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MECHANISTIC STUDIES ON THE REPRESSION OF CELLULAR REACTION TO HYPOXIA AS CAUSE FOR CHRONIC COMPLICATIONS IN DIABETES

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my parents,
and Marcus.

”Kunskap är tungt att lära men lätt att bära”

- A quote from my childhood

Popular Science Summary

DIABETES AND IMPAIRED OXYGEN SENSING

Diabetes is a syndrome arising from a group of disorders with the common denominator that the blood glucose levels are too high. When exposing the organs in the body to high blood glucose levels over a longer period of time, some of the organs will eventually be damaged, resulting in what is known as diabetes complications. These diabetes complications cause a lot of personal suffering and premature death, and therefore need to be addressed. Today there is no efficient treatment aiming directly at the diabetes complications. This is because the question of exactly *how* the high blood glucose levels damage the organs is still unresolved. Since there is an inadequate knowledge of how, it is difficult to design drugs that interrupt these harmful effects caused by the glucose.

In recent years, however, we have started to understand one way by which the high blood glucose levels damage the organs. Throughout the body, there is a lower amount of oxygen than in the surrounding atmosphere. This is normal. For individuals with diabetes, however, the levels of oxygen are even lower. The lack of oxygen results in a state termed hypoxia. Normally, hypoxia would elicit a response in the cells, so that they work harder to restore the oxygen balance. This response is mainly orchestrated by a powerful molecule termed hypoxia-inducible factor (HIF). However, HIF does not function properly when exposed to high blood glucose levels, and consequently the cells of individuals with diabetes do not respond adequately to hypoxia. This has been connected with organ damage in diabetes.

This thesis has aimed to explore the inadequate reaction to hypoxia in the following organs affected by diabetes complications; the eyes, kidneys and non-healing diabetic wounds. We have found that when HIF function is improved by different measures, the organ damage is reduced in eyes and kidneys. This seems to be at least partly mediated by a normalized oxygen utilization in the cells, which is one of HIF’s normal functions. In diabetic wound healing, we have also looked at other factors, termed microRNA, that are normally involved in settings of low oxygen. We have found that they too do not function well in diabetes, and by restoring them to normal levels we were able to improve diabetic wound healing.

To conclude, this thesis provides insights into how organ damage caused by elevated glucose levels could potentially be inhibited. This has been done by shedding further light on how the blood glucose damages the cells. With our findings, together with those of others within this field, we believe that we are one step closer to being able to develop treatments that directly target the organ damage in individuals with diabetes. This has the potential to benefit hundreds of millions of people all over the world who are living with diabetes.

ABSTRACT

Diabetes Mellitus is an ongoing epidemic, causing individual suffering as well as constituting an enormous burden to society and healthcare systems globally. Much of diabetes-associated morbidity and mortality are caused by chronic diabetes complications, for which there today is no specific treatment due to an incomplete understanding of the underlying disease mechanisms. In the last two decades, the dysregulated cellular response to hypoxia has emerged as a pathogenic mechanism driving the development of diabetes complications. This work has therefore aimed to investigate the dysregulated hypoxic response further in the setting of diabetes microvascular complications.

In **paper I**, we have investigated the influence of the *HIF-1A* Pro582Ser polymorphism on the risk of developing diabetic retinopathy. We found that the *HIF-1A* Pro582Ser polymorphism confers a 95% risk reduction of developing severe diabetic retinopathy even when adjusting for traditional risk factors such as diabetes duration, hyperglycemia and hypertension. Moreover, we have further characterized the biological effects of this polymorphism and found that it increased HIF-1α transcriptional activity over a large spectrum of glucose concentrations despite being sensitive to hyperglycemia-induced degradation.

Paper II explores the role played by a dysregulated HIF-1α in diabetic nephropathy and its interplay with oxidative stress, which is known to be an important pathogenic factor in the development of kidney injury in diabetes. We found that the repressed HIF-1α signaling in diabetic kidney contributes to excess ROS production through increased mitochondrial respiration. Pharmacologic or genetic approaches to restore HIF-1α function attenuated ROS overproduction despite persistent hyperglycemia which prevented kidney injury and improved renal function.

In **paper III**, we have explored the regulation and function of the hypoxamiR-210 in diabetic wound healing. We found that diabetes repressed miR-210 *in vitro* and in *vivo* (human and mice wounds), which is pathogenic since local reconstitution of miR-210 improved wound healing in diabetic mice. Reconstitution of miR-210 resulted in restored cellular metabolism secondary to a decrease in mitochondrial respiration and ROS production and increased glycolysis, which improved cellular migration.

In **paper IV**, we investigated miR-34a in the setting of diabetic wound healing. We found that it was hypoxia-responsive in macrophages and keratinocytes, and overall repressed by high glucose levels both in vitro and *in vivo* (diabetic wounds). This repression was p53-independent and mediated on the transcriptional level. Local reconstitution of miR-34a in the wound resulted in improved wound healing in a diabetic mice model.

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I. Rajamand Ekberg N*, **Eliasson S***, Li YW, Zheng X, Chatzidionysiou K, Falhammar H, Gu H, Catrina S-B.

Protective Effect of the *HIF-1A* Pro582Ser polymorphism on Severe Diabetic Retinopathy.

Journal of Diabetes Research. 2019. 2019:2936962.

II. Zheng X*, Narayanan S*, Xu C*, **Eliasson Angelstig S**, Grünler J, Zhao A, di Toro A, Bernardi L, Mazzone M, Carmeliet P, Del Sole M, Solaini G, Forsberg E, Zhang A, Brismar K, Schiffer T, Rajamand Ekberg N, Botusan IR, Palm F, Catrina S-B.

Repression of Hypoxia-Inducible Factor-1 Contributes to Increased Mitochondrial Reactive Oxygen Species Production in Diabetes

Manuscript

III. Narayanan S, **Eliasson Angelstig S**, Xu C, Grünler J, Zhao A, Zhu W, Xu Landén N, Ståhle M, Zhang J, Ivan M, Maltesen RG, Botusan IR, Rajamand Ekberg N, Zheng X[#], Catrina S-B[#].

HypoxamiR-210 accelerates wound healing in diabetic mice by improving cellular metabolism

Communications Biology. 2020. 3(1):768.

IV. **Eliasson Angelstig S***, Narayanan S*, Zhao A*, Grünler J, Xu C, Li D, Xu Landén N, Ståhle M, Botusan IR, Rajamand Ekberg N, Zheng X[#], Catrina S-B[#].

Downregulated miR-34a in diabetic wounds contributes to delayed wound healing

Manuscript

**Shared first authorship*

[#]*Equally contributed authors*

SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

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

   Proceedings of the National Academy of Science (PNAS). 2019. 116(14): 6985-6994.

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   miR-19a/b and miR-20a Promote Wound Healing by Regulating the Inflammatory Response of Keratinocytes

   Journal of Investigative Dermatology 2021. 141(3):659-671.

**Shared first authorship*

CONTENTS

LIST OF ABBREVIATIONS

4-HNE	4-Hydroxynonenal
AGE	Advanced glycation end-products
Ago	Argonaute
ATP	Adenosine triphosphate
Bcl-2	B-cell lymphoma 2
BMI	Body mass index
CDK4/6	Cyclin-dependent kinase 4/6
cDNA	Complementary DNA
COX	Cyclooxygenase
CTAD	C-terminal transactivation domain
DAMP	Damage-associate molecular pattern
DFU	Diabetic foot ulcer
DFX	Deferoxamine
DME	Diabetic macular edema
DMOG	Dimethyloxalylglycine
DN	Diabetic nephropathy
DR	Diabetic retinopathy
E2F3	E2F transcription factor 3
ECM	Extracellular matrix
eNOS	Endothelial nitric oxide synthase
ELK-1	ETS like-1 protein
EMT	Epithelial-to-mesenchymal transition
EPC	Endothelial progenitor cell
ESRD	End-stage renal disease
ETC	Electron transport chain
FADH	Flavin adenine dinucleotide
FIH-1	Factor inhibiting HIF-1
GFB	Glomerular filtration barrier
GFP	Green fluorescent protein
GFR	Glomerular filtration rate

GSH	Glutathione
HbA1c	Glycated hemoglobin
HDF	Human dermal fibroblast
HDL	High-density lipoprotein
HDMEC	Human dermal microvascular endothelial cell
HEK293A	Human embryonic kidney 293A (cells)
HIF	Hypoxia-inducible factor
HMBS	Hydroxymethylbilane synthase
HRE	Hypoxia responsive element
IGF-1	Insulin-like growth factor 1
IL-1	Interleukin-1
IL-6	Interleukin-6
ISCU	Iron-sulfur cluster assembly enzyme
KIM-1	Kidney injury marker-1
LDL	Low-density lipoprotein
LGR4	Leucine-rich-repeat-containing G-protein coupled receptor 4
MGO	Methylglyoxal
MET	Mesenchymal Epithelial transition proto-oncogene
mIMCD-3	Mouse inner medullary collecting duct-3
miRNA	microRNA
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
mRNA	Messenger RNA
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NDUFAF4	NADH dehydrogenase 1α subcomplex 4
NF-κB	Nuclear factor kappa B
NO	Nitric oxide
NOS	Nitric oxide synthase
NPDR	Non-proliferative diabetic retinopathy
NTAD	N-terminal transactivation domains

OCR	Oxygen consumption rate
PAMP	Pathogen-associated molecular pattern
PBGD	Porphobilinogen deaminase
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDH	Pyruvate dehydrogenase
PDK1	Pyruvate dehydrogenase kinase 1
PDR	Proliferative diabetic retinopathy
PHD	Prolyl hydroxylase domain
PKC	Protein kinase C
RISC	RNA-induced silencing complex
ROS	Reactive oxygen species
RT-qPCR	Quantitative reverse transcription PCR
SDF-1α	Stromal cell-derived factor 1α
SDHD	D-subunit of succinate dehydrogenase
siRNA	Small interfering RNA
SIRT1	Sirtuin 1
STAT3	Signal transducer and activator of transcription 3
STZ	Streptozotocin
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TCA	Tricarboxylic acid (cycle)
TGFβ	Transforming growth factor-β
TNF-α	Tumor necrosis factor-α
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
VHL	von Hippel-Lindau
VU	Venous ulcer
WT	Wild-type
XO	Xanthine oxidase

1 BACKGROUND

1.1 DIABETES

1.1.1 Epidemiology and etiology

Diabetes has reached epidemic proportions and constitutes a major medical, social and economic burden. The global prevalence of diabetes is expected to increase from 463 million adults in 2017 to a staggering 693 million people in 2045. Approximately five million deaths were attributable to diabetes in 2017 (1).

Diabetes is a group of metabolic disorders, where the diagnostic feature is chronically elevated blood glucose levels due to insufficient insulin action. The lack of insulin action results in extensive disturbances in large parts of the metabolic system, including carbohydrate, lipid and protein metabolism. Diabetes can be broadly classified into two main types depending on disease etiology, although rare cases of diabetes with specific etiologies do exist. Clinical presentation ranges from asymptomatic patients to fulminant cases with profound metabolic disturbances, leading to ketoacidosis, coma and even death if no treatment is commenced (2).

1.1.1.1 Type 1 diabetes

Type 1 diabetes (T1D) results from an autoimmune destruction of beta cells in the pancreas that is triggered by a combination of genetic and environmental factors. Following the destruction of the beta cells, an absolute insulin deficiency develops and clinical presentation may be dramatic and life-threatening. T1D usually presents in younger patients but may occur at any age (2). The incidence of T1D varies across the world, with only 0.1/100,000 individuals per year in Asia whereas Scandinavia has one of the highest incidences in the world, 30/100,000 per year (3). The incidence of T1D is slowly increasing, although the reasons for this are unclear (4).

1.1.1.2 Type 2 diabetes

Type 2 diabetes (T2D) is characterized by insulin resistance of multifactorial origin combined with impaired insulin production. T2D has a marked hereditary pattern, where multiple risk alleles in combination with an unhealthy lifestyle increase the risk of developing T2D. T2D typically affects older individuals, although recently it has been shown to be increasing also in adolescents (5). The majority of the patients (>90%) that contribute to the dramatic increase in diabetes incidence have T2D. This is the consequence of lack of exercise, increased consumption of calorie-dense foods and increased prevalence of obesity which is seen in large parts of the world (6). The clinical picture of T2D is strongly heterogeneous, with nearly half of the patients being unaware of their condition (1). If the disease progresses, patients may develop an absolute insulin deficiency similar to that of T1D due to pancreatic beta cell stress and, subsequently, beta cell death. However, some patients have a marked insulin deficiency already early in the disease course (2).

1.1.1.3 Other types of diabetes

During pregnancy, insulin resistance increases and glucose metabolism is altered. This may result in overt hyperglycemia in predisposed individuals, which is termed gestational diabetes. It is estimated to affect 13.9% of all pregnancies and results in few clinical symptoms (7). However, even a relatively mild hyperglycemia may have consequences for the infant and mother and gestational diabetes is therefore closely monitored and treated. Usually, it resolves after delivery but mothers are at increased risk of developing T2D later in life (2).

Rare types of diabetes include monogenic diabetes, where single genetic abnormalities may result in impaired beta cell function or disturbed insulin action. Moreover, there are also various disorders and conditions that can result in secondary diabetes, such as pancreatic disease, liver disease and exposure to drugs, toxins and viral infections (2).

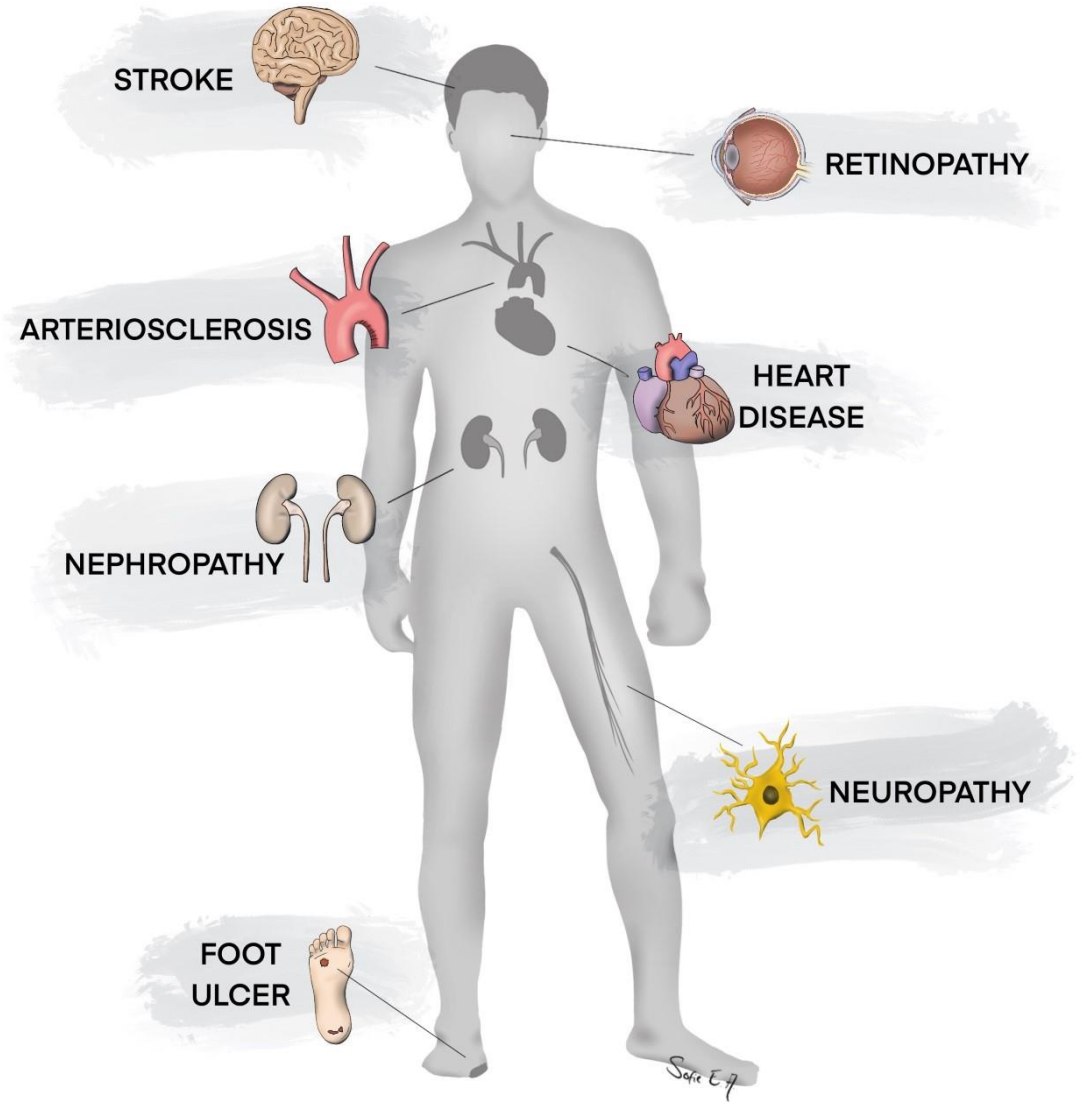

Fig. 1. Diabetes complications. Diabetes may result in chronic complications affecting different organs in the body. These complications are divided into macrovascular complications (such as stroke, coronary heart disease and peripheral arterial disease) and microvascular complications (such as nephropathy, neuropathy and retinopathy). Impaired diabetic wound healing results from a mixture of small and large vessel disease (8, 9).

1.1.2 Diabetes complications – a therapeutic challenge

The morbidity caused by diabetes is an enormous challenge for the healthcare system. Diabetes accounts for 10% of the global health expenditure, and it is increasing most rapidly in low- and middle income countries (1). Much of this burden is due to chronic complications of diabetes. These complications are divided into macrovascular complications, such as stroke, coronary heart disease, and microvascular complications, such as nephropathy, neuropathy and retinopathy (Fig. 1), and represent a major therapeutic challenge (10). Current therapies focus on maintaining a good metabolic control, which has been shown to slow the development of diabetes complications (11). However, these therapies are only partly successful in preventing the development of diabetes complications since they seldom achieve a perfect metabolic control and due to side effects as severe hypoglycemia. As of today, no efficient therapy directed towards diabetes complications is available (12). This urges for the development of therapeutic strategies based on the identification of pathogenic mechanisms for chronic diabetes complications.

1.1.3 Pathophysiology

Hyperglycemia is the main driving force in the development of diabetes complications (11, 13). However, despite extensive research efforts during the last decades, the knowledge about the pathogenic mechanisms induced by hyperglycemia remains incomplete.

Many hypotheses behind the hyperglycemia-induced tissue damage have been proposed. These hypotheses include increased formation of advanced glycation end-products (AGEs) (14), polyol pathway activation (15), increased activity of protein kinase C (PKC) isoforms (16), hexosamine pathway activation (17), oxidative stress (18), altered lipoprotein metabolism (19) and changed cytokine and growth factor expression (20, 21). The general view has been that these altered pathways are at least partly interconnected and overlapping. In 2001, Brownlee et al proposed a unifying hypotheses, suggesting that mitochondrial overproduction of ROS in hyperglycemia is the initiating event behind most of the major pathways previously shown to be pathologically relevant for diabetes complications (22). This view became somewhat of a paradigm in diabetes research but has since then become challenged (23). Despite promising pre-clinical trials and a bulk of evidence indicating a pathogenic role for ROS in diabetes complications, antioxidative therapy has had limited effect in human studies (24-27).

In recent years, hypoxia, hypoxia-inducible factors (28) and hypoxia-responsive microRNAs (29-31) have emerged as dysregulated and pathogenic factors in diabetes. In this thesis work, we have therefore focused on further exploring the contribution of these mechanisms to the development of diabetes complications.

1.2 DIABETES COMPLICATIONS

1.2.1 Diabetic retinopathy

1.2.1.1 The retina

The retina is a light-sensitive membrane that coats the inner eyeball. It is composed of the outer pigmented part (pigment epithelium) and the inner neural part, which receives light rays and transmits impulses to the brain via the optic nerve. The outer 1/3 of the retina receives its blood supply from the choroid while the inner 2/3 of the retina is supplied by the central retinal artery. The central retinal artery enters the eyeball together with the optic nerve at the optic disc and sends branches over the internal surface of the retina. The macula is situated about three millimeters temporal to the optic disc. The fovea, which is responsible for acute vision, is located in the center of the macula (Fig. 2).

1.2.1.2 Pathophysiologic changes in diabetic retinopathy

Diabetic retinopathy (DR) is the most common chronic diabetes complication (32), affecting about one third of people with diabetes (33), and it is the leading cause of blindness in working-age adults (34).

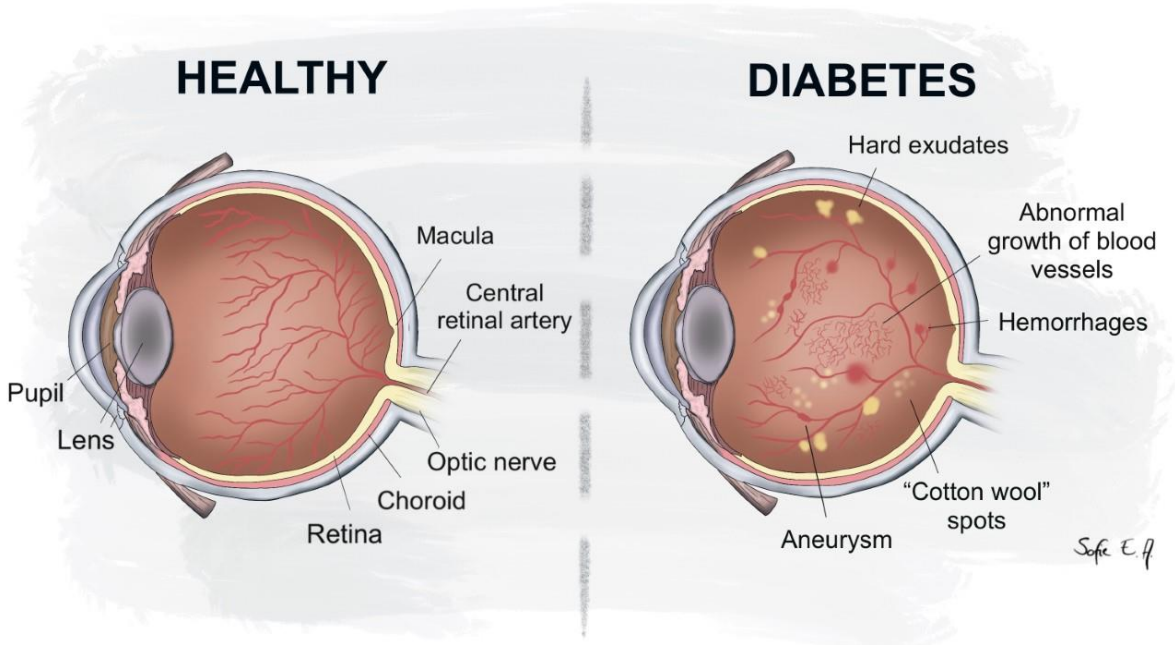

Fig. 2. Diabetic retinopathy. Typical changes in diabetic retinopathy include microaneurysms, "cotton wool" spots (resulting from infarcted neurons), hemorrhages, pathological neovascularization and hard lipid exudates. Diabetic retinopathy may also affect the macula, leading to diabetic macular edema.

DR can be broadly divided into two categories; the early stage of non-proliferative diabetic retinopathy (NPDR), and the advanced stage of proliferative diabetic retinopathy (PDR). The hallmarks of NPDR include microaneurysms, intraretinal hemorrhages, venous caliber abnormalities, lipid exudates and non-perfused capillaries with resultant infarction of neurons, represented as cotton wool spots (Fig. 2). Depending on the severity of these vascular lesions, NPDR can be further divided into mild, moderate and severe NPDR (35).

With progression to PDR, pathological neovascularization of vessels into the preretinal space will occur (36). In parallel to these processes, DR can also affect the macula. It is then called diabetic macular edema (DME), which is the most common cause of blindness in diabetes (37). DME may arise at any stage of DR but is most common in the advanced stages (32).

The microvasculopathy in DR is well characterized and it is clear that retinal pericytes and arteriolar smooth vascular cells are damaged and undergo apoptosis early in the development of DR (38). Other pathological vascular alterations include a disturbed blood retinal barrier function, leading to leakage of macromolecules into the interstitial space of the retina. This can be seen relatively short after disease onset (39). Furthermore, the capillary basement membranes have been seen to thicken, which might contribute to the disturbed communication between endothelial cells and pericytes and the impaired autoregulation by the capillaries (40). Later in the progression of DR, capillary endothelial cells die, leading to acellular capillaries which contributes to the progressive ischemia seen in DR (41).

It has become increasingly clear that DR is not uniquely a microvasculopathy. DR affects the retinal neurovascular unit as a whole, with detrimental effects on the relation between neuronal cells, glia and blood vessels that integrate to form the normal retinal function (42). These cells regulate the perfusion to meet the high metabolic requirements of the retinal neuropile, but diabetes leads to a dysregulation of this process early in the disease course (36). Moreover, there is also a retinal neurodegenerative process, which might even occur before any vascular abnormalities are present (43). Neural apoptosis and reactive gliosis are now considered hallmarks of DR, and retinal neurodegeneration might even mediate retinal microvasculopathy (40).

Furthermore, a low-grade chronic inflammation contributes to DR. Many different immune cells are activated in early DR, and several pro-inflammatory cytokines are dysregulated. An enhanced leukocyte-endothelial interaction leads to leukostasis with physical obstruction of the capillaries and ischemia (44), but also to activation of resident microglia and infiltration of monocytes to the retinal interstitium contributing to a pro-inflammatory environment (40).

The retina normally has very low levels of oxygen, which decrease even more shortly after disease onset (45). This is the consequence of a reduced oxygen extraction (46) and decreased retinal perfusion due to a constriction of major arteries and arterioles (47). Furthermore, despite the progressive retinal ischemia, the intra-retinal re-vascularization is very limited due to the impaired angiogenesis, which is a common feature in diabetes. This is in contrary to the advanced proliferative stage of DR, which occurs after decades of vasodegeneration where the ischemia-mediated angiogenic stimulus can be tremendously high, leading to uncontrolled and pathological growth of new vessels (40) .

1.2.1.3 Risk factors for diabetic retinopathy

Several systemic features of diabetes influences the risk of DR. Intensive glycemic control has been shown to delay initiation and progression of DR (48). Dyslipidemia and hypertension have also been shown to influence the risk of DR (49). However, the incidence

and progression of DR may vary among patients with similar risk factors (50). Moreover, it has been observed that even though all ethnicities are affected by DR, some populations are at increased risk (51), and there is also a familiar clustering of DR (52-54). This suggests the existence of a strong genetic component in the pathogenesis for DR. Indeed, the heritability of DR was estimated to be 27% in one study (55), and even higher (52%) for a more severe form (56). We have further investigated the genetic contribution to DR in paper I in this thesis.

1.2.2 Diabetic nephropathy

1.2.2.1 The kidney

The kidneys are about 12 centimeters in length, located in the left and right retroperitoneal space. They control the volume of body fluids, electrolyte concentrations, acid-base balance, remove toxins and synthesize certain hormones. The smallest functional units are the nephrons, which are composed of the glomeruli, Bowman's capsule and the renal tubule. The blood filtration process occurs over three layers termed the glomerular filtration barrier (GFB): the glomerular endothelial cells, the glomerular basement membrane and foot processes of the podocytes lining the capsule. Once the filtrate enters the tubuli, 99% of the fluid will be reabsorbed through a series of passive and active processes along with transportation of electrolytes and metabolic waste products before becoming the final urine.

1.2.2.2 Diabetic nephropathy – a silent killer

Diabetic nephropathy (DN) is now the leading cause of end-stage renal disease (ESRD) in Western populations (57) and a strong risk factor for cardiovascular mortality in diabetes (58). DN presents clinically as increasing albuminuria with a subsequent progressive drop in glomerular filtration rate (GFR). Microalbuminuria is present in 30% of both type 1 and 2 diabetes patients (59, 60). Without treatment, roughly half of diabetes patients will develop macroalbuminuria which leads to a tenfold increased risk of developing ESRD compared to patients with normoalbuminuria (61). In both T1D and T2D, patients do not develop albuminuria in the absence of hyperglycemia and glycemic control is the most important factor for the development of overt DN (62). Except glucose control, risk factors for DN are hypertension, obesity and genetic factors (63, 64).

1.2.2.3 Pathophysiologic changes in diabetic nephropathy

The classic morphologic changes seen in DN include glomerular basement membrane thickening, nodular glomerulosclerosis, arteriolar hyalinosis and mesangial matrix expansion (Fig. 3). Biopsies from young and lean patients with T1D often show these changes. However, older patients with T2D often also have signs of other pathogenic changes independent of diabetes such as primary glomerulopathies, ageing-related nephropathy or previous acute kidney injuries (65).

DN, with renal fibrosis as the final outcome, is the result of several pathogenic mechanisms that are activated in diabetic kidneys. These mechanisms include renal hemodynamic changes

with an overactive renin-angiotensin aldosterone system, inflammatory processes, ischemia, glucose metabolism abnormalities and associated oxidative stress (66).

The hemodynamic changes start immediately upon disease onset. Hyperglycemia induces glomerular hyperfiltration, leading to an upregulation of glucose transporters in the proximal tubules. These glucose transporters also cotransport sodium, resulting in reduced levels of sodium in the distal tubule and at macula densa, thereby activating the local intrarenal renin-angiotensin aldosterone system. The resulting dilation of the afferent arteriole and constriction of the efferent arteriole leads to glomerular hypertension (67).

Thickening of the glomerular basement membrane is an early sign in DN, caused by the abnormal turnover of extracellular matrix (ECM) components (68). These changes in the structure of ECM is a direct effect of hyperglycemia, which leads to mesangial cell proliferation and matrix production (69) mediated by the expression of transforming growth factor-β (TGFβ) and vascular endothelial growth factor (VEGF) (70). Hyperglycemia also contributes to an abnormal turnover of ECM by affecting the function of matrix metalloproteinases. Eventually, the mesangial cell expansion leads to glomerular hypertrophy (71).

Persistent hyperglycemia also causes endothelial cell dysfunction, which leads to dysfunction of the glomerular endothelium with increased vascular leakage and reduction of the GFB function (72). Concurrently, hyperglycemia damages the endothelial glycocalyx, partly mediated by the production of reactive oxygen species (ROS) that inhibit the generation of heparin sulfate (73), which further increases the vascular permeability (74). Furthermore, the podocytes with their crucial role for maintaining GFB function are also injured by hyperglycemia. This is seen as a podocyte hypertrophy, foot process effacement and finally podocyte loss (75). Podocyte loss, which is irreversible, leads to macroproteinuria and contributes to glomerulosclerosis with its subsequent loss of nephrons (76).

The kidneys have a high metabolic demand and are second only to the heart with regards to oxygen consumption per mass, which is much due to the large amount of adenosine triphosphate (ATP) needed to drive the sodium-glucose cotransporters in the renal tubules (77). However, the kidneys develop a profound and progressive hypoxia that can be seen already three days after diabetes onset in experimental animals, preceding the onset of typical markers of DN such as albuminuria (78). The hypoxia develops as a consequence of increased flux through the sodium-glucose cotransporters, increased mitochondrial uncoupling (79), glomerular and vascular lesions and reduced production of the vasodilator nitric oxide (NO) (80). The tubuli are especially vulnerable to hypoxia, leading to apoptosis (81), and also to stimulation of extracellular matrix expansion, which further reduces the supply of oxygen, thereby creating a vicious circle. The tubular hypoxia has also been observed to lead to secondary glomerulopathy, thereby being a key driver in the progression of DN (82). Moreover, hypoxia in itself, without confounders such as hyperglycemia, high blood pressure or oxidative stress, results in proteinuria and morphological damages typical of DN (83). These observations have contributed to a shift from an almost entirely

"glomerulocentric view" in DN to a recognition of tubulopathy and profound hypoxia as early pathogenic events and key drivers in DN (82). The crucial role played by hypoxia in DN will be discussed further later in the thesis.

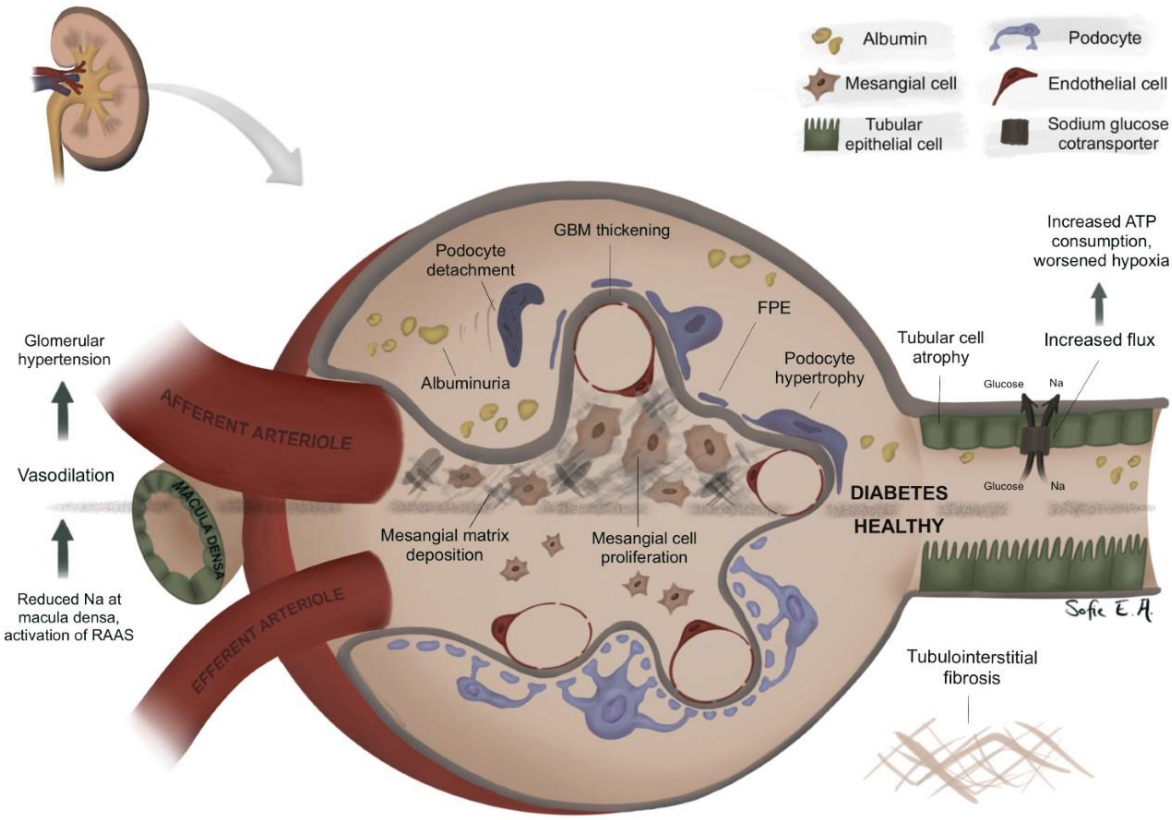

Fig. 3. Diabetic nephropathy. Pathologic glomerular changes in diabetic nephropathy are depicted in the upper half of the glomerulus. Diabetes results in increased mesangial cell proliferation and hypertrophy, increased matrix deposition, podocytopathy, albuminuria, tubular cell atrophy and tubulointerstital fibrosis. The hyperglycemia-induced hyperfiltration leads to increased flux through sodium glucose cotransporters that results in increased ATP consumption and worsened kidney hypoxia. This also leads to reduced sodium levels at macula densa, which activates the local RAAS and causes dilation of the afferent arteriole, constriction of the efferent arteriole (not shown) and glomerular hypertension. ATP, adenosine triphosphate; FPE, foot-process effacement; GBM, glomerular basement membrane; Na, natrium; RAAS, renin-angiotensin-aldosterone system.

1.2.3 Diabetic foot ulcer

1.2.3.1 Wound healing

Wound healing is a complex process, requiring the coordination of multiple cell types. It is precisely regulated to quickly restore barrier function and protect us against the hostile environment. Normal wound healing consists of four separate stages; hemostasis (the first hours following injury), inflammation (day 1-3), proliferation (day 4-21) and remodeling (lasting from three weeks up until one year) (84).

During the hemostasis phase, the injury leads to immediate activation of the intrinsic and extrinsic clotting cascade. There is a rapid vasoconstriction and thrombocyte aggregation to prevent excessive blood loss, which leads to hypoxia (85). A blood clot is formed, serving as a matrix that enables different cell types to migrate to the wound area (84).

In the inflammatory phase, the tissue injury activates several different intracellular signaling pathways in the resident cells, i.e. keratinocytes, macrophages, dendritic cells and mast cells. This is mediated via danger signals, which are expressed following tissue injury. Danger signals can be categorized as damage-associate molecular patterns (DAMP) and pathogen-associated molecular patterns (PAMP) (86). DAMP consist of intracellular material that is exposed when cells undergo apoptosis whilst PAMP are molecules specifically derived from pathogens (86). Danger signals activate pattern recognition receptors, leading to the induction of various genes, such as chemokines, cytokines and antimicrobial peptides (87). As a consequence of this, neutrophils are recruited to the wound site early in the inflammatory phase. The endothelial cells will also express adhesion molecules, which facilitate neutrophil adhesion and extravasation (88). The chemokine gradient leads the neutrophils to the wound site, where they stay around 2-5 days. The neutrophils perform phagocytosis and release cytokines that amplify the inflammatory response (89). Simultaneously monocytes start to migrate into the wound area. There they develop into mature macrophages. The macrophages continue the phagocytosis and also release growth factors in order to recruit keratinocytes, endothelial cells and fibroblasts (90).

During the proliferative phase, the surface gets re-epithelialized, the vascular network is restored and granulation tissue is formed. This requires the coordinated actions of keratinocytes, endothelial cells and fibroblasts. The keratinocytes that are adjacent to the wound begin to migrate to cover the wound. In order to generate more keratinocytes, epithelial stem cells and keratinocytes from the basal layer of the wound edge start to proliferate (91). Simultaneously, granulation tissue is formed, which consists of fibroblasts, macrophages, granulocytes, blood vessels and collagen bundles. The granulation tissue replaces the provisional wound matrix formed during hemostasis, which to some extent leads to a recovery of the structure and function of the skin (92). This enables cell adhesion, migration and growth during wound repair. Fibroblasts are key players in this process, migrating from nearby dermis, or being generated from fibrocytes if the wound process is long-lasting (93).

The wound develops a pronounced hypoxic environment, due to the disruption of vessels and increased oxygen demand during wound healing (94). The restoration of blood vessels is therefore crucial for the wound healing process. Blood vessels can develop from already existing ones, called angiogenesis, or develop de novo from bone marrow-derived progenitors, which is called vasculogenesis (95).

The final stage is the remodeling phase. The fibroblasts are stimulated by mechanical tensions and cytokines to become myofibroblasts, which are able to contract the wound (96). The collagen which was quickly produced in the granulation tissue is replaced with collagen of a higher tensile strength (97). However, the skin is only able reach around 80% of the tensile strength it had before injury (98).

1.2.3.2 Impaired wound healing in diabetes

Diabetic wound healing is impaired, which can result in diabetic foot ulcers (DFUs). DFUs are associated with considerable morbidity and mortality. Patients with DFUs have a mortality rate of 16.7% after one year and 50% after five years, which is a mortality similar to that of colon cancer (99). The lifetime incidence for diabetic patients to develop foot ulcers is 25% (100), and it estimated that every 30 seconds someone in the world loses an extremity because of diabetes (101). Despite extensive knowledge and competence in multidisciplinary teams (composed of endocrinologists, orthopedic surgeons, vascular surgeons and podiatrists), the treatments that can be offered to patients with DFUs are limited and of poor efficacy (102).

1.2.3.3 Risk factors for diabetic foot ulcers

DFUs are usually resulting from repeated pressure to an area exposed to high vertical or shear stress in patients with peripheral neuropathy. The motor neuropathy leads to foot deformity and biomechanical abnormalities, which results in abnormal pressure distribution over the foot. Due to recurring pressure, keratosis and callus formation occurs. Repetitive pressure on the areas with callus damage the foot tissues, leading to ulcer formation. Furthermore, the sensory neuropathy leads to a loss of protective sensation, whereby harmful stimuli may be unnoticed. Autonomous neuropathy leads to dysfunctional sweat glands, which makes the skin dry and prone to ulceration (103).

Peripheral artery disease contributes in almost 50% of the patients to the occurrence of DFU (8). In diabetes patients, microcirculatory defects are present early in the disease course, including thickening of basement membranes, reduced capillary size and arteriolar hyalinosis (9). In addition, there is an accelerated arteriosclerosis in diabetes patients. The chronic hyperglycemia also leads to dysfunction of the endothelium and smooth muscle, leading to constriction of the vessels. Ultimately, this contributes to the development of occlusive arterial disease with ischemia and ulcer formation in diabetes (9).

1.2.3.4 Mechanisms behind disturbed diabetic wound healing

Once the skin barrier has been breached, diabetic patients are at high risk of developing a chronic ulcer due to the impaired wound healing. The mechanisms underlying the delayed wound healing in diabetes are complex and only partly understood. The mechanisms include a prolonged inflammatory response (104), dysregulation of key cellular players (105, 106), reduced growth factor signaling (107-109), decreased angiogenesis (110, 111), increased susceptibility to infections (112) and tissue hypoxia (113) (Fig. 4).

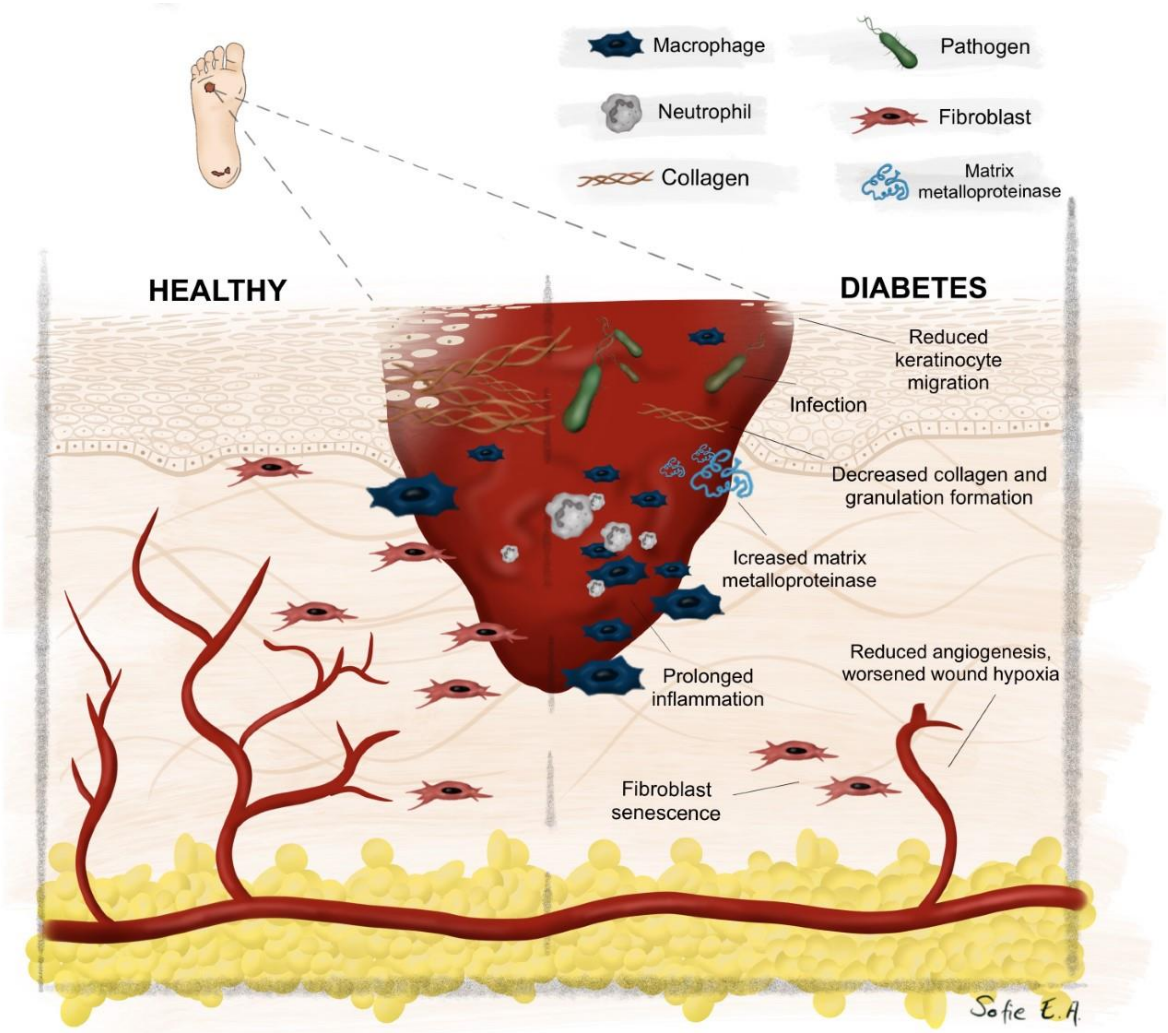

Fig. 4. Diabetic wound healing. The pathologic changes leading to impaired diabetic wound healing include reduced keratinocyte migration, increased susceptibility to infections, decreased formation of granulation tissue, increased matrix metalloproteinase action, prolonged inflammation, fibroblast senescence, reduced angiogenesis and increased tissue hypoxia.

Several key cellular players in diabetic wounds are dysregulated. Keratinocytes and fibroblasts show decreased migration and proliferation (105, 106). Insulin-like growth factor 1 (IGF-1), TGF-β and platelet-derived growth factor (PDGF), which stimulate migration and proliferation of fibroblasts, keratinocytes and endothelial cells, are reduced in human wounds (107-109). Diabetic wounds also have reduced expression of stromal cell-derived factor 1α (SDF-1α), which leads to an impaired mobilization of bone marrow-derived endothelial progenitor cells (EPCs). This results in reduced angiogenesis (110, 111). Moreover, there is also an increased expression of matrix metalloproteinases (MMP) (114). These elevated levels of MMPs lead to the degradation of extracellular matrix components, such as fibronectin, cytokines and growth factors, which disrupts the wound healing process (115).

Diabetic tissues have a chronic low-grade inflammation and an extended inflammatory response following injury, which further impairs wound healing. This might inhibit the transition from the inflammatory phase to the proliferative phase (90). The pro-inflammatory M1 to anti-inflammatory M2 macrophage ratio, which is a typical indicator in chronic wounds, is elevated in diabetic skin (116). There is also an increase of pro-inflammatory

cytokines, including tumor necrosis factor-α (TNF- α), interleukin-1 (IL-1) and interleukin-6 (IL-6) (104). Moreover, diabetic wounds have an abundance of neutrophils, which appear to delay the wound healing rate (117).

Infection is an important factor contributing to delayed diabetic wound healing. More than half of diabetic ulcers become infected (112). Diabetic patients have an increased susceptibility to infections because of several immune defects (118), such as dysfunction of the endothelium that disturbs the recruitment of inflammatory cells and impairment of macrophage and neutrophil function (119).

Wounds are profoundly hypoxic (94), but the cellular response to hypoxia in diabetes is impaired (120). Therefore, hypoxia has emerged as a novel pathogenic mechanism in DFU, which will be discussed in further detail below.

1.3 HYPOXIA AND HYPOXIA-INDUCIBLE FACTORS

1.3.1 Hypoxia

Hypoxia is defined as a condition with reduced oxygen levels due to a reduced supply and/or increased consumption of oxygen. Normal oxygen levels in the human body range from 1% to 11% (121); consequently, hypoxia is a relative term. Hypoxia occurs physiologically and plays an important role in tissue homeostasis and development. It is also involved in the pathogenesis of diseases such as ischemic conditions, tumors and diabetes (121, 122).

1.3.2 HIF signaling

The cellular response to tissue hypoxia is mediated by the transcription factor hypoxia-inducible factor (HIF) (Fig. 5). HIF is a heterodimer that consists of oxygen-regulated α subunits and a constitutively expressed β subunit (122). Three α subunits have been identified, of which HIF-1α is expressed ubiquitously. HIF-2α has a limited tissue distribution and is similar to HIF-1α in structure and function while HIF-3α counteracts the function of HIF-1α and HIF-2α and thereby works as a repressor (120).

Under normoxic conditions, HIF-1α is hydroxylated at two proline residues (Pro402 and Pro564) by prolyl hydroxylase domain (PHD) proteins (123) which require oxygen, iron, and α-ketoglutarate as cosubstrates (124). The hydroxylated HIF-1α is recognized by the von Hippel-Lindau (VHL) tumor suppressor protein which mediates the ubiquitylation of HIF-1α that targets HIF-1α for proteasomal degradation (125). PHDs are inhibited in hypoxic conditions which protects HIF-1α from degradation (122). Since the discovery of the ingenious oxygen-dependent PHD-mediated regulation of HIF-1, also termed the canonical regulation of HIF-1, a great number of additional regulatory pathways have been identified. These non-canonical pathways may act independently of external oxygen concentration (123). However, PHDs appear to play a major role for the regulation of HIF-1α protein stability since inhibition of PHD function is able to stabilize HIF-1α in normoxia (126).

In addition to HIF-1α stability, oxygen levels also regulate the transactivation activity of HIF-1α. HIF-1α has two transactivation domains: the N-terminal (NTAD) and C-terminal (CTAD) transactivation domains (120). The CTAD activity is inhibited in normoxia through the action of factor inhibiting HIF-1 (FIH-1), which hydroxylates an asparagine residue in CTAD so that the recruitment of co-activator CBP/p300 is inhibited. In contrast, NTAD transactivation activity is largely coupled to protein stability (120).

Once stabilized, HIF-1α moves to the nucleus where it will form a heterodimer with HIF-1β on the hypoxia-responsive element (127) within the promotor of target genes (128). The genes activated by HIF-1 mediate local and systemic adaptation to hypoxia by regulating angiogenesis, erythropoiesis, metabolism, vasodilation, pH, migration, proliferation and autophagy (122). Recently, HIF-1 has also been shown to regulate a wide array of microRNAs (miRNAs) and long non-coding RNAs, which are used to further modulate the hypoxic response (129).

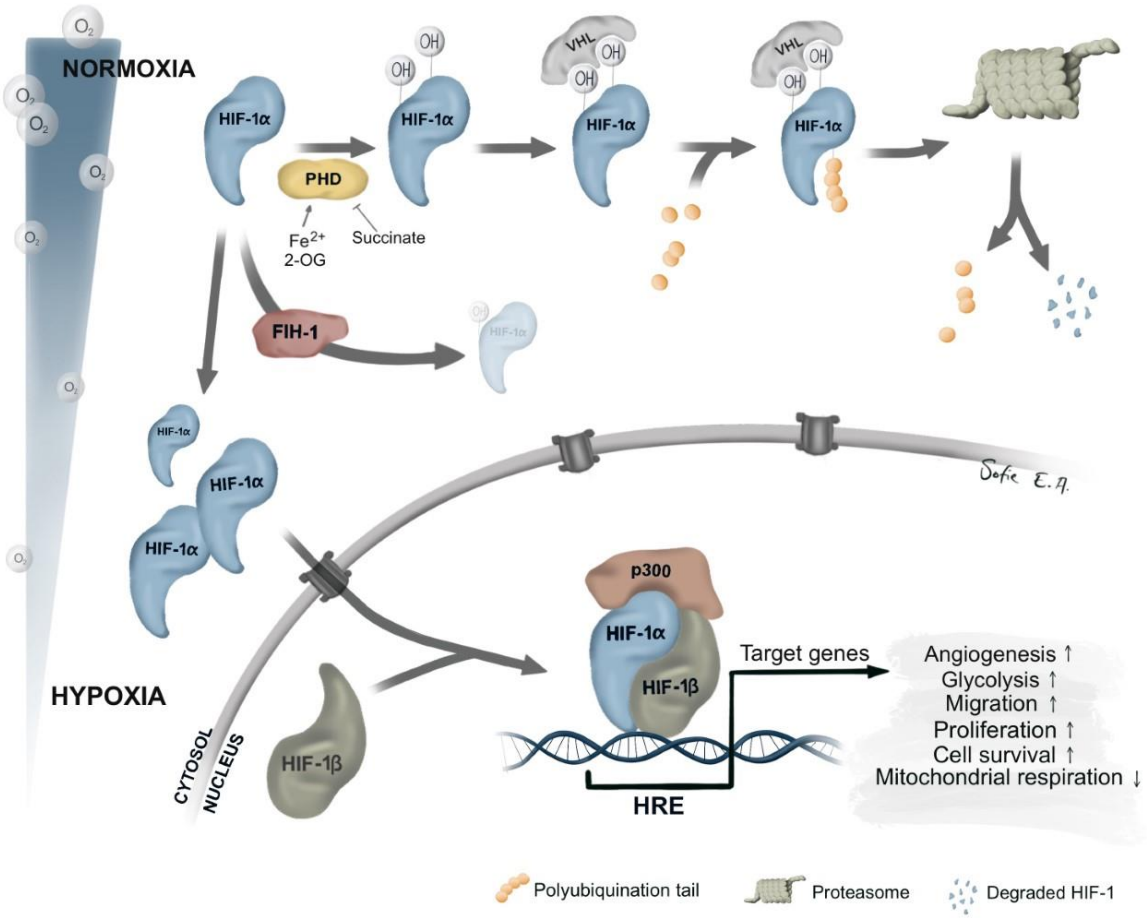

Fig. 5. Canonical regulation of HIF-1 activity. Under normoxic conditions, HIF-1α is hydroxylated by PHD at two prolines residues (Pro402 and Pro564). PHD requires Fe^{2+} and 2-OG as cofactors. Once hydroxylated, HIF-1α is recognized by VHL and marked for proteasomal degradation. HIF-1α can also be hydroxylated by FIH-1 in the presence of oxygen, which inhibits HIF-1α transactivation activity. In hypoxic conditions, HIF-1α is stabilized and translocates to the nucleus where it forms a heterodimer with HIF-1β and binds to the HRE of its target genes together with p300 and other coactivators. 2-OG, 2-oxoglutarate; FIH-1, factor inhibiting HIF-1; HIF, hypoxia-inducible factor; HRE, hypoxia responsive element; PHD, prolyl hydroxylase domain; VHL, von Hippel-Lindau tumor suppressor protein.

1.3.3 HIF signaling is dysregulated in diabetes

Even though hyperglycemia is the primary causative factor for chronic complications of diabetes, emerging evidence have shown that hypoxia is present in retina (130), kidney (131), skin (132) and wound (126). Despite a profound hypoxic environment, previous studies have shown that hyperglycemia diminishes HIF-1α function resulting in a deregulated hypoxia response in diabetes (126, 127, 133-136). This deregulated cellular response to hypoxia has been observed in all diabetic tissues investigated in diabetic animals and humans, which suggests that it plays an important role for the development of diabetes complications (120).

The molecular basis for the dysregulated HIF-1 signaling in hyperglycemia is incompletely understood. It has been shown that the repressive action of hyperglycemia is exerted both on the protein stability and on the transactivation level (126). Our group has previously shown that the reduced HIF-1α protein levels under hyperglycemic conditions can be partly restored by inhibition of PHDs, implying an increased susceptibility of HIF-1α towards the canonical proline hydroxylation in hyperglycemia (134). In cardiac myocytes, it has been shown that diabetic dyslipidemia results in reduced levels of succinate leading to increased activity of PHD. Supplementation with succinate was able to restore HIF-1α levels in hyperglycemia (136). However, HIF-1α protein stability is only partially increased in fibroblasts following inhibition of PHDs (134), which indicates that other mechanisms are involved in the HIF-1α destabilization in hyperglycemia. Osmotic challenge can be one mechanism by which hyperglycemia destabilizes HIF-1α. Administration of mannitol is able to mimic the hyperglycemia-induced destabilization of HIF-1α protein in fibroblasts and endothelial cells (134). However this is not a general mechanism since mannitol does not modulate HIF-1α protein levels in bovine aortic smooth muscle cells (127). Additional data has shown that the dicarbonyl metabolite methylglyoxal (MGO), which is increased in hyperglycemia, is able to promote proteasomal degradation of HIF-1α independent of VHL and the proline hydroxylation of HIF-1α. However, normalization of MGO levels through overexpression of glyoxylase 1 is only partially successful in stabilizing HIF-1α (137). In addition, intracellular levels of MGO required for HIF destabilization are two to three times higher than those resulting from hyperglycemia (120).

On the transactivation level, MGO has been shown to modify HIF-1α, thereby preventing heterodimerization and binding of HIF-1α to its promoters (138). Moreover, MGO decreases the binding of coactivators to HIF-1α through covalent modification of its coactivator p300, resulting in a decreased transactivation of HIF-1α (133). Hyperglycemia has been observed to repress the activity of both NTAD and CTAD of HIF-1α, independently of HIF-1α protein stability (126). A genetic variant of HIF-1α (HIF-1α Pro582Ser) has an increased transactivation activity in hyperglycemia compared with wild-type HIF-1α, even though it does not confer protection against proteasomal degradation of HIF-1α mediated by hyperglycemia (131). To summarize, the mechanisms behind the repressive effect of hyperglycemia on HIF-1 transactivation activity and protein stability are complex and not yet completely understood.

1.3.3.1 HIF and diabetic retinopathy

As previously described, hypoxia plays a key role in the pathogenesis of DR. HIF-1 target genes are crucial for normal retinal development, adequate retinal function, vasculature stability and vision maintenance. Several HIF-1 targets have been found to be essential for the protection of the retina in DR (139). It is known that the target genes of HIF-1 enhance oxygen and glucose delivery, anti-oxidization, neovascularization, anti-inflammation and neurotrophy (122). This indicates that manipulation of HIF-1 signaling might provide a new therapeutic target. Few efforts have been done to treat retinal hypoxia in DR, even though it has a key pathogenic role (140). We have further investigated the role of HIF-1 in diabetic retinopathy in paper I in this thesis.

1.3.3.2 HIF and diabetic nephropathy

Kidneys develop hypoxia early in the course of diabetes. Renal hypoxia has been shown in adolescents with T1D (141), early stage nephropathy in T2D (142) and as soon as three days after diabetes induction in experimental animals (78). Studies on HIF-1α expression in diabetic kidneys have found conflicting results, pointing towards a cell- and compartment-dependent regulation of HIF-1α. Glomeruli were found to have an increased expression of HIF-1α, which resulted in profibrotic signaling and glomerulosclerosis, whereas HIF-1α has been reported to be repressed in tubular cells (143, 144). However, activation of HIF-1 via chemical compounds attenuated the development of DN in animal models of diabetes (145, 146). Interestingly, tubular hypoxia has been proposed to be the major contributor to tubular atrophy and interstitial fibrosis, which further aggravates glomerular disease in DN (82). Moreover, the *HIF-1A* Pro582Ser with a more active HIF-1α variant in diabetes protects against development of diabetic nephropathy (131). This may indicate that HIF-1 activation has a positive net effect in DN, although more studies are warranted. The role of HIF-1 in DN is further investigated in paper II in this thesis.

1.3.3.3 HIF and diabetic wound healing

Wounds are profoundly hypoxic due to a disrupted vascular supply and increased oxygen consumption during the wound healing process (94). HIF-1 signaling has been shown to be critically involved in virtually all wound healing stages, for example by promoting angiogenesis through the induction of cytokines and growth factors such as VEGF and SDF-1 (147). In line with this, adenoviral-mediated transfer of HIF-1α improves tissue limb perfusion and angiogenesis in animal models of diabetic peripheral ischemia (148, 149). Moreover, in diabetic chronic wounds, HIF-1α expression has been reported to be downregulated in comparison to equally hypoxic chronic venous ulcers, which contributes to the development of diabetic foot ulcer (126). The clinical relevance of this finding is demonstrated by the improved wound-healing rate following different measures to stabilize HIF-1α (126, 150). Targeting the HIF-1 signaling pathway therefore holds promise for a new therapeutic approach for diabetic wound healing.

1.4 OXIDATIVE STRESS

1.4.1 Mitochondria

1.4.1.1 Mitochondrial function

Mitochondria are cytosolic organelles, initially stemming from bacteria, which can be found in all cells except for erythrocytes. In line with their bacterial origin, they possess double membranes and their own mitochondrial DNA. Mitochondria are responsible for oxidative phosphorylation that produces ATP from fuels such as glucose, fatty acids and amino acids. This is mediated by a complex process where electrons from NADH and $FADH_2$, which are generated during oxidative phosphorylation, travel through complexes I-IV in the electron transport chain (Fig. 6). Oxygen functions as the final electron acceptor in a series of redox reactions, resulting in a proton gradient over the inner mitochondrial membrane. The ATP synthase (complex V) uses this electrochemical gradient to produce ATP (151).

Apart from the generation of ATP, mitochondria are involved in other cellular processes, such as calcium homeostasis, regulating apoptosis, cell cycle progression and steroid hormone synthesis (152). The ATP gradient may also be used for non-shivering thermogenesis by the use of uncoupling protein 1 (UCP1), which dissipates the proton gradient (termed “proton leak”) and generates heat instead of ATP. UCP2 and UCP3 may also release the proton gradient, but their actions appear to be mainly focusing on reducing the production of reactive oxygen species (ROS) in conditions of increased oxidative stress (153).

1.4.1.2 Oxidants and antioxidants

During the oxidative phosphorylation, 1-4% of oxygen physiologically undergoes conversion to incomplete reduced oxygen, resulting in reactive oxygen species (ROS) that are highly reactive and have a short half-life (154). The primary ROS produced in the body is superoxide anion, which is immediately converted to another ROS, hydrogen peroxide, by the enzyme superoxide dismutase (SOD) (155). ROS generation can rise following increased electron flux through the ETC secondary to increased supply of substrates, which generates an elevated voltage gradient that blocks the electron transfer to complex III (156). This causes the electrons to instead react with oxygen, thereby forming superoxide (157). In addition to mitochondrial ROS generation, ROS are also produced from various other sources, including cytosolic nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, xanthine oxidase (XO), uncoupled endothelial nitric oxide synthase (NOS), myeloperoxidase (MPO), cyclooxygenase 2 (COX-2) and endoplasmic reticulum stress (recently reviewed in 23). Cells can also be exposed to exogenous oxidants such as cigarette smoke, ozone, ionizing radiation and heavy metal ions. The cellular mechanism to neutralize oxidants consists of a partly overlapping system of antioxidants, which include non-enzymatic and enzymatic antioxidants. The non-enzymatic antioxidants include vitamins (vitamin A, B, C and E), uric acid and glutathione (GSH), where GSH is the predominant soluble antioxidant. Among the enzymatic antioxidants are SOD, catalase and glutathione peroxidase (154).

ROS has for long been considered as metabolic by-products but accumulating evidence is now indicating that ROS function as critical signaling molecules (termed redox signaling) in vital cellular processes such as proliferation, differentiation, survival, apoptosis and repair (158). Moreover, ROS is also implicated in the regulation of HIF-1 activity (120), which will be further discussed below.

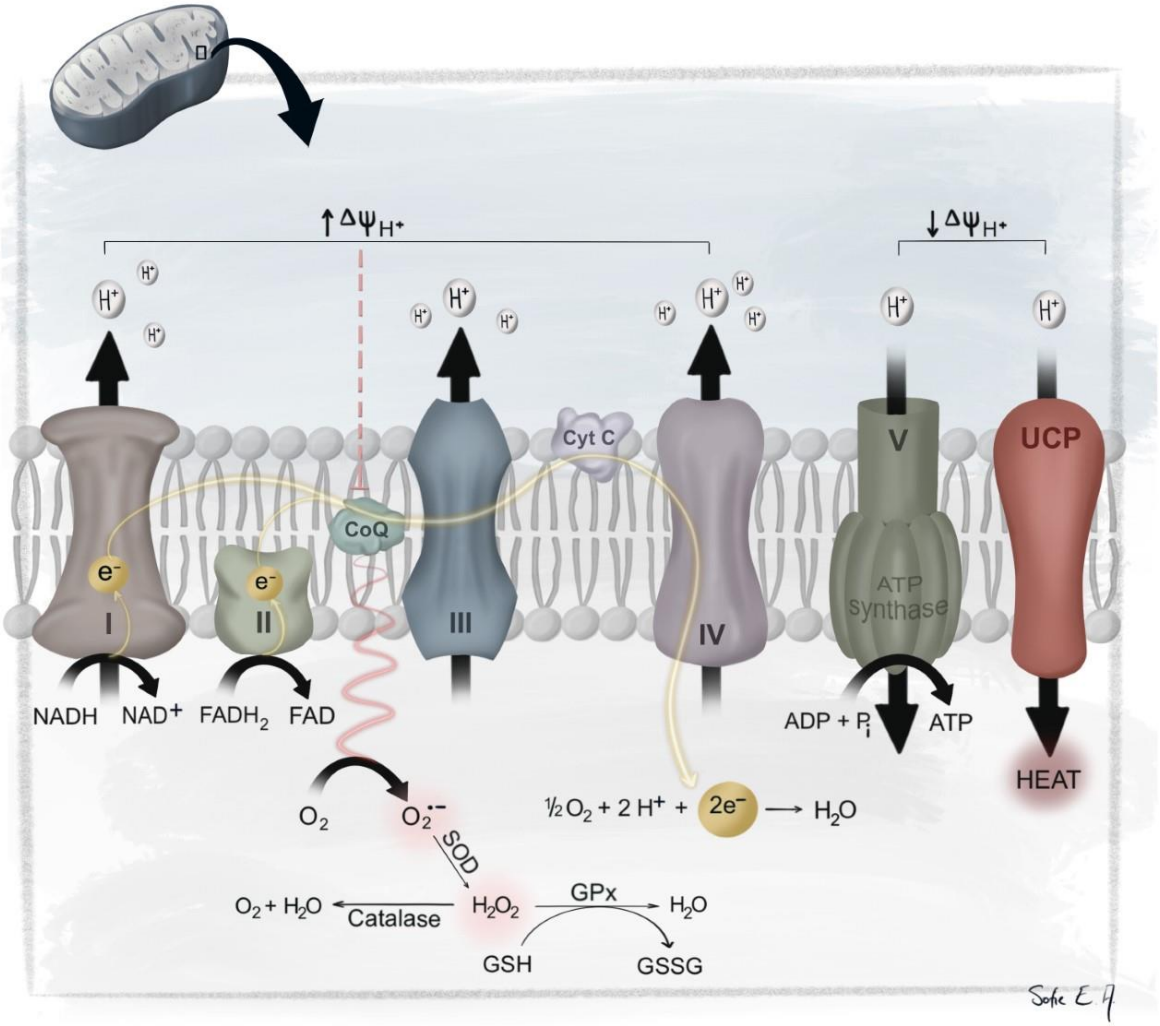

Fig. 6. ROS production during mitochondrial respiration. NADH and $FADH_2$ leave their electrons at complex I and II of the ETC. When the electrons are transferred through the ETC, some of their energy is used to pump H^+ into the intermembrane space. This results in a voltage gradient ($\Delta\Psi$) across the inner mitochondrial membrane, which drives the ATP synthase (complex V) and generates ATP. If the voltage gradient reaches a critical threshold due to increased electron flux, electrons cannot move from coenzyme Q to complex III (red dashed line). The electrons will instead react with oxygen, generating superoxide anions, which are extremely short-lived ROS. Superoxide anions are converted by SOD to another ROS, hydrogen peroxide, which can be neutralized either by the catalase or glutathione systems. UCP can reduce the voltage gradient by transporting H^+ back to the matrix ("proton leak"), which will activate the ETC and reduce ROS formation. ADP, adenosine diphosphate; ATP, adenosine triphosphate; CoQ, coenzyme Q; Cyt C, cytochrome C; ETC, electron transport chain; $FADH_2$, flavin adenine dinucleotide; GPx, glutathione peroxidase; H^+, proton; H_2O_2, hydrogen peroxide; SOD, superoxide dismutase; NADH, nicotinamide adenine dinucleotide; $O_2^{\bullet-}$, superoxide anion; P_i, inorganic phosphate; ROS, reactive oxygen species; UCP, uncoupling protein.

1.4.2 Oxidative damage in diabetes

Oxidative damage arises when there is an unbalance between oxidants and antioxidants in favor of the former, so that the antioxidant systems are overwhelmed resulting in oxidative stress and altered redox signaling. Oxidative stress leads to ROS-mediated damage to DNA, lipids and proteins (159). Moreover, oxidative stress leads to the induction of various pro-inflammatory cytokines, thereby enhancing inflammation which further promotes ROS production (160). Not surprisingly, oxidative stress has been shown to be involved in multiple disease progresses, including carcinogenesis (161), neurodegeneration (162), atherosclerosis (163) and aging (164).

As mentioned above, mitochondrial ROS formation can increase due to an increased delivery of substrates (165). ROS formation can also increase due to a reduced availability of O_2, for reasons that are yet not entirely clear (166). In diabetes there is both an elevated nutrient flux through the electron transport chain (ETC) (165) as well as hypoxia (28), and oxidative stress has indeed been shown to play a key pathogenic role in diabetes complications (167, 168). Mitochondrial ROS has been proposed as a unifying mechanism leading to the activation of several other pathogenic pathways in the development of diabetic complications; increased polyol pathway flux, increased formation of AGEs, activation of PKC and overactivity of the hexosamine pathway. Moreover, it also inhibits two enzymes with anti-atherosclerotic effects, endothelial nitric oxide synthase (eNOS) and prostacyclin synthase (169). Thus, it has been proposed that by targeting excessive mitochondrial ROS production, other downstream pathogenic signaling pathways might also be inhibited (22). Despite this, antioxidative therapy has had limited or even detrimental effects for the prevention of diabetes complications in clinical studies (24-27). This might be due to an incomplete understanding of the mechanisms of action underlying the antioxidants and the optimal way of administering these drugs. It has therefore been suggested that targeting the source behind the generation of increased ROS might prove more efficient than the non-specific use of systemic antioxidants (170). At the pre-clinical level, the evidence is contradictory. Antioxidant therapy has yielded beneficial effects on diabetes complications (171, 172), but there are also preclinical studies showing that diabetes complications may occur in the absence of increased oxidative stress (173-175). This indicates that excessive mitochondrial ROS production is not the only initiating factor in the development of complications. Taken together, ROS appears to play a more complicated role in the pathogenesis of diabetes complications than previously appreciated. Therefore, there is a need to further characterize the physiological and pathological relevance of ROS in diabetes complications as well as to investigate novel pathogenic pathways interacting with ROS in order to find a specific treatment for diabetes complications.

1.4.3 HIF, ROS and cellular bioenergetics

Previously it was believed that cells change to glycolytic metabolism in hypoxia because oxygen was not available for oxidative phosphorylation. However, long before oxygen levels are limiting for oxidative phosphorylation, cells start to switch to glycolytic metabolism (176). This is done because hypoxia induces excessive formation of ROS at the ETC (177), which leads to cellular dysfunction and death (159). The metabolic switch is mediate by HIF-1, which functions as a master regulator of redox homeostasis (176). HIF-1 exerts its protective functions by enhancing glycolytic enzymes (178-180) and expression of antioxidants (176), as well as suppressing the ETC (181-183), mitochondrial biogenesis (184) and the oxygen-demanding fatty acid oxidation (185). The protective effect of HIF-1 in hypoxic conditions was shown in mouse embryo fibroblasts lacking HIF-1 function that were unable to switch to glycolytic metabolism in hypoxia and therefore died secondary to oxidative stress. However, the cells could be rescued either by treatment with antioxidant or forced expression of pyruvate dehydrogenase kinase 1 (PDK1) (180, 186). PDK1 is a direct target of HIF-1 and inhibits the electron transport flux by preventing pyruvate from entering the tricarboxylic acid cycle (TCA cycle) (180). Moreover, HIF-1 also strongly upregulates the hypoxamiR-210, which mirrors the function of HIF-1 and enforces the switch to glycolytic metabolism in hypoxia (29). HypoxamiR-210 will be further discussed below.

The relationship between HIF-1 and ROS is bidirectional, since ROS signaling also affects HIF-1 function. Although the picture is unclear, most reports indicate an activating role of ROS on HIF-1 function (187). For instance, it has been shown that inhibition of mitochondrial complex III prevents activation of HIF-1 during a hypoxic challenge (177, 188) and that overexpression of glutathione peroxidase or catalase can prevent the stabilization of HIF-1α (188). The connection between HIF-1 and ROS and their role in the development of diabetes complications are further investigated in paper II in this thesis.

1.5 MICRORNAS

1.5.1 microRNA biogenesis

MicroRNAs (miRNAs) are short non-coding RNAs that regulate gene expression post-transcriptionally (189). They bind to the 3'-untranslated regions (3'-UTR) of their target messenger RNAs (mRNA), thereby leading to mRNA destabilization, translational repression or both (190). The generation of mature miRNAs is a multistage process (Fig. 7). Initially, miRNAs are transcribed as pri-miRNAs by RNA polymerase II or III. Thereafter, pri-miRNAs are cleaved by the RNase II enzyme Drosha, resulting in a pre-miRNA of around 70 nucleotides in length. The pre-miRNA is thereafter transported to the cytosol via the transport protein Exportin where it undergoes the second cleavage by the RNase Dicer. This final cleavage results in the mature miRNA and its antisense strand. Mature miRNAs will together with Argonaute (Ago) proteins form the RNA-induced silencing complex (RISC), through which it silences target mRNAs (191) (Fig. 7). There have also been reports on other modes of gene repression, such as non-canonical binding of mRNA to 5'UTR and the coding

regions of mRNAs (192). Moreover, under certain conditions, miRNAs may also enhance gene expression (193-195). It is also worthwhile to mention that since miRNA function is depending on the coexpressed target mRNAs, the effects of the same miRNA can vary across cell and tissue types depending on the current transcriptome, resulting in even opposite effects in different organs (196).

It is estimated that more than 60% of human protein-coding genes have pairing to miRNAs, indicating that miRNAs have powerful regulatory roles in cellular processes and need to be tightly controlled (197). However, several miRNAs have recently been found to be differentially expressed, with a potential causative role, in diabetes and its related complications (31, 198-200). Moreover, miRNAs have been shown to be involved in each step of wound healing (201, 202). Therefore we have investigated the role of two miRNAs with crucial functions for normal wound healing in the pathogenesis of DFU.

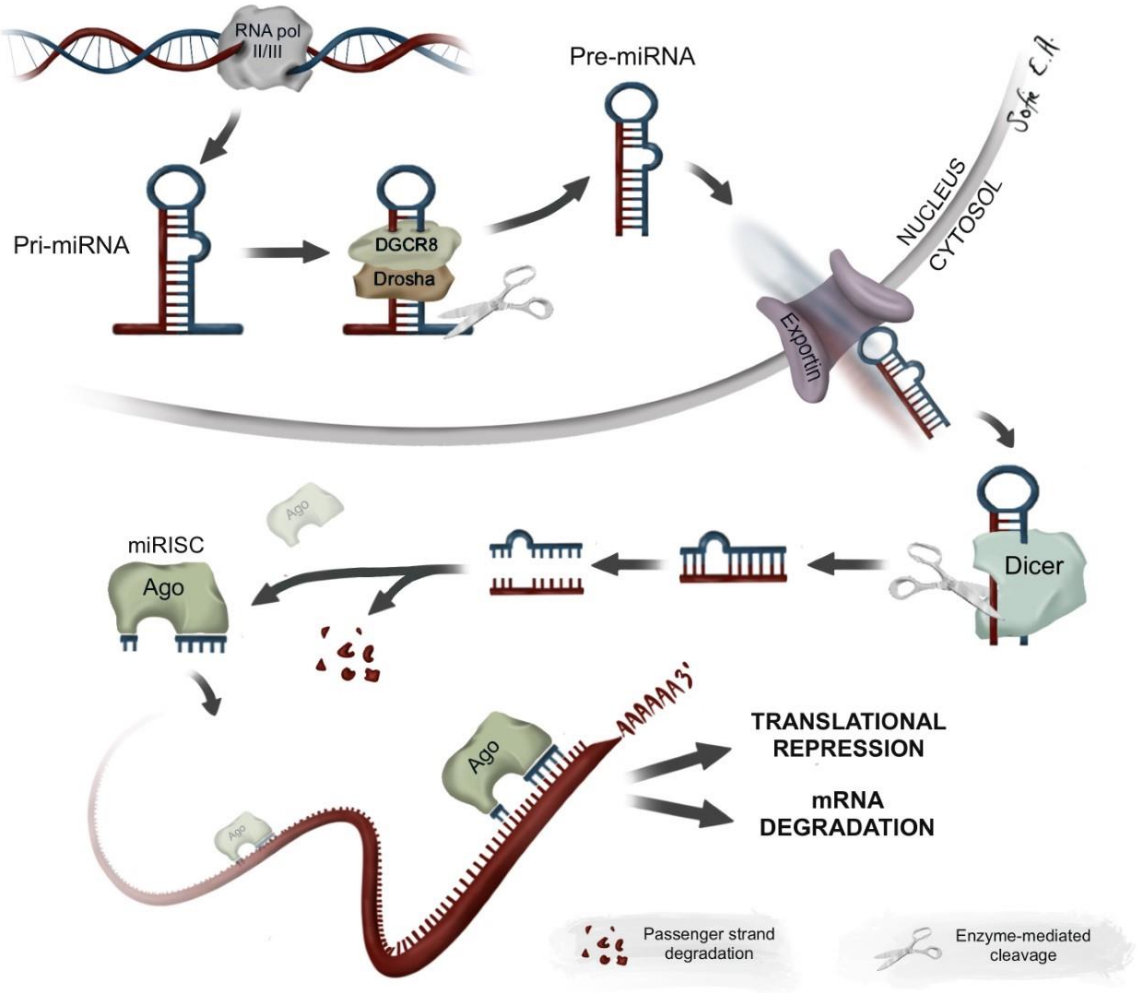

Fig. 7. miRNA biogenesis. A pri-miRNA transcript is produced after transcription of the miRNA gene by RNA polymerase II or III. The pri-miRNA transcript is then cleaved by Drosha and DGCR8 into pre-miRNA. The pre-miRNA is subsequently transported to the cytosol by Exportin and processed by Dicer to generate the mature miRNA. Mature miRNA binds to Ago protein and forms the miRISC. miRISC binds to complementary regions on mRNA which leads to translational repression or mRNA degradation. Ago, argonaute; DGCR8, DiGeorge Syndrome Critical Region 8; miRISC, miRNA-induced silencing complex; miRNA, microRNA.

1.5.2 miR-210

Over 50 hypoxia-responsive miRNAs (hypoxamiRs) have been characterized in the recent years, including miR-210. Of these miRNAs, miR-210 stands out as being the only miRNA that is consistently and strongly upregulated in basically all cell types in contrast to most hypoxamiRs that are more cell specific (203, 204). This suggests that miR-210 is the dominant responder to hypoxic stress (205). miR-210 contains an HRE on the proximal miR-210 promoter and is dependent on HIF-1α to be activated. The miR-210 promoter is to a large extent evolutionary conserved across species, which indicates the importance of hypoxia regulating miR-210 (184, 206). Furthermore, miR-210 is also involved in HIF-1 regulatory mechanisms, with evidence of both positive and negative feedback regulation depending on cellular context. This is probably important for the fine-tuning of the hypoxic response (207).

1.5.2.1 Functions of miR-210

As mentioned previously, HIF-1 is central in inducing a metabolic switch during hypoxic conditions (176). miR-210 reflects HIF-1 function by directly targeting the iron-sulfur cluster assembly enzymes (ISCU)-1 and ISCU2, which are essential for formation of iron-sulfur clusters. Iron-sulfur clusters are present in various enzymes in the TCA cycle and are also necessary for the function of the mitochondrial respiratory complexes in the electron transport chain (208, 209). Furthermore, miR-210 also directly targets several integral components of the mitochondrial electronic transport chain, such as nicotinamide adenine dinucleotide (NADH) dehydrogenase 1α subcomplex 4 (210) and D-subunit of succinate dehydrogenase (SDHD) (211). In line with this, miR-210 upregulates glycolysis in hypoxic conditions (210). Several reports have found that glycolysis promotes angiogenesis (212) and other cellular processes during tissue repair and regeneration (213, 214), indicating that miR-210 might have a vital role in wound healing. Moreover, it is known that in addition to energy metabolism, miR-210 also regulates the expression of several genes important for tissue regeneration such as cell proliferation, apoptosis and angiogenesis (205). miR-210 was found to modulate endothelial cell response (215) and promote angiogenesis in ischemic tissues (216, 217). miR-210 also stimulates migration and proliferation in both normal and transformed cells (215, 218-220).

Emerging evidence is now suggesting that miR-210 is dysregulated in diabetes. For example, AGE-induced repression of miR-210 has been shown to aggravate diabetic cardiomyopathy via mitochondrial dysfunction (221). In diabetic kidneys, miR-210 was indicated to contribute to DN by promoting aerobic glycolysis (222). miR-210 has also been shown to be differentially expressed in diabetic and control subjects (223). One study report increased miR-210 in the serum of T2D patients, which had inhibitory effects on the function and number of endothelial progenitor cells (224). In non-diabetic settings, miR-210 inhibits keratinocyte proliferation and thereby impairs wound healing (225), but its role in diabetic wound healing is less clear. Since miR-210 regulates several cellular functions important for wound healing and is dysregulated is diabetes, we aimed to investigate its role in DFU. This work is presented in paper III.

1.5.3 miR-34a

The miR-34 family is composed of three miRNAs; miR-34a, miR-34b and miR-34c. These miRNAs are clustered on two different chromosomes, where miR-34a is located on chromosome 1p36 and has its own primary transcript. miR-34a is the most prevalent miRNA from the miR-34 family, except in testis and lung where miR-34b/c are more abundant (226). There are 512 predicted target genes of miR-34a, whereof 109 have been validated experimentally (227).

miR-34a was originally identified in 2007 as a direct target of the tumor suppressor protein p53 via a p53 responsive element in its promotor (226). Oncogene stress, DNA damage or other stresses, such as hypoxia, are able to activate p53, which leads to up-regulation of miR-34a (228). Moreover, miR-34a forms a positive feedback-loop where it activates p53 via its negative regulation of sirtuin 1 (SIRT1) (229). Even though miR-34a expression seems to be mainly regulated by p53, it has been detected in conditions with inactivated p53 (230). This indicates the existence of p53-independent regulation of miR-34a. For example, other members of the p53-family such as p63 and p73 have also been shown to positively regulate miR-34a through the same p53-specfic binding region (229). Apart from the activation mediated by the p53-family, miR-34a can also be epigenetically regulated. miR-34a contains a prominent CpG island in the genomic region upstream of the p53 binding site in the promotor, which has been shown to be heavily methylated in several cancer forms, resulting a downregulation of miR-34a expression (231). Nuclear factor-κB (NF-κB) is another important transcription factor for miR-34a expression. It contains three conserved binding sites in the miR-34a promotor (119). miR-34a has been reported to be upregulated by hypoxia (232, 233), and has been found to be under direct influence of HIF-1α in one study (234). Furthermore, various other factors, such as ELK-1 (235), IL-6-induced STAT3 (236), SNAIL (237) and C/EBPα (238), have also been shown to modulate miR-34a expression.

1.5.3.1 Functions of miR-34a

miR-34a functions have mostly been characterized in the field of cancer where it has emerged as a tumor suppressor. Overexpression of miR-34a induces apoptosis, cell-cycle arrest and cellular senescence, and miR-34a has been suggested to be a strong mediator of tumor suppression by p53 (226). miR-34a targets various mRNAs with functions in DNA damage response and cell-cycle control, such as Cyclin E2, MET, CDK4/6 and Bcl-2 (239). Moreover, miR-34a has been reported to be involved in the control of epithelial-to-mesenchymal transition (EMT) (234), proliferation (240), differentiation (241), angiogenesis (242) and autophagy (243). Consistent with miR-34a's reported anti-oncogenic effects, miR-34a has been reported to be downregulated in a majority of cancers (226). miR-34a was the first miRNA to reach clinical studies and was used in a phase 1 study for various solid tumors. However, the study was terminated in advance due to severe immune-related events (134).

Apart from its role in cancer, miR-34a has also been discovered to be involved in various other diseases processes. For example, miR-34a has been implicated to play a role in cardiovascular disease (228), neuropsychiatric and cognitive disorders (244, 245), kidney disease (30) and metabolic liver disease (246).

miR-34a has recently been found to be dysregulated in diabetes, although the picture is unclear and contradictory reports exist even from within the same organ. For example, it has been shown to be repressed in whole tissue lysate from kidneys affected by DN (30), but upregulated in glomeruli from diabetic mice (247). The role of miR-34a in DFU has not been studied. In normal wound healing, the results are opposing. miR-34a inhibition has been shown to delay wound healing through enhanced inflammation mediated via IL-6/STAT3 activation (248). However, in another study, miR-34a overexpression increased inflammation through LGR4, resulting in prolonged wound healing (249). Since miR-34a controls various cellular processes important for wound healing, such as proliferation, differentiation and migration (226), we decided to investigate its role in diabetic wound healing. Our work is presented in paper IV in this thesis.

2 RESEARCH AIMS

The overall aim of this thesis work has been to investigate the dysregulated cellular response to hypoxia as a cause for chronic microvascular complications in diabetes. The ultimate goal has been to identify new potential therapeutic targets for tomorrow's drug therapies, since we today lack efficient treatment due to an incomplete understanding of the underlying pathogenic mechanisms activated in diabetes complications.

To this end, we have had four specific aims:

I. To investigate the role of the *HIF-1A* Pro582Ser polymorphism for the development of diabetic retinopathy

II. To investigate how the dysregulated HIF-1 signaling interacts with oxidative stress in the development of diabetic nephropathy

III. To investigate miR-210 and cellular metabolism in diabetic wound healing

IV. To investigate the regulation and function of miR-34a in diabetic wound healing

3 MATERIALS AND METHODS

3.1 HUMAN STUDIES

3.1.1 Genetic association study

Paper I. All patients (*n* =1492) with T1D at the endocrinology department at Karolinska University Hospital from October 2011 to May 2014 were invited to participate in the study. No exclusion criteria were used. 703 patients chose to participate in the genetic analysis and left blood samples. The genetic analysis of the polymorphism was done using TaqMan Allelic Discrimination assay on the ABI 7300 system (Applied Biosystems). Blood samples for HbA1c, total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglycerides were analyzed at the routine hospital laboratory. All subjects were also examined by an ophthalmologist at St. Erik Eye Hospital where the severity of DR was determined.

3.1.2 Human skin and wound biopsies

Paper III, IV. 9 patients with DFU (age: 69.8 ± 3.2 years old; HbA1c: 60.13 ± 4.45mmol/mol), 13 age-matched normoglycemic control subjects (age: 68.1 ± 2.7 years old; HbA1c: 39.62 ± 0.84 mmol/mol) and 8 patients with venous ulcer (VU) (age: 81.0 ± 9.7 years old) were recruited at the endocrinology and dermatology departments at Karolinska University Hospital after having given their informed consent. Biopsies from the control skin and the edge of the DFU and venous ulcers (VU) were taken using a 4 mm biopsy punch after local anesthesia. The biopsies were stored in either liquid nitrogen or RNAlater^{TM} (ThermoFisher Scientific) before being analyzed in experiments.

3.1.3 Intermittent hypoxia

Paper II. 13 patients with T1D 28.9 ± 7.2 years old; 53.8% male and 46.2% female; HbA1c: 74.4 ± 11.8 mmol/mol (9.0 ± 1.1 %); BMI: 24.3 ± 4.0 kg/m^2) and 11 healthy, age-matched controls (30.5 ± 8.5 years old; 54.5 % male and 45.5% female; HbA1c: 35.6 ± 2.6 mmol/mol (5.4 ± 0.2 %); BMI: 24.3 ± 4.0 kg/m^2) participated in the study. Diabetes duration was 10-20 years and the patients and had not developed any diabetes complications. They were exposed to intermittent hypoxia for 1 hour by breathing air with 13% O_2 for 6 minutes and 21% O_2 for another 6 minutes, which was repeated 5 times. Blood samples were taken before and immediately after the intermittent hypoxia for analysis of ROS levels.

3.2 ANIMAL STUDIES

3.2.1 Mice models

Paper II-IV. BKS(D)-Leprdb/JOrIRj (db/db) mice and their corresponding normoglycemic control mice were purchased from Janvier Labs. Db/db mice with HbA1c levels >55 mmol/mol or blood glucose >15 mM when HbA1c levels were between 45 and 55 mmol/mol were included in the analysis. Mice were allocated into groups according to their age, HbA1c

or blood glucose levels. The mice were housed 5-8 per cage in a 12-hour light/dark cycle at 22°C. They were provided standard laboratory food and water ad libitum.

In paper II, the mice received intraperitoneal injection (i.p.) DMOG to analyze mitochondrial function. PHD2-haploinsufficient (PHD2+/-) mice and their wild-type (WT) littermates were generated as previously described (250) and made diabetic using streptozotocin (STZ) i.p. injections. They received STZ injections at 50 mg/kg body weight one time per day for 5 days and were diabetic for at least 6 weeks before sacrifice. Urine was collected from mice bladders after sacrifice and stored in liquid nitrogen. The urine was analyzed for amount of albumin and creatinine using a DCA Vantage Analyzer (Siemens Healthcare GmbH) with the corresponding test cartridges DCA Microalbumin/Creatinine ACR urine test (01443699, Siemens Healthcare GmbH).

3.2.2 Wound model

Paper III-IV. The mice were wounded after anesthesia, shaving and thorough cleaning with 70% ethanol. Two full-thickness wounds on the mice backs extending through the panniculus carnosus were created on either side of the dorsal midline using a 6-mm biopsy punch. miRNA mimic or negative control mimic were injected intra-dermally at four different places in the wound edges on each wound on the day of wounding as well as after 6 days post-surgery. The wounds were photographed on the day of wounding and then every other day. A circle of known area was included in each picture and placed next to the wounds to correct the measurement for distance between the camera and the wound. The area of the wound was then evaluated blindly using ImageJ software, version 1.32 (N.I.H., USA). The animals were euthanized on day 8 after wounding and the wounds were harvested and placed in RNAlater™ (ThermoFisher Scientific) or frozen in liquid nitrogen.

3.3 CELL CULTURE WORK

3.3.1 Primary cells and cell lines

Paper I-IV. Primary human keratinocytes and human dermal microvascular endothelial cells (HDMECs) (PromoCell) were cultured in their special medium provided by PromoCell. Human embryonic kidney 293A (HEK293A) cells were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and F-12 medium supplemented with FCS (10%), penicillin (50 IU/mL), and streptomycin sulfate (50 mg/mL) from Invitrogen. Primary human dermal fibroblasts (HDFs, ATCC), mouse inner medullary collecting duct-3 (mIMCD-3) cells (ATCC) and the macrophage cell line RAW 264.7 (ATCC) were cultured in DMEM supplemented with 100 IU/mL penicillin and streptomycin (ThermoFisher Scientific) and 10% heat-inactivated FBS (Invitrogen). Passages 4–9 of the primary cells were used for experiments. All of the cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C in a cell culture incubator. Cells were cultured under normoxic [21% (vol/vol) O2] or hypoxic (1% O_2) conditions in Hypoxia Workstation Invivo$_2$ (MedicalExpo).

3.3.2 Plasmid constructs

Paper I, II, IV. Plasmids with FLAG-fused HIF-1α with P402A/P564A mutations were further modified, generating the mutation P583S, with the QuickChange site-directed mutagenesis kit (Stratagene). DYEnamic sequencing kit (Amersham Biosciences Corp.) was used to screen for positive mutants. The Renilla luciferase reporter was obtained from Promega Corp. The plasmids with HRE-driven luciferase reporter, GFP and GFP-HIF-1α have been described previously (251). Plasmids encoding the miR-34a promoter with wild-type (P1) or mutated (M53) p53-binding site coupled to luciferase reporters were obtained from Addgene.

3.3.3 RNA interference and miRNA overexpression

Paper II-IV. siRNA directed towards mouse VHL (Flexitube Gene Solution GS22346) was purchased from Qiagen and used together with AllStars negative control siRNA (Ambion). Custom stabilized miRIDIAN mmu-microRNA-210-3p mimic (C-310570-5) and mmu-microRNA-34a-5p mimic (C-310529-07) were used together with negative control mimic #1 (CN-001000-01), all purchased from Dharmacon.

3.3.4 Transient transfection

Paper I-IV. Transient transfection was performed using Lipofectamine reagent (Invitrogen) and Lipofectamine RNAiMAX (Life Technologies) according to the instructions of the company. After 16-24 hours, the cells were exposed to different conditions (5.5 or 30 mM glucose in 1% O_2 or 21% O_2) for 24-48 hours before being harvested and further analyzed.

3.4 IN VITRO ASSAYS

3.4.1 Dual-luciferase reporter assay

Paper I, II, IV. After harvest, luciferase activity was measured using the Dual Luciferase Assay System (Promega) on the GloMax Luminometer (Promega) according to the manufacturer's instructions. The firefly luciferase activity (which was coupled to HRE or miR-34a promoter) was normalized to Renilla luciferase activity and expressed as relative luciferase activity. In paper I, the HRE-driven luciferase reporter gene was coexpressed together with *HIF-1A* (P402A/P564A) or *HIF-1A* (P402A/P564A/P583S).

3.4.2 Migration assay

Paper III. Wound healing was studied *in vitro* using the migration assay. Collagen coating (50 µg/uL) was applied to 24-well plates overnight. Plates were thereafter blocked with 3% BSA for 2 hours before seeding with HDFs. Cells were transfected with miR-210 mimic or negative control as described above when they reached 90% confluency. A scratch was made in each well using a micropipette tip after 24 hours and the cells were treated with 5.5 mM or 30 mM glucose and maintained in 1% O_2 or 21% O_2 for another 16 hours. Triplicates were used for each condition. Mitomycin C (10 µg/ml) was used in the medium to prevent cell proliferation. Photos were taken immediately after the scratch had been done and 16 hours

later using the EVOS XL Core Cell Imaging System (ThermoFisher Scientific). ImajgeJ v1.47 software (N.I.H., Bethesda, MD, USA) was used to quantify the migration.

3.4.3 Cellular apoptosis analysis

Paper II. The degree of apoptosis mIMCD-3 cells following exposure to normoglycemia/hyperglycemia and normoxia/hypoxia was assessed using Annexin V-FITC / 7-AAD kit (Beckman Coulter). The cells were treated with Annexcin V-FITC and 7-AAD for 15 minutes in the dark. Thereafter, they were analyzed using flow cytometry on a CyanTM ADP analyser (Beckman Coulter). The results were calculated as the percentage of Annexin V – positive and 7-AAD – negative apoptotic cells.

3.5 LABORATORY METHODS

3.5.1 RNA purification and analysis of gene expression

Paper II-IV. Total RNA, including microRNAs, was extracted from cells and from tissues using a miRNeasy RNA extraction kit (Qiagen). cDNA was produced using High-Capacity cDNA Reverse Transcription Kit to detect mRNA and pri-miRNA expression whereas TaqMan microRNA Reverse Transcription kit and TaqMan Advanced miRNA cDNA Synthesis kit were used to detect miRNA expression. All cDNA synthesis kits were purchased from ThermoFisher Scientific. Quantitative RT-PCR was performed on a 7300/7900 Real-Time PCR System (Applied Biosystems) or QuantStudio 6/7 Flex Real-Time PCR System (Applied Biosystems) using SYBR Green Master Mix or Taqman Universal Master Mix II (with UNG) (ThermoFisher Scientific). Actin, PBGD and HMBS were used as internal controls for mRNA or pri-miRNA expression while snoRNA55, miR-16, miR-23a, miR-24 and miR-191 were used as internal controls for miRNA detection. The gene expression was calculated using either the standard curve method or delta-delta Ct methods.

3.5.2 Protein extraction and western blotting

Paper I, II. Cells and kidney biopsies were homogenized and lysed using a buffer with 50 mM Tris-HCl (pH 7.4), 180 mM NaCl, 0.2% NP-40, 20% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 5 mM β-mercaptoethanol, and a protease inhibitor mix (Complete-Mini; Roche Biochemicals). Bradford Protein Assay (Bio-Rad) was used to determine protein concentration. The lysates were separated by SDS-PAGE and blotted onto nitrocellulose membranes. TBS buffer (50 mM Tris pH 7.4 and 150 mM NaCl) containing 5% milk was used to block the membranes before incubation with primary antibodies in TBS buffer containing 1% milk. In paper II, anti-HIF-1α (1:500, NB100-479; Novus Biologicals), anti-Histone H3 (1:5000, ab1791; Abcam), anti-KIM-1 (1:500, NBP1-76701; Novus Biologicals) and anti-α-tubulin (1:1000, MAB11106; Abnova) were used as primary antibodies followed by incubation with IRDye 800 goat anti-rabbit or IRDye 680 goat anti-mouse secondary antibodies (LI-COR). The membranes were then scanned with Odyssey Clx Imaging System (LI-COR). In paper I, anti-FLAG M2 (F3165, Sigma-Aldrich) or anti-β-

actin (ab6276, Abcam) antibodies were used followed by incubation with anti-mouse or anti-rabbit IgG-horseradish peroxidase conjugate (Amersham Biosciences Corp.). The membranes were then visualized using enhanced chemiluminescence (Amersham Biosciences Corp.).

3.5.3 Nuclear extraction

Paper II. After harvest, the cells were incubated on ice for 10 minutes in hypotonic buffer with 10 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM PMSF, 0.5 mM dithiothreitol, and protease inhibitor mix (Complete-Mini; Roche Biochemicals). Once the cells had swollen, the nuclei were released using a Dounce homogenizer. The nuclei were centrifuged down and resuspended using a buffer with 20 mM Tris (pH 7.4), 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 0.02 M KCl. Soluble nuclear proteins were then released from the nuclei by addition of a buffer with 20 mM Tris (pH 7.4), 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 0.6 M KCl. The nuclear extracts were thereafter centrifuged and dialyzed in a dialysis buffer with 20 mM Tris (pH 7.4), 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM dithiothreitol, and protease inhibitor mix. The resulting nuclear extracts were then processed as the cell lysates described above.

3.5.4 Histology

Paper III. Formalin-fixed and paraffin-embedded (FFPE) sections from wounds were used for histology. The sections were deparaffinized and rehydrated. Thereafter, staining was done with hematoxylin and eosin. Image analysis and quantification of the granulation area were done using Image Pro Premier software v9.2 (Media Cybernetics). A minimum of 2 images per slide were analyzed and there were 3-5 slides for each condition.

After deparaffinization and rehydration of another set of FFPE sections, Masson-Goldner Trichrome staining (Merck Millipore) was used to quantify the amount of collagen in the wounds. Leica DM3000 LED fluorescence microscope was used for image acquisition and quantification was done using Image Pro Premier software v9.2 (Media Cybernetics).

3.5.5 TUNEL staining

Paper II. In Situ Cell Death Detection Kit (Sigma Aldrich/Roche) was used to evaluate apoptosis in kidneys. FFPE sections were deparaffinized, rehydrated and blocked with 3% H_2O_2 to quench endogenous peroxidase activity. The sections were permeabilized with 0.1% Triton X-100, 0.1% sodium citrate solution and blocked with 3% BSA in PBS. TUNEL mixture was applied to the sections for one hour at 37°C before the sections were treated with 0.1% Sudan Black-B solution to quench autofluorescence. The sections were thereafter counterstained with DAPI before mounting. Leica TCS SP8 confocal microscope (Leica Microsystems) was used to acquire images. Image analysis was done with the Image-Pro Premier v9.2 software (Media Cybernetics).

3.5.6 Fluorescent immunohistochemistry

Paper II, III. FFPE sections were deparaffinized and rehydrated. Antigen retrieval was done in a microwave (800W for 10 minutes) using citrate buffer. After blocking the sections were blocked with either goat serum or 5% BSA in PBS. Incubation with primary antibodies was done overnight at 4˚C before incubation with a fluorochrome-conjugated secondary antibody for 1 hour in room temperature. The sections were treated with 0.1% Sudan Black-B solution (Sigma) for 10 minutes to reduce the autofluorescence followed by counterstaining with DAPI for 3 minutes before mounting. Image acquisition was done using Leica TCS SP5 and SP8 confocal microscope (Leica Microsystems). The images were analyzed with Image-Pro Premier v9.2 (Media Cybernetics) and ImageJ v1.47 software.

In paper III, pimonidazole solution (Hypoxyprobe™-1 Omni Kit, Hypoxyprobe, Inc.) was i.p. administered to mice at a dosage of 60 mg/kg body weight 90 minutes before harvest to enable detection of hypoxia in kidneys. A RED PE dye-conjugated antibody directed towards the pimonidazole adducts was then used on the kidney sections according to the Hypoxyprobe™ RED PE Kit protocol.

3.5.7 In situ hybridization

Paper III. To detect miR-210, Exiqon miRCURY locked nucleic acid (LNA)-DIG labelled probe was applied to the FFPE as previously described, with some alterations (252). FFPE sections were deparaffinized and the RNAs were demasked using 15 µg/mL proteinase K treatment for 10 minutes at 37°C. The probes were hybridized with Mmu-miR-210-3p specific LNA-probes (Exiqon) at a concentration of 250 nM for 2 hours at 53°C. Thereafter the sections were washed with a series of Saline-Sodium Citrate buffer with decreasing concentrations, followed by incubation with an alkaline phosphatase (AP)-conjugated antibody specific to DIG (Roche). miR-210 was then visualized by adding an AP substrate, NBT/BCIP (ThermoFisher). Slides were counterstained using nuclear fast red stain (Vectorlabs).

3.5.8 Cellular respirometry

Paper II. Mitochondrial function in mice kidneys was assessed using high resolution respirometry (Oxygraph 2k, Oroboros) as previously described (253). Respirometry was performed in respiration medium containing EGTA (0.5 mM), MgCl2 (3 mM), K-lactobionate (60 mM), taurine (20 mM), KH_2PO_4 (10 mM), HEPES (20 mM), sucrose (110 mM) and fatty acid-free BSA (1 g/L). Pyruvate (5 mM) and malate (2 mM) were added to measure state 2 respiration, followed by the addition of ADP (2.5 mM) to measure complex I mediated maximal respiratory capacity (state 3 respiration). Complex I + II-mediated maximal oxidative phosphorylation was evaluated after adding succinate (10 mM). Leak respiration was measured in the presence of pyruvate (5 mM), malate (2 mM) and oligomycin (2.5 µM). Mitochondrial protein content was used for normalization, which was measured using the DC Protein Assay kit (Bio-Rad).

Paper III. Seahorse XF Analyzer (Agilent Technologies) was used to determine basal oxygen consumption rate (OCR), extracellular acidification rate (ECAR) and ATP production rate, using the XF Cell Mito Stress Test kit, XF Glycolytic Rate Assay kit and XF Real-Time ATP Rate Assay kit. The sensor cartridges used for measuring the oxygen flux were equilibrated in an XF Calibrant for 16-24 hours before the experiment in an incubator with 0% CO_2. When analyzing tissues, the wounds were carefully dissected and rinsed before being put at the bottom of an XF24 Islet Capture Microplate and covered with mesh. When cells were used, they were seeded into an XF24 or XFe96 Cell Culture Microplate. The plates were then run according to optimized protocols. The results were normalized using protein concentration or cell numbers.

3.5.9 ATP levels in tissue

Paper III. ATP detection assay kit (Cayman Chemical) was used to determine ATP levels in wounds. The tissues were lysed using the ATP detection sample buffer. The lysate was thereafter incubated with a reaction mix containing ATP detection assay buffer, D-luciferin and luciferase. After 20 minutes, luminescence was measured in a Glomax Luminometer (Promega). ATP concentration was determined using a standard curve method and normalized to protein concentrations.

3.5.10 Lactate assay

Paper III. Lactate Colorimetric Assay kit (Biovision) was used to quantify lactate production. homogenized in lactate assay buffer provided in the kit and lactate levels were measured and normalized to protein levels.

3.5.11 ROS detection

3.5.11.1 Electron Paramagnetic Resonance

Paper II, III. Electron Paramagnetic Resonance (EPR) Spectroscopy (254) was used to measure ROS levels in blood and cells.

In paper II, blood samples were mixed with spin probe 1-hydroxy-3-carboxy-pyrrolidine (CPH, 200 µM) in EPR-grade Krebs HEPES buffer supplemented with 25 mM Deferoxamine (DFX) and 5 mM diethyldithiocarbamate (DETC), and incubated at 37°C for 30 minutes before being frozen in liquid nitrogen. EPR measurements were carried out using a table-top EPR spectrometer (Noxygen Science Transfer & Diagnostics GmbH). All data were converted to absolute concentration levels of CP radical (mmol O_2^-/min/µg) using the standard curve method. All chemicals and reagents for EPR Spectroscopy were obtained from Noxygen Science Transfer & Diagnostics GmbH.

In paper III, cells were treated according to the experimental design. At the end of the experiment, the media was removed and cells were washed with PBS and incubated with 1-hydroxy-3- methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) buffer for 30 minutes. After incubation, cells were collected in CMH buffer and frozen in liquid nitrogen prior to

measurement. The ROS levels were measured using a CMH spin probe (200 µM). The rest of the procedure was as outlined above.

3.5.11.2 4-Hydroxynonenal measurement

Paper II, III. 4-Hydroxynonenal (4-HNE) protein adduct levels were measured in frozen kidney and wound lysates using commercially available kits (OxiSelect STA-838 and STA-310, Cell Biolabs, San Diego, CA, USA) according to the manufacturer's instructions.

3.5.11.3 Flow cytometry

Paper II. mIMCD-3 cells were stained with MitoSOX™ Red Mitochondrial Superoxide Indicator (Thermo Fisher Scientific). A working concentration of 5µM was used, and cells were incubated at 37°C for 10 minutes protected from light. Cells were thereafter trypsinized and resuspended in Krebs HEPES buffer. The analysis was done with flow cytometry on a CyanTM ADP analyzer (Beckman Coulter) using FlowJo software. Mitochondrial ROS levels were expressed as percentage of MitoSOX Red fluorescence intensity.

3.6 STATISTICAL ANALYSIS

Paper I. Kruskal-Wallis test was used to test if there were differences between the DR groups. Univariate logistic regression analyses were done with DR as the dependent variable and a number of demographic and disease variables were used as independent variables. The results from the univariate logistic regression and correlation analyses (Pearson's and Spearman's correlations) were used to select variables for the multivariate logistic regression analyses, which was performed with the Enter method. Tests for linearity, interactions and goodness of fit were done. $P<0.05$ was considered as statistically significant. All statistical analyses were done with SPSS IBM Statistics 24.

Paper II-IV. Kolmogorov-Smirnov test was used to analyze normality of distribution. If normally distributed, differences between groups were analyzed using Student's t-test, one-way or two-way ANOVA followed by Bonferroni's post hoc test. If not normally distributed, statistics were analyzed using Mann-Whitney U test and Kruskal Wallis test as appropiate. Outliers identified using Grubbs' test were excluded from analysis. All the *in vitro* experiments were performed for a minimum of three times. $P<0.05$ was considered as statistically significant. The data is presented as mean ± standard error of the mean (SEM). All statistical analysis was made in GraphPad Prism software.

3.7 ETHICAL CONSIDERATIONS

The experimental procedure in animals was approved by the North Stockholm Ethical Committee for the Care and Use of Laboratory Animals. The necessity and the number of animals that were used have been carefully considered, where the advantages of the outcomes of the experiment were balanced against the suffering of the animals. We have strictly followed the three Rs (reduction, replacement and refinement). The studies using human material was reviewed and approved by the Regional Ethical Committee of Stockholm.

Written informed consent was received from participants prior to inclusion in the study. All the human materials have been handled and stored according to biobank regulations. The studies were conducted according to the Declaration of Helsinki's principles.

4 RESULTS

4.1 PAPER I – THE ROLE OF HIF-1A IN DIABETIC RETINOPATHY

Previous work by our group established HIF-1α as pathologically downregulated in diabetic wounds (134), which had functional relevance since activation of HIF-1α resulted in improved wound healing in diabetic mice (126). Interestingly, our group also found that a *HIF-1A* polymorphism (*HIF-1A* Pro582Ser) that was more resistant to the repressive effect of hyperglycemia conferred protection against DN (131). This polymorphism was also found by others to protect against DFU (255). However, the role of this polymorphism had not been studied in the setting of DR. We therefore decided to perform a genetic association study, where we analyzed whether the *HIF-1A* Pro582Ser polymorphism affected the risk of developing DR.

4.1.1 Clinical characteristics

In total 703 patients accepted to be included in the study, whereof 148 (21%) had no DR, 373 (53%) had mild or moderate NPDR and 182 (26%) had severe NPDR and PDR. Different disease variables and demographic factors are shown in table 1. When the patients were grouped based on their polymorphism status (CC – no polymorphism; CT – heterozygous for the polymorphism; TT – homozygous for the polymorphism), the incidence of different stages of DR varied, so that the genotype TT had the lowest incidence of severe DR. Moreover, there were no significant differences in traditional factors known to increase the risk for DR (Table 2). Encouraged by these observations we moved on to the logistic regression analysis.

Table 1. Clinical characteristics.

	Patients with no DR (n = 148)	Patients with mild-moderate NPDR (n = 373)	Patients with severe NPDR/PDR (n = 182)	P value
Women/men (n)	68/80	156/217	81/101	0.65
Ethnic origin, Caucasian vs. other (n), (%)	145 (98.0)	370 (99.2)	179 (98.3)	0.47
Age (years)	44.9 ± 1.3 (19-86)	46.2 ± 0.8 (20-86)	52.8 ± 1.0 (25-86)	<0.001
BMI (kg/m^2)	25.8 ± 0.4 (15.5-40.0)	25.6 ± 0.2 (16.4-45.3)	25.8 ± 0.3 (18.3-42.5)	0.55
Diabetes duration (years)	21.2 ± 1.0 (3-66)	27.1 ± 0.6 (8-73)	36.1 ± 0.9 (12-69)	<0.001
HbA1c (%)	7.7 ± 0.09 (5.5-12.5)	8.1 ± 0.0.5 (5.6-12.1)	8.6 ± 0.1 (4.7-12.7)	<0.001
HbA1c (mmol/mol)	61.1 ± 1.0 (37-113)	65.4 ± 0.6 (38-109)	70.7 ± 1.1 (28-115)	
e-GFR (mL/min/1.73 m^2)	97.4 ± 1.6 (23.6-133)	96.9 ± 1.1 (18-140)	83.4 ± 1.7 (7.2-140)	<0.001
TG (mmol/L)	0.9 ± 0.06 (0.18-7.7)	0.9 ± 0.0 (0.2-5.5)	1.1 ± 0.1 (0.3-6.7)	<0.001
Cholesterol (mmol/L)	4.8 ± 0.07 (2.7-8.4)	4.7 ± 0.0 (2.0-7.7)	4.7 ± 0.1 (2.6-8)	0.45
LDL (mmol/L)	2.7 ± 0.06 (1.1-5.2)	2.7 ± 0.0 (1.1-5.4)	2.7 ± 0.1 (1.0-5.8)	0.38
HDL (mmol/L)	1.7 ± 0.04 (0.8-3.8)	1.6 ± 0.0 (0.6-4.2)	1.6 ± 0.0 (0.5-3.7)	0.032
Systolic blood pressure (mmHg)	125.9 ± 1.2 (90-170)	127.2 ± 0.8 (85-180)	132.6 ± 1.2 (90-180)	<0.001
Diastolic blood pressure (mmHg)	73.2 ± 0.7 (50-95)	74.2 ± 0.5 (40-100)	72.2 ± 0.8 (40-100)	0.083

Data are presented as mean ± SEM (range). The differences between the three groups were tested using the Kruskal-Wallis test. DR, diabetic retinopathy; NPDR, nonproliferative diabetic retinopathy; PDR, proliferative diabetic retinopathy; BMI, body mass index; HbA1c, glycated hemoglobin; e-GFR, estimated glomerular filtration rate; TG, triglycerides; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

Table 2. Clinical characteristics of patients with different *HIF-1A* variants.

	Total	CC	CT	TT	*P* value
N	703	591	87	25	
Women/men (*n*)	305/398	260/331	34/53	11/14	0.688
Age (years)	47.6 ± 0.6	48.8 ± 0.6	47.0 ± 1.8	45.7 ± 2.3	0.586
BMI (kg/m^2)	25.5 ± 0.1	25.5 ± 0.2	25.8 ± 0.5	25.6 ± 0.9	0.752
Diabetes duration (years)	28.2 ± 0.5	28.2 ± 0.5	28.1 ± 1.4	26.8 ± 3.0	0.638
HbA1c (mmol/mol)	65.9 ± 0.5	65.8 ± 0.5	65.7 ± 1.4	66.8 ± 3.3	0.895
e-GFR (mL/min/1.73 m^2)	92.6 ± 0.9	93.9 ± 0.9	90.9 ± 2.5	92.5 ± 5.3	0.376
Height (cm)	174.0 ± 0.4	173.9 ± 0.4	174.7 ± 1.1	175.6 ± 2.4	0.762
Systolic blood pressure (mmHg)	128.3 ± 0.6	128.2 ± 0.6	128.9 ± 1.7	130.1 ± 3.5	0.898
Diastolic blood pressure (mmHg)	73.4 ± 0.4	73.4 ± 0.4	73.3 ± 1.0	74.2 ± 1.8	0.828
TG (mg/dL)	0.92 ± 0.03	0.94 ± 0.03	0.8 ± 0.04	0.9 ± 0.08	0.730
Cholesterol (mmol/L)	4.7 ± 0.03	4.7 ± 0.04	4.7 ± 0.09	4.8 ± 0.2	0.966
LDL (mmol/L)	2.7 ± 0.03	2.7 ± 0.03	2.7 ± 0.07	2.8 ± 0.1	0.252
HDL (mmol/L)	1.6 ± 0.02	1.6 ± 0.02	1.6 ± 0.06	1.5 ± 0.09	0.242
Smoking (*n*)	85 (12%)	74 (13%)	9 (10%)	2 (8%)	0.689
Antihypertensive treatment (*n*)	321 (46%)	276 (47%)	34 (39%)	11 (44%)	0.511

Data are shown as mean ± SEM. The differences between the three groups were tested using the Kruskal-Wallis test. CC, CT, and TT are the genotypes of the *HIF-1A* Pro582Ser polymorphism. ns, nonsignificant; BMI, body mass index; HbA1c, glycated hemoglobin; e-GFR, estimated glomerular filtration rate; TG, triglycerides; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

4.1.2 The *HIF-1A* Pro582Ser polymorphism reduces the risk of severe DR

We first performed a univariate analysis where we found that the TT genotype reduced the risk with 84% (OR = 0.16, 95% CI: 0.03-0.76) of developing severe NPDR/PDR. However, other factors also influenced the risk of developing severe NPDR/PDR such as age, diabetes duration, HbA1c, systolic blood pressure, triglycerides and HDL. We therefore corrected for these factors in a multivariate analysis. Interestingly, the risk reduction mediated by the TT genotype became even more pronounced and increased to 95% (OR = 0.05, 95% CI: 0.003-0.91). There was no significant association between the TT genotype and the risk for mild-moderate NPDR.

4.1.3 Biological effects of the *HIF-1A* Pro582Ser polymorphism

Given the pronounced effect of the *HIF-1A* Pro582Ser polymorphism on the risk of developing severe DR we next set out to investigate the mechanisms behind this finding. Previous work by our group has shown that the HIF-1α Pro582Ser polymorphism has a reduced protein stability in hyperglycemia (131). We decided to further investigate this and therefore generated two plasmids encoding mutated *HIF-1A.* The first plasmid had mutated *HIF-1A* at position 402 and 564, where prolines were exchanged for alanines (*HIF-1A* P402A/P564A). The prolines 402 and 564 are responsible for the canonical hydroxylation mediated by PHDs, which have a major role in the oxygen-dependent degradation of HIF-1α (124). These mutations thus make HIF-1α inaccessible to PHDs. The second plasmid that we generated also had the current polymorphism, in addition to the two proline mutations above (HIF-1α P402A/P564A/P582S). The comparison of these plasmid constructs thus enabled us to investigate whether the *HIF-1A* Pro582Ser polymorphism has a separate role on the non-canonical regulation of HIF-1 stability in hyperglycemia. HEK293A cells were transfected with these plasmid constructs, which were equally expressed in normoxic and

normoglycemic conditions. The HIF-1α P402A/P564A/P582S was still sensitive to the hyperglycemia-induced destabilization of HIF-1α in hypoxia. We further analyzed this polymorphism on the transactivation level using a dual luciferase reporter assay where the plasmids were cotransfected with an HRE reporter gene. We found that the HIF-1α P402A/P564A/P582S conferred an increased transcriptional activity, despite a preserved sensitivity to the destabilizing effect of hyperglycemia on the protein level.

In summary, we have found that the *HIF-1A* Pro582Ser polymorphism, encoding a HIF-1α more resistant to the repressive effect of hyperglycemia, protects against development of severe DR. This protective effect is independent of other traditional risk factors for DR. Moreover, we bring further insight into how this specific polymorphism increases HIF-1α function, which is through an increased transactivation activity.

4.2 PAPER II – HIF-1A, ROS AND DIABETIC NEPHROPATHY

The repression of HIF-1α in hyperglycemia has been reported to be involved in the pathogenesis of DFU (113), DN (79) and now also DR, as shown by the previous study. However, the mechanisms behind the detrimental effects of impaired HIF-1 signaling in DN remain to be elucidated. It is known that oxidative stress plays important roles in the development of DN (256), and among its many functions HIF-1α also protects the cells against oxidative stress-induced damage by reducing ROS production in hypoxia (166). We thus hypothesized that the reduced HIF-1α signaling in DN might mediate its detrimental effect via an impaired regulation of ROS production. We decided to investigate this hypothesis in the setting of DN.

4.2.1 Repression of HIF-1 contributes to increased ROS production

As an *in vitro* model of DN, we used mouse inner medulla collecting tubular cells (mIMCD-3). We found that HIF-1α protein stability and function were repressed by hyperglycemia in hypoxic conditions, which could be rescued by inhibition of PHDs using dimethyloxalylglycine (DMOG). This was also seen *in vivo*, where HIF-1α was repressed in kidneys from a mouse model with type 2 diabetes (the db/db mouse) and streptozotocin (STZ)-induced T1D mice. Restoration of HIF-1α function by using either DMOG or genetically modifying PHD2 function using PHD2-haploinsufficient ($PHD2^{+/-}$) mice resulted in increased HIF-1α function. ROS levels were found to be increased in hypoxic and hyperglycemic conditions both *in vitro* and *in vivo*, which correlated with the decreased HIF-1α function.

Having established the repressed HIF-1 signaling and increased ROS production both *in vitro* and *in vivo*, we next set out to investigate whether there was a functional link between these observations. We started by treating mIMCD-3 cells with DMOG. Interestingly, treatment with DMOG was able to normalize the mitochondrial ROS production in hyperglycemic conditions. To further validate our findings, we silenced VHL, which mediates the degradation of HIF-1α (125), using siRNA. This also resulted in reduced ROS levels in hyperglycemia. Stimulated by these findings we moved on to an *in vivo* model and treated db/db mice and their controls with DMOG. In the db/db mice, treatment with DMOG resulted in normalized ROS levels. We next investigated ROS production in STZ-induced diabetic $PHD2^{+/-}$ (with increased HIF-1α protein stability) and compared them to their wild-type controls. The PHD2 haplodeficiency led to decreased renal ROS levels, further confirming our findings.

4.2.2 HIF-1 activation improves mitochondrial respiration and kidney function

The generation of ROS is increased in diabetes due to increased flux through the ETC (165). We therefore decided to investigate mitochondrial function in our animal models. Characterization of mitochondrial function in the kidneys from these two animal models of diabetes showed an increased proton leak as well as an increased mitochondrial respiration.

However, restoration of HIF-1α function resulted in normalized mitochondrial respiration. These findings correlated with a down-regulation of PDK1 in diabetic kidneys, which was upregulated following HIF-1α activation. PDK1 is a direct target of HIF-1α and inhibits the flux through the TCA cycle, resulting in decreased mitochondrial respiration (180). This might provide an explanation for how HIF-1α activation exerts its protective effects in DN.

Given the reduced ROS levels following HIF-1α activation, we next proposed to assess whether this had any functional relevance. Indeed, the activation of HIF-1α function led to improved kidney function and reduced renal injuries. Both animal models demonstrated decreased albuminuria as well as decreased markers of DN as shown by reduced apoptosis and expression of kidney injury marker-1 (KIM-1).

4.2.3 Hypoxia increases circulating ROS levels in diabetes

We also investigated the effect of systemic hypoxia on ROS production in human T1D patients and their healthy age-matched controls. The subjects were exposed to intermittent hypoxia (13% O_2) for one hour and ROS levels were measured in peripheral blood. Interestingly, the hypoxic challenge increased ROS in subjects with diabetes, but not in the controls subjects without diabetes.

To summarize, we have shown that HIF-1α signaling is repressed in DN, which leads to mitochondrial overproduction of ROS. The production of ROS can be normalized following pharmacologic and genetic measures to restore HIF-1α activity, leading to improved renal function and reduced kidney injury. Our conclusion is presented schematically below (Fig. 8).

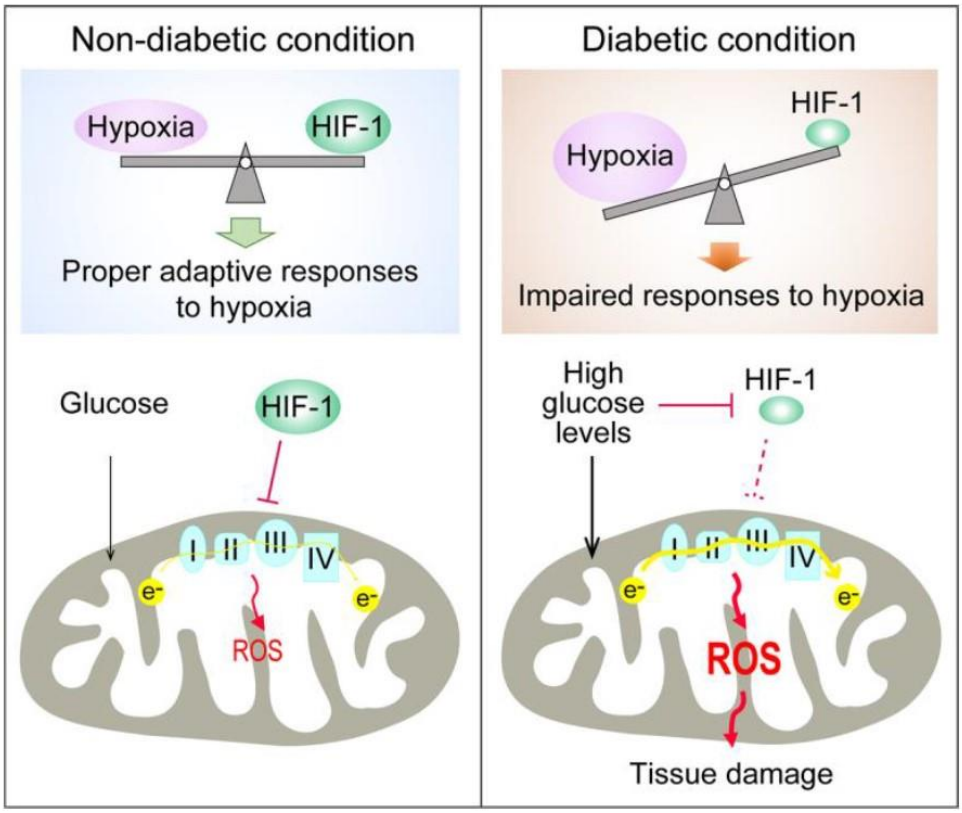

Fig. 8. Schematic illustration of how HIF-1 and ROS interplay in diabetes. HIF-1, hypoxia-inducible factor 1; ROS, reactive oxygen species. For full figure legend please refer to paper II.

4.3 PAPER III – MIR-210, CELLULAR METABOLISM AND WOUND HEALING

As shown by paper I and II and previous work by our group, the repression of HIF-1 signaling has a clear pathogenic role in diabetes microvascular complications (28). However, how HIF-1 signaling should be targeted is a matter open to debate. In DR, HIF-1 signaling can be seen as a double-edged sword, where both too little and too much has negative effects (139, 257). Moreover, in DN there are also reports indicating a detrimental role for HIF-1 (79). In skin and connective tissue, an overactivation of HIF-1 has been implicated to be involved in fibroproliferative disorders and systemic sclerosis (258, 259). Since HIF-1 is known to regulate more than 1000 genes in human (260), having a narrower therapeutic window might provide a safer alternative with fewer adverse effects. In the setting of wound healing, the hypoxamiR-210 represents such an alternative, regulating around 80 genes (29). It is strongly induced by HIF-1α and regulates several targets essential for wound healing (29). In paper III, we have therefore characterized miR-210 in the setting of DFU in order to investigate its potential role as a therapeutic target.

4.3.1 miR-210 is repressed in diabetic wound healing

We started by investigating the behavior of miR-210 in diabetic conditions. Similarly to paper II, we characterized this in an *in vitro* model with relevance for the complication being studied, i.e. in cell with key roles in diabetic wounding (fibroblasts, endothelial cells and keratinocytes). We found that miR-210 was upregulated by hypoxia, but in line with being a HIF-1α target, it was also repressed in hyperglycemia. This was seen in all cell types studied. We also investigated miR-210 *in vivo*, using human biopsy material and the db/db mouse model. In humans we found that miR-210 was lowered in DFUs compared to chronic venous ulcers. This was further confirmed in the db/db mouse, where the expression of miR-210 was lowered in diabetes compared to non-diabetic wounds.

4.3.2 Treatment with miR-210 in diabetic wounds

Having confirmed that miR-210 was indeed dysregulated in diabetic wounds, we next assessed whether this observation had any functional relevance. To this end, we generated a mouse model of diabetic wound healing using the db/db and wild-type mouse. The mice were wounded on their backs and received local injection with miR-210 mimic or placebo. The results showed that treatment with miR-210 improved wound healing rate in db/db mice, but not in the wild-type mice. We found that treatment with miR-210 mimic improved several processes important for wound healing. miR-210 mimic increased the cellular proliferation, angiogenesis, granulation tissue and collagen deposition and reduced inflammation in the wounds compared to treatment with control mimic.

4.3.3 miR-210 reprograms cellular metabolism

Next, we investigated how miR-210 mediates its beneficial effects in diabetic wound healing. miR-210 is known to regulate cellular energy metabolism, together with its effects on cellular processes that require a large amount of energy (i.e., proliferation, apoptosis, angiogenesis).

We therefore decided to investigate whether local treatment with miR-210 affected cellular metabolism. Indeed, we found that two of the miR-210 targets that regulate mitochondrial function, ISCU and SDHD (208, 209, 211), were downregulated following miR-210 mimic treatment. In line with the effects of these target genes (204), miR-210 mimic treatment normalized the increased oxygen consumption rate (OCR) to the non-diabetic control mice levels. This was followed by a reduction in ROS levels.

We further validated our findings in fibroblasts, which are the predominant cells in the wound tissue (261). Similarly, to the *in vivo* results, miR-210 mimic treatment in hypoxic and hyperglycemic conditions reduced ISCU and SDHD, decreased OCR and ROS levels and increased cellular migration. Moreover, miR-210 increased the glycolysis in these cells and increased the proportion of ATP generated from glycolysis than from mitochondrial respiration. Highly relevant functionally, when glycolysis was inhibited, the positive effect of miR-210 on fibroblast migration was abrogated.

In summary, in this study we have found that miR-210 represents an attractive therapeutic target in order to restore the repressed cellular reaction to hypoxia. miR-210 was successfully delivered to the wounds, without any systemic increase in miR-210. This resulted in improved wound healing, at least partly because of improved cellular function in hypoxia due to the metabolic reprogramming induced by miR-210. These findings are presented schematically below (Fig. 9).

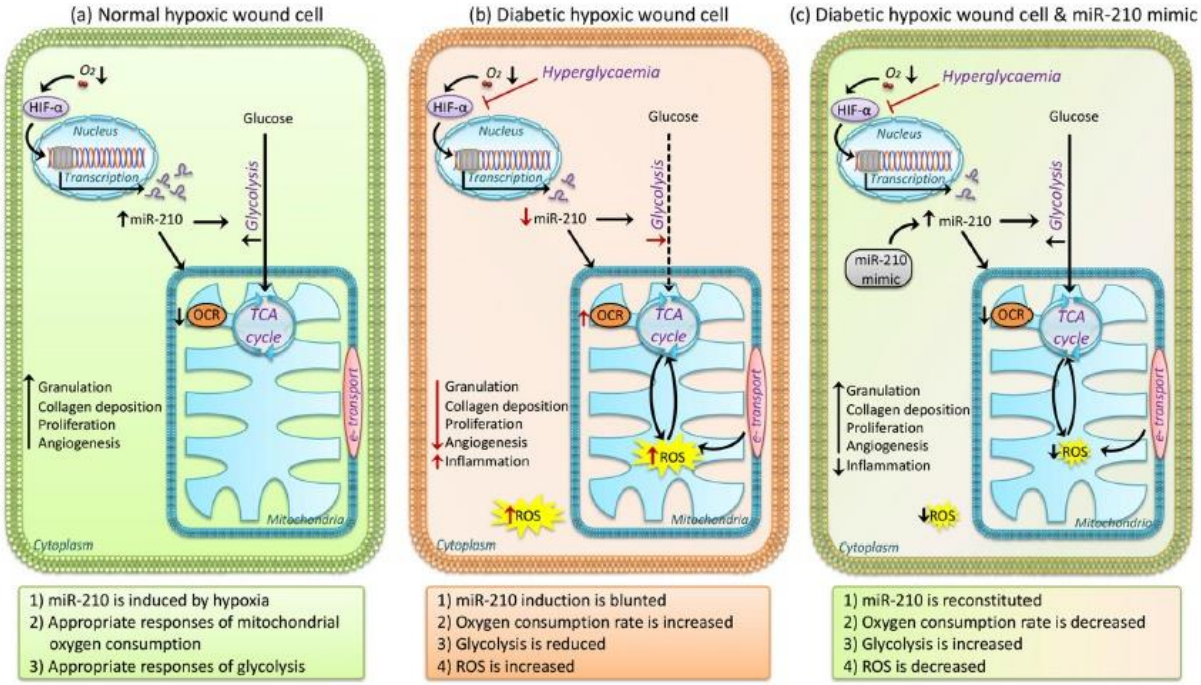

Fig. 9. Summary of the altered metabolism in diabetic wounds before and after miR-210 reconstitution. HIF, hypoxia-inducible factor; OCR, oxygen consumption rate; ROS, reactive oxygen species; TCA, tricarboxylic acid (cycle). For full figure legend please refer to paper III.

4.4 PAPER IV – MIR-34A AS A TARGET IN DIABETIC WOUND HEALING

In order to find attractive therapeutic targets for the treatment of diabetes complications we continued our search in the field of miRNAs, given the recent findings that a wide array of miRNAs are dysregulated in diabetes (31, 198-200). miR-34a, found to be upregulated in hypoxia (232, 233), is one of these miRNAs. Moreover, miR-34a has also been shown to be involved in the regulation of cellular functions important for wound healing (262). We therefore decided to investigate the role of miR-34a in diabetic wound healing.

4.4.1 miR-34a is repressed in diabetes in a cell type-dependent manner

We started by investigating whether miR-34a was repressed in diabetes. To this end, we analyzed the expression of miR-34a in biopsies from non-healing DFUs and compared the expression to venous ulcers (VU). We found that miR-34a indeed was repressed in DFU compared to VU. Moreover, the expression of miR-34a was higher in VU compared to control skin, while miR-34a levels were similar when comparing wound and skin tissue in diabetes.

We next moved on to analyzing miR-34a *in vitro*, investigating the regulation of miR-34a in a hypoxic environment and thereby mimicking the oxygen-depleted environment of wounds. We used cells with relevance for wound healing. Hypoxia led to an upregulation of miR-34a in macrophages and keratinocytes, which was abrogated in high glucose conditions. Interestingly, we found that miR-34a was not regulated in endothelial cells and fibroblasts in diabetic conditions.

4.4.2 miR-34a is repressed by hyperglycemia at the transcriptional level

Having established the dysregulated miR-34a pattern, we next investigated the mechanisms behind this observation. We pursued our investigation in macrophages and found that miR-34a was similarly dysregulated on the pri-miRNA level, being upregulated by hypoxia but repressed when the cells were also exposed to hyperglycemia. The same regulation was confirmed using a miR-34a promoter reporter assay. This suggests that hypoxia induces the transcription of miR-34a, but the transcription is inhibited in hyperglycemia.

Since p53 is the major inducer of miR-34a expression and also is modulated by glucose (263, 264), we investigated the role of p53 in regulating miR-34a in diabetic conditions using a luciferase reporter driven by miR-34a promoter in which p53 binding site was mutated. As expected, the mutation of p53 binding site decreased the expression of miR-34a. However, it did not affect the induction of miR-34a by hypoxia or the inhibition by hyperglycemia. This indicates that the dysregulation of miR-34a in diabetic conditions is mediated by p53-independent mechanisms.

4.4.3 Repressed miR-34a contributes to impaired diabetic wound healing

We next investigated whether the observed repression of miR-34a in DFUs and macrophages and keratinocytes had functional relevance. We analyzed the expression of miR-34a in a

wound healing model using the db/db mouse. Similar to the human data, we found that miR-34a was repressed in db/db wounds compared to control wounds. We then injected miR-34a locally to the wounds and found that miR-34a mimic improved wound healing exclusively in the db/db mice, but not in the non-diabetic mice (Fig. 10).

In summary we have demonstrated that miR-34a is dysregulated in diabetic wounds, both in humans and in mice. The dysregulation of miR-34a was found to be cell type-specific, being present only in macrophages and keratinocytes. The observed repression by hyperglycemia in hypoxic conditions is independent of p53. Moreover, the repressed miR-34a has functional relevance since reconstitution of miR-34a mimic improves wound healing rate in db/db mice.

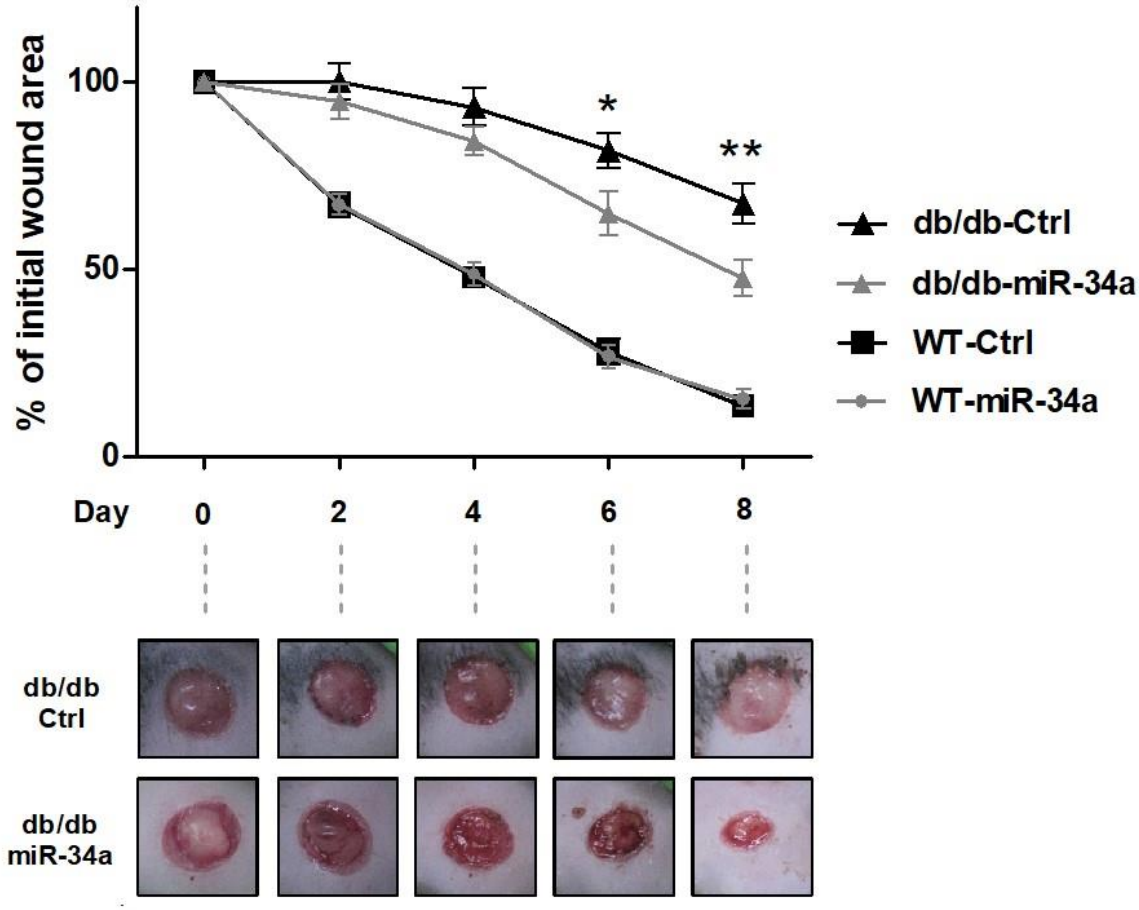

Fig. 10. Local reconstitution with miR-34a improves wound healing in db/db mice. Ctrl, placebo treatment; miR-34a, miR-34a mimic treatment, WT, wild-type. For full figure legend please refer to paper IV.

5 DISCUSSION

In this thesis work, the aim has been to investigate the role of the dysregulated hypoxic response in diabetic microvascular complications, i.e. diabetic retinopathy, diabetic nephropathy and diabetic wound healing. The ultimate goal has been to identify new potential therapeutic targets since we today lack efficient treatment for diabetes complications due to an incomplete understanding of the underlying pathogenic mechanisms.

In paper I, we found that the *HIF-1A* Pro582Ser polymorphism, encoding a HIF-1α more resistant to the repressive effect of hyperglycemia, protects against development of severe DR. This finding was in perfect agreement with previous studies showing a protective effect of the *HIF-1A* Pro582Ser polymorphism on the development of DFU (255) and DN (131). To the best of our knowledge, our study is the first showing that the hyperglycemia-induced repression of HIF-1α has pathological relevance in DR, thereby involving the repression of HIF-1α in yet another microvascular complication of diabetes.

The finding that a more active HIF-1α protects against severe DR is highly interesting given the many observations showing a detrimental effect of HIF-1α in DR, and inhibition of the HIF-1 target gene VEGF is now routinely used in the clinic (257). However, even though the detrimental role of HIF-1 in the proliferative phase of DR is well-known, adequate HIF-1 function in the early stages might be protective. HIF-1 is essential for normal retinal development, vasculature stability, proper retinal function and vision maintenance and several target genes of HIF-1 are important for the protection of the retina in DR (139). Patients that are carriers of the *HIF-1A* Pro582Ser polymorphism may therefore be able to better adapt to the rapidly developing hypoxia following diabetes onset (45), which might slow down the progression to severe DR as indicated by our study. Relatively little effort has been put into treating retinal hypoxia despite its key pathogenic role (140).

A limitation of our study is the few individuals with the genotype TT, as a result of the relative infrequency of this polymorphism in our study population (minor allele frequency for HIF-1A C>T was 0.071). Future studies would therefore benefit from multicenter pooling of data and it would also be interesting to analyze the frequency of this polymorphism and its relation to DR in other populations since some ethnicities are at increased risk of DR (51). Liu et al recently published a similar study on Chinese subjects where no association was found between the *HIF-1A* Pro582Ser polymorphism and DR. However, none of the diabetes subjects (n=299) had the genotype TT and the degree of DR was not specified (265).

Encouraged by the findings in paper I as well as by previous reports on a role played by HIF-1α in DN (79), we further investigated HIF-1α in DN. In paper II, we found that the impaired HIF-1α signaling in diabetes contributes to renal dysfunction, and that the mitochondrial overproduction of ROS in DN can be normalized by HIF-1α activation both *in vitro* and *in vivo*. Our results indicate that the reduced mitochondrial ROS production following HIF-1 activation might be mediated by PDK1, which has previously been shown to inhibit pyruvate dehydrogenase (PDH) activity, thereby resulting in reduced ETC substrate availability (180).

Even though HIF-1 appears to play a protective role for acute kidney injury (266), the role of chronically activated HIF-1 signaling in kidneys is less clear. There exist different reports on HIF-1 activation in diabetic kidneys, with evidence of both increased (267) and repressed HIF-1 signaling (79, 268). However, it is important to correct for tissue oxygenation, since the adequate level of HIF-1 activation can only be determined when comparing equally hypoxic conditions. Excessive HIF-1 signaling in kidneys have been shown to activate profibrotic pathways, resulting in interstitial fibrosis (269). While just one study found that HIF-1 inhibition in an OVE26 type 1 diabetes mice model resulted in reduced albuminuria (270) most investigation point towards a protective effect of HIF-1 activation in diabetes kidneys (131, 146, 271). This is believed to be resulting from the ability of HIF-1 to restore the oxygen homeostasis (79). The profound hypoxic environment that develops rapidly in kidneys upon diabetes onset (78) has been shown to result in nephropathy (83). The hypoxia is largely resulting from the massive flux through the sodium glucose cotransporters in the tubuli and from increased mitochondrial uncoupling as a way to protect the cells from lethal oxidative stress-induced injury, which results in inefficient oxygen utilization and further aggravation of renal hypoxia (79). Therefore, a restored HIF-1 signaling has the potential to inhibit this vicious circle, as supported by the findings from our study. It has also been speculated that HIF-1 has different actions in different cell types in the kidney depending on the expression pattern of HIF-1 signaling components (79). For example, the activation of the HIF-1/ADAM17 signaling pathway in the mesangial cells in the glomeruli have been shown to be profibrotic, whereas the tubuli lack this enzyme (267). However, our study indicates that the net effect of HIF-1 activation is protective for renal function, but the possible detrimental effect of HIF-1 signaling in glomeruli is an important observation and warrants further studies (79).

Oxygen concentrations in the body physiologically range between 1-11% (121), which leads to HIF-1 signaling constantly being activated. All diabetic organs prone to diabetes complications have been shown to have a decreased oxygen tension (28), which in combination with an impaired HIF-1 signaling likely contributes to increased ROS levels. Oxidative stress causes tissue damage and has been shown to be involved all diabetes complications (169, 272). Reducing ROS levels by targeting HIF-1α as in the present study might therefore provide a new treatment strategy, not only for DN but also for other end-organ complications of diabetes.

The control subjects in our study, which have an intact HIF-1 signaling machinery, showed no increase in ROS levels following exposure to hypoxia, as opposed to the T1D subjects where the ROS levels increased. The impaired HIF-1α signaling in the T1D subjects might provide a plausible explanation for this observation. We have therefore further pursued these findings and are currently conducting a study where T1D subjects are randomized to receiving either the HIF-1α stabilizer deferoxamine (DFX) or placebo following exposure to intermittent hypoxia, using the same protocol as in paper II (ClinicalTrials.gov identifier: NCT02174731). The results from this ongoing study will be highly relevant for the development of new strategies targeting HIF-1α in diabetes.

However, targeting of HIF-1α might as well have its difficulties. HIF-1α is known to regulate more than 1000 genes, thereby with the risk to have effects going far beyond the desired ones for treatment of diabetes complications. Detrimental effects of an overactivated HIF-1α has been reported in organs and cell types affected by diabetes complications (257-259, 269), mirroring the pleiotropic effects of this central transcription factor. Moreover, overactivated HIF-1 is known to be a central mechanism in tumorigenesis (273). Therefore, it is worthwhile to dissect the hypoxic signaling machinery to identify potential targets with a narrower therapeutic window. This led us into the investigation of miRNAs as drug targets in diabetes in paper III and IV.

In paper III, miR-210 proved to be powerfully induced by hypoxia but also repressed by concurrent hyperglycemia, in line with it being under direct influence of HIF-1α. This was seen both in cells, db/db mouse and human biopsies. The use of DMOG led to rescued expression of miR-210 in combined hypoxic and hyperglycemic conditions, emphasizing the tight connection between HIF-1 and miR-210 expression. Moreover, reconstitution of miR-210 was able to improve wound healing in the db/db mice model. The downstream effects of restored miR-210 expression were secondary to the improved metabolic balance which optimized the cellular energy utilization and thereby improved cellular functions.

Interestingly, the usage of miR-210 mimic also reduced ROS levels, reminiscing of the findings in paper II where restoration of HIF-1α activity in diabetes reduced ROS levels and thereby reduced renal damage and improved kidney function. Despite the well-established link between oxidative stress and organ-damage in diabetes (169), it has been notoriously difficult to translate this knowledge into clinical practice. This is considered to be due to an incomplete understanding of its role played in health and disease as well as difficulties to target the source of the pathological overproduction of ROS in diabetes (23). In that light, our findings that a restored HIF-1 and miR-210 expression at least partly mediates its beneficial effects in diabetes microvascular complications by reducing the oxidative stress is highly interesting.

Excessive expression levels of miR-210 have been shown to inhibit keratinocyte proliferation through its target E2F3 (225) and impair healing of ischemic wounds in normal mice (274). This finding underscores the notion that miR-210 has different effects depending on context and degree of hypoxia, since miR-210 was found to stimulate proliferation in other experimental models (215, 218, 220, 275). The role of miR-210 in diabetic wound is relatively unexplored. However, Dallas et al found that inhibition of miR-210 and inhibition of PHD2 improved diabetic wound healing in db/db mice (276). The discrepancies between this study and ours might be caused by variations in pharmacokinetics and wound model, but also by the fact that the biological effects of miR-210 are a combination of direct and indirect effects mediated by HIF-1α with which it has complex interactions (207). This might also be an explanation for why Dallas et al. did not find an additive effect on wound healing when they combined PHD2 inhibition together with miR-210 inhibition since both treatments act through a common HIF-1 signaling pathway. It is also important to bear in mind that

treatment with miR-210 mimic might have its own biological effects, independent of HIF-1 signaling, as was found in our investigation. Our study together with the one by Dallas et al together emphasize the important role played by the HIF-1/miR-210 axis in diabetic wounds, and further studies are warranted.

In paper IV, we investigated the regulation of miR-34a and its role in diabetic wound healing. miR-34a was much alike miR-210 induced by hypoxia and repressed by concurrent hyperglycemia both *in vitro* and *in vivo*, however only in certain cell types as opposed to miR-210. Continuing with miR-34a, we found that the dysregulation in diabetes was p53-independent. Given the repressed HIF-1 signaling in diabetes and the fact that the miR-34a promoter contains an HRE (234), it would therefore be highly interesting to go beyond p53 and investigate if HIF-1 causes the dysregulated miR-34a expression. Preliminary results (not included in the manuscript) indicate that the repressed miR-34a expression in diabetic wounds can indeed be rescued by restored HIF-1 signaling, since treatment with DMOG in db/db wounds normalize miR-34a expression. However, an HIF-independent regulation in hypoxia is also to be taken in account. For example, miR-210 has been shown to also be upregulated in mouse embryonic fibroblasts lacking functional HIF-1, which was mediated by the p53 and protein kinase B (Akt) pathways (277). We are currently pursuing our findings and further investigating the regulation by miR-34a in hypoxia, encouraged by the results showing that local miR-34a reconstitution improves diabetic wound healing.

miR-34a was the first miRNA to reach phase 1 clinical trials as an anti-cancer therapy, although the study had to be terminated in advance due to several immune-related adverse effects (278). However, that treatment was systemic while treatment with miR-34a and miR-210 in diabetic wounds could be performed locally. Local treatment with miRNA has the big advantage of most likely conferring less side effects due to limited spread of the drug, as showed in paper III where miR-210 was not taken up systemically.

It would be of interest to investigate miR-34a in the context of other diabetes complications. However, care has to be taken when extrapolating the results from our study to other organs. The regulation of miR-34a was cell type-dependent, only being present in macrophages and keratinocytes but not in fibroblasts and endothelial cells. Moreover, as is the case for many miRNAs (200), the actions of miR-34a have been reported to be different depending on context and dosage. For example, miR-34a was reported to both increase and decrease cardiomyocyte proliferation (279, 280). In keratinocytes, supraphysiological doses of miR-34a promoted apoptosis and senescence in addition to promoting differentiation, whereas moderate (around two-fold increase) levels of miR-34a selectively promoted differentiation (281). It has been shown that expression levels of miR-34a vary greatly even within the same organ (282), indicating that miR-34a modulation might have different results in different tissues. Finally, even though paper IV shows a positive effect for miR-34a in diabetic wound healing, the mechanism behind this is yet unknown and might not be relevant for other diabetes complications. Taken together, our findings are highly interesting in the setting of

diabetic wound healing but more studies are needed in order to be able to assess whether miR-34a could be used as a therapeutic target in a broader perspective.

miRNAs have been found to be stable in virtually all possible biological fluids as RNA-protein complexes despite being stored in extreme conditions, making them well suited as biomarkers (200). Interestingly, miR-34a and miR-210 have been suggested as biomarkers in diabetes. For example, both exosomal miR-34a and miR-210 in urine could detect DN with albuminuria (283). miR-210 was found to be increased both in urine and plasma from young T1D patients, possibly being an early indicator of development of diabetes complications (284). In DR, miR-210 was upregulated and correlated with severity of retinopathy (275). Serum miR-34a levels were associated with insulin resistance and obesity in human pre-diabetic and diabetic subjects (285). The widespread dysregulation of these miRNAs thereby make them excellent candidates as biomarkers but also reflect their potential pathogenic involvement both in the development of diabetes and its complications.

Although beyond the scope for this thesis work, it is interesting to speculate about HIF-1 signaling as a therapeutic target in diabetes macrovascular complications. Cardiovascular disease is the most common cause of death among diabetes patients, and their prognosis after myocardial infarction or heart failure is poor (286). HIF-1 signaling has been shown to be crucial for recovery of the heart following ischemia (185), but patients with poor metabolic control have lowered HIF-1 activity (287). This leads to diabetic hearts being less tolerant towards hypoxia with a reduced ability to adapt in the longer perspective (288). Restoring HIF-1 signaling by using a PHD inhibitor was able to improve the recovery of the heart after a hypoxic insult (136, 289). The cardioprotective effects of HIF-1 are at least partly mediated by modulation of the cellular metabolism, where glycolysis and glucose oxidation are promoted (290) and ROS production decreased (166). This protective mechanism of HIF-1 activation is similar to that identified in paper II and III in this thesis work, indicating that our findings might hold relevance for also other diabetes complications.

Taken together, our studies have contributed to shedding light on the dysregulated hypoxic signaling in diabetes complications. Together with the work of others, we are starting to have a clearer picture of how HIF-1 signaling could potentially be modulated to treat and prevent diabetes complications. Targeting HIF-1 signaling topically in DFUs seem especially promising, given the positive effects in numerous preclinical trials (126, 133, 291-293). Moreover, HIF-1 activation in diabetes has been reported to prevent development of DN (79, 145), peripheral neuropathy (294) and protect the ischemic heart (136). Short-term induction of HIF-1 systemically is unlikely to have adverse effects and could potentially be used in diabetes for relatively acute hypoxic insults (for example, myocardial infarction or stroke). Already now, DFX is allowed for treatment as an iron chelator and the PHD2 inhibitor Roxadustat was recently approved for treating anemia in patients with chronic kidney disease (295). Additional HIF activators are currently being investigated in clinical trials. However, care has to be taken when it comes to long-term treatment with HIF-1 activators since systemic HIF-1 activation has at least theoretical potential adverse effects (296). Moreover,

chronically activated HIF-1 signaling is known to play an oncogenic role (273). In that sense, further investigation of miRNAs, with a more narrow therapeutic window, that are involved in dysregulated hypoxic signaling might provide an interesting therapeutic alternative for the treatment of diabetes complications.

6 CONCLUSIONS

We have investigated the dysregulated hypoxia signaling in microvascular complications of diabetes. Our results are presented in paper I-IV and based on them, we draw the following conclusions:

- The *HIF-1A* Pro582Ser polymorphism confers a 95% risk reduction of developing severe diabetic retinopathy even when adjusting for traditional risk factors. The *HIF-1A* Pro582Ser polymorphism encodes a HIF-1α variant that is still sensitive to non-canonical degradation pathways in hyperglycemia, but confers a higher transcriptional activity.

- HIF-1α is repressed in diabetic nephropathy, which leads to increased mitochondrial respiration and ROS production. Pharmacologic and genetic measures to restore HIF-1α function in diabetic nephropathy normalize the ROS levels and protect against kidney injury.

- miR-210 expression is repressed in diabetic wound healing. Local reconstitution of miR-210 to an animal model of diabetic wound healing improves wound healing and reduces ROS production. Treatment with miR-210 also increases glycolysis with positive effects for energy utilization and cellular function.

- miR-34a is repressed in diabetic wound healing, which is mediated by p53-independent mechanisms on the transcriptional level. Treatment with miR-34a locally in the wounds improves wound healing in db/db mice.

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