations at room temperature. The next day, the sections were incubated with secondary antibody for two hours at room temperature followed by three washes in PBS before being incubated with DAPI (1:500 dilution, Thermo Fisher Scientific, USA) at room temperature for ten minutes. Images were captured using Zeiss LSM 710 confocal microscope or Leica DM5500 Microscope (Leica Microsystems, USA), and quantified using Image J (National Institutes of Health, USA). The details of primary antibodies used in tissue histology and their relevant time points are indicated in **Table 6**.

Table 6 Details of primary antibodies used in tissue histology

Primary antibody for tissue staining				
Antibody	Origin	Dilution	Source	Time point
LRG1	Rabbit	1:100	Proteintech, USA	Normal skin, day one and three
CD31	Rat	1:100	BD Bioscience	Normal skin, day three and seven
Vimentin	Rat	1:100	R&D Systems, USA	Normal skin
CD11b	Rat	1:100	Miltenyi Biote, Germany	Day one
F4/80	Rat	1:100	Bio-Rad, USA	Day one and three
Ly6G	Rat	1:100	BD Bioscience, USA	Day one
MPO1	Rabbit	1:100	Abcam, USA	Day one
Ki67	Rabbit	1:100	Abcam, USA	Day one

The details of secondary antibodies are listed in **Table 7**.

Table 7 Details of secondary antibodies

Secondary antibody			
Antibody	Origin	Dilution	Source
Alexa Fluor ® 488 conjugated	goat anti-rabbit	1:200	Thermo Fisher Scientific, USA
Alexa Fluor ® 488 conjugated	goat anti-rat	1:200	Thermo Fisher Scientific, USA
Alexa Fluor ® 594 conjugated	goat anti-rabbit	1:200	Thermo Fisher Scientific, USA
Alexa Fluor ® 594 conjugated	goat anti-rat	1:200	Thermo Fisher Scientific, USA

2.5 Statistical Analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad Software, Inc.). Results are depicted as mean $\pm$ standard error of mean (SEM).

For cell-based experiments with at least three biological repeats ($n \geq 3$) and animal studies with at least a number of three ($n \geq 3$), statistical analysis was performed using Mann-Whitney U test, or one way/two-way analysis of variance (ANOVA) followed by multiple comparisons test. Differences were deemed statistically significant for p -values < 0.05 .

Chapter 3 LRG1 is required for normal cutaneous wound healing

3.1 Background and Rationale

Our previous study has demonstrated that LRG1 is expressed in the skin vasculature of normal human skin tissue [122]. However, the expression level and pattern of LRG1 in other types of skin cells of normal skin tissue and during the wound healing process remain to be elucidated. To understand the role of LRG1 in wound healing, we aim to characterize the expression of LRG1 at different stages of wound healing and evaluate the impact of *Lrg1*-deficiency on wound closure.

3.2 Results

3.2.1 LRG1 is expressed by multiple cells types in normal skin tissue

To study the role of LRG1 in wound healing, we first evaluated the expression pattern of LRG1 in normal human skin by immunohistochemistry staining. Our study showed that LRG1 is not only expressed in the skin vasculature but also expressed in fibroblasts and keratinocytes (**Figure 5A-D**). The expression pattern of LRG1 in human skin was further confirmed by immunofluorescent staining with an LRG1-specific antibody and cell-specific markers in mouse skin. Our study revealed that LRG1 is expressed in Vimentin⁺ dermal fibroblasts (**Figure 5E**) and platelet endothelial cell adhesion molecule (CD31/PECAM-1)⁺ endothelial cells in normal mouse skin (**Figure 5F**). Similarly, the epidermis, which is made of closely packed keratinocytes, is also positive for LRG1 (**Figure 5G**).

Figure 5. LRG1 expression in normal human and murine skin. **A**, Immunohistochemistry staining of normal human skin with an LRG1-specific antibody shows the expression of LRG1 in keratinocytes (arrow) and fibroblasts (arrowhead). Scale bar: 50µm. **B**, Immunohistochemistry staining of normal human skin with an LRG1-specific antibody shows the expression of LRG1 in the vasculature. Scale bar: 50µm. **C-D**, Immunohistochemistry staining of normal human skin with secondary antibody only shows no positive signal in keratinocytes, fibroblasts or the vasculature. Scale bar: 50µm. **E**, Immunofluorescence staining of normal mouse skin with specific antibodies against LRG1 and CD31 shows the expression of LRG1 in endothelial cells. Scale bar: 20µm. **F**, Immunofluorescence staining of normal mouse skin with specific antibodies against LRG1 and Vimentin shows the expression of LRG1 in fibroblasts. Scale bar: 10µm. **G**, Immunofluorescence staining of normal mouse skin with an LRG1-specific antibody shows the expression of

LRG1 in the epidermis outlined by dashed white lines. Scale bar: 30µm. All images are representative.

3.2.2 LRG1 is differentially expressed at different phases of cutaneous wound healing

Cutaneous wound healing is defined by three overlapping phases: inflammation, proliferation and remodeling [4]. In murine excisional wound healing model, the inflammatory phase starts with the recruitment of neutrophils and monocytes immediately after injury, which gradually diminishes and reaches basal level five days after injury [215]. The late stage of inflammatory phase (day three to day four) overlaps with proliferative phase and involves re-epithelialization, angiogenesis and fibroplasia till day ten after injury [215]. The remodeling process starts from day five and gradually increases until weeks to months after injury [215]. To understand the association of LRG1 and different biological processes during wound healing, we conducted qRT-PCR analysis in wound tissues collected at different time points during cutaneous wound healing in mice.

qRT-PCR analysis showed that LRG1 transcript level is significantly increased one day (inflammatory phase) after the injury, which is maintained till day three (inflammatory and proliferative phase) (**Figure 6A**). Interestingly, LRG1 mRNA level was significantly reduced 14 days after the injury, coinciding with the tissue remodeling phase (**Figure 6A**). The up-regulated expression of LRG1 on day one and day three was further confirmed by Western blot analysis (**Figure 6B**).

Figure 6. LRG1 expression during cutaneous wound healing in a mouse model of excisional wound healing. **A**, qRT-PCR analysis of LRG1 transcript levels at different stages of excisional wound healing in mice, $n \geq 3$. **B**, Western blot analysis of LRG1 protein levels at different stages of excisional wound healing in mice, $n = 3$. Data are expressed as mean $\pm$ SEM. Significance was determined by one-way ANOVA followed by Tukey's multiple comparisons test. *: $p < 0.05$.

Having demonstrated an increased expression of LRG1 at the inflammatory phase of wound healing, we went on exploring the cellular source of LRG1 in injured skin tissue. Immunofluorescence staining showed that the infiltrating CD11b⁺ myeloid cells are the major source of LRG1 in the wound bed one day after the injury (**Figure 7A**). This observation was further confirmed by the observation of overlapping LRG1 and neutrophil marker Ly6G⁺ at the same time point (**Figure 7B**). Interestingly, LRG1 was absent in the F4/80⁺ macrophages in the wound bed one day after injury (**Figure 7C**).

Figure 7. LRG1 is expressed in infiltrated neutrophils but not macrophages in the wound bed. **A**, Immunofluorescence staining detecting LRG1 (green), CD11b (red) and DAPI (blue) in wounded C67BL/6 mouse skin tissue one day after injury. **B**, Immunofluorescence staining detecting LRG1 (green), Ly6G (red) and DAPI (blue) in wounded C67BL/6 mouse skin tissue one day after injury. **C**, Immunofluorescence staining detecting LRG1 (green), F4/80 (red) and DAPI (blue) in wounded C67BL/6 mouse skin tissue one day after injury. All images are representative. All scale bars: 20µm.

We further demonstrated that LRG1 is highly enriched in keratinocytes at the migrating tongue and endothelial cells proximal to the wound sites of day three wounds (**Figure 8A-B**).

A

B

Figure 8. LRG1 is expressed in activated keratinocytes and endothelial cells in the wound bed three days after injury. **A**, Immunofluorescence staining detecting LRG1 (green) and DAPI (blue) in normal skin and day three wound tissues of C67BL/6 mice. **B**, Immunofluorescence staining detecting LRG1 (green), CD31 (red) and DAPI (blue) in normal skin and day three wound tissues of C67BL/6 mice. All images are representative. All scale bars: 30µm.

3.2.3 TNF- α induces LRG1 expression via the NF- κ B pathway

Inflammation plays a central role in wound healing, especially during the first three days in a mouse model of excisional wound healing. The upregulation of LRG1 during inflammatory phase prompted us to carry out the *in silico* analysis of a 2kb nucleotide sequence near the transcription initiation site of the LRG1 gene. Two NF- κ B binding sites located between -87 to -97 and +350 to +360 were identified (**Figure 9A**). NF- κ B is known to be activated by numerous inflammatory cytokines, such as TNF- α and interleukins [216].

TNF- α is a master regulator of inflammation [217]. It is significantly induced at the inflammatory phase of cutaneous wound healing [218]. Our study showed that TNF- α could significantly increase LRG1 transcript and protein levels in human dermal microvascular endothelial cells (HDMECs) and keratinocytes (HaCaT cells) (**Figure 9B-C**). ChIP assay was conducted to further validate the transcriptional control of LRG1 by TNF- α /NF- κ B by demonstrating the association between NF- κ B and the *LRG1* promoter region, which was further enhanced by TNF- α treatment (**Figure 9D-E**). Together, these data provided evidence that LRG1 is a direct target gene of TNF- α in endothelial cells and keratinocytes, which might contribute to the increased LRG1 levels during the inflammatory phase of cutaneous wound healing.

Figure 9. TNF- α induces LRG1 expression in HDMECs and HaCaT Cells via the NF- κ B pathway. **A**, Schematic diagram shows the *In silico* analysis of the 2kb sequence near the TSS of the *LRG1* promoter containing two NF- κ B binding sites. **B**, Western blot analysis shows increased LRG1 protein levels in 10ng/ml TNF- α -treated HaCaT cells and HDMECs, $n \geq 3$. **C**, qRT-PCR shows increase LRG1 transcript levels in 10ng/ml TNF- α -treated HaCaT cells and HDMECs, $n \geq 3$. **D**, RT-qPCR analysis of TNF- α -induced association between NF- κ B/p65 and the *LRG1* promoter in HDMECs following ChIP assay, $n=4$. **E**, DNA agarose gel electrophoresis analysis following ChIP for NF- κ B/p65 and the *LRG1* promoter association in TNF- α treated HDMECs. Data are expressed as

mean $\pm$ SEM. Significance was determined by Mann-Whitney U test. ***: $p < 0.001$, **: $p < 0.01$.

3.2.4 *Lrg1*-deficiency leads to delayed cutaneous wound healing

After identifying the source of LRG1 and its transcriptional control during wound healing, we went on investigating the impact of *Lrg1*-deficiency on cutaneous wound healing. As expected, *Lrg1*^{-/-} mice showed a delayed wound closure as compared to their age-matched wild-type counterparts (**Figure 10**), indicating a critical role of LRG1 in the wound healing process.

Figure 10. Wound closure in wild-type and *Lrg1*^{-/-} mice. Representative images (left) and quantification (right) of wound closure in wild-type and *Lrg1*^{-/-} mice. Data are expressed as mean $\pm$ SEM. $n \geq 3$. Significance was determined by Mann-Whitney U test between genotypes at different time points. ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$. Scale bar: 1mm.

3.3 Discussion

Our previous study demonstrated an endothelial cell-specific LRG1 expression in human normal skin tissue [122]. Besides endothelial cells,

we showed that LRG1 is expressed in keratinocytes and fibroblasts of both mouse and human normal skin tissues.

All three cell types are activated during cutaneous wound healing and are essential for effective wound closure [4]. We further showed an elevated Lrg1 expression in mouse skin one day after injury, a stage featured by prominent immune cell influx, largely neutrophils [219]. We also showed that neutrophils, but not macrophages serve as a major source of Lrg1, which is consistent with previous observations in a mouse model of myocardial infarction [131]. Furthermore, there is an increased LRG1 expression in neutrophils subjected to calcium ionophore and phorbol myristate acetate (PMA)-mediated activation [132]. Interestingly, it was reported that the neutrophil-derived LRG1 is differentially glycosylated *in vitro* [132]. It is not clear whether LRG1 isoforms with different levels of glycosylation have different functions.

Infiltrated immune cells produce large amounts of pro-inflammatory cytokines and chemokines, including TNF- α , one of the most important pro-inflammatory cytokines mediating the wound healing process [220, 221]. TNF- α activates its downstream gene expression through transcription factor NF- κ B [222]. Similar to what was demonstrated in endothelial cells [130], our data provided evidence that LRG1 is a direct target gene of TNF- α in keratinocytes and endothelial cells (**Figure 11**). TNF- α might be responsible for elevated LRG1 expression in day three wound tissue, where there is an active migration and proliferation of keratinocytes and endothelial cells in an attempt to regenerate disrupted skin tissues.

Figure 11. TNF- α regulates LRG1 in keratinocytes and endothelial cells. TNF- α binds to TNF receptor 1, activating its canonical NF- κ B pathway. The recruitment of NF- κ B to LRG1 promoter starts the transcription of LRG1.

Other than TNF- α , numerous signaling pathways are known to activate NF- κ B, including members of the IL-1 and the Toll-like microbial pattern recognition receptors (TLRs) [216]. Since keratinocytes express two types of IL1 receptors and IL17-receptor A [223, 224], it will be interesting to know whether other NF- κ B activators, including interleukins and microbial products, can trigger LRG1 expression in keratinocytes during the wound healing process.

Peroxisome proliferator-activated receptor- β/δ (PPAR β/δ) is a member of the nuclear hormone receptor superfamily transcription factors that are collectively involved in the control of lipid homeostasis [225]. It has been shown to regulate LRG1 gene expression in skin fibroblasts [226]. Elevated PPAR β/δ expression was observed at the inflammatory phase

of wound healing [227]. Furthermore, PPAR β/δ was reported to regulate NF- κ B's downstream gene expression by direct interaction with NF- κ B [228, 229]. It will be of interest to study whether PPAR β/δ and NF- κ B work together in regulating LRG1 expression during the wound healing process.

In addition to the healing of epidermis, the underlying dermis must be reconstituted, which involves the formation of granulation tissue that is composed mainly of fibroblasts that migrated from the healthy dermis at the wound margins [230]. These activated fibroblasts contribute to the synthesis, bundling and alignment of collagen fibers [231], which reach the highest level on day 14 of wound closure. Interestingly, LRG1 expression was reduced on day 14, however, the significance of which is not clear. Finally, our study showed a delayed wound healing in *Lrg1*^{-/-} mice suggesting an important role of LRG1 for an effective healing process. In the proposed model, TNF- α derived from infiltrated immune cells trigger LRG1 expression in keratinocytes and endothelial cells *via* activating NF- κ B signaling pathway upon skin injury.

Chapter 4 The role of LRG1 at the inflammatory phase of wound healing

4.1 Background and rationale

Our results so far have demonstrated an elevated LRG1 expression at the inflammatory phase of cutaneous wound healing. In response to inflammatory cytokines, endothelial cells will undergo structural and functional changes to facilitate further immune cell infiltration [232]. LRG1 was previously demonstrated to affect endothelial cells functions [122, 130]. The role LRG1 in endothelial cell-mediated immune cell response during wound healing is investigated in this chapter.

4.2 Results

4.2.1 There is reduced neutrophil recruitment in the wound bed of *Lrg1*^{-/-} mice

As there is significant induction in LRG1 expression in day one wound tissue and neutrophils serve as a major source of LRG1 at the inflammatory phase of wound healing, we first characterized the number of neutrophils in wild-type and *Lrg1*^{-/-} wounds one day after injury. MPO serves as a reliable marker for tissue infiltrating neutrophils [233]. Our study demonstrated a significant reduction in the number of MPO⁺ neutrophils in *Lrg1*^{-/-} wounds (**Figure 12A**). Neutrophil number starts to decline after day one and macrophage population increases gradually to reach peak on day three after injury [234]. Interestingly, macrophage infiltration in day three wounds was not affected by the deletion of *Lrg1* (**Figure 12B**). Together, these data demonstrated an impaired inflammatory response in *Lrg1*^{-/-} mice during cutaneous wound healing.

Figure 12. There is an impaired immune cell infiltration in *Lrg1*^{-/-} mice. **A**, Representative images (left) and quantification (right) of neutrophil number per high power field (HPF) in day one wounds from wild-type and *Lrg1*^{-/-} mice. Tissues were stained against MPO (green), and DAPI (Blue), $n \geq 3$. **B**, Representative images (left) and quantification (right) of the percentage of F4/80 positive area over the total microscopic area in day three wounds from wild-type and *Lrg1*^{-/-} mice. Tissues were stained against F4/80 (red), and DAPI (Blue), $n \geq 3$. Data are expressed as mean

± SEM. Significance was determined by Mann-Whitney U test. *p<0.05; n.s: not significant. Scale bars: 30µm.

4.2.2 LRG1 modulates blood vessel leakage *in vivo* and *in vitro*

Neutrophil extravasation is a multistep cascade [11]. Disruption of endothelial cell junctional complex formation and subsequent increase in blood vessel permeability contribute to the increased infiltration of neutrophils [202]. To understand the underlying mechanism of compromised neutrophil infiltration in *Lrg1*^{-/-} mice, we first characterized the impact of LRG1 on skin vessel leakage in wild-type mice using Mile's assay. Our study showed that subcutaneous delivery of recombinant rLRG1 treatment led to a significant increase in skin vessel leakage as demonstrated by the increased level of extravasation of circulating Evans Blue dye (**Figure 13A**). VEGF is a powerful vascular permeabilizing agent that has a profound physiological role during the wound healing process [186, 235]. Our study showed that the VEGF-induced blood vessel leakage was significantly compromised in *Lrg1*^{-/-} mice as compared to that in wild-type controls (**Figure 13A**).

The endothelium serves as a selective semi-permeable barrier and its permeability is controlled by cell junctions, in particular, endothelial adherens junctional complex, VE-cadherin [236]. The availability of VE-cadherin at the plasma membrane is instrumental in maintaining vascular integrity and homeostasis [237]. To investigate the molecular and cellular mechanism of LRG1-induced vessel leakage, HDMECs were treated with rLRG1 and subjected to immunofluorescence staining with a specific antibody targeting VE-cadherin. Our study showed that rLRG1 significantly disrupted the membrane distribution of VE-cadherin (**Figure 13B**). Consistent with this observation, rLRG1 led to a significant increase in the amount of FITC-dextran leaked through the HDMEC monolayer in an *in vitro* Transwell-based permeability assay, to a similar extent as VEGF-treated HDMECs (**Figure 13C**). It was previously reported that VEGF mediates the disruption of endothelial cell junctional complex

through src family kinase (SFK) [238]. We further showed an elevated level of phosphorylated SFK in rLRG1-treated HDMECs as demonstrated by Western blot analysis (**Figure 13D**). In summary, our data provided compelling evidence for the promoting role of LRG1 in vascular permeability and LRG1 may exert its function through regulating the phosphorylation of SFK.

Figure 13. LRG1 regulates vessel leakage via SFK pathway. **A**, Representative images (left) and quantification (right) of Miles' assay demonstrating reduced vascular leakage in the skin of *Lrg1*^{-/-} mice as indicated by the level of extravasated circulating Evans Blue dye, $n \geq 3$. Scale bars: 1mm. **B**, Representative images of HDMEC monolayer stained with anti-VE-cadherin (Green), Phalloidin (Red) and DAPI (Blue). Scale bar: 65 μ m. **C**, FITC-dextran transwell permeability assay on rLRG1 or rVEGF treated HDMECs, $n \geq 3$. **D**, Representative Western blots of p-SFK, SFK and GAPDH expression levels in rLRG1-treated HDMECs, $n = 3$.

Data are expressed as mean $\pm$ SEM. Significance was determined by one-way or two-way ANOVA followed by Tukey's multiple comparisons test or Mann-Whitney U test. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.0001$.

4.3 Discussion

The endothelium is a selective semi-permeable barrier that controls the movement of macromolecules and even cells between blood and surrounding tissues [239]. In response to injury, an acute inflammatory response triggers the loosening of endothelial junctions to allow inflammatory cells, plasma proteins and fluid to travel from circulation to the injury site [240, 241]. In this chapter, the infiltration of immune cells was studied in skin tissues of *Lrg1*^{-/-} mice after injury. The infiltration of neutrophils is compromised in *Lrg1*^{-/-} mouse which is, at least in part, due to the reduced vessel permeability at the early phase of wound healing. Interestingly, macrophage infiltration is not affected in *Lrg1*-deficient mice. Although infiltration of mononuclear-macrophages and neutrophils share similar mechanisms [242], the expression levels of adhesion molecules and chemotactic factors affect the types of phagocytes that infiltrate the injured tissue [243]. Our data indicated a cell-type-specific role of LRG1 in mediating immune cell infiltration.

Mile's assay was used to further elucidate LRG1's role in vessel permeability *in vivo* and demonstrated that rLRG1 significantly induces the skin vessel leakage. Consistently, deletion of LRG1 attenuates the VEGF-induced skin vessel leakage. However, the vessel permeability has not been examined in the wound healing process. It will be of interest to study whether vessel permeability is changed in *Lrg1*^{-/-} mice during the wound healing process.

To achieve increased permeability, endothelial adherens junctional protein, mainly VE-cadherin, will be disrupted upon activations [244, 245]. Immunofluorescence staining showed that rLRG1 could disrupt the

membrane organization of VE-cadherin in HDMECs. This was further supported by *in vitro* FITC-dextran permeability assay. Mile's assay showed VEGF-induced skin vessel permeability is significantly attenuated in *Lrg1*^{-/-} mice. Src family kinases (SFK) have been implicated in VEGF-induced endothelial hyper-permeability [238, 246]. Similar to VEGF, rLRG1 also promotes the phosphorylation of SFK. However, it is not clear whether LRG1 exerts its function in a VEGF-dependent manner. In addition, LRG1 is known to interact with Endoglin [122]. A recent study demonstrated that Endoglin is able to bind to VEGFR2 on the cell surface and prevent its degradation [247]. It will be interesting to know whether LRG1-mediated activation of SFK is dependent on Endoglin.

Besides increased vessel permeability, endothelial cells express numerous cell surface adhesion proteins to capture neutrophils and facilitate their rolling on the endothelium. It will be of interest to study whether LRG1 regulates the expression of cell-surface adhesion proteins in both neutrophils and endothelial cells during the wound healing process. The recruitment of neutrophils in skin wounds is also determined by a plethora of inflammatory chemokines and cytokines. It will be interesting to assess whether LRG1 mediates the expression and action of other inflammatory factors during wound healing.

Chapter 5 The role of LRG1 in re-epithelialization of wound healing

5.1 Background and rationale

One of the critical steps of wound healing is the regeneration of wound epidermis, which is achieved by a combined action of keratinocyte migration and proliferation at the wound edge [39]. TGF β 1 strongly inhibits keratinocyte growth but induces keratinocyte differentiation towards a migratory phenotype [248]. Our previous data demonstrated a regulatory role of LRG1 on endothelial TGF β 1 signaling. However, its role in keratinocyte behaviors remains to be elucidated.

5.2 Results

5.2.1 There is a delayed re-epithelialization in *Lrg1*-deficient mice

To further characterize the delayed wound healing in *Lrg1*^{-/-} mice, we studied the rate of re-epithelialization at different time points during the wound healing process. Our data showed a delayed rate of re-epithelialization in *Lrg1*^{-/-} mice as compared to that in wild-type controls on day three and four after injury (**Figure 14**).

Figure 14. Impaired re-epithelialization in *Lrg1*^{-/-} mice. Representative images (up) and quantification (bottom) of wound tissues at day three and four after injury in wild-type and *Lrg1*^{-/-} mice. The advancing epithelial tongue is delineated by white dotted lines. Data are expressed as mean $\pm$ SEM. Significance was determined by Mann-Whitney U test; $n \geq 3$; * $p < 0.05$. n.s: not significant. Scale bars: 125 μ m

5.2.2 LRG1 promotes keratinocyte wound healing *in vitro*

Next, the impact of LRG1 on keratinocyte behaviors was examined *in vitro* using scratch wound healing assay. Our study demonstrated that LRG1-overexpressing HaCaT cells were able to close the gap at a much faster rate, whereas HaCaT cells subjected to siRNA-mediated LRG1 took a significantly longer time to close the gap as compared to scrambled siRNA-treated control cells (**Figure 15A**). It is well established that both migration and proliferation contribute to wound closure in this assay. To further determine the key factor contributing to the promoting effect of LRG1, keratinocyte proliferation was blocked by mitomycin C, a potent anti-proliferative drug. Interestingly, the impact of LRG1 on keratinocyte wound closure was completely lost when keratinocyte proliferation is inhibited by mitomycin C (**Figure 15B**). Together, our data provided evidence that the promoting role of LRG1 in re-epithelialization was predominantly contributed by its influence on cell proliferation rather than migration.

Figure 15. LRG1 promotes keratinocyte wound healing *in vitro*. **A**, Representative images (left) and quantification (right) of scratch wound healing assay on confluent HaCaT cells subjected to siRNA-mediated LRG1 knockdown or transfection with pcDNA3.1-LRG1 $n \geq 3$. **B**, Representative images (left) and quantification (right) of scratch wound healing assay on confluent HaCaT cells subjected to siRNA-mediated LRG1 knockdown or transfection with pcDNA3.1-LRG1 with mitomycin C pre-treatment, $n \geq 3$. Data are expressed as mean $\pm$ SEM. Significance was determined by one way ANOVA followed by multiple comparisons test. **: $p < 0.01$, ***: $p < 0.001$, n.s: not significant. Scale bar: 110 μ m.

5.2.3 LRG1 increases keratinocyte viability through attenuating TGF β pathway

Ki67 is a human nuclear protein that is strictly associated with proliferating cells [249]. Besides the reduced rate of re-epithelialization, the percentage of proliferating Ki67⁺ keratinocytes at day three wound edge is dramatically reduced in *Lrg1*^{-/-} mice (**Figure 16A**). Consistent with the reduced re-epithelialization rate and percentage of proliferating keratinocytes, the thickness of the newly formed epithelium in the healing area of day five wound is significantly reduced in *Lrg1*^{-/-} mice (**Figure 16B**). Consistent with the observation *in vivo*, LRG1-overexpressing HaCaT cells demonstrated an increased viability as compared to pcDNA3.1 plasmid transfected control cells (**Figure 16C**). On the contrary, HaCaT cells subjected to siRNA-mediated LRG1 knockdown showed a reduced viability (**Figure 16C**). Cyclin D1 is a central regulator of proliferation [250, 251]. Our data revealed reduced cyclin D1 level in LRG1 knockdown HaCaT cells as demonstrated by Western blot analysis, whereas an increased cyclin D1 expression was observed in LRG1-overexpressing keratinocytes (**Figure 16D**).

The canonical TGF β /Smad2/3 signaling pathway plays a critical role TGF β 1-mediated growth suppression in keratinocytes [252, 253]. Consistent with the reduced keratinocyte viability in LRG1 knockdown HaCaT cells, there is an elevated level of TGF β 1-induced phosphorylation of Smad2 in LRG1 knockdown HaCaT cells (**Figure 16E**). Together, these data suggested that LRG1 promotes re-epithelialization by attenuating the anti-proliferative TGF β /Smad signaling pathway in keratinocytes.

Figure 16. LRG1 mediates keratinocyte proliferation through the TGF β signaling pathway. **A**, Diagram indicating the location of representative images (Upper), representative images (bottom left) and quantification (bottom right) of Ki67 positive epithelial cells at day three wound edge. Tissues were stained against Ki67 (green) and DAPI (Blue), $n \geq 3$. Scale bar: 30 μm . **B**, Diagram indicating the location of representative images (Upper), representative images (bottom left) and quantification (bottom right) of the epithelium thickness in the healing area of day five wound, $n \geq 3$. Scale bar: 25 μm . **C**, Fold change of the number of pcDNA3.1-LRG1 overexpressing HaCaT cells normalized to pcDNA3.1 transfected control cells and LRG1 siRNA treated HaCaT cells normalized to scramble siRNA treated control cells, $n \geq 3$. **D**, Representative Western blot analysis of cyclin D1 in LRG1-

overexpressing, knockdown or control HaCaT cells, $n \geq 3$. **E**, Representative Western blot analysis of p-Smad2, Smad2 and GAPDH in HaCaT cells transfected with scramble siRNA or LRG1 siRNA, $n \geq 3$. Data are expressed as mean $\pm$ SEM. Significance was determined by Mann-Whitney U test or one way ANOVA followed by multiple comparisons test. *: $p < 0.05$.

5.3 Discussion

One of the key hallmarks of successful wound healing is the closure of the disrupted epidermis. Our study demonstrated a reduced rate of re-epithelialization in day three and day four wound tissues and a significant reduction in the thickness of newly formed epidermis in day five wound tissues of *Lrg1*^{-/-} mice. We further demonstrated a promoting role of LRG1 in keratinocyte proliferation. TGF- β 1 is one of the most potent inhibitors of keratinocyte proliferation and it exerts its function through the canonical Smad2/3 pathway [252]. Transgenic mice expressing a dominant negative TGF β RII in keratinocytes show increased keratinocyte proliferation and accelerated wound healing [254]. Consistent with LRG1's pro-proliferative effect, there is an elevated Smad2 phosphorylation in *LRG1* knockdown keratinocytes. Our previous data demonstrated that LRG1 is able to bind to the ubiquitously expressed TGF β type I receptor, ALK5, however, the significance of LRG1 and ALK5 interaction remains to be elucidated [122]. TGF β 1 mainly signals through the ALK5 receptor in keratinocytes [85]. In the follow-up study, we will investigate the interaction between LRG1 and different TGF β receptors in keratinocytes and its functional consequence.

Both proliferation and migration of keratinocytes contribute to the re-epithelialization during wound healing [176]. Besides its potent anti-proliferation role, over-activation of TGF β /Smad2 signaling pathway retards the migration of keratinocytes during wound healing [253]. While LRG1 attenuates the TGF β 1-induced activation of Smad2, LRG1 knockdown or overexpressing keratinocytes showed no difference in

terms of wound closure *in vitro* in the absence of proliferation. Despite this, we could not exclude the involvement of LRG1 in keratinocyte differentiation *in vivo*. Further investigation of keratinocyte differentiation markers, such as Keratin 10, Keratin 2, and LM-3A32, is necessary to address this question.

Furthermore, TGF β signaling is highly complex and context-dependent, and the role of TGF β 1 in re-epithelialization is still contradictory. The crosstalk between canonical and non-canonical TGF β 1 signaling transducers, as well as its interaction with other signaling pathways define the final cellular response to TGF β 1 treatment. For instance, it has been demonstrated that TGF β 1 inhibits keratinocyte growth in a PKC α -dependent manner [255]. Other studies also showed that TGF β 1-induced stress fiber formation and mesenchymal transition of keratinocytes require the activation of Rho-like GTPases, RhoA and PI3K/Akt/mTOR pathway [117]. It is worth exploring the impact of LRG1 in TGF β 1-regulated activations of PKC α , RhoA and PI3K/Akt/mTOR in keratinocyte migration and proliferation. It is possible that the fine-tuning of LRG1 expression level will affect the outcome of TGF β 1-mediated re-epithelialization. These proposed experiments will help us understand the contradictory roles of TGF β 1's in re-epithelialization.

It has been recently reported that LRG1 can directly bind to EGF receptor and activate phosphorylation of p38 signaling in pancreatic cancer [256], and it is known that EGF and EGF receptor are the master regulators of re-epithelialization [257]. It will be interesting to investigate whether LRG1 can regulate EGF pathway in re-epithelialization.

In addition, it is important to point out the differences between HaCaT cells and primary keratinocytes. As immortalized keratinocytes, HaCaT cells have been reported to be metabolically different from primary human

keratinocytes [258, 259]. Primary human and murine keratinocytes will be used to confirm our observation in HaCaT cells.

Collectively, we demonstrated a critical role of LRG1 in re-epithelialization during wound healing. LRG1 promotes keratinocyte proliferation by attenuating the canonical TGF β /Smad2/3 signaling

Chapter 6 The role of LRG1 in angiogenesis in wound healing

6.1 Background and rationale

During wound healing, capillaries from adjacent healthy skin tissue invade the fibrin/fibronectin-rich provisional matrix and organize themselves into a complex microvascular network that spread throughout the granulation tissue within a few days [50]. Under physiological conditions, new blood vessel formation is a tightly controlled process mediated by dynamic interactions between different types of cells, angiogenic growth factors and extracellular matrix components. Deficient angiogenesis contributes to impaired wound healing [50].

LRG1 was previously demonstrated to play a critical role in pathological angiogenesis leading to the development of multiple vasoproliferative diseases, including diabetic retinopathy, cancer, and osteoarthritis [122, 130, 260]. However, LRG1's involvement in physiological neovascularization during tissue regeneration is poorly defined. In this chapter, the role of LRG1 in dermal microvascular endothelial cell function and angiogenesis was examined.

6.2 Results

6.2.1 There is a reduced vessel density in the wounds of *Lrg1*^{-/-} mice

Angiogenesis occurs during the few days after skin injury [261]. Vessel density reaches a peak at day seven before blood vessels start to regress in a mouse model of excisional wound healing created by 4mm punches [262]. To understand the role of LRG1 in physiological neovascularization

during wound healing, vessel density in day seven wounds was examined in wild-type and *Lrg1*^{-/-} mice. Skin vasculature was labeled by endothelial cell-specific marker CD31. Our study demonstrated a significant reduction in vessel density in the granulation tissue of *Lrg1*^{-/-} mice as compared to wild-type controls (**Figure 17**).

Figure 17. Vessel density in the granulation tissue of wild-type and *Lrg1*^{-/-} mice. Representative images (left) and quantification (right) of the percentage of blood vessels in the granulation tissue normalized to the total wound area in wild-type and *Lrg1*^{-/-} mice seven days after injury. Tissue was stained against CD31 (green), and DAPI (Blue), $n \geq 3$. Data are expressed as mean $\pm$ SEM. Significance was determined by Mann-Whitney U test. * $p < 0.05$. Scale bar: 40 μ m.

6.2.2 LRG1 promotes dermal microvascular endothelial cell proliferation, migration and tube formation *in vitro*

To further understand LRG1's role in skin angiogenesis, rLRG1-treated HDMECs were subjected to MTS assay with VEGF, one of the most potent angiogenic factor, being used as a positive control. Our study

showed that rLRG1 was able to increase the viability of HDMECs to a similar extent as VEGF (**Figure 18A**). Consistently, an increased expression of a cell cycle maker, cyclin D1, was observed in rLRG1-treated HDMECs (**Figure 18B**). To further understand the underlying mechanism of rLRG1-induced increase in cell viability, HDMECs were subjected to immunofluorescence staining with an antibody specific for cell proliferation marker, Ki67. Our study showed that both rVEGF and rLRG1 lead to a significant increase in the percentage of Ki67-positive HDMECs (**Figure 18C**). Endothelial cell migration is essential for the successful establishment of new blood vessels during wound healing [50]. This process is regulated by chemotactic, haptotactic, and mechanotactic stimuli and requires the degradation of ECM enveloping the vascular cells [263]. In this study, Transwell migration assay was used to study the motility of HDMECs. Our data showed that LRG1 significantly promotes the migration of HDMECs to a similar extent as VEGF (**Figure 18D**). Capillary tube formation in Matrigel requires proliferation and migration of endothelial cells to form a three-dimensional vascular network. Our study showed that rLRG1 promotes HDMECs to form tube-like structures (**Figure 18E**).

Figure 18. rLRG1 promotes endothelial cell activation and angiogenesis *in vitro*. **A**, MTS assay was used to test the viability of VEGF and rLRG1-treated HDMECs, n=5. **B**, Representative Western blot analysis of cyclin D1 expression in rLRG1 treated HDMECs, n=3. **C**, Representative images of HDMECs subjected to immunofluorescence staining (left) with Ki67 antibody (Red) and DAPI (Blue) and quantitative analysis (right) of the percentage of Ki67 positive cells, n=3. Scale bar: 100µm. **D**, Representative images (left) and quantification (right) of transwell migration assay of HDMECs subjected to VEGF and rLRG1

treatments, n=3. Scale bar: 100 μ m. **E**, Representative images (left) and quantification (right) of Matrigel tube formation by HDMECs subjected to VEGF and rLRG1 treatments, n=3. Scale bar: 250 μ m. Data are expressed as mean $\pm$ SEM. Significance was determined by one way ANOVA followed by multiple comparisons test. *: p<0.05, **: p<0.01, ***: p<0.001.

6.2.3 LRG1 exerts its pro-angiogenic function by activating TGF β signaling pathway

LRG1 was previously reported to promote the switch from the angiostatic T β RII-ALK5/Smad2/3 pathway towards the pro-angiogenic T β RII-ALK1/Smad1/5/8 pathway in macrovascular HUVECs [122]. As angiogenesis is a highly tissue-specific process, the impact of LRG1 on TGF β 1 signaling pathway in microvascular HDMECs was investigated in this study.

Unlike that in HUVECs, rLRG1 is able to induce the phosphorylation of canonical TGF β 1 signaling pathway transducers, Smad1/5/8 in HDMECs in the absence of exogenous TGF β 1 (**Figure 19**). However, the promoting effect of rLRG1 was completely lost in the presences of ALK1 inhibitor (LDN193189), ALK5 inhibitor (SB431542) and TGF β 1 neutralizing antibody, suggesting TGF β 1 is required for the action of LRG1 in HDMECs (**Figure 19**).

Figure 19. LRG1 regulates the endothelial TGF β signaling pathway. Representative images of Western blots showing the activation of canonical TGF β 1 signaling pathway after the treatment with rLRG1 in the absence or the presence of ALK1 inhibitor, LDN193189, ALK5 inhibitor, SB431542 or a TGF β 1 neutralizing antibody, n=4.

6.3 Discussion

Although LRG1 is widely implicated in pathological angiogenesis, this study, for the first time, revealed a promoting role of LRG1 in physiological angiogenesis during the wound healing process. Reduced vessel density was observed in the day seven wounds of *Lrg1*^{-/-} mice. Most data on LRG1-regulated angiogenesis were carried out in HUVECs, a type of macrovascular endothelial cells, in the past. In this study, skin-derived primary microvascular endothelial HDMECs were used. Consistent with previous studies in HUVECs, rLRG1 significantly increases HDMEC proliferation, migration, and the ability to form tube-like structures.

LRG1 is known to enhance the TGF β 1-induced phosphorylation of Smad1/5/8 in HUVECs. Interestingly, Western blot analysis indicated that rLRG1 could activate the Smad1/5 activation in the absence of exogenous TGF β 1. Yet, blocking TGF β 1 by a TGF β 1 neutralizing antibody significantly reduces the LRG1-induced phosphorylation of Smad1/5, suggesting the promoting effect of LRG1 on Smad1/5 depends on TGF β 1. The difference in endogenous TGF β 1 level in different types

of endothelial cells might be responsible for the differential effect of LRG1 in HUVECs and HDMECs.

The previous study demonstrated that blocking Smad1/5/8 using LDN-193189 abrogated LRG1-induced Matrigel tube formation in HUVECs [122], indicating the angiogenic role of LRG1 is dependent on canonical TGF β 1/Smad1/5/8 pathway. However, besides its role in regulating the canonical TGF β 1 signaling pathway, studies in chapter 4 revealed that LRG1 induces SFK phosphorylation in HDMECs as well. Activation of SFK is required for not only VEGF-induced vessel permeability but also angiogenesis [264]. Furthermore, extensive crosstalk between SFK and TGF β 1 was reported in non-endothelial cells [265-267]. It will be interesting to know whether LRG1 modulates angiogenesis by mediating the crosstalk between SFK and TGF β 1 pathway.

The maturation and stabilization of newly formed blood vessels require the involvement of pericytes [268], which has not been explored in this study. It will be interesting to investigate whether perivascular cells coverage is altered in the wounds from *Lrg1*^{-/-} mice. In addition, excessive vessels need to be removed from the wound bed at the late stage of wound healing in a process called blood vessel pruning [61, 261]. This is a significant part of physiological angiogenesis. It will be interesting to see whether LRG1 mediates vessel pruning at the later stage of wound healing by regulating EC survival.

Taken together, this chapter demonstrated an important role of LRG1 in physiological angiogenesis in the normal wound healing process, and LRG1 exerts its function by regulating the TGF β /smad1/5/8 signaling pathway.

Chapter 7 The role of LRG1 in ECM formation and remodeling in wound healing

7.1 Background and rationale

Formation of ECM is essential for proper wound healing since it provides the scaffold guiding the migration of different cellular components into the wound bed, serves as a reservoir of key growth factors and cytokines, generates contractile force bringing wound edge together, and provides mechanical strength supporting the newly formed skin tissue [202]. In response to tissue injury, fibroblasts are recruited into the wound bed from adjacent health skin tissues to produce ECM [47]. Next, pro-fibrotic growth factors, mainly TGF β 1, stimulate fibroblast proliferation and fibroblast-to-myofibroblast differentiation, which is critical in ECM production and wound contraction [269].

Decreased ECM production is the hallmark of non-healing wounds in diabetic patients [270]. However, underlying molecular mechanisms remain to be elucidated. In this chapter, the role of LRG1 in ECM homeostasis and skin fibrosis was studied under both physiological and pathological conditions using different *in vitro* and *in vivo* models.

7.2 Results

7.2.1 There is an increased fibrotic gene expression in *Lrg1*^{-/-} wounds

To study the role of LRG1 in ECM formation and remodeling, we conducted qRT-PCR in day seven wounds and demonstrated increased

expressions of α -SMA and FN1 mRNA in the wound bed of *Lrg1*^{-/-} mice (**Figure 20A-B**). These data suggested a potential role of LRG1 in ECM remodeling during wound healing.

Figure 20. There is an increased fibrotic gene expression in wounds of *Lrg1*^{-/-} mice. A-B, qRT-PCR results show fibronectin and α -SMA mRNA levels in day seven wounds from wild-type and *Lrg1*^{-/-} mice, $n \geq 3$. Data are expressed as mean $\pm$ SEM. Significance was determined by Mann-Whitney U test. *: $p < 0.05$.

7.2.2 rLRG1 attenuates the TGF β 1-induced activation of dermal fibroblasts

The promoting effects of TGF β 1 on the proliferation of fibroblasts are well established [109, 271]. To study the role of LRG1 in TGF β 1-induced dermal fibroblast proliferation, normal human dermal fibroblasts (NHDFs) were treated with TGF β 1 and/or rLRG1. While TGF β 1 led to a drastic increase in the percentage of Ki67 positive cells, rLRG1 significantly attenuated the pro-mitotic effect of TGF β 1 on dermal fibroblasts (**Figure 21A**). Western blot analysis demonstrated that TGF β 1 induces the expression of CTGF in NHDFs, whereas rLRG1 drastically attenuates the TGF β 1-induced CTGF expression (**Figure 21B**).

A

B

Figure 21. rLRG1 attenuates the TGFβ1-induced activation of NHDFs.

A, Representative images (left) and quantification (right) of the percentage of Ki67 positive NHDFs subjected to treatment with 200ng/ml rLRG1 in the presence or absence of 10ng/ml TGFβ1. NHDFs were stained with Ki67 (red) and DAPI (Blue). $n=3$. Scale bar: 100μm. **B**, Representative Western blot of whole cell lysates probed with CTGF and GAPDH antibodies, $n\geq 3$. Data are expressed as mean $\pm$ SEM. Significance was determined by one-way ANOVA followed by Tukey's multiple comparisons test. **: $p<0.01$, ****: $p<0.0001$.

7.2.3 rLRG1 attenuates the TGF β 1-induced myofibroblast differentiation

Myofibroblasts play key roles in ECM remodeling and fibrotic scar formation [272, 273]. In addition, the contractility generated by myofibroblasts is responsible for bringing the wound edge together [274, 275]. Targeting myofibroblast differentiation is an attractive strategy for the treatment of impaired wound healing and excessive fibrosis. Gel contraction assay was conducted and showed that rLRG1 significantly suppresses the TGF β 1-induced collagen gel contraction (**Figure 22A**). Western blot analysis demonstrated that the TGF β 1-induced expression of α -SMA is drastically reduced in NHDFs co-treated with rLRG1 (**Figure 22B**).

A

B

Figure 22. rLRG1 attenuates the TGFβ1-induced myofibroblast differentiation. **A**, Representative images (left) and quantification (right) of collagen I gel contraction by NHDFs treated with 200ng/ml rLRG1 in the presence or absence of 10ng/ml TGFβ1, $n \geq 3$. Scale bars: 250μm. **B**, Representative Western blot of whole cell lysates probed with α-SMA and GAPDH antibodies, $n \geq 3$. Data are expressed as mean $\pm$ SEM. Significance was determined by one-way ANOVA followed by Tukey's multiple comparisons test. ****: $p < 0.0001$, *: $p < 0.05$.

7.2.4 rLRG1 attenuates the TGF β 1-induced activation of canonical and non-canonical signalling transducers in fibroblasts

TGF β 1 mediates the activation of fibroblasts through both canonical and non-canonical signaling transducers. Besides Smad2/3 [276, 277], the non-canonical TGF β 1 signaling transducers, such as Akt/PI3K and p38/MAPK, are shown to play important roles in skin fibrosis [278-280]. Western blot analysis showed that TGF β 1 induces the phosphorylation of Smad2/3 in NHDFs, whereas the addition of rLRG1 drastically reduces the TGF β 1-induced Smad2/3 phosphorylation 60minutes after the treatment (**Figure 23A**). Similarly, rLRG1 significantly reduced the TGF β 1-induced p38 and Akt phosphorylation at 60 minutes after TGF β 1 treatment (**Figure 23B**).

Figure 23. rLRG1 attenuates the TGF β 1 signaling in NHDFs. A, Representative Western blot of whole cell lysates probed with antibodies for canonical TGF β signaling transducers, $n \geq 3$. **B,** Representative Western blot of whole cell lysates probed with antibodies for non-canonical TGF β signaling transducers. NHDFs were treated with 200ng/ml rLRG1 in the presence or absence of 10ng/ml TGF β 1, $n \geq 3$.

7.2.5 LRG1 overexpressing NHDFs are less responsive to TGF β 1-induced fibroblast activation

Having established the inhibitory role of rLRG1 on TGF β -induced activation of NHDFs, we went on testing the responsiveness of LRG1-overexpressing NHDFs to TGF β 1-induced activation. LRG1 overexpression in NHDFs was confirmed by qRT-PCR analysis (**Figure 24A**). Consistent with the observation using rLRG1, LRG1 overexpressing NHDFs are less responsive to the TGF β 1-induced expression of CTGF and α SMA as compared to pcDNA3.1 transfected control cells (**Figure 24B**). In addition, overexpressing LRG1 in NHDFs significantly attenuates the TGF β 1-induced collagen I gel contraction (**Figure 24C**).

Figure 24. LRG1 overexpression attenuates the TGF β 1-induced fibroblast activation. A, qRT-PCR analysis of LRG1 mRNA in LRG1 overexpressing NHDFs. B, Representative Western blot demonstrating the expression of CTGF and α SMA in LRG1 overexpressing NHDFs in the presence or absence of 10ng/ml TGF β 1, $n \geq 3$. C, Representative images (left) and quantification (right) of collagen I gel contraction mediated by pcDNA3.1 or pcDNA3.1-LRG1 overexpressing NHDFs in the presence or absence of 10ng/ml TGF β 1, $n \geq 3$. Scale bars: 250 μ m. Data are expressed as mean $\pm$ SEM. Significance was determined by unpaired,

by Mann-Whitney U test or two-way ANOVA followed by Tukey's multiple comparisons. *: $p < 0.05$, **: $p < 0.01$.

7.2.6 *Lrg1*-deficiency leads to increased responsiveness to the TGF β 1-induced fibroblast activation

Having demonstrated an inhibitory effect of LRG1 on TGF β 1-induced NHDF activation, we next characterized the impact of *Lrg1*-deficiency on TGF β 1-mediated fibroblast activation using mouse embryonic fibroblasts (MEFs) isolated from wild-type and *Lrg1*^{-/-} mice. Our study demonstrated a higher basal contractility generated by MEFs isolated from *Lrg1*^{-/-} mice as compared to those generated by MEFs isolated from wild-type mice, which was further enhanced by the treatment of TGF β 1 (**Figure 25A**). In addition, the increased contraction generated by *Lrg1*^{-/-} MEFs could be reversed by the treatment with exogenous rLRG1 (**Figure 25B**). qRT-PCR and Western blot analyses further showed that *Lrg1*^{-/-} MEFs express higher CTGF at both mRNA (**Figure 25C**) and protein levels (**Figure 25 D**).

Figure 25. *Lrg1*-deficiency leads to an exacerbated response to TGF β 1-induced fibroblast activation. A, Representative images (left)

and quantification (right) of collagen I gel contraction induced by wild-type and *Lrg1*^{-/-} MEFs in the presence or absence of 10ng/ml TGFβ1, n≥3. **B**, Representative images (left) and quantification (right) of collagen I gel contraction induced by *Lrg1*^{-/-} MEFs with the presence or absence of 200ng/ml rLRG1, 10ng/ml TGFβ1, or the combination of both. n≥3. **C**, qRT-PCR analysis of CTGF expression in wild-type and *Lrg1*^{-/-} MEFs in the presence or absence of 10ng/ml TGFβ1, n≥3. **D**, Western blot analysis of CTGF expression in wild-type and *Lrg1*^{-/-} MEFs in the presence or absence of 10ng/ml TGFβ1, n≥3. Data are expressed as mean ± SEM. Significance was determined by two-way ANOVA followed by Tukey's multiple comparisons test. *: p<0.05, **: p<0.01. All scale bars: 250μm.

7.2.7 *Lrg1*-deficiency leads to increased cell content and CTGF expression in bleomycin-induced skin fibrosis

LRG1's effect in skin fibrosis was further examined using a bleomycin-induced skin fibrosis model *in vivo*. After 28 days of subcutaneous injection of bleomycin, there was a significant increase in cell density in subcutaneous adipose tissue of wild-type mice, which was further increased in bleomycin-treated *Lrg1*^{-/-} mice (**Figure 26A-B**). Consistently, there is an increased CTGF expression in bleomycin-treated *Lrg1*^{-/-} mice skin as compared to their wild-type counterparts (**Figure 26C**).

Figure 26. *Lrg1*-deficiency leads to exacerbated bleomycin-induced increase in subcutaneous cellular content and CTGF expression. **A-B**, Representative images (left) and quantification (right) of the cell density in hypodermis area of wild-type and *Lrg1*^{-/-} mouse skin after bleomycin treatment. $n \geq 6$. **C**, qRT-PCR analysis of CTGF expression in wild-type and *Lrg1*^{-/-} mouse skin after bleomycin treatment, $n \geq 3$. Data are expressed as mean $\pm$ SEM. Significance was determined by two-way ANOVA followed by Tukey's multiple comparisons test. ***: $p < 0.001$, ****: $p < 0.001$. Scale bar: 25 μ m

7.3 Discussion

Dysfunctional ECM formation and remodeling are critical in the pathogenesis of diabetic foot ulcers and skin scarring [57, 270]. TGF β 1 is the most potent growth factor mediating ECM homeostasis and it exerts its function mainly through the canonical Smad2/3 pathway [116]. Similar to what we observed in keratinocytes, LRG1 strongly inhibits the TGF β 1-induced activation of Smad 2/3 in fibroblasts.

Besides the canonical TGF β signaling, the non-canonical pathways of TGF β 1, including p38 MAPK and Akt pathways were also regulated by LRG1 in fibroblasts. It is well-established that non-canonical TGF β pathways also play an important role in mediating fibrotic responses [107]. For example, p38 MAPK has been proven to be required for TGF β 1-induced collagen synthesis in dermal fibroblasts and myofibroblast differentiation in tenon fibroblasts. In addition, it is also reported that Akt is required for fibroblast survival and myofibroblast differentiation. Collectively, our data suggested a universal suppressive effect of LRG1 on fibroblastic TGF β 1 pathways.

Besides its important role in skin wound healing, fibroblast activation is involved in wound healing processes in other organs, including lungs, livers, heart and kidneys [281]. An over-activated fibrotic response is a major cause of organ failure and is responsible for 45% of all deaths in the industrialized world [282]. Despite the fact that the anti-fibrotic role of LRG1 may be detrimental in diabetic wound healing, targeting LRG1 may serve as an attractive strategy for the treatment of other fibrotic diseases. Taken together, this study suggested that LRG1 attenuates the TGF β 1-induced fibroblast activation during the wound healing process.

Chapter 8 Elevated LRG1 contributes to delayed wound healing in diabetes

8.1 Background and rationale

Elevated plasma LRG1 levels were observed in a few diabetic vascular complications, including retinopathy [147], peripheral artery disease [283], and chronic kidney disease [284]. Having established the role of LRG1 in normal wound healing, the role of LRG1 in diabetic wound healing was further investigated in this chapter.

8.2 Results

8.2.1 There is an increased LRG1 expression in the serum and wound tissues of DFU patients

To establish the association between LRG1 and DFU, serum LRG1 level in DFU patients was measured by ELISA assay. Consistent with the observation in other diabetic vascular complications, DFU patients showed a significantly higher level of LRG1 in serum as compared to diabetic patients without DFU or individuals with venous ulcer (**Figure 27A**). Consistently, LRG1 level is significantly higher in the wound margin of DFU as compared to that of venous ulcers (**Figure 27B**).

Figure 27. Increased LRG1 expression in patients with DFU. **A**, Serum LRG1 concentration in patients with DM alone, venous ulcer and DFU. $n \geq 6$. **B**, Representative images (top) and densitometry analysis (bottom) of Western blot demonstrated an increased LRG1 protein level in wound tissues harvested from patients with venous ulcers and DFU. $n \geq 6$. Data are expressed as mean $\pm$ SEM. Significance were determined by one-way ANOVA followed by Tukey's multiple comparisons test or Mann-Whitney U test. * $p < 0.05$, ** $P < 0.01$.

8.2.2 Elevated LRG1 level is associated with delayed wound healing in diabetic mice

To further validate the association between LRG1 and diabetic wound healing, streptozotocin (STZ)-induced type I diabetes was established in both wild-type and *Lrg1*^{-/-} mice. Fasting blood glucose (FBG) was significantly increased in STZ-injected mice, and no difference in FBG has been observed between wild-type and *Lrg1*^{-/-} mice under normal and diabetic conditions (**Figure 28A**). Mice with FBG higher than 200mg/dL were included in the study. Consistent with previous studies [164],

delayed wound healing was observed in STZ-induced diabetic mice (**Figure 28B**), suggesting a successful establishment of diabetic wound healing model.

LRG1 expression at different stages during the wound healing process was examined in this model. qRT-PCR results demonstrated elevated LRG1 mRNA levels on day one, day three and day 14 during diabetic wound healing as compared to those in normoglycemic control group (**Figure 28C**). Immunofluorescence staining of LRG1 showed a higher percentage of LRG1-positive keratinocytes in the epithelial migrating tongue in diabetic wounds (**Figure 28D**). Taken together, an elevated LRG1 expression is associated with delayed wound healing in diabetes.

Figure 28. There is an increased LRG1 expression in wound tissue of diabetic mice. A, Measurement of fasting blood glucose in STZ treated wild-type and *Lrg1*^{-/-} mice. **B,** Assessment of wound closure in wild-type mice subjected to STZ-induced diabetes, $n \geq 3$. **C,** qRT-PCR analysis of LRG1 expression at different time points during wound healing in normal and diabetic mice, $n \geq 3$. **D,** Representative images (left) of LRG1 staining and quantification of LRG1-positive keratinocytes (right) in day three wounds of normal and diabetic mice. Scale bar: 30 μ m. Data are expressed as mean $\pm$ SEM. Significance was determined by two-way ANOVA followed by Tukey's multiple comparisons test or by Mann-Whitney U test between groups at different time points. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$.

8.2.3 There is an accelerated wound closure in *Lrg1*^{-/-} mice subjected to STZ-induced diabetes

Having established the association between elevated LRG1 expression and delayed wound healing in diabetic mice, we next studied the rate of wound closure in wild-type and *Lrg1*^{-/-} mice subjected to STZ-induced diabetes. Intriguingly, despite the delayed wound healing in *Lrg1*^{-/-} mice under physiological conditions, *Lrg1*^{-/-} mice were resistant to diabetes-induced impairment in wound healing (**Figure 29**).

Figure 29. Wound closure in wild-type and *Lrg1*^{-/-} mice subjected to STZ-induced diabetes. Representative images (left) and quantification (right) of wound size in wild-type and *Lrg1*^{-/-} mice subjected to STZ-induced diabetes. Data are expressed as mean $\pm$ SEM. $n \geq 3$. Significance was determined by Mann-Whitney U test between genotypes at different time points. *: $p < 0.05$. Scale bar: 2 mm.

8.2.4 There is a reduced NETosis in *Lrg1*^{-/-} mice subjected to STZ-induced diabetes

As demonstrated in figure 29, the biggest difference in wound closure between wild-type and *Lrg1*^{-/-} mice was observed three days after injury, the time point when there is an active inflammatory response. Elevated NETosis has been reported as the major contributor to delayed wound healing in diabetes and removal of NETs by DNase I completely rescues the delayed wound closure in diabetic mice [164]. LRG1 has been reported to regulate myelopoiesis *via* regulating TGFβ signaling [132], and we have demonstrated that infiltrating neutrophils act as a major source of LRG1 at the inflammatory phase of wound healing. To understand the role of LRG1 in NETosis, lysates from day three wounds of diabetic wild-type and *Lrg1*^{-/-} mice were subjected to Western blot analysis for citrullinated histone H3 (H3Cit), a specific marker for NETs [285]. Our data showed a significant reduction in H3Cit levels in the diabetic wounds of *Lrg1*^{-/-} mice (**Figure 30A**). To further investigate the role of LRG1 in NETosis, neutrophils were isolated from the bone marrow of wild-type and *Lrg1*^{-/-} mice and subjected to treatment with calcium ionophore A23187 (A23187), a potent stimulant of NETosis [286]. Our study demonstrated that A23187 induces a significant increase in NETosis in wild-type neutrophils, whereas *Lrg1*^{-/-} neutrophils are resistant to A23187-induced NETosis (**Figure 30B**). When neutrophils undergo NETosis, they release their DNA content into the extracellular environment [287]. Consistent with our previous observation, *Lrg1*^{-/-} neutrophils treated with A23187 release less cell-free DNA as compared to wild-type control (**Figure 30C**). On the other hand, rLRG1 induces human peripheral neutrophils to release cell-free DNA in human neutrophils (**Figure 30D**). ERK pathway was previously reported to mediate NETosis in response to external stimuli [288, 289]. Western blot analysis showed a significant activation of ERK pathway in human neutrophils treated with rLRG1 (**Figure 30E**). Together, these data

suggested a critical role of LRG1 in NETosis, possibly through the ERK signaling pathway, during wound healing in diabetes.

Figure 30. LRG1 regulates NETosis via ERK pathway. **A**, Representative Western Blot shows a reduced H3Cit expression in day three wounds of *Lrg1*^{-/-} mice subjected to STZ-induced diabetes, $n \geq 3$. **B**, Representative images (left) and quantification (right) of H3Cit staining in bone marrow-derived neutrophils isolated from wild-type and *Lrg1*^{-/-} mice after A23187 treatments, $n \geq 3$. Scale bars: 20 μ m. **C**, Quantification of cell-free DNA by Sytox Green in bone marrow-derived neutrophils isolated from wild-type and *Lrg1*^{-/-} mice after A23187 treatments, $n \geq 3$. **D**, Quantification of cell-free DNA measured by Sytox Green in human neutrophils subjected to rLRG1 treatment, $n \geq 3$. **E**, Representative Western blot of p-ERK, ERK, H3Cit and GAPDH in human neutrophils subjected to rLRG1 treatment. Data are expressed as mean $\pm$ SEM. Significance was determined by Mann-Whitney U test or two-way ANOVA followed by Tukey's multiple comparisons test. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$.

8.3 Discussion

Under physiological conditions, LRG1 modulates the function of multiple cell lineages required for efficient healing process. Consistently, *Lrg1*^{-/-} mice showed a delayed wound closure as compared to their wild-type counterparts. Intriguingly, elevated LRG1 levels are observed in both plasma and wound tissues of diabetic patients with DFU and diabetic mice. *Lrg1*^{-/-} mice are resistant to diabetes-induced delay in wound closure. Together, our study suggests that both overproduction and insufficient LRG1 are detrimental for efficient healing process. We further demonstrated that the most significant difference in wound closure between wild-type and *Lrg1*^{-/-} mice subjected to STZ-induced diabetes occurs three days after injury, a time point when there is an active inflammatory response. The presence of LRG1 in the wound infiltrating neutrophils prompted us to look into the role of LRG1 in neutrophil function. NETosis has been implicated as an important part of neutrophil innate immunity [15]. However, excess NETosis leads to numerous of autoimmune and inflammatory disorders, including delayed wound healing in diabetic patients [164]. Our study showed a reduced NETs marker, H3Cit, in day three wound tissues of *Lrg1*^{-/-} mice subjected to STZ-induced diabetes. Furthermore, *Lrg1*-deficient neutrophils are resistant to calcium ionophore (A23187)-induced NETs formation. However, whether LRG1 can affect NETosis triggered by other stimulus, including phorbol 12-myristate 13-acetate (PMA), LPS, glucose, and pro-inflammatory cytokines, remain to be elucidated. The activation of the MEK/ERK pathway has been implicated in a variety of NETosis molecular mechanisms [16] and our data provided evidence that rLRG1 activated NETosis *via* ERK pathway. However, the specific receptors for LRG1-mediated activation of ERK during NETosis remain to be identified. A previous study demonstrated that human FcγRIIIb-induced NETs formation requires transforming growth factor-β-activated kinase 1 (TAK1) in the ERK-dependent manner [289]. Since LRG1 regulates TGFβ

signaling pathway in multiple cell types involved in wound healing, it will be of interest to see whether LRG1-activated NETs formation is dependent on TAK1 and FcγRIIIb.

We previously demonstrated that LRG1 affects neutrophil recruitment at the inflammatory phase of wound healing in healthy control mice. It is possible that the accelerated wound healing in *Lrg1*^{-/-} mice under diabetic conditions is caused by reduced neutrophil infiltration as well as diminished NETosis activation.

Chapter 9 Conclusion and future direction

9.1 Conclusion and proposed model of LRG1's role in normal and diabetic wound healing

Cutaneous wound healing is one of the most complicated biological processes in the human body. In this study, we elucidated the roles of LRG1 in normal and diabetic wound healing. During the normal wound healing process, neutrophils are one of the major sources of cytokines, growth factors and soluble mediators that regulate the functions of immune cells, fibroblasts, endothelial cells and keratinocytes [7]. Our study showed that neutrophils also secrete a large amount of LRG1, which regulates NETosis by activating ERK signaling.

LRG1 is triggered by TNF- α in keratinocytes and endothelial cells. This induction is dependent on the classical NF- κ B activation by TNF- α . We further showed that LRG1 increases skin vessel leakage and disrupts VE-cadherin of endothelial cells *via* activating SFK signaling pathway, which may facilitate transendothelial migration of neutrophils. Indeed, the wound infiltrating neutrophils is significantly reduced in *Lrg1*^{-/-} mice. In addition, LRG1 promotes keratinocyte proliferation *via* attenuating TGF β /Smad2/3 signaling pathway. Deletion of LRG1 significantly compromises re-epithelialization in excisional wound healing model by reducing keratinocyte proliferation in the wound edge. LRG1 was previously implicated in abnormal angiogenesis [122]. Our study provided evidence that LRG1 is also involved in physiological neovascularization during wound healing and LRG1 exerts its function through mediating the TGF β /Smad1/5/8 pathway in HDMECs. Similar to what we observed in keratinocytes, LRG1 attenuates TGF β /Smad2/3 signaling pathway in fibroblasts and reduces TGF β 1-induced fibroblast activation. Reduced LRG1 expression observed in day 14 wound tissues when there is an active ECM remodeling is possibly due to the need for activating

fibroblasts. The dynamic and cell-type dependent functions of LRG1 orchestrate wound healing under normoglycemic conditions, and our study demonstrated a delayed wound healing in non-diabetic *Lrg1*^{-/-} mice. Under diabetic conditions, prolonged inflammation results in increased LRG1 level, which induces NETs formation *via* activating ERK pathway, therefore, leading to delayed wound healing.

Taken together, LRG1 regulates normal wound healing by spatially and temporarily mediating the TGF β signaling pathway in keratinocytes, endothelial cells and fibroblasts, but excessive LRG1 leads to increased NETs formation, ultimately delaying wound healing in diabetes (**Figure 31**).

Figure 31. Proposed a working model of LRG1's role in normal and diabetic wound healing. During normal wound healing skin injury, infiltrated neutrophils, activated keratinocytes and endothelial cells secrete LRG1. Secreted LRG1 increases vessel leakage to facilitate recruitment of neutrophils in inflammatory phase, promotes angiogenesis and re-epithelialization during proliferative phase. Due to its inhibitory effects on fibroblast activation, LRG1 expression starts to decline with the increase of ECM deposition and tissue remodeling. Under diabetic conditions, LRG1 is further upregulated and promotes NETosis, which halts the wound healing in inflammatory stage.

9.2 Crosstalk of LRG1 and ENG in wound healing

The complex role of LRG1 in wound healing is contributed by its function in regulating TGF β signaling pathway, which affects multiple cell types during the wound healing process. The cell-dependent regulation by LRG1 possibly results from the presence of ENG in different cell types. It has been reported that ENG is expressed in low levels in quiescent skin and significantly up-regulated in endothelial cells and basal-like keratinocytes upon tissue injury [290]. However, ENG expression in human wound fibroblasts is not up-regulated during the wound healing process [291]. A previous study has demonstrated that LRG1 binds to ENG and switches the endothelial TGF β pathway from ALK5/Smad2/3 to ALK1/Smad1/5/8 [122]. In addition, the critical role of ENG in wound healing was further supported by the findings that the wound repair is impaired in adult *Endoglin* heterozygous mice due to defective re-epithelialization and compromised keratinocyte proliferation [292]. Although it has not been investigated in the wound healing context, it is known that ENG is a critical player in angiogenesis. Significant defects in vascular repair are observed in *Endoglin* heterozygous mice [291]. In addition, another study revealed that *Endoglin* heterozygous mice shows increased fibroblast accumulation during wound healing [293]. ENG's beneficial roles in re-epithelialization and angiogenesis, but a detrimental role in fibroblast activation are highly similar to LRG1's role in wound healing. Therefore, it will be of interest to study whether ENG and LRG1 work together to regulate the wound healing process.

9.3 Future directions

Although our study proves that LRG1 is pro-angiogenic in wound healing, which is considered as a beneficial function, the detrimental effects of LRG1 in inducing NETosis play a predominant role in diabetic wound healing. This is supported by the fact that accelerated wound closure is

associated with reduced NETosis formation in *Lrg1*^{-/-} diabetic wounds as compared to their wild-type counterparts. It is of interest to study whether the removal of NETosis using DNase can reverse or attenuate the detrimental effect of LRG1 in diabetic wound healing.

Extensive investigations of LRG1's role in vessel permeability, re-epithelialization, angiogenesis and fibroblast activation have been done in the normoglycemic wound healing process. However, whether LRG1 can affect some of those processes in diabetic wound healing remains to be elucidated. For example, re-epithelialization starts at three days following injury in our mouse model of wound healing. Hyperproliferative and non-migratory keratinocytes are one of the hallmarks of DFU. Considering the important role of LRG1 in keratinocyte proliferation during the normal wound healing process, it is plausible that the elevated LRG1 levels observed in diabetes contribute to a hyperproliferative phenotype of keratinocytes. Although LRG1 did not affect keratinocyte migration in *in vitro* studies, it is worthy investigating the contribution of LRG1 to the non-migratory phenotypes under diabetic conditions. In addition, LRG1 expression is maintained at high levels at the late phase wound healing under diabetic conditions. Considering the anti-fibrotic role of LRG1, this may contribute to reduced ECM deposition in diabetic wounds, also a characteristic feature of DFU.

Under the diabetic condition, it is worthwhile to explore LRG1's potential to be a biomarker of diabetic wound healing. Currently, it is difficult to objectively assess the status of wound healing based on biological assay [294]. Serum/Plasma LRG1 has been proposed to be a valid biomarker for many diseases [142, 295, 296]. This study demonstrated that LRG1 is significantly upregulated in the serum of DFU patients and wound margins from diabetic foot ulcers. The accessibility of peripheral venous blood indicates that LRG1 might be an ideal systemic biomarker for disease diagnosis and prognosis. Therefore, further investigations are required to

find out whether there is a correlation between LRG1 expression level and healing outcome.

The beneficial and detrimental roles of LRG1 in normal and diabetic wound healing raise the concern of LRG1's therapeutic potential in diabetic wound healing. Although angiogenic growth factors including VEGF and PDGF have been demonstrated to facilitate diabetic wound healing in animal models [194, 297, 298], topical application of recombinant LRG1 might not be beneficial due to its role in NETs formation as NETosis is one of a causative factor leading to delayed wound healing in diabetes. Targeting LRG1 using small interfering RNA or neutralizing antibodies will be potential approaches of reducing NETosis in diabetic foot ulcers. Although deactivation of LRG1 may result in other abnormalities like reduced re-epithelialization and angiogenesis, NETosis and prolonged inflammation play a more predominant role in the pathogenesis of diabetic foot ulcers. Blocking LRG1 at the early stage might still be helpful in preventing wounds from becoming arrested in the inflammatory phase of healing.

9.4 The implications of LRG1 in other skin diseases

Psoriasis is a chronic inflammatory skin disease associated with striking epidermal growth and angiogenesis [298, 299]. A recent study reported an increase in mRNA level of LRG1 in the lesional region from patients with psoriasis patients as compared to the non-lesional region (GDS4602 / 228648_at/LRG1) [300]. Considering the important roles of LRG1 in keratinocyte proliferation process, angiogenesis, and vessel leakage, it is possible that LRG1 is also involved in the pathogenesis of psoriasis.

Angiogenesis is vital in the growth and metastasis of skin cancers. Hence, targeting angiogenesis has a great potential in treating numerous solid cutaneous tumors such as squamous cell carcinoma, melanoma and

basal cell carcinoma [301]. LRG1 has been implicated in the progress of several cancers due to its role in tumor angiogenesis [118, 136, 140, 143]. However, whether LRG1 is involved in cutaneous tumors is hitherto unknown. Besides angiogenesis, it has been demonstrated in this study that LRG1 could affect the TGF β /Smad2 signaling pathway in epithelial cells as well. TGF β /Smads activation drives EMT in invasion and metastasis of carcinomas. Targeting the EMT process is also a potential way to suppress tumor metastasis [302]. Collectively, the remarkable similarities between wound healing and cancer indicate a potential role of LRG1 in skin cancers.

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