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A MULTIFACTORIAL APPROACH TO TARGETING SIGNALING PATHWAYS IN DIABETIC FOOT ULCERS

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A Multifactorial Approach to Targeting Signaling Pathways in Diabetic Foot Ulcers

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“If you can’t explain it simply, you don’t understand it well enough”

– Albert Einstein

ABSTRACT

Diabetic foot ulcers (DFU) are one of the most debilitating complication of diabetes that adversely impacts the health, economics and quality of life of the afflicted individual. The primary pathogenic factor of DFU is hyperglycemia, and its negative effects on normal signaling pathways is still being investigated. As such, there is no specific therapy that could target the underlying dysregulations caused by hyperglycemia. So, it is important to delve into various pathways that are altered by hyperglycemia in diabetic foot in order to successfully establish novel treatment paradigms.

Wound healing consists of various phases where different cellular processes such as cell proliferation, migration, angiogenesis and apoptosis coordinate to achieve a swift healing of the wound. In my thesis, I have investigated several signaling pathways that play key roles in wound healing and are profoundly disturbed by hyperglycemia in diabetes.

Notch signaling pathway is an important pathway where receptors and ligands from juxtaposed cells activate signal transduction. Upon activation, an intracellular domain of Notch (NICD) translocates to the nucleus and initiates the transcription of specific targets to control cell proliferation, cell migration, angiogenesis, differentiation and apoptosis. In paper-I, we show that Notch1 is activated in human and rodent skin and several processes central to wound healing are impaired in response to hyperglycemia in a Notch1 dependent manner. Mechanistically, we show that hyperglycemia activates a Dll4-Notch1 feedforward loop that impairs wound healing in diabetes. Inhibition of Notch signaling by chemical and genetic approaches improved wound healing in diabetic mice significantly.

IGF-I is a growth hormone that is expressed in every cell of our body. The circulating IGF-I is however derived mainly from the liver. IGF-I promotes wound healing and its levels are decreased in diabetic wounds. However, the contribution of circulating IGF-I to wound healing is unknown. In Paper II, we generated a liver-specific IGF-I knockout mice and induced diabetes in these mice to study the effect of liver-derived IGF-I on wound healing. We found that the lack of liver-derived IGF-I did not affect healthy wound healing. Although diabetes delayed wound healing, there was no difference between knock-out mice and control mice. In addition, the processes contributing to wound healing were not altered by the liver-derived IGF-I deficiency. In summary, we found that a lack of liver-derived IGF-I did not affect wound healing. Future therapies using IGF-I can be designed to be delivered locally since systemic IGF-I therapy is known to carry risks of unfavorable side-effects.

In papers-III and IV, I have investigated the roles of miRNA-210 and miRNA-34a in diabetic wound healing respectively. miR-210 is induced by transcription factor HIF-1 in response to hypoxia. miR-210 mirrors HIF function in hypoxia by regulating important processes such as cell proliferation, migration, apoptosis, metabolism

and angiogenesis. We found that miR-210 expression is reduced in diabetic wounds and locally reconstituting miR-210 using mimics improves diabetic wound healing significantly. miR-210 reconstitution led to a reduction in the oxygen consumption rate in the wounds that led to a decrease in ROS levels in the wound tissue. This metabolic reprogramming by miR-210 ultimately resulted in the improvement in different cellular processes central to wound healing.

miR-34a plays important roles in cell cycle and DNA repair. Importantly, miR-34a has been shown to regulate Notch1 directly. Although there are contrasting reports on their function in hypoxia and diabetes, their role in diabetic wound healing has not been elucidated. In paper-IV, we show that miR-34a was reduced in DFUs and in the wounds of diabetic mice. We also found that a long exposure to hypoxia increased miR-34a expression exclusively in keratinocytes but exposing cells to high glucose decreased its expression in hypoxia. Reciprocally, Notch1 expression levels increased in keratinocytes under hypoxic and high glucose levels in a time-dependent manner. Finally, we found that diabetic wounds injected with miR-34a mimic showed significantly lower expression of Notch1, directly correlating with paper-I, indicating that reconstitution of miR-34a could be a potential therapeutic strategy for diabetic wounds.

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I. Zheng X*, **Narayanan S***, Sunkari VG*, Eliasson S, Botusan IR, Grünler J, Catrina AI, Radtke F, Xu C, Zhao A, Ekberg NR, Lendahl U, Catrina SB.

Triggering of a Dll4-Notch1 loop impairs wound healing in diabetes.

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Deficiency of liver-derived insulin-like growth factor-I (IGF-I) does not interfere with the skin wound healing rate.

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III. **Narayanan S**, Eliasson S, Xu C, Grünler J, Zhao A, Zhu W, Landén NX, Ståhle M, Botusan IR, Ekberg NR, Zheng X*, Catrina SB*

HypoxamiR-210 accelerates wound healing in diabetes by improving mitochondrial energy metabolism.

Manuscript

* equally contributed authors

IV. **Narayanan S***, Eliasson S*, Grünler J, Xu C, Li D, Landén NX, Ståhle M, Botusan IR, Ekberg NR, Zheng X#, Catrina SB#

Downregulated miR-34a contributes to the increased Notch1 signaling in diabetic wounds.

Manuscript

*, # equally contributed authors

LIST OF PUBLICATIONS NOT INCLUDED IN THESIS

I. Zheng X, **Narayanan S**, Zheng X, Luecke-Johansson S, Gradin K, Catrina SB, Poellinger L, Pereira TS.

A Notch-independent mechanism contributes to the induction of Hes1 gene expression in response to hypoxia in P19 cells.

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MicroRNA-132 enhances transition from inflammation to proliferation during wound healing.

Journal of Clinical Investigation. 2015. 125(8):3008-26.

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III. Li X*, Li D*, Wang A, Chu T, Lohcharoenkal W, Zheng X, Grünler J, **Narayanan S**, Eliasson S, Herter EK, Wang Y, Ma Y, Ehrström M, Eidsmo L, Kasper M, Pivarcsi A, Sonkoly E, Catrina SB, Ståhle M, Xu Landén N.

MicroRNA-132 with Therapeutic Potential in Chronic Wounds.

Journal of Investigative Dermatology. 2017. 137(12):2630-2638.

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LIST OF ABBREVIATIONS

5'-UTR	5'-Untranslated region
ADAM	A-disintegrin and metalloproteinase
AGEs	Advanced glycation end-products
AGO	Argonaute
ALS	Acid labile subunit
AMDCC	Animals Models for Diabetes Complications Consortium
Bcl-2	B-cell lymphoma 2
BrdU	5-Bromo-2'-deoxyuridine
BSA	Bovine serum albumin
CD11b	Cluster of differentiation molecule 11b
CD31	Cluster of differentiation 31
CDK4/6	Cyclin-dependent kinase 4/6
cDNA	Complementary DNA
CSL	CBF1-suppressor of Hairless-LAG1
CXCR4	C-X-C chemokine receptor type 4
DAPI	4',6-diamidino-2-phenylindole
DAPT	*tert*-Butyl(2S)-2-[[(2S)-2-[[2-(3,5-difluorophenyl)acetyl]amino]propanoyl]amino]-2-phenylacetate
DFU	Diabetic foot ulcers
DGCR8	DiGeorge syndrome chromosomal (or critical) region 8
Dll4	Delta-like 4
E2F1	E2F transcription factor 1
ECM	Extracellular matrix
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ELK1	ETS like-1 protein
eNOS	Endothelial Nitric oxide synthase
EPC	Endothelial progenitor cells
FALEC	Focally amplified long non-coding RNA in epithelial cancer
FFPE	Formalin-fixed paraffin-embedded
FGF	Fibroblast growth factor
FIH-1	Factor inhibiting HIF-1
Foxp1	Forkhead box protein P1

GCKR	Glucokinase regulatory protein
GH	Growth hormone
GLUT2	Glucose transporter 2
GPD1-L	Glycerol-3-phosphate dehydrogenase 1-like
GSI	Gamma-secretase inhibitor
GTP	Guanosine triphosphate
HbA1c	Glycated hemoglobin A1C
HDF	Human dermal fibroblasts
HDMEC	Human dermal microvascular endothelial cells
Herp	Homocysteine-induced endoplasmic reticulum protein
Hes	Hairy and enhancer of split
HIF-1	Hypoxia inducible factor-1
HMGB1	High mobility group box 1
HOTAIR	HOX transcript antisense RNA
HRE	Hypoxia responsive element
IDF	International diabetes federation
IGF-I	Insulin-like growth factor-1
IGFBP	Insulin-like growth factor binding protein
IGFR	Insulin-like growth factor receptor
IL-1	Interleukin-1
ISCU	Iron-Sulphur cluster assembly enzyme
K14	Keratin 14
KDM	Histone lysine demethylase
KGF	Keratinocyte growth factor
KHB	Kreb's henseleit buffer
LGR4	Leucine-rich-repeat-containing G-protein coupled receptor 4
MALAT1	Metastasis associated lung adenocarcinoma transcript 1
MAML1	Mastermind-like transcriptional activator 1
MET	Mesenchymal Epithelial transition proto-oncogene
MIB	Mindbomb
miRISC	microRNA-induced silencing complex
MMPs	Matrix metalloproteinases
MNT	Max's Next Tango (Max-binding protein)
mRNA	Messenger RNA
NAD	Nicotinamide adenine dinucleotide

NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NEUR	Neuralized
NEXT	Notch extracellular truncation
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	Nerve growth factor
NICD	Notch intracellular domain
NMYC	Neuroblastoma MYC transcription factor
NO	Nitric oxide
Nrarp	Notch regulated ankyrin repeat protein
OCR	Oxygen consumption rate
Oct-4	Octamer-binding transcription factor 4
ORF	Open reading frame
PAS	Per-Arnt-Sim domain
PBGD	Porphobilinogen Deaminase
PBS	Phosphate buffered saline
PCNA	Proliferation cell nuclear antigen
PDGF	Platelet derived growth factor
PEST	Proline (P)-Glutamic acid (E)- Serine (S)-Threonine (T) sequence
PHD	Prolyl hydroxylase
PKC	Protein kinase C
PPARγ	Peroxisome proliferator-activated receptor gamma
RAD52	RAD52 Homolog, DNA repair protein
ROS	Reactive oxygen species
RT-PCR	Real-time polymerase chain reaction
SDF-1	Stromal-derived factor 1
SDHD	Succinate dehydrogenase subunit D
SIRT1	Sirtuin 1
SKIP	Ski-interacting protein
STZ	Streptozotocin
TCA	Tricarboxylic acid cycle
TGF-β	Transforming growth factor-β
T_h17	T helper 17 cells
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VHL	von Hippel Lindau protein

1 BACKGROUND

1.1 Diabetes – an epidemic

Diabetes mellitus is a heterogeneous disease characterized by a chronic increase in the blood glucose levels. The International Diabetes Federation (IDF) estimates that 1 in 11 adults are affected by diabetes and 50% of the afflicted are undiagnosed. It is estimated that the number of patients with diabetes will increase to 642 million by 2040[1]. Obesity, sedentary lifestyle and increase in the age of the population are the main contributing factors to the increase in the prevalence of diabetes[1]. Diabetes has an important economic burden that will increase from US $1.3 billion in 2015 to $2.1 trillion in 2030[2].

1.1.1 Pathophysiology of diabetes and its complications

Diabetes is broadly classified as type 1 diabetes and type 2 diabetes. The type 1 diabetes results from a total insulin deficiency caused by the destruction of the pancreatic β-cells due to an autoimmune attack[3]. Type 2 diabetes is characterized by an insufficient insulin production by the β-cells to maintain normal blood glucose levels and insulin resistance[3].

Prolonged exposure of cells to hyperglycemia is the major contributor to diabetes-associated complications[6,7]. Increased uptake of glucose by the cells leads to an increased mitochondrial ROS production, that results in the 4 damaging pathways common to all the complications of diabetes – increased flux through polyol pathway, production of Advanced Glycation End-products (AGE), increased production of Protein Kinase C (PKC) and the increased production of hexosamine intermediates[4]. Besides, the increase in AGE and low-density lipoprotein levels induce ROS production from NADPH oxidase in the endothelial cells[5]. Hyperglycemia dependent glycosylation of endothelial Nitric oxide synthase (eNOS) and an increase in arginase, which competes with eNOS for the substrate, leads to decrease in eNOS activity. This leads to the production of reactive nitrogen species[6]. Ultimately, all of these lead to microvascular complications (retinopathy, nephropathy, and neuropathy) (Figure 1).

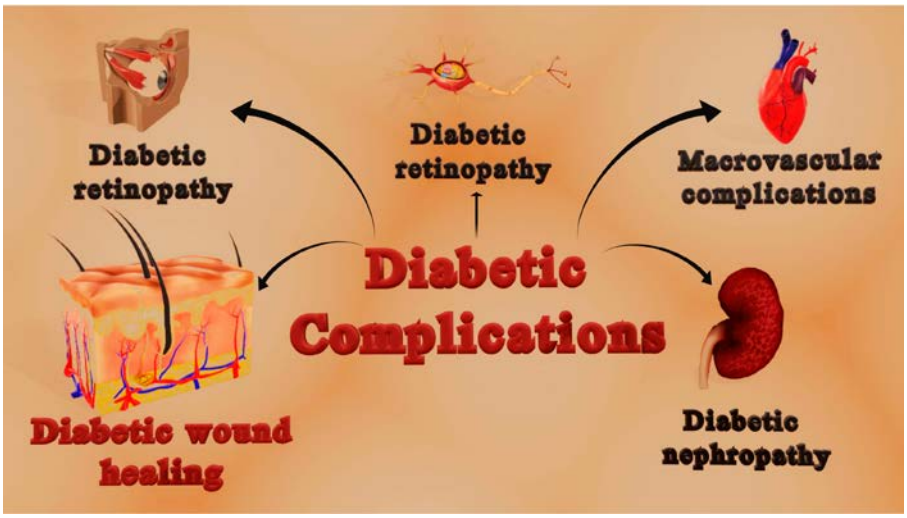

Figure 1. *A graphic representation of the different complications of diabetes.*

1.2 Wound healing

Healing of a wound is a complex process coordinated by various molecular events leading to wound closure and scar formation[7,8,9,10]. The events that occur immediately after a cutaneous injury can be divided into four overlapping phases: coagulation and hemostasis, inflammation, proliferation and remodeling[7,8]. The appropriate and coordinated progression of these processes is critical to a normal and effective wound healing. Impairment in wound healing occurs when one or several underlying molecular processes in the different phases are deregulated. A brief overview of the molecular events during these phases is presented below (Figure 2).

1.2.1 Coagulation and hemostasis

A cutaneous injury damage the epidermal and dermal layers of the skin, exposes the underlying tissue, disrupts the blood vessels and results in an outflow of the blood into the tissue. Within minutes after the injury, the vascular smooth muscle cells rapidly contract and constrict the damaged blood vessels, preventing the leakage of the blood[10]. This is followed by the release of clotting factors from the platelets which contributes to the formation of a blood clot, followed by hemostasis. It will further provide a provisional matrix for the cells to migrate in the wound[7,11]. The sequence of events during the activation of platelets and formation of the clot provides the transition to the next phase of healing. For instance, platelets activate the complement system and release a variety of growth factors such as platelet-derived growth factors (PDGF), epidermal growth factor (EGF) and transforming growth factor-β (TGF-β) which act as signaling molecules to recruit neutrophils, macrophages, endothelial cells

and fibroblasts[8,10]. During the formation of the clot, the polymerization of fibrinogen to fibrin yields fragments such as fibrinopeptides A and B, which also act as signaling molecules to recruit inflammatory cells to the wound[7].

1.2.2 Inflammation

Neutrophils are the first immune cells that arrive at the wound site after hemostasis. Various chemokines produced during clotting attract the neutrophils from circulation by inducing the expression of adhesion molecules on endothelial cells[12]. The neutrophils adhere to these adhesion molecules, roll along the endothelium and extravasate into the wound site[12]. Neutrophils play an important role in protecting the wound from external factors such as bacteria and foreign bodies. They also release cytokines that amplify the inflammatory response. In about 3 to 5 days, the neutrophils are cleared and monocytes arrive at the wound site[12]. A lack of adequate levels of immune response or prolonged activation of the inflammatory phase could lead to a lack of progression to the proliferation phase and hence could affect the wound healing.

1.2.3 Proliferation

This phase is characterized by the migration of fibroblasts to the wound site, secretion of matrix components and formation of the granulation tissue. Migration and proliferation of fibroblasts is facilitated by various growth factors like PDGF secreted by inflammatory cells. Once in the wound site, fibroblasts in the presence of transforming growth factor-β (TGF-β), attach to the fibrous proteins in the extracellular matrix (ECM) and differentiate into myofibroblasts[13]. The extracellular matrix (collagen, hyaluronan, proteoglycans, etc.) produced by the fibroblasts replaces the provisional matrix formed by the platelets. This collagen matrix provides the basis for migration of more fibroblasts and contributes to the contraction of the wound[14]. Moreover, the hypoxia caused by the disruption of the blood vessels at the wound site stimulates the production of vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) by macrophages[15]. This promotes the proliferation and migration of endothelial cells into the wound with secondary formation of capillaries within the injured site. The macrophages, fibroblasts, the nascent endothelium along with the matrix of collagen, fibrinogen and fibronectin collectively constitute the granulation tissue[16,14]. With the accumulation of newer collagen matrix, the neovascularization diminishes and as the healing proceeds, the granulation tissue matures to form a scar.

1.2.4 Epithelialization and tissue modeling

As the granulation tissue becomes dense, the keratinocytes around the wound edges are activated by various chemokines such as interleukin-1 (IL-1) and TGF-β[11,17]. The activated keratinocytes migrate over the injured site and proliferate to induce epidermis regenration[17]. The proliferating fibroblasts in the granulation tissue also produce

keratinocyte growth factor (KGF), which activates the migration and proliferation of keratinocytes[18]. This process is called epithelialization and is vital for regeneration of the epidermis. Meanwhile, as the granulation tissue matures, the extracellular matrix is degraded by matrix metalloproteinases and a new layer of collagen is secreted. The newly secreted collagen I has a higher tensile strength compared to the initially secreted collagen III and hence, helps retain the toughness and texture of unwounded skin after healing[12]. This remodeling includes even the contraction of the wound mediated by fibroblasts[14,12]. A careful balance between the degradation and synthesis of matrix components is important for a successful remodeling of the wound. As the wound heals, excess fibroblasts, macrophages and endothelial cells undergo apoptosis followed by a decrease of blood flow at the wound site. The remaining granulation tissue ultimately matures into a scar to complete the wound healing process.

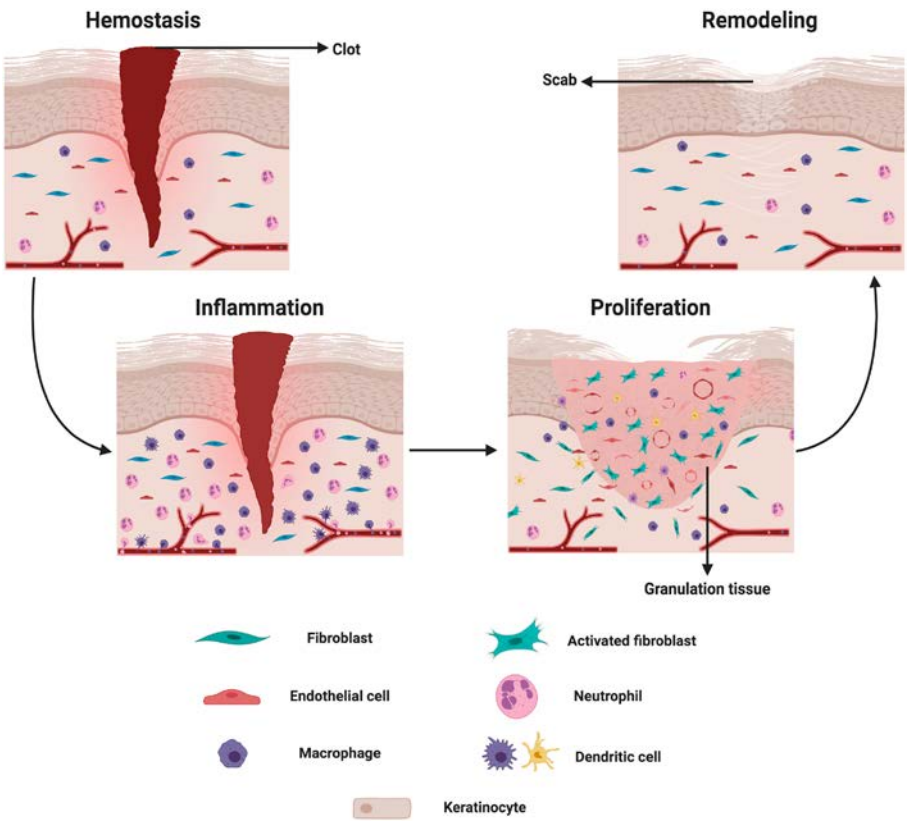

Figure 2. *A flow diagram showing the different stages of wound healing.*

1.3 Diabetic wounds

1.3.1 Molecular aspects of impaired wound healing in diabetes

The factors that contribute to impairment in wound healing in diabetes are complex. That includes various intrinsic (neuropathy, angiopathy, hyperglycemia) and extrinsic factors (infection, pressure on the wound site)[7]. Beside the traditional "pathogenic triad" that comprise neuropathy, ischemia and trauma, there are several other players that contribute to impaired wound healing in diabetes.

1.3.2 The pathogenic triad

Neuropathy – remains the primary cause for more than 60% of the diabetic foot ulcers[19]. Nerve cell damage affects the lower extremities in several ways[20]. Sensory neuropathy is often manifested by a lack of sensation in patients where trauma is left unnoticed, which progressively worsens due to repeated physical stress. The loss of motor neurons creates an imbalance between the flexion and extension of the affected foot. This could lead to anatomic foot deformities that cause abnormal pressure points[7]. Autonomic neuropathy leads to dysfunctional sweat glands and the resulting dryness of the feet that makes the skin more fragile[19].

Vascular abnormalities – Both macrovascular disease[21] and microangiopathy contributes to lower blood flow to the wound area. Patients with diabetes also have impaired mobilization of bone marrow-derived endothelial progenitor cells (EPC) and defective docking to the wound area (Figure 3)[22,23].

Trauma – Physical trauma is frequently the initiating factor in the development of a foot ulcer. Because of the lack of sensory input, a trauma may go unnoticed. Continuous pressure on a deformed area with lack of sensation can also end up in an ulcer[7].

1.3.3 Other molecular factors affecting diabetic wound healing

Infection is also an important pathogenic factor that contributes to delay in the healing of an established foot ulcer. Infections are also the primary reasons for amputations in patients with diabetic foot ulcers[24]. Patients with diabetes are more susceptible to infections due to several immune defects[9] i.e. endothelial dysfunction that prevents proper homing of inflammatory cells[24], repression of the function of macrophages and neutrophils (Figure 3)[25]. Pleotropic growth factors such as IGF-I, TGF-β and PDGF, which are important for the migration and proliferation of endothelial cells, keratinocytes and fibroblasts have been shown to be reduced in biopsies from human diabetic foot ulcers (Figure 3)[26-28].

Moreover, cell-type specific growth factors such as nerve growth factor (NGF), keratinocyte growth factor (KGF) have also been found to be decreased in the patients with diabetes ulcers [29,30]. Expression of matrix metalloproteinases (MMPs) is also

impaired in diabetes and contributes to defective wound healing[31,25]. MMPs play a vital role in regulating the degradation of extracellular matrix (ECM) and for making the growth factors and other molecules to be available for the cells that participate in wound healing. Epigenetic factors influence the progenitor cells in the bone marrow which results in a pro-inflammatory phenotype in macrophages, adversely affecting wound healing in diabetes[32].

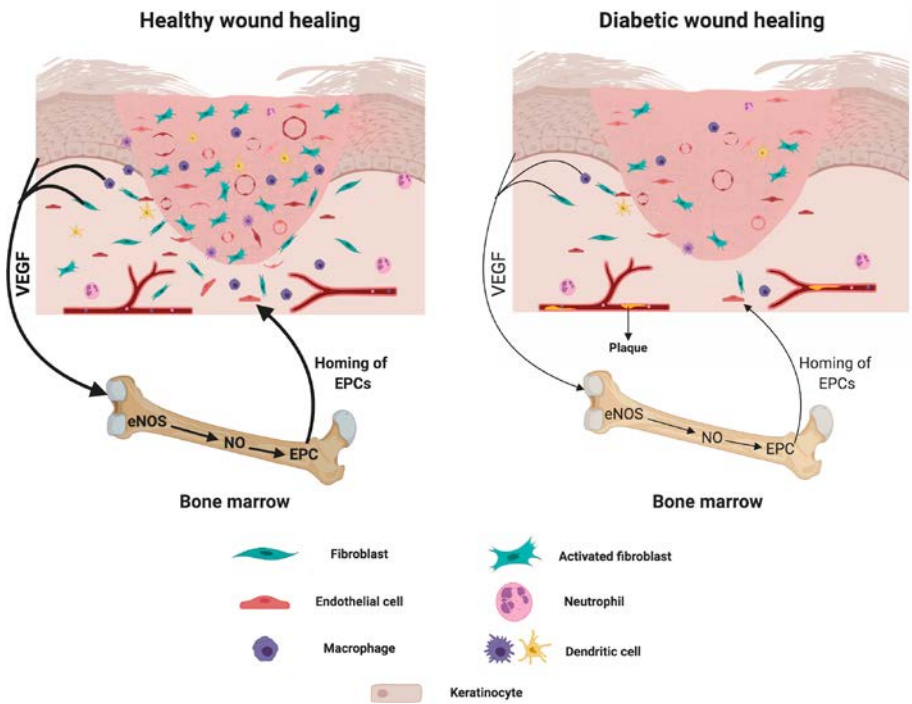

Figure 3. *Depiction of defective wound healing in diabetes with impaired proliferation of fibroblasts, a decrease in immune cells and poor signaling that leads to reduced recruitment of EPCs and poor angiogenesis.*

1.3.4 Importance of targeting signaling pathways in diabetic wound healing

Progress has been made in the management of diabetic foot ulcers in recent times by different approaches such as management of infection, offloading[33-35].

There is however a lack of a specific therapy for diabetes foot ulcers. However several specific defects were identified in different phases of wound healing in diabetes such as inflammation[36,37], angiogenesis[38,39], hypoxia response[40,41,42] and epithelialization[43,26] and hopefully will generate targeted therapies.

1.4 Notch signaling pathway

Notch is an important signaling pathway that plays vital role in cell fate decisions in the developmental program of the metazoans. Notch signaling regulates cell proliferation, differentiation, angiogenesis and apoptosis in a context dependent manner[44,45]. The importance of Notch pathway is highlighted in various developmental diseases caused by genetic loss of Notch or the components of the pathway. More importantly, aberrantly activated Notch signaling in adults may also form the pathogenesis of various diseases including hereditary diseases, cancer, cardiovascular disease, and kidney disease[46]. Thus, Notch has become an important therapeutic target of clinical significance.

The Notch pathway has a unique mechanism of signal transduction. The pathway is activated by cell-to-cell contact of Notch receptors and its corresponding ligands (Figure 4). In mammals, the Notch receptor consists of 4 paralogues (Notch 1 to 4) and ligands Delta-like (Dll1, Dll3 and Dll4) and Jagged (Jag1 and Jag2)[44]. Upon activation, the receptor undergoes a series of cleavages that results in an activated intracellular domain of Notch (NICD) that directly translocates into the nucleus where it displaces the transcriptional repressor bound to DNA binding protein CBF1-suppressor of Hairless-LAG1 (CSL) and along with co-activator Mastermind-like transcriptional activator 1 (MAML1), induces the transcription of target genes[45]. The series of cleavages upon ligand binding is mediated first by ADAM proteases which cleaves the Notch receptor at site S2 leaving an intermediate called Notch Extracellular Truncation (NEXT). This becomes the substrate for γ-secretase enzyme, that cleaves the NEXT progressively from site S3 into the middle of the transmembrane domain at S4 resulting in the generation of NICD[44]. The recruitment of NICD to CSL displaces the corepressor, that is initially bound to CSL. The trimeric complex formed by CSL, NICD and MAML1 is essential for Notch-dependent transcription. In addition, this complex recruits Ski-interacting protein (SKIP), a transcriptional coregulator, to the promoter. In turn, MAML1 recruits the histone acetylase p300, which promotes the assembly of initiation and elongation complexes[47]. Assembly of these complexes induces the transcription of Notch target genes such as Hes and Herp family of transcription factors, cell cycle regulator p21 and Notch regulated ankyrin-repeat protein (Nrarp)[48,49].

1.4.1 Functions of Notch1 pathway

Notch signaling pathway is one of the most evolutionarily conserved pathways among metazoans. It is prominently active during development and in the self-renewing tissues of adults and influences vital cell fate decisions by controlling gene expression[45]. However, Notch signaling is strictly context specific, which is underlined by the distinct functions and effects of its ligands and receptors. In addition, any small changes – increase or decrease in its expression levels may lead to disease conditions[50]. Moreover, the cooperation of Notch with other signaling pathways in a particular milieu dictates the amplitude and extent of its signaling[51,52].

Notch signaling operates through different modes of actions during development. For instance, during cell division, Notch is capable of amplifying its signaling differentially and promote asymmetric inheritance of its ligands in the daughter cells to promote *lineage specification*[47,53-56]. Notch signaling can also segregate different populations of cells through induction of Notch receptor and ligand in corresponding different populations through *boundary resolution*[57,58]. In addition, Notch also autoregulates itself in the same cell using *cis-inhibition*, where the receptor is inhibited by the ligands produced by the same cell[59,60].

Notch is able to switch cells into proliferative and apoptotic states depending on the context. However, the ability of Notch to regulate cell cycle is dictated by the requirements in differentiation[53]. Notch also plays important role in angiogenesis. The endothelial cells express receptors Notch1, 4 and ligands Delta-like 1, 4 and Jagged1, of which Notch1 and Dll4 play crucial role in angiogenic sprouting. The sprouting is led by endothelial tip cells which expresses high levels of Dll4 and is located at the protruding front directed towards to angiogenic cues[61].

1.4.2 Regulation of Notch1 signaling

The Notch signaling pathway is a complex machinery with a seemingly simple activation mechanism. Apart from the signaling cues, Notch signaling is also regulated by factors within as well as from outside of its core machinery.

Regulation of receptor-ligand interaction – Since each Notch molecule can generate a signal by ligand binding once, the foremost regulation of Notch signaling is through the availability of the receptors and ligands. Although there may be overlapping expression levels of different receptors and ligands in a cell, the spacial and temporal expression of receptor and ligand is regulated by various cues and interaction with other signaling pathways, which are still being unraveled[44,45].

Regulation by ligand/receptor endocytosis – After engaging with the Notch receptor, the ligand undergoes endocytosis. This generates a pulling force, that exposes the site of proteolytic cleavage on the receptor. The endocytosis of the ligand is carried out by E3 ubiquitin ligases Neuralized (NEUR) and Mindbomb (MIB). Hence, the levels of these ubiquitin ligases in the cells can regulate the extent of endocytosis of the ligands, thereby affecting the signal strength[45].

Receptor glycosylation – Notch receptors are glycoproteins, which means their EGF repeats can be modified by glycosylation by O-fucose and O-glucose moieties. The amount of fucosylation determines the choice of ligand binding to the receptor and hence controls the signaling[45]. Moreover, fucosylation appears to be essential for Notch signaling events that requires regulation by Fringe glycotransferases, enzymes that extend O-fucose with more sugar moieties[62].

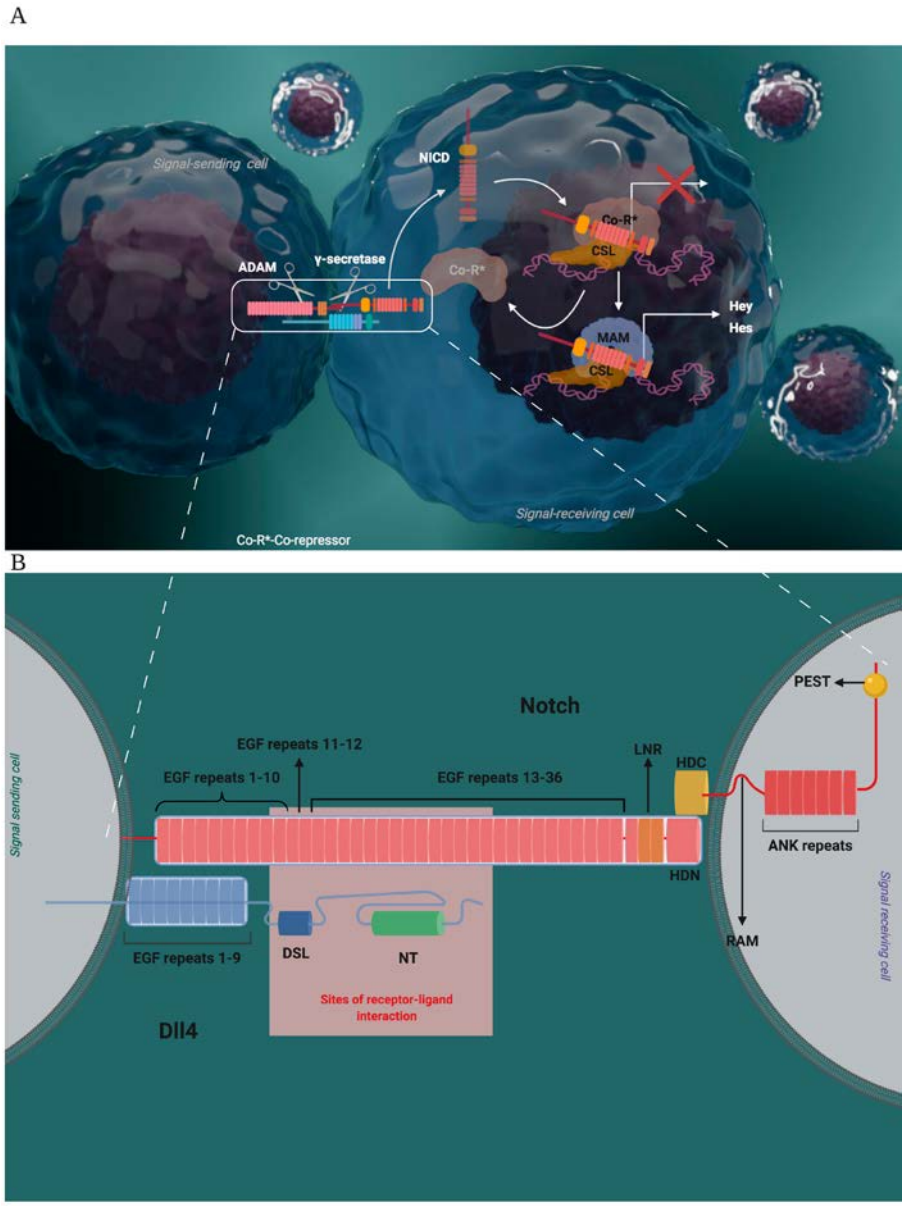

Figure 4. *(A) Schematic representation of the induction of Notch signaling pathway; (B) The organization of the domains of Notch receptor and Dll4 ligand.*

Other regulatory factors – NICD stability is tightly regulated by the phosphorylation of the PEST domain which targets NICD to proteasome degradation upon recruitment of the SCF/Sel10/FBXW7 E3 ubiquitin ligase complex[63]. NICD transactivation activity is not only regulated by the availability of CSL and co-activators that are recruited to the promoter of Notch1 target genes, but also regulated by crosstalk with other signaling pathways, such as HIF-1 [52] and FIH-1[64]. Apart from that, various epigenetic factors such as chromatin modification and histone re-arrangements can affect the target specificity of Notch signaling[47].

1.4.3 Notch1 in diabetes and diabetes complications

Both activated and reduced Notch signaling have been reported in various tissues in diabetes. Notch pathway has been shown to be involved in various diabetic complications, but with different trends. For example, deletion of Notch1 in the podocytes of diabetic mice ameliorated the kidney function[65]. Also, inhibition of Notch by DAPT, an inhibitor of γ-secretase, improved neovascularization of diabetic mice after hindlimb ischemia[66]. However, in retinal capillaries, diabetes-induced increase in Jag1 expression leads to Notch1 inhibition in endothelial cells causing diabetic microvasculopathy in the eyes[67].

1.4.4 Notch1 and wound healing

Notch signaling plays important roles in the development and postnatal physiology of the skin[68-73] and is involved in normal wound healing through the regulation of angiogenesis, cell migration and inflammation[37,74]. Notch inhibition in non-diabetic animals has either no effect[75] or even delaying effect[74,76] of the wound healing rate.

Notch signaling is increased in monocytes from patients with type 2 diabetes and in wound macrophages from a high-fat diet induced diabetic mouse model which contributes to the increased inflammation in diabetic wound[37]. Notch inhibition in macrophage improves wound healing in high-fat diet induced diabetic mice[37]. In this thesis, I have also presented our recent findings that hyperglycemia triggers a Dll4-Notch1 feedback loop in diabetic skin and local Notch1 inhibition improves healing exclusively in diabetic but not in non-diabetic animals[77]. The beneficial effects of Notch inhibition on diabetic wound healing are confirmed in another recent publication[78].

1.5 IGF-I signaling pathway

The insulin-like growth factors (IGFs) are a class of molecules homologous to insulin and exert pleiotropic effects on almost all cells of the body during fetal, neonatal and adult development and homeostasis[79,80]. Induced by the growth hormone, IGFs mediate

long-term actions in terms of cell growth, differentiation and cell survival while insulin mainly possess metabolic activity. The importance of IGF signaling is realized when its defective signaling can lead to insulin resistance and metabolic disease.

IGFs are mainly two isoforms IGF-I and IGF-2 and they act as ligands to IGF-I, IGF-2 and insulin receptors. IGF receptors (IGFR) are also highly homologous to insulin receptor. IGFs and insulin can bind to both insulin and IGF receptors with varying affinities[79]. In addition, IGF signaling is regulated by a family of IGF-binding proteins (IGFBPs). There are six types of IGFBPs which are capable of binding IGFs with equal or greater affinity than the IGF receptors. A vast majority of IGFs in the serum are found to be bound in a ternary complex consisting of IGF, IGFBP and an acid labile subunit (ALS). Binding to IGFBPs increases the half-life of IGFs to around 25 minutes as opposed to free-form which has a half-life of 10 minutes. IGFBP3 is the most prevalent binding protein found in the serum with the highest concentration. In general, IGFBPs regulates the availability of IGFs that bind to the IGF receptor[81].

1.5.1 Physiological roles of IGF-I

Although IGF-I is mainly produced by the liver to act in an endocrine fashion in the peripheral organs, every tissue is capable of producing local IGF-I for autocrine or paracrine purposes. A detailed description of the roles of IGF-I in different tissues is depicted in figure 5. The expression levels of IGF-I has been shown to be higher in fetal lung, muscle and stomach than it is postnatally. Moreover, the importance of IGF-I signaling during fetal development is highlighted by mutation in IGF-IR resulting in both intrauterine and postnatal growth impairment[82]. The role of IGF-I in fetal development is seen as independent of and synergistic to the role of the growth hormone.

IGF-I is produced in peaks that coincides with neural progenitor proliferation and differentiation and neurite outgrowth[83]. In addition, it has been shown that IGF-I could cross the blood-brain barrier by the presence of IGF-IR in the brain capillary endothelial cells. Other evidences point to the role of liver-derived IGF-I in regulating the clearance of brain amyloid-β levels and its potential implications in Alzheimer's disease[84]. The role of IGF-I in kidney development and function is underscored not only by the expression of IGF-I and IGF-IR but also by the examples of animal models developing renal disease by IGF-I disruption[85]. Furthermore, administration of IGF-I to rodents and humans increases kidney growth, renal blood flow and glomerular filtration rate[86]. IGF-I is very important for the cardiovascular system as well and IGF-I and IGF-IR are expressed in the myocardium, aortic smooth muscle cells and endothelial cells. Studies have shown that IGF-I is a potent vasodilator and this effect may be partly mediated by increased NO release from the endothelium[87]. Also, insufficient levels of IGF-I plays significant roles in vascular diseases such as atherosclerosis[88].

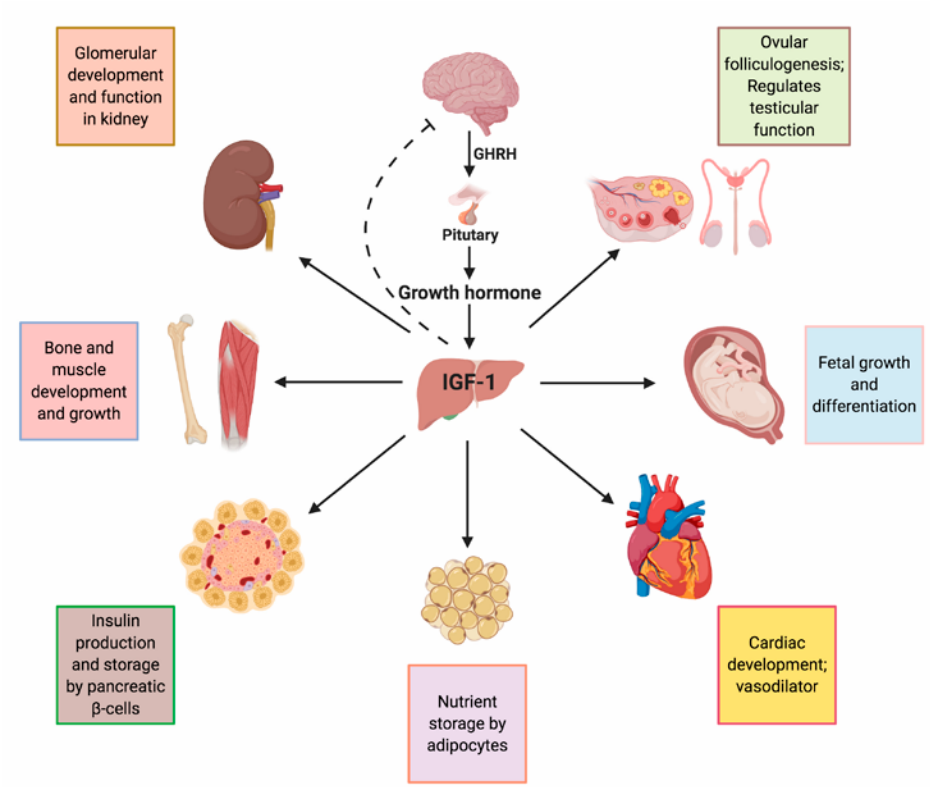

Figure 5. *An illustration of the various physiological roles of IGF-I in human body.*

1.5.2 IGF-I regulation in diabetes

It is well-known that low circulating levels of IGF-I are associated with reduced insulin sensitivity and risk of glucose intolerance and type 2 diabetes[89]. Meta-analysis of genome-wide data for continuous diabetes-related traits in non-diabetic subjects reported two loci, IGF-I and GCKR, with significant association for fasting insulin levels[90]. Moreover, liver-specific IGF-I knockout mice revealed insulin resistance and hyperinsulinemia that may be reversed by administering recombinant IGF-I[91]. IGF-I seems to have a glucose-lowering effect similar to that of insulin, since patients with type 2 diabetes responded to IGF-I treatment with improved glucose tolerance, hyperinsulinemia and hyperlipidemia[92]. Despite its positive effects, many clinical trials have reported adverse effects such as cataract, renal hypertrophy and neoplastic formations from long term recombinant human IGF-I treatment albeit well tolerated and easily managed without treatment discontinuation. Hence, care should be taken and more efforts should be made to further assess its safety as a long-term treatment option for diabetes.

1.5.3 IGF-I and wound healing

As a growth promoting molecule, IGF-I is considered to be important in promoting wound healing. Reports have shown that overexpression of locally acting IGF-I under K14 promoter improves murine wound healing[93]. In addition, the effects of IGF-I *in vitro* on keratinocytes and fibroblasts seem to improve proliferation and migration, which aids in the healing[94-96]. Given its deficiency in metabolic diseases, cells isolated from diabetic foot ulcers and diabetic mouse wounds had reduced IGF-I levels[26,97]. Moreover, the individual contributions of liver-derived and locally produced IGF-I to wound healing is not clear. Evidences from abnormal wound settings such as the ones in diabetes is also not helpful since both liver-derived and locally produced IGF-I levels are reduced in diabetes. Thus, in my second paper, I have investigated the effects of endocrine IGF-I in both normal and diabetic wound healing.

1.6 Hypoxia and hypoxia inducible factors

Hypoxia is a state where the demand for oxygen exceeds its supply. In order to cope with such limited oxygen levels, all metazoans have evolved a complex mechanism of regulation by a family of proteins called Hypoxia inducible factors (HIF)[98,99]. HIF consists of two subunits, a tightly regulated α subunit and a constitutively expressed β subunit. The α subunit exists in three different isoforms. The HIF-1α isoform is ubiquitously expressed whereas HIF-2α and HIF-3α are tissue specific[100]. While HIF-1 and HIF-2 coordinate similar functions, they have also been shown to regulate unique gene expression patterns according to their localization[101-103]. The regulation and function of HIF-3 is not clearly understood. The helix-loop-helix-PAS domains in both the α and β subunits facilitate heterodimerization and DNA binding.

HIF-1α is regulated at various levels to ensure proper oxygen homeostasis. Under normal oxygen conditions, HIF-1α protein is hydroxylated at specific proline residues by proline hydroxylases (PHDs)[104,105]. In the presence of oxygen, iron and 2-oxoglutarate, PHDs use one atom from molecular Oxygen to insert into the proline residues and the other atom combines with α-oxoglutarate to form succinate and CO_2. In its hydroxylated form, HIF-1α binds to the ubiquitin ligase von Hippel Lindau (VHL), which targets it for proteasomal degradation[104,105]. HIF function is also regulated by factor inhibiting HIF (FIH-1) by the hydroxylation of asparagine residues of HIF-1α to prevent the recruitment of transcriptional coactivators[106,107]. During hypoxia, lack of oxygen renders the hydroxylases inactive and a stabilized HIF-1α dimerizes with HIF-1β and binds to specific regions in the DNA called hypoxia responsive elements (HRE). This is followed by the activation of the transcription of genes involved in angiogenesis, erythropoiesis, proliferation and glucose metabolism and helps the cells adapt to the hypoxic environment[98,99,108]. Moreover, HIF-1 also regulates a host of microRNAs and long non-coding RNAs such as miR-23, miR-24, miR-26, miR-27, miR-103, miR-107, miR-181, miR-210, miR-213, H19, MALAT1, HOTAIR and FALEC, which are used as fine-tuning mechanisms of the hypoxia response[109].

1.6.1 HIF-1 and wound healing

A nascent wound microenvironment experiences acute hypoxia due to the damage to the blood vessels and a rapid increase in the consumption of oxygen by the cells metabolically activated to heal the wounds. This leads to the stabilization of HIF-1α which plays important roles in promoting wound healing. However, HIF-1 activation has been shown to be impaired in diabetic wounds[110, 111]. Accordingly, HIF-1 protein levels and function are downregulated in the wounds of diabetic mice[112]. This also corresponds to low HIF-1 levels in the biopsies from patients with diabetic foot ulcers as compared to those from control subjects[112]. Moreover, local stabilization of HIF-1 on the wounds of diabetic mice through chemical inducers has been proven to be effective in improving wound healing[110]. Hence, a deep insight on the mechanisms of HIF-1 inhibition in diabetes would unlock a potential therapeutic strategy for treating chronic diabetic wounds.

1.7 MicroRNAs and wound healing

Of all the genomic DNA, only about 2% of it is encoded into proteins. About 97 to 98% of it is transcribed into non-protein coding RNAs which consists of two broad categories of small and long non-coding RNAs based on their lengths. microRNAs (miRNAs) are a major class of small RNAs which are 21-23 nucleotide long oligonucleotides that negatively regulates gene transcription by translation inhibition or mRNA degradation[113-115]. In some cases, miRNAs can also bind to the open reading frame (ORF) sequences and the 5'-UTR, leading to gene activation rather than repression[116]. miRNA-based regulation of gene expression is being unraveled in every biological process from development to tissue homeostasis. In addition, dysregulated miRNA expression has been shown to be pathogenic for various diseases.

1.7.1 microRNA biogenesis

miRNAs are transcribed from miRNA genes by RNA polymerase II into about 1kb long transcript called primary-miRNA (pri-miRNA) (Figure 6)[113,114]. This transcript is then processed to a 65 nucleotide long premature-miRNA (pre-miRNA) by a complex called microprocessor consisting of an RNase III enzyme Drosha and its cofactor DGCR8. This transcript is then transported to the cytoplasm through a Ran-GTP dependent nuclear export factor, Exportin-5. In the cytoplasm, the pre-miRNA is further processed by another RNase III enzyme Argonaute (AGO) to form a miRNA-induced silencing complex (miRISC). This involves the cleavage of one of the strands of the duplex and the other one forming the mature miRNA[113-115].

1.7.2 microRNAs in wound healing

Since the discovery of miRNA's role in diseases, an increasing number of reports have revealed the importance of miRNAs in regulating different physiological processes

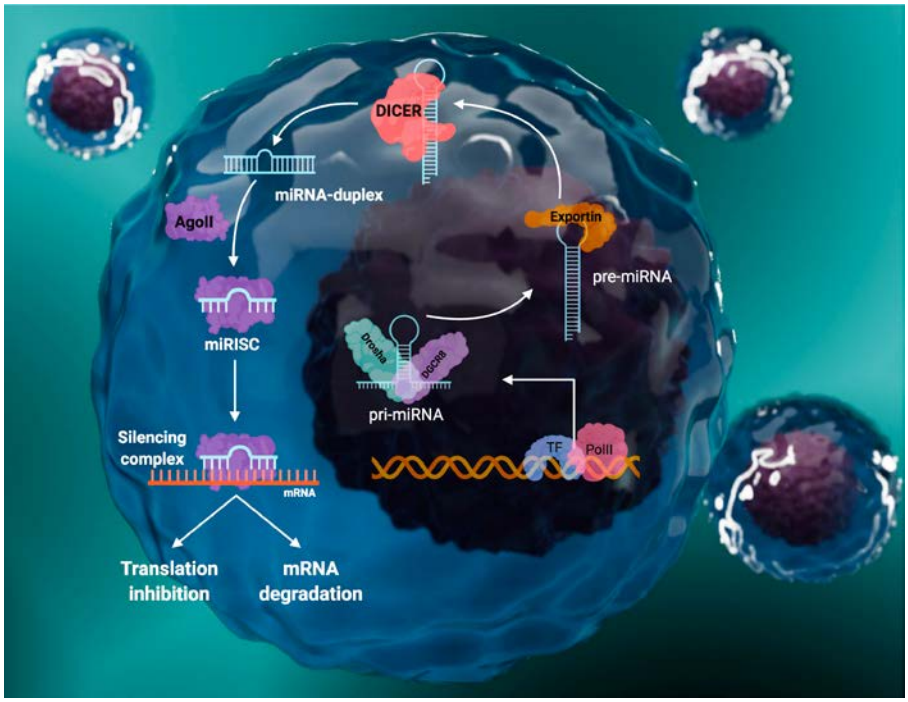

Figure 6. *A description of microRNA biogenesis.*

including wound repair. Studies have shown that miRNAs are involved in each stage of wound healing. For example, miR-146a and miR-155 produced by macrophages regulates differentiation of monocytes to macrophages[117,118]. miR-146a has even been implicated in the inflammatory phase of diabetic wounds in mice. An array of miRNAs including miR-21, -203 and -205 has been associated with migration and proliferation of keratinocytes[116]. Therefore, it is of interest to explore the possibility of more miRNAs that could regulate processes in wound healing and to study their potential role in the pathogenesis of diabetic foot ulcers. In my thesis, I have investigated the role of miR-210 and miR-34a in diabetic wound healing.

1.7.3 microRNAs regulated by hypoxia and HIFs

microRNAs that are induced in response to hypoxia are called "hypoxamiRs". There are different modes of regulation of positive and negative feedback loops of miRNAs acting upstream as well as downstream of HIF and sometimes even independent of HIF to reinforce hypoxic adaptation. For instance, in endothelial cells, miR-424 stabilizes HIFα isoforms through targeting cullin2 to promote angiogenesis[119]. miRNAs such as miR-20b and miR-199a, which normally target HIF transcript to repress its expression, are repressed in hypoxia[120,121]. An example of negative feedback regula-

tion is hypoxamiR-155, which is induced by HIF in hypoxia and it contributes to resolution of HIF effects in intestinal epithelial cells by targeting HIF transcript during prolonged hypoxia[122].

It is also becoming clear that other transcription factors apart from HIF can also regulate miRNA expression during hypoxia. For example, p53, which is induced by hypoxia promotes miR-34, miR-15a and miR-16a expression[123]. Hypoxia can also induce NF-κB, a transcription factor that controls inflammation. miRNAs such as miR-146a and miR-21 are induced by NF-κB, which regulates inflammation in hypoxia[123]. Among all of the hypoxia-induced miRNAs, the miRNA-210 stands out as the most robust hypoxamiR regulated by HIF. miR-210 not only mirrors HIF function by performing synergistic functions, but also regulates HIF expression in different contexts. I have expanded on the role of miR-210 below.

1.7.4 microRNA-210

Also known as the master hypoxamiR, miR-210 is regulated by both HIF-1 as well as HIF-2[124]. The HRE element found 40bp upstream of the transcription start site of miR-210 is highly conserved across species, highlighting the importance of its regulation under hypoxia during evolution. It is also noteworthy that adjacent to the HRE elements, there are a number of binding sites for other transcription factors including E2F1, Oct4 and PPARγ, which may indicate its role in cell cycle, metabolism and stem cell biology where hypoxia plays a vital role[124].

1.7.4.1 Functions of miR-210

The main function of HIF in hypoxia is to conserve energy and reduce oxidative stress by inhibiting the TCA cycle and inducing glycolytic enzymes. To that effect, miR-210 reflects HIF function non-redundantly by downregulating iron-Sulphur cluster scaffold proteins (ISCU) [125]. Iron-Sulphur clusters are essential components of TCA cycle enzymes including aconitase and mitochondrial complexes I, II and III [126]. miR-210 further alters mitochondrial dynamics in hypoxia by targeting the D-subunit of succinate dehydrogenase (SDHD) [127]. SDH is the only enzyme complex taking part in TCA cycle, oxidizing succinate to fumarate as well as being part of the electron transport chain as complex II. Thus, miR-210 represents a profound fine-tuning mechanism of the mitochondrial function mediated by HIF (Figure 7).

The role of miR-210 in angiogenesis is highlighted by its down modulation of the receptor tyrosine kinase ligand EphrinA3 in endothelial cells[128]. However, the role of miR-210 in cell cycle progression remains to be a double-edged sword. While it targets the transcription factor E2F3, which is involved in the G1/S progression[129], thereby inducing cell cycle arrest, it also downregulates the repressive partner of transcription factor c-Myc, MNT, leading to cell cycle progression[130]. miR-210 also targets DNA repair protein RAD52 in hypoxia[131]. Interestingly, there have also been reports of miR-210

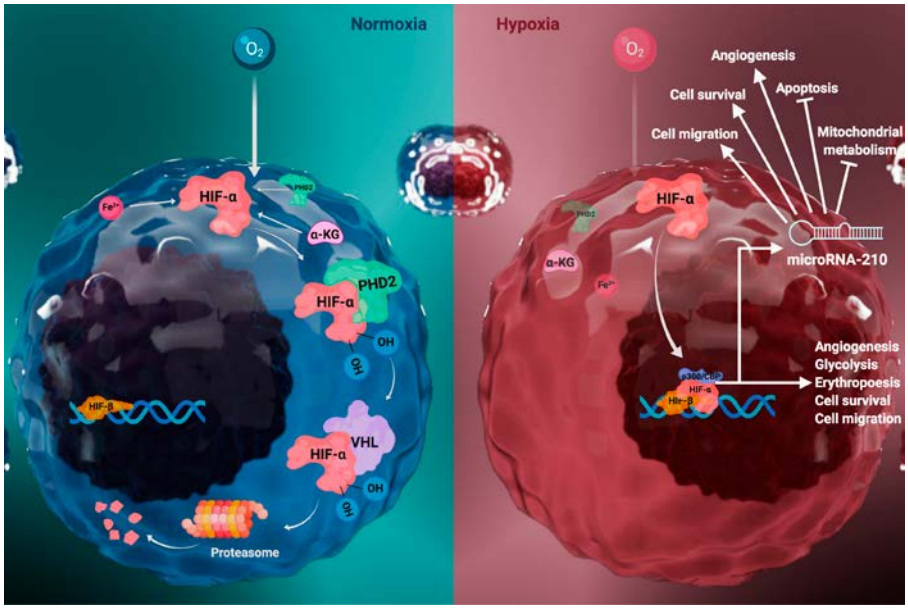

Figure 7. *A schematic showing the induction and various roles of miR-210 in response to hypoxia.*

being both positive as well as negative regulator of HIF-1α itself in different contexts. One report showed miR-210 to repress HIF-1α and promote TH17 cell differentiation, thus, reducing the extent of inflammation in an experimental model of colitis[132]. Another report claimed miR-210 to repress Glycerol-3-phosphate dehydrogenase 1-like (GPD1-L), which indirectly stabilizes HIF-1α by modulating PHD2 levels[133].

1.7.4.2 miR-210 and wound healing

From the perspective of wound healing, a study has shown that HIF mediated upregulation of miR-210 repressed keratinocyte proliferation in an ischemic wound model in mice[134]. Further, an interesting study has revealed that by inhibiting both PHD2 and miR-210 with oligos diabetic wound healing can be improved, although specific mechanism is unclear[135]. Considering the role of HIF in wound healing and given its profound regulation of miR-210, it is tempting to investigate further the mechanism of miR-210 regulation in diabetic wound healing.

1.7.5 microRNA-34a

miR-34 family of miRNAs consists of three processed miRNAs – miR-34a, miR-34b and miR-34c. They are encoded by two different genes – miR-34a is transcribed as its own transcript while miR-34b and miR-34c share a common primary transcript[136]. While

miR-34b/c is mainly expressed in the lungs, miR-34a is ubiquitously expressed[136]. In the context of cancer, miR-34a is considered as a tumor suppressor since it is a direct target of p53 and is a potent inducer of apoptosis[137]. However, several studies have uncovered various roles for miR-34a in different cellular processes.

1.7.5.1 Functions of miR-34a

The majority of miR-34a targets belong to genes that control cell cycle and DNA damage response such as NMYC, CDK4/6, Cyclin E2, MET and Bcl-2 (Figure 8)[138,139]. Naturally, since p53 is mutated/deleted in many types of cancers, miR-34a is also inactivated either as a consequence of p53 mutation or as a direct mutation. Moreover, miR-34a mediates a feedforward loop with p53 by targeting the NAD-dependent deacetylase SIRT1, which acts on p53 itself and represses the expression of various apoptotic proteins[140]. It is reported that apart from p53, miR-34a is also regulated by ELK1[141]. miR-34a regulates B-cell development by targeting Foxp1, which induces the differentiation of pro-B-cell to pre-B-cell progenitor[142].

There has been conflicting reports of the effect of hypoxia and high glucose on miR-34a. While some reports claim that miR-34a is induced in cardiomyocytes and hepatocytes exposed to hypoxia-reoxygenation[143,144], it seems to be downregulated in the tubular epithelial cells of the kidney[145]. In addition, miR-34a has been shown to be increased in the testes, cochlear hair cells, dorsal root ganglion, foot pad tissue, sciatic nerve and the serum of diabetic mice and patients[146-149]. miR-34a was also upregulated in the mouse microvascular endothelial cells contributing to impaired angiogenesis in diabetes[150]. Although, miR-34a was reduced in the kidneys of diabetic patients compared to that of non-diabetic subjects[145].

Interestingly, miR-34a is a direct regulator of Notch pathway by targeting different receptors and ligands including Notch1, Jag1, Dll1 (Figure 8)[145,151,152]. In addition, miR-34a regulates inflammation by targeting several pro-inflammatory molecules like NF-κB and HMGB1[153,154].

1.7.5.2 miR-34a and wound healing

The studies on the role miR-34a in wound healing and its effect in diabetes are scarce. However, the few studies that do involve miR-34a has surprisingly implicated miR-34a in impaired wound healing in diabetes. For example, fibroblasts cultured from DFUs seem to be differentially express miR-34a promoting senescence and cell differentiation and inhibiting cell movement and proliferation, resulting in an overall impairment of healing[155]. Furthermore, in venous ulcers, miR-34a assumes a pro-inflammatory role by targeting the anti-inflammatory LGR4 in keratinocytes [156]. Given its anti-inflammatory role in various other contexts, it is therefore tempting to declutter the exact role of miR-34a in the context of diabetic foot ulcers.

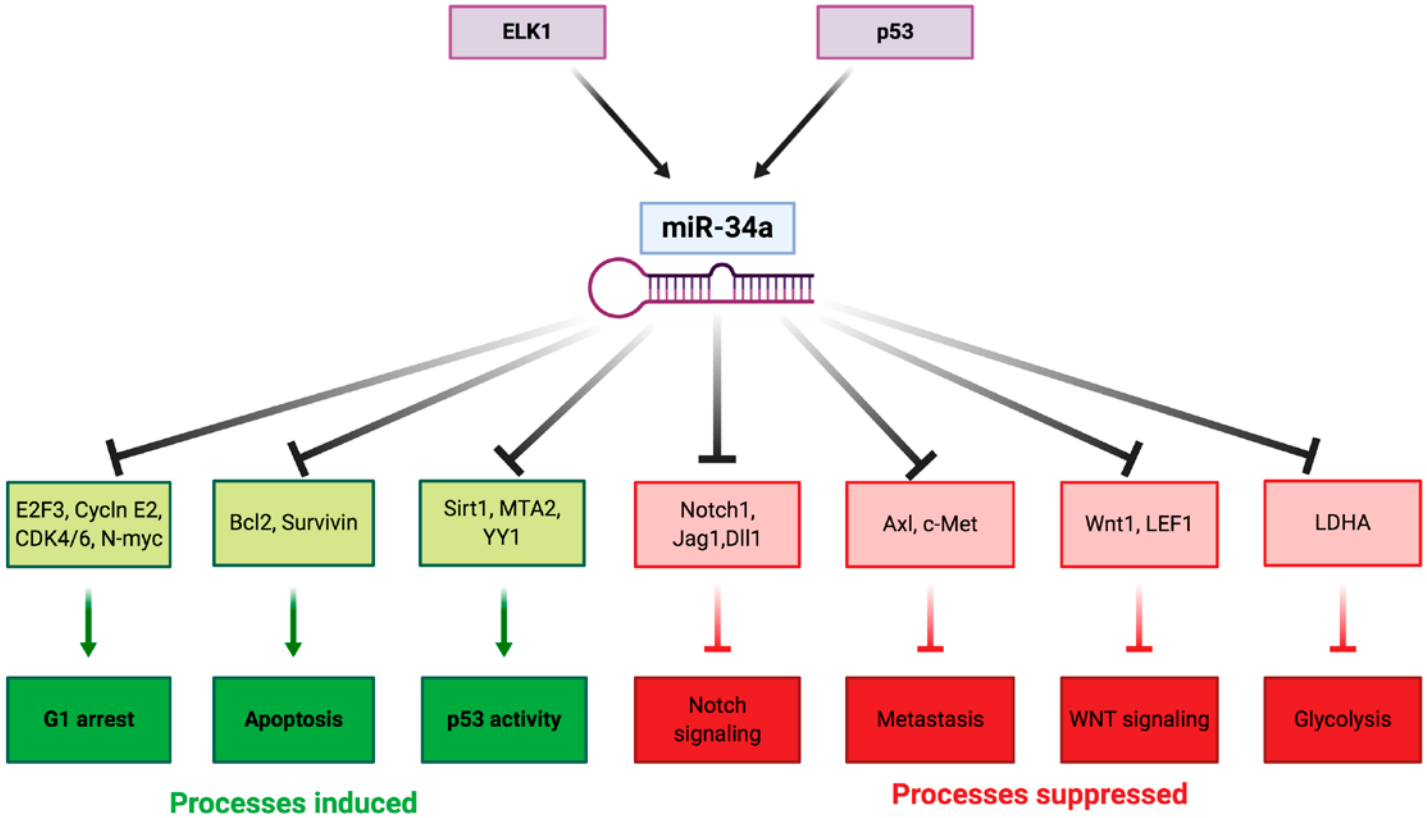

Figure 8. *A schematic representation of different miR-34a targets and their effect on cellular processes.*

2 AIMS AND OBJECTIVES

The main objectives of my thesis were to understand different mechanisms by which hyperglycemia impairs wound healing in diabetes. Using *in vitro* (primary cell lines) *in vivo* (mouse models) methods together with exploration of wounds from patients with diabetes, I specifically focused on the following specific aims:

1. Determine the role of Notch signaling in diabetic wound healing.
2. Understand the role of liver-dervied IGF-I in healthy and diabetic wound healing.
3. Investigate the regulation and function of miR-210 in diabetic wound healing.
4. Uncover the role of miR-34a and its specific regulation in diabetic wound healing.

3 METHODS

3.1 Animals

C57BL/KsJm/*Leprdb* (db/db) diabetic mice and their heterozygous control mice were obtained from Charles River (Belgium). Db/db mice are a widely used mouse strain to study diabetes and its complications. The loss of Leptin receptor by a point mutation renders the leptin signaling defective, which leads to obesity in these mice as early as 6 weeks of age, that worsens gradually. These mice develop obesity, insulin resistance, hyperglycemia and hyperlipidemia and features a decrease in plasma insulin at week 15, indicating β-cell dysfunction. Thus, db/db mice represent a severe type 2 diabetic phenotype, suitable to study molecular pathologies underlying diabetic complications[157].

Diabetes was induced in *KRT14-Cre;Notch1*$^{fl/fl}$ and LI-IGF-I-/- mice and their heterozygous littermates with age of 8-10 weeks by injected 50mg/kg Streptozotocin (STZ) prepared in sodium citrate buffer (i.p.) daily for 5 consecutive days according to the instructions from AMDCC (Animals Models for Diabetes Complications Consortium). STZ is a glucosamine-nitrosourea compound, which is used as a chemotherapeutic agent in pancreatic cancer. It induces β-cell apoptosis by preferential accumulation through entry through GLUT2 transporter due to its structural similarity to glucose[158]. The mice became diabetic 2 weeks after injection. The mice were kept diabetic for another 2 weeks before starting the experiment (14-15 weeks).

3.2 Ethical considerations

All studies were performed in accordance with the ethical permits approved by the institutional ethical review committee at Karolinska Institutet, Sweden. Skin and wound biopsies from patients and controls were obtained after agreeing and providing their informed consent.

3.3 Wound model

Following blood glucose and HbA1c control, the mice were anesthetized with 3% isoflurane. The hair at the back of the mice was shaved using an electric shaver followed by the application of depilatory cream. The skin was rinsed with alcohol and two full-thickness wounds were made extending through the panniculus carnosus on either side of the dorsal midline, using a 6mm biopsy punch. For topical treatments: paper-I: 100uL of DAPT (100μM), a transparent dressing (Tegaderm) was applied to cover the treatment on the wounds. For paper III and paper IV, miR-210 mimic, miR-34a mimic and their respective controls (0.125 nmol/wound) was injected intradermally around the wound edges on the day of wounding and 6 days after wounding. Following wounding, the mice were housed individually and injected with buprenorphine (0.03 mg/kg) (i.p.) for the first 2 days twice a day to relieve any physical distress possibly caused by the surgical procedure.

For the measurement of the wound healing rate, the wounds were photographed using a digital camera every alternate day until 95% closure of the wounds. A circular reference was used to correct for the distance between the wound and the camera. The wound area was calculated using the ImageJ software version 1.32 (N.I.H., U.S.A.), corrected for the area of the reference circle and expressed in percentage of initial wound area. For analysis of histology, IHC, mRNA, miRNA or protein expression, oxygen consumption rate (OCR) measurement, the wounds were harvested 8 days after wounding and processed appropriately according to requirement.

3.4 Histology

Histology was performed on formalin-fixed, paraffin-embedded sections (5 μm). After deparaffinization and rehydration, the slides were stained with hematoxylin and eosin and the granulation tissue area of the wound was measured. Image analysis and quantification was performed using smart segmentation feature on Image Pro Premier software v9.2 (Media Cybernetics). At least 3 images from each slide were evaluated and each condition had 3-5 slides. Granulation was measured as ratio of the number of cells to the total area of the granulation layer in an image.

Masson-Goldner trichrome staining is used in histology to visualize connective tissues by using three different stains to color different subsets of tissues. Weigert's iron hematoxylin is used to generate dark blue/black nuclei, Azophloxine and Tungstophosphoric acid Orange G is used to stain cytoplasm, erythrocytes and muscles with a red/pink and light green SF solution to counterstain connective tissue including collagen. FFPE sections of wounds were deparaffinized with 2 passes in Xylene for 3 minutes each and rehydrated in sequential passes of 100% and 95% Ethanol for 3 minutes, 2 times each. The slides were treated according to manufacturer's instructions to obtain Masson-Goldner Trichrome staining (Merck Millipore). Images of the staining were obtained using Leica DM3000 LED fluorescence microscope using the transmitted light. The collagen stained areas in the wounds were analyzed and quantified using the Smart Segmentation feature on Image Pro Premier software v9.2 (Media Cybernetics). At least 2-4 images from 3-4 tissues from each condition were analyzed. Collagen staining was expressed as the percentage of area stained by collagen (green).

3.5 Fluorescent immunohistochemistry

The tissues to be analyzed were snap-frozen at the time of animal sacrifice and stored in liquid nitrogen until use. The tissues were sectioned at 7μm thickness and stored at -80 °C until staining. Before the staining, the slides were thawed and fixed sequentially with 50% acetone for 30 s and 100% acetone for 5 min. For FFPE sections, the sections were deparaffinized and rehydrated, and antigen retrieval was performed in a microwave (800 W for 20 min) by using citrate buffer. The slides were then washed with PBS-T (0.1% Tween) three times for 3 min each. The sections were blocked with goat serum or 5% BSA in PBS for 30 min at room temperature (RT), then incubated

with primary antibodies overnight at 4 °C. After four times washing with PBS-T for 5 min each, the sections were incubated with fluorochrome-conjugated secondary antibody for 1h at RT in the dark. After washing four times for 5 minutes each, the slides were treated with 1 μg/mL DAPI (Life Technologies) in PBS for 5 min at RT. The FFPE sections were treated with 0.1% Sudan Black-B solution for 10 min to quench autofluorescence. The sections were then mounted and stored at 4 °C. The fluorescent images were acquired by using a Leica DM3000 LED fluorescence microscope, an LSM Meta 510 confocal microscope (Zeiss), or a Leica TCS SP5 confocal microscope (Leica Microsystems). Image analysis was performed by using Image-Pro Premier (Version 9.2) and ImageJ (Version 1.47) software.

3.6 Cell culture

Primary human dermal fibroblasts (HDFs) (ATCC, USA) were cultured in Dulbecco's Modified Eagle's medium (DMEM; 5.5 mM glucose) supplemented with 100 IU/ml penicillin and streptomycin, and 10% heat-inactivated FBS (Invitrogen). Primary human keratinocytes and HDMECs (PromoCell) were cultured in medium provided by PromoCell. The cells were maintained in a humidified atmosphere with 5% CO_2 at 37 °C in a cell culture incubator, and passages 4 to 9 were used for experiments. Cells were exposed to normal glucose (5.5mM) or high glucose (30mM) conditions and cultured under normoxic [21% (vol/vol) O_2] or hypoxic (1% O2) conditions in Hypoxia Workstation INVIVO2 (MedicalExpo).

3.7 RNA interference and miRNA overexpression

siRNA oligonucleotides against human *Notch1*, *Notch2*, *Notch3*, *Notch4*, *Dll4* and their respective negative controls (Sigma, ThermoFisher Scientific) were used. Keratinocytes and HDMECs were transfected with siRNAs using Lipofectamine RNAiMAX transfection reagent (ThermoFisher Scientific) or HiPerfect Transfection reagent (Qiagen), according to the manufacturer's protocol. 24 hours after transfection, the cells were appropriately treated and harvested.

HDFs and keratinocytes were transfected with miR-210 mimic and miR-34a mimic or negative control mimic respectively using Lipofectamine RNAiMAX (Life Technologies) according to manufacturer's instructions. The cells were harvested 24-48 hours after transfection, total RNA was extracted and the expression levels of miR-210 and miR-34a were analyzed.

3.8 RNA purification and quantitative RT-PCR

Total RNA, including microRNAs, was extracted from the cells and tissues using a miRNeasy RNA extraction kit (Qiagen). To detect mRNA expression, High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific) was used. All quantitative RT-PCR was performed on a 7300 Real-Time PCR System or QuantStudio 6 and 7

Flex Real-Time PCR System (Applied Biosystems) using SYBR Green Master Mix or Taqman Gene Expression Assays (ThermoFisher Scientific). The internal controls for mRNA expression were *PBGD and Actin*.

3.9 MicroRNA detection

TaqMan microRNA Reverse Transcription kit was used to synthesize cDNA for detecting miR-210, snoRNA55 and U6 snRNA using TaqMan miRNA assays (ThermoFisher Scientific). U6 and SnoRNA55 snRNA were used as internal controls in cells and mouse tissues, respectively. To detect miR-210 expression in human wounds, cDNA was produced using TaqMan Advanced miRNA cDNA Synthesis Kit (ThermoFisher Scientific) and microRNA expression was detected using TaqMan Advanced miRNA assays (ThermoFisher Scientific), where the average of miR-16, miR-23a, and miR-24 were used as internal controls.

3.10 *In vitro* migration assay

HDF and keratinocyte migration was studied using the scratch assay. The cells were plated in 24-well plates that were coated with collagen (50 µg/uL) overnight and blocked with 3% BSA in PBS for 2 hours. At 90% confluency, HDF cells were transfected with the miR-210 mimic or negative control mimic as mentioned above. 24 hours after transfection, a scratch was generated in each well with a micropipette tip. The cells were rinsed and treated with normal (5.5 mM) or high glucose (30 mM) medium supplemented with 0.2% FBS and the plates were placed in normoxia or hypoxia. Mitomycin C (10 µg/mL) (Roche) was included in the media to prevent cell proliferation. Digital pictures were obtained immediately after scratching and after 16 hours using EVOS XL Core Cell Imaging System (ThermoFisher Scientific). Images were analyzed using ImageJ v1.47 software (N.I.H., Bethesda, MD, U.S.A.). The experiment was done in triplicates and 3 images from each replicate were used for analysis. On each image, the distance between the two sides of the scratch were measured at certain intervals using ImageJ and the mean of distance was calculated. Migration rate was calculated as the difference between the mean of distances at 0 hour (Distance_0h) and 16 hours (Distance_16h) divided by the distance at 0 hour ((Distance_0h – Distance_16h)/ Distance_0h). The migration rate for each condition was expressed as percentage to the control condition and was expressed as Relative Migration.

3.11 Cell proliferation assay

To measure the effect of miR-210 mimic on proliferation, HDF cells were seeded in 6 cm^2 dishes and were transfected with miR-210 mimic or negative control mimic as mentioned above. After 24 hours, the cells from each condition were trypsinized and plated on to 96-well plates. 24 hours after seeding, the cells were treated with normal (5.5 mM) or high (30 mM) glucose levels in serum-free media and placed under

hypoxic or normoxic conditions. Cell proliferation was measured the next day using the BrdU Cell Proliferation ELISA kit (Abcam; ab126556). BrdU (provided with the kit) was added 16 hours before fixing the cells. After fixation, standard protocol according to the manufacturer was performed to measure the proliferation of cells and were expressed in terms of the percentage of BrdU incorporation.

3.12 Measurement of oxygen consumption rate by Seahorse

The effect of miR-210 on the oxygen consumption rate (OCR) in fresh wound tissue and cells was measured using the Seahorse XF Analyzer (Agilent Technologies). The sensor cartridges used for measuring the oxygen flux was equilibrated in an XF Calibrant (Agilent Technologies) 16-24 hours before the experiment in a 0%-CO_2 37 °C incubator. The granulation tissue from the wound was carefully dissected and rinsed with unbuffered Krebs-Henseleit buffer (KHB) media. The tissue was placed at the bottom of the XF24 Islet Capture Microplate (Agilent technologies) and covered with a mesh. 450µL KHB medium was added to each well containing the tissue and equilibrated in a 0%-CO_2 incubator for 30 minutes. Then, the cartridge was placed on the assay plate and run in the XF analyzer using an optimized protocol to measure basal OCR. For analysis in cells, HDF cells were transfected with negative control mimic or miR-210 mimic as explained above. After 24 hours treatment with normal or high glucose levels in normoxia or hypoxia, 30,000 cells from each condition were seeded onto an XF24 Cell Culture Microplate (Agilent Technologies). Basal OCR was analyzed using XF analyzer.

3.13 Statistics

Differences between groups were analyzed using Student t-test, one-way ANOVA followed by Tukey's post hoc test or two-way repeated measures ANOVA. $P<0.05$ was considered statistically significant. All the *in vitro* experiments were performed at least 3 times. All the data are presented as mean ± standard error of the mean (SEM).

4 RESULTS AND DISCUSSIONS

4.1 Paper-I

Triggering of a Dll4–Notch1 loop impairs wound healing in diabetes

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4.1.1 Notch1 is activated in diabetic skin

As discussed earlier, Notch signaling is context-specific, "goldilocks" pathway and sensitive to changes in its expression levels[50]. Hence, it was of interest to investigate Notch signaling in the diabetic skin. We detected an increase in activated Notch1 intracellular domain (N1ICD) levels in the skin of patients with diabetes and diabetic mice. This increase was mainly observed, but not restricted to the epidermis. Further, the gene expression levels of Notch targets (Hey1, Hey2, Hes1 and Hes5) were also upregulated in the human and mouse diabetic skin. Interestingly, the Notch ligand *Dll4*, but not *Jagged1,* was found to be increased in the skin of diabetic human and mouse skin. The preferential regulation of specific ligands for various functions in response to different environmental cues is typical of Notch pathway[46]. In addition several other genes regulated by the Notch pathway, assessed by a PCR array analysis were upregulated in the diabetic mouse skin compared to their controls. These genes play important roles in processes central to wound healing such as angiogenesis, differentiation, proliferation, apoptosis and inflammation.

4.1.2 Hyperglycemia induced Notch1 signaling impairs wound healing in diabetes

We have therefore investigated the Notch 1 signaling in different cells types important for wound healing: human dermal fibroblasts (HDF), human keratinocytes and human dermal microvascular endothelial cells (HDMEC). Exposing these cells to high glucose concentration increased Notch1 signaling as reflected by increase in its target *Hey1* expression levels in all cells studied. Moreover, this effect was specific to Notch1 and not the other receptors (as confirmed by the siRNA silencing of Notch1) was abrogated when the cells were exposed to the γ-secretase inhibitor (GSI), DAPT. Activation of Notch1 by high glucose had also negative effects on the migration of keratinocytes and fibroblasts and on the tube formation of the endothelial cells that are processes central for wound healing. DAPT treatment releases the inhibitory effect of hyperglycemia on these processes, suggesting a pivotal functional role for Notch1 in of wound healing.

In order to further investigate the role of activated Notch signaling in diabetic wound healing, two GSIs (DAPT and L-685 458) were tested locally in wounds of diabetic db/db mice and both improved wound healing rate. However, the GSIs did not affect the healing rate of non-diabetic mice suggesting that the increased Notch signaling in diabetic wounds are pathogenic. In non-diabetic wounds, where the Notch signaling is low, if any, inhibition of Notch signaling has no functional consequence. In perfect agreement with this other investigations have shown either no effect or delaying of wound healing when Notch signaling is inhibited[75,76]. Several processes were improved by Notch inhibition in diabetic wounds: increased proliferation and angiogenesis (shown through PCNA and lectin staining respectively). DAPT treatment was also followed by an increased levels of angiogenic factors such as CD31, VEGFR2 and VEGFR3 and of chemokine and receptors which facilitate recruitment of endothelial progenitor cells such as SDF-1 and CXCR4. This, again, reflects the profound consequences of an increased Notch signaling for diabetic wounds.

4.1.3 Notch1 activates a positive-feedback loop to amplify its signaling through Dll4

The increase in the mRNA levels of *Dll4* in the skin of diabetic patients and mice, was followed by a corresponding increase in the protein levels, specifically in the epidermis. Furthermore, the *Dll4* expression in primary keratinocytes was increased by exposure to high glucose, effect that was abolished by DAPT treatment. Moreover, silencing Notch1, but not Notch2 and Notch3 was sufficient to abolish the induction of *Dll4* by high glucose concentration, confirming the direct relation between Notch1 and Dll4 in diabetes.

4.1.4 Inhibiting Notch signaling leads to improvement in wound healing in diabetic mice

We further investigated the relevance of the above suggested Dll4-Notch1 loop by generating a mouse model where Notch1 is specifically abolished in the keratinocytes (*KRT14-Cre;Notch1$^{fl/fl}$*). While the ablation of Notch1 in the epidermis did not affect the expression of other receptors, it significantly reduced the overall Notch signaling as shown by the dramatic decrease in *Hes1* and *Dll4* levels. While induction of diabetes was followed by an expected increase in the Notch signaling in the skin of the wild-type mice, minimal if Notch signaling was detected in the skin of diabetic *KRT14-Cre;Notch1$^{fl/fl}$* mice. Moreover, the wound healing rate in the diabetes-induced *KRT14-Cre;Notch1$^{fl/fl}$* mice was higher than the corresponding diabetes-induced wild-type mice.

These findings reveal a specific Dll4-Notch1 feedforward loop that contributes to impaired wound healing in diabetes. The specificity of the loop offer the basis for development of a specific Notch1 inhibitor to be used in therapy avoiding the potentially systemically risks of a pan inhibition of Notch receptors in the skin [159].

4.2 Paper-II

Deficiency of liver-derived insulin-like growth factor-1 (IGF-I) does not interfere with the skin wound healing rate

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4.2.1 Liver-derived IGF-I does not affect cutaneous wound healing

IGF-I promotes wound healing through its pleiotropic effects on endothelial progenitor migration and keratinocyte and fibroblast proliferation. However, the relative contributions of locally secreted IGF-I and systemic IGF-I produced by the liver is not characterized. To that end, we generated a liver-specific IGF-I knockout mice (LI-IGF-I-/-) by a Mx-cre 31 interferon inducible mouse model[160,161]. These mice had a 75% reduction in the serum IGF-I. This was followed by a compensatory increase in the serum growth hormone (GH) levels, which however, did not affect the expression of IGF-I, IGF-2 or IGF-IR in the skin. Induction of diabetes further decreased the serum IGF-I levels in these mice, in concordance with other observation in diabetes[162,163]. The wound healing rate in the healthy LI-IGF-I-/- mice was identical to corresponding wild-type mice. Diabetes was followed by a decrease in the wound healing rate of the same magnitude in both LI-IGF-I-/- mice and in wild-type mice. Taken together a 75% reduction of circulating IGF-I does not affect the wound healing rate that is in concordance with the lack of the effect on the postnatal body growth which is normal and was proposed to be affected just when the IGF1 serum levels reach 10-25%[164].

4.2.2 Processes central to wound healing are not affected by the deficiency of liver-derived IGF-I

The processes of angiogenesis, proliferation and inflammation were analyzed by immunohistochemistry. The loss of IGF-I in LI-IGF-I-/- mice did not lead to any significant difference in any of these processes when compared to the wild-type wounds. The density of granulation layer was also comparable between wild-type and LI-IGF-I-/- wounds. Since the Mx-cre 13 interferon inducible model also targets other cells like macrophages, apart from hepatocytes, the distribution of IGF-I in different types of cells was evaluated by the co-localization of IGF-I with markers for different cells (CD31 for endothelial cells and CD11b for leukocytes such as macrophages, monocytes and lymphocytes in the epidermis). Indeed, the expression of IGF-I was significantly reduced in CD11b positive leukocytes in the LI-IGF-I-/- wounds compared to the wild-type wounds at seven days post wounding while no difference was found at the beginning or at the end of the wound healing process. No difference was

found in the IGF-I expression in CD31 positive cells of the skin/wound. Overall, this study shows that a pathological decrease in the serum-derived IGF-I levels found in both type 1 and type 2 diabetes, probably does not contribute to impaired wound healing. This is important in confirming that a local delivery of IGF-I could suffice to improve wound healing in diabetes and could help avoid the negative consequences of a systemic IGF-I delivery.

4.3 Paper-III

HypoxamiR-210 accelerates wound healing in diabetes by improving mitochondrial energy metabolism

Sampath Narayanan, Sofie Eliasson, Cheng Xu, Jacob Grünler, Allan Zhao, Wan Zhu, Ning Xu Landén, Mona Ståhle, Ileana Ruxandra Botusan, Neda Rajamand Ekberg, Xiaowei Zheng*, Sergiu-Bogdan Catrina*. * equally contributed authors.

4.3.1 miR-210 is inhibited in diabetic wounds

HIF-1 is a master regulator of hypoxia in cells and it regulates more than 1000 coding and non-coding genes in humans. Although, we and others have shown that chemically or genetic recovering HIF function improved diabetic wound healing[110,111], there are potential risks in targeting a large subset of factors, some of which, may not be relevant for wound healing. For instance, a chronic/hyper-activation of HIF-1 has been implicated in fibroproliferative diseases such as keloid and hypertrophic scars and systemic sclerosis[165,166]. So, it is important to find a narrower therapeutic window to target the hypoxia signaling machinery.

miR-210 represents an attractive target in that sense, since it is a robust target of HIF-1 and regulates a variety of targets relevant to wound healing. We confirmed the regulation of miR-210 by hypoxia-in the functionally relevant primary keratinocytes, dermal fibroblasts and human dermal microvascular endothelial cells in a time-dependent manner. However, exposing to high glucose diminished the hypoxia-induced expression of miR-210 in these cells. Similarly with the *in vitro* data, miR-210 was induced in the wounds of non-diabetic mice compared to unwounded skin, but not in the wounds from diabetic db/db mice. Concordantly, miR-210 expression was reduced in diabetic foot ulcers in diabetic patients compared to venous ulcers from age-matched non-diabetic subjects.

4.3.2 Local reconstitution of miR-210 in the wounds improve wound healing in diabetes

In order to further dissect the role of miR-210 in diabetic wound healing, we injected a stabilized form of miR-210 locally on wound edges in db/db mice. The reconstitution of miR-210 improved wound healing specifically in the diabetic mice significantly.

Investigation of the wounds revealed an increase in cellular proliferation (evaluated by Ki67), angiogenesis (evaluated by CD31) and an overall increase in the density of the granulation layer. An increase in the deposition of collagen and a decrease in the immune cell infiltrates (evaluated by CD11b) was also observed. This is in agreement with previously reported roles of miR-210 in cell proliferation, migration and angiogenesis[128,130,167]. The miR-210 targets, Iron-Suphur cluster assembly enzyme (ISCU) and Succinate dehydrogenase subunit D (SDHD), which were overexpressed in diabetic wounds were also significantly lowered by miR-210 administration. As mentioned earlier, these two proteins play important roles in the TCA cycle and electron transport chain and miR-210 was reported to limit the flux through mitochondria with secondary reduction of ROS formation, through these two targets[125,168]. In agreement with these studies, reconstitution of miR-210 lead a normalization of oxygen consumption rate (OCR), which was abnormally increased in the control-treated db/db wounds and was followed by a decrease in the ROS production in db/db wounds that was increased compared with the control-non diabetic wounds.

4.3.3 miR-210 induces metabolic reprogramming in diabetic wounds

The majority of the fraction of the granulation tissue consists of fibroblasts. Given the timing of the effect of mi-210 and the important role of fibroblasts in remodeling the tissue, the effect of miR-210 mimic transfection was investigated in fibroblasts exposed to hypoxia and high glucose. miR-210 overexpression significantly reduced the expression levels of *ISCU* and *SDHD,* which led to the normalization of OCR, consistent with the *in vivo* observation. This also led to a normalization of ROS production and an improvement in the overall function measured by an increase in proliferation and migration following miR-210 overexpression. An increased oxygen consumption results from hypoxia and a defective response to it[169]. A resulting increase in ROS production is a systemic problem inherent to all diabetic complications, including wound healing[170]. Thus, our finding that miR-210 could decrease oxygen consumption and ROS production in diabetic wounds by modulating mitochondrial function is a promising therapeutic target to treat diabetic foot ulcers.

4.4 Paper-IV

Downregulated miR-34a contributes to the increased Notch1 signaling in diabetic wounds

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4.4.1 miR-34a is downregulated in diabetic foot ulcers

While the role of miR-34a is not well characterized in diabetic skin and wounds, the fact that miR-34a targets Notch1 and the detailed pathological activation of Notch1 in diabetic wounds make miR-34a, a compelling molecule to be investigated. We found that miR-34a expression levels in diabetic foot ulcers were significantly reduced compared to venous ulcers from age-matched non-diabetic controls. While miR-34a levels were higher in the venous ulcers compared normal skin, DFUs showed similar levels of miR-34a compared to diabetic skin. Consistently, miR-34a levels were also reduced in the wounds from db/db mice compared to that of the non-diabetic controls.

4.4.2 Hyperglycemia inhibits miR-34a specifically in keratinocytes

In vitro analysis revealed that miR-34a was modulated by hypoxia or high glucose exclusively in keratinocytes. Interestingly, literature agrees that miR-34a is one of the highly expressed miRNAs in the epidermis[171]. In keratinocytes, miR-34a was induced by 48 hours of exposure to hypoxia and this upregulation was diminished when exposed to high glucose and hypoxia. However, the negative effect of high glucose on miR-34a was found exclusively in hypoxia, not in normoxia. This shows that miR-34a is affected not just by the cell type but also by the condition exposed. Clearly, in diabetic wounds, the miR-34a is regulated in opposite ways by hypoxia and hyperglycemia. Ultimately, this could explain the dysregulation of miR-34a in DFU as compared to venous ulcers.

4.4.3 miR-34a-dependent Notch upregulation in keratinocytes

Interestingly, the high glucose mediated reduction in miR-34a levels in hypoxia is coupled to a gradual time-dependent increase in the levels of Notch1 in high glucose and hypoxia. Concomitantly, in diabetic wounds locally injected with miR-34a, the levels of Notch1 were significantly reduced. This shows a reciprocal regulation of Notch1 and miR-34a, confirming that Notch1 could be a target of miR-34a in diabetic wounds. This provides evidence for miR-34a repression in epidermis as causal factor for the activation of Notch1 signaling in diabetic wounds shown in paper-I. Future studies will assess the effect of miR-34a on wound healing rate and mechanism of its regulation in various processes of wound healing.

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