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Protective Effect of Modified Human Fibroblast Growth Factor on Diabetic Nephropathy

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PROTECTIVE EFFECT OF MODIFIED HUMAN FIBROBLAST GROWTH FACTOR ON DIABETIC NEPHROPATHY

(Thesis format: Monograph)

by

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Graduate Program in Pathology

A thesis submitted in partial fulfilment of the requirements for the degree of
Master of Science

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London, Ontario, Canada

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THE UNIVERSITY OF WESTERN ONTARIO
SCHOOL OF GRADUATE AND POSTDOCTORAL STUDIES

Abstract

Oxidative stress is a key mechanism causing Diabetic Nephropathy (DN). Acidic fibroblast growth factor (aFGF) is known to confer protection from oxidative stress. However, it also has significant angiogenic activity. Hence, we have generated a mutated human acidic FGF (maFGF), with intact antioxidant properties but devoid of angiogenic activities. Recent evidence shows that maFGF treatment prevented diabetic cardiomyopathy and further in vitro studies suggest that this prevention is mediated by suppression of cardiac oxidative stress, hypertrophy and fibrosis. We hypothesized that maFGF treatment has a protective effect in DN.

We show that maFGF treatment did not affect body weight and blood sugar levels in a type 1 diabetic mouse model. However maFGF prevented renal functional alterations in diabetes and decreased renal hypertrophy following long-term diabetes. maFGF also prevented diabetes–induced DNA damage, upregulation of angiotensinogen, oxidative stress marker heme oxygenase 1, and alteration of endothelial nitric oxide synthase (eNOS). Surprisingly, it failed to prevent upregulation of the fibrogenic cytokine transforming growth factor β1 mRNA expression. However, long term administration of maFGF partially prevented diabetes-induced extracellular matrix proteins accumulation. Further analyses showed similar results in high glucose-induced alterations in podocytes and microvascular endothelial cells. Likewise, maFGF showed prevention of diabetes-induced decreased nitric oxide (NO) production and apoptosis in vivo and in vitro. These results were consistent with the prevention of long term diabetes- induced down regulation of eNOS enzyme. Data from these experiments suggest that the preventative effects of maFGF treatment in DN are probably via alteration of NO production, and indicate a potential therapeutic role of maFGF in DN.

Keywords: modified acidic FGF, acidic FGF, diabetic nephropathy, oxidative stress, nitric oxide.

Dedication

I dedicate this thesis to my admirable mentors who have educated me with their guidance and have made me a better professional with new perspectives to approach science and even Life.

To my family and friends who inspire me to engage in my goals.

Acknowledgments

I would like to gratefully acknowledge my supervisor Dr. Subrata Chakrabarti for his eager support and continued assistance throughout the course of my Master's research. I thankfully acknowledge the opportunity I received to work in his lab and the possibility to open new horizons in my graduate career. The support and above all the patience Dr. Chakrabarti portrayed over the past two years have been and will continue to be indispensible for my professional development. My special thanks to Dr. Zia Khan, Associate Professor, Dr. Chandan Chakraborty and Dr. Tianqing Peng, my advisors, for their valuable suggestions during the course of this project. Also my sincere gratitude to Dr. Biao Feng and Dr. Shaly Chen, Research Associates, for the experience and knowledge imparted to me. Special gratitude is extended to my colleagues Michael Ruiz and Anu Thomas for their immeasurable help and feedback; and Yanan, Rokhsana and Andrew for greatly supporting me during the course of my project.

Finally, I would dearly like to thank my family for their relentless encouragement and support and my friends for believing in me. Also, I would like to acknowledge the department of Pathology: Tracey, Linda, Kathilyn, who have always been very supportive and patience. I thank you all for the learning provided to me during this journey.

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List of Abbreviations

8-OHdG 8-hydroxy-2-deoxyguanosine

ADA American diabetes association

aFGF acidic fibroblast growth factor

AGE advanced glycation end products

AGT angiotensinogen

ANOVA analysis of variance

AP-1 activating protein 1

Bak-1 Bcl-2 homologous antagonist/killer

BCA bicinchoninic acid assay

Bcl-2 B-cell lymphoma 2

bFGF basic fibroblast growth factor

BSA bovine serum albumin

CIHR Canadian institutes of health research

Col 1α(IV) Collagen 1α(IV)

DAN 2, 3-diaminonaphthalene

DAPI 4',6-diamidino-2-phenylindole

DCFDA 2',7' (DCF)–dichlorofluorescin diacetate

DEPC diethylpyrocarbonate

DM diabetes mellitus

DN diabetic nephropathy

ECL enhanced chemiluminescence

ECM extracellular matrix

EDTA Ethylenediaminetetraacetic acid

ELISA enzyme-linked immunosorbent assay

eNOS endothelial nitric oxide synthase

ERK extracellular signal-regulated kinase

ET-1 endothelin 1

FBS fetal bovine serum

FGFs fibroblast growth factors

FITC Fluorescein isothiocyanate

FN fibronectin

GAPDH glyceraldehyde 3-phosphate dehydrogenase

GFR glomerular filtration rate

HG high glucose, 25mM D-glucose

HMVEC human microvascular endothelial cells

HO heme oxygenase

HRP horseradish peroxidase

WST-1 Water soluble Tetrazolium salts

iNOS inducible nitric oxide synthase

IP intraperitoneal

L-NAME L-N^G-Nitroarginine methyl ester

maFGF modified acidic fibroblast growth factor

MAPK mitogen-activated protein kinase

NO nitric oxide

ONOO$^-$ peroxynitrite

SOD superoxide dismutase

NADPH nicotinamide adenine dinucleotide phosphate

NFκB nuclear factor kappa B

NG normal glucose, 5mM D-glucose

PAS periodic acid-schiff

PBS phosphate-buffered saline

PI3K phosphatidylinositide 3-kinase

PKC protein kinase C

PVDF polyvinylidene difluoride

qPCR quantitative polymerase chain reaction

RIPA radioimmunoprecipitation assay

RNS reactive nitrogen species

ROS reactive oxygen species

RS1 recognition site 1

RS2 recognition site 2

RT-PCR reverse transcription polymerase chain reaction

SDS-PAGE Sodium dodecyl sulfate- Polyacrylamide gel electrophoresis

STZ streptozotocin

TdT terminal deoxynucleotidyl transferase

TGFβ transforming growth factor β

TUNEL terminal deoxynucleotidyl transferase dUTP nick end labeling

VEGF vascular endothelial growth factor

WHO world health organization

Chapter 1: Introduction

1.1 Diabetes Mellitus

Diabetes Mellitus (DM) is a metabolic disorder that exhibits a defective or deficient insulin secretory response, glucose underutilization, and hyperglycemia [1]. It is classified in two major types according to its etiology: type 1 DM (insulin dependent) and type 2 DM (non insulin dependent). The etiology of type 1 DM is associated with absolute lack of insulin, which is caused by a reduction of β cells in the pancreas due to autoimmune reactions. It is also called juvenile diabetes as the onset of this disease is before 20 years of age. Alternatively, type 2 DM is characterized by insulin resistance (that happens when cells fail to use insulin properly) and sometimes is combined with an absolute lack of insulin, mainly when the disease is advanced. This is the result of the failure of pancreatic beta cells over time to secrete more insulin to compensate hyperglycemia in the insulin-resistant state [2]. Type 2 DM is associated with overweight or obesity and usually appears after 30 years of age, although the trend of younger people with this disease is increasing [1, 2, 3]. There are other types of diabetes namely gestational diabetes that occurs when pregnant women develop a high blood glucose level without having a previous history of diabetes. This third type of diabetes usually resolves after the birth of the baby [1].

The treatment strategy for the various types of diabetes differs, however monitoring and control of blood glucose levels, keeping a healthy diet and regular physical activity is equally important. In the case of type 1 DM, insulin injections are required and islet cell transplant can be performed to patients with specific requirements. Various medications and/or insulin can be used to treat type 2 or gestational DM: antihyperglycemic, hypoglycemic agents and insulin sensitizers such as the family of Biguanide (metformin), sulphonylureas (glibenclamide), Thiazolidinediones (pioglitazones) and others [3].

In type 1 and type 2 diabetes the net effect is a chronic disorder of carbohydrate, fat and protein metabolism with resulting chronic complications affecting the blood vessels, kidneys, eyes and nerves [1]. These secondary complications, which include coronary and cerebrovascular disorders, peripheral arterial disease, nephropathy and retinopathy, are the main cause of the increased morbidity and mortality in the diabetic population.

Hence, the study of the molecular pathophysiology of diabetes complications can bring up insights for potential treatments to manage these complications.

1.2 Epidemiology

Diabetes Mellitus is one of the fastest-growing health issues in the world, affecting around 387 million people in the planet [4]. As a consequence of a life-style consisting in lack of exercise, an unhealthy diet and overweight/obesity, DM is reaching epidemic proportion in some regions. North America and the Caribbean is the region with higher prevalence with an estimate of 39 million people with this disease.

According to the American Diabetes Association (ADA), the prevalence of diabetic and prediabetic cases in the United States has increased since 2010 and diabetes remained the seventh cause of death by the same year [5]. In Canada, the overall prevalence of diabetes has increased gradually although incidence has been stable in the last decade [6]). The relative increase in prevalence accounts mainly for Canadians in the working age group and it is associated with overweight and obesity. This causes a burden to the health care system as individuals with diabetes are estimated to create three to four times more health care expenses than individuals without diabetes [5, 6]. Some examples include the increased risk of hospitalization with cardiovascular disease (over 3 times), end- stage renal disease (12 times) and non-traumatic lower limb amputations (20 times) of diabetic individuals compared to non diabetic population [5, 6].

Diabetic nephropathy (DN) is one of the significant long-term complications in terms of morbidity and mortality for individual patients with diabetes. It is estimated that about 25-40% of diabetic patients develop DN within 20-25 years of the onset of diabetes [7]. In fact, diabetes is responsible for 30-40% of all end-stage renal diseases in North America [8, 9].

1.3 Diabetes Nephropathy and pathophysiologic changes

Diabetic nephropathy is a clinical syndrome characterized by the following [10]:

- Persistent albuminuria (>300 mg/d or >200 µg/min)
- Progressive decline in the glomerular filtration rate (GFR)
- Elevated arterial blood pressure

At the tissue level, various cell types are involved in DN. They include glomerular podocytes, mesangial cells and endothelial cells, tubular epithelium, and interstitial fibroblasts [9, 11]. However, similar to the other diabetic complications, DN is essentially a microvascular disease in which endothelial dysfunction serves as a key event in its development and progression.

Additionally, DN develops as a result of the confluence of hemodynamic and metabolic perturbations [9, 12, 13]. The pathophysiologic changes comprise hyperfiltration, hyperperfusion and microalbuminuria in the very early stages followed by worsening of renal functions. This is linked with diffuse thickening of the glomerular and tubular basement membranes, as well as mesangial matrix and tubule-interstitial compartment expansion caused by increased deposition of extracellular matrix proteins and mesangial cell hypertrophy. The progressive expansion of the mesangium in the later stages restricts the capillary surface available for filtration and, as a consequence, the glomerular filtration rate falls. In addition, another pathologic correlates are glomerular podocyte alteration and loss. This is exhibited through effacement of foot processes and decreased number of podocytes (detachment of podocytes from the glomerular membrane) due to apoptosis. These changes ultimately progress to proteinuria, glomerulosclerosis and tubulointerstitial fibrosis [9, 11-14].

1.4 Pathophysiologic mechanisms in DN

Similar to the other microvascular complications of diabetes , hyperglycemia is the major factor which causes damage to the discussed cell types by increasing the production of

reactive oxygen species (ROS) [9, 14, 15]. Moreover, DN is thought to result from interactions involving metabolic and hemodynamic factors [9, 13]. Hyperglycemia increases superoxide production by the mitochondrial electron transport chain. Increased ROS inhibits glycolysis by interfering with the activity of the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) impelling glucose to other pathways which also induce oxidative stress [14]. These specific glucose dependent metabolic pathways are activated within the endothelial cells and include increased flux of polyols and hexosamines, increase activity of protein kinase C (PKC) and generation of advanced glycation end products (AGE). As a result, other cellular events triggered are increased activity of transforming growth factor β (TGF-β)- Smad-MAPK and G-proteins, altered expression of cyclin kinases and their inhibitors, and of matrix degrading enzymes and their inhibitors [9, 11, 12].

Hemodynamic factors implicated in DN include elevation of systemic and intraglomerular pressure and activation of various vasoactive factors pathways including the renin-angiotensin system and endothelins [7, 12, 13]. The altered hemodynamic changes act in concert with metabolic pathways to activate intracellular second messengers which affect gene expression. Some of the consequences are increased prosclerotic cytokine TGF-β1, transcription factors such as NF- κB that activates local inflammatory responses and various growth factors such as the permeability enhancing VEGF. Ultimately, these molecular mechanisms lead to the progression of the functional and structural changes that lead to end stage renal failure [9, 11, 14] (Figure 1.1).

1.4.1 Oxidative stress in DN

Oxidative stress occurs when the amount of reactive oxygen species (ROS) and reactive nitrogen species (RNS) are more than the defensive system can remove [15, 16]. In DN, there are a number of enzymatic and non enzymatic sources of ROS including auto oxidation of glucose, transition metal-catalyzed Fenton reactions, advanced glycation end products formation, polyol pathway flux, mitochondrial respiratory chain
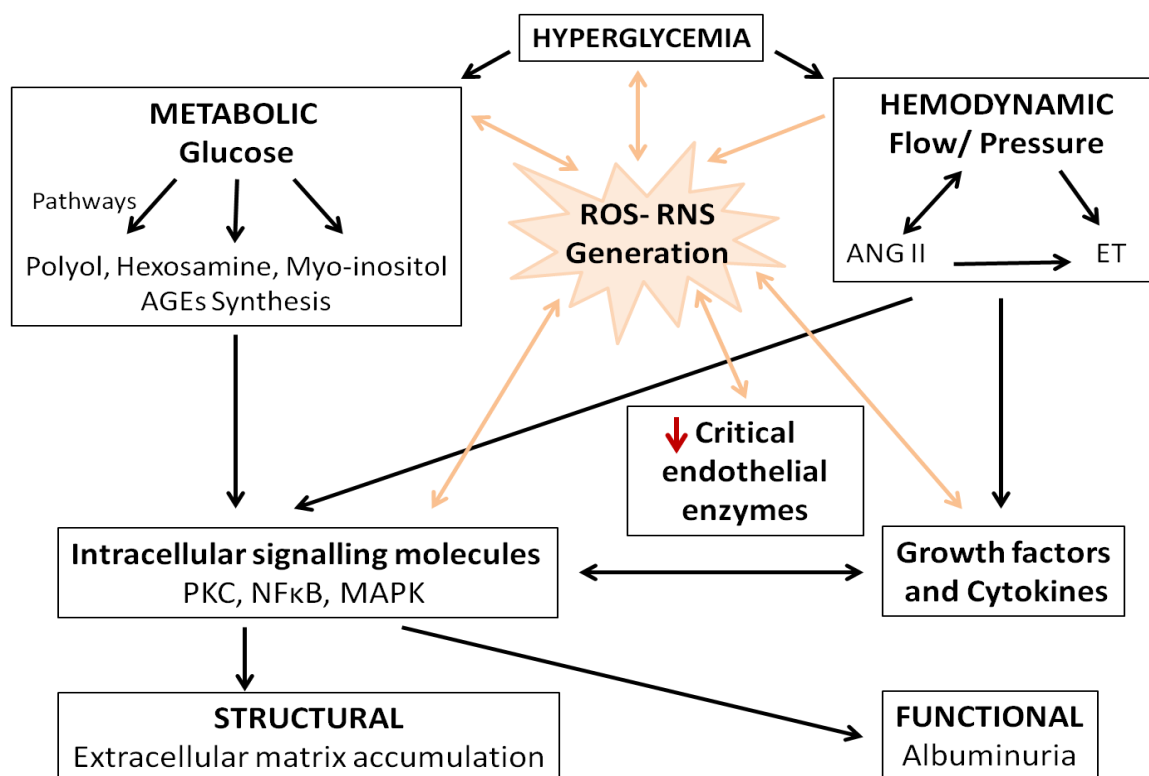
Figure 1.1 Scheme of the Pathophysiological mechanisms of DN. Metabolic and hemodynamic processes are induced by hyperglycemia and act in concert activating different pathways with altered expression of genes and cellular dysfunction. Oxidative stress from ROS and RNS generation is enhanced by hyperglycemia-induced metabolic and hemodynamic processes and is important for the formation of mediators causing structural alterations and functional damage (Figure adapted from reference # [13]).

deficiencies, xanthine oxidase activity, peroxidases, nitric oxide synthase and (NADPH) oxidase [15]. The mitochondrial respiratory chain is thought to be a major source of excess ROS in diabetes in response to hyperglycemia and a key initiator for the pathogenic pathways [14, 15]. Furthermore, a number of functional enzymes within the mitochondria are particularly susceptible to ROS mediated damage, leading to altered ATP synthesis, cellular Ca^{2+} dysregulation, and induction of mitochondrial permeability transition, all of which predispose the cell to necrosis or apoptosis [15].

In addition, overproduction of superoxide has shown to be associated with endothelial dysfunction by activating proinflammatory signals and inactivating important enzymes involved in vascular homeostasis such as endothelial Nitric Oxide Synthase (eNOS) [17, 18]. Therefore, oxidative stress suppresses Nitric Oxide (NO) bioavailability which increase generation of ROS and lead to oxidative damage to lipids, proteins, aminoacids and DNA [17]. This decreased NO bioactivity is also thought to be due to quenching by increased ROS production. NO is inactivated through its reaction with ROS to form peroxynitrite ($ONOO^-$) which is toxic and exhibits direct oxidative reactivity. Hence, the rate of NO production and its reactivity relies on the rates of ROS generation in the tissues [17].

1.4.2 Nitric Oxide and endothelial dysfunction in DN

Nitric Oxide is considered the single most important factor for maintaining vascular endothelial function and vascular tone relaxation [17, 19]. Several studies have shown that NO produced by endothelial cells through eNOS plays a major role for maintaining vascular function and homeostasis, and that decreased NO production and bioavailability in effect contribute to endothelial dysfunction in diabetes [17, 20-21]. Moreover, the development and progression of DN is associated with alterations in eNOS expression and activity [17, 22]. The fact that lack of eNOS can contribute to both glomerular and tubulointerstitial fibrosis suggests that disruption of NO signaling in the kidney may be a major factor in the development of DN [17].

Nitric Oxide is a gaseous free radical molecule which is synthesized by the action of the enzymes Nitric Oxide Synthase (NOS) using as substrate L-arginine and yielding L-

citrulline. It plays several physiological roles in the kidney, including control of renal and glomerular hemodynamics, promoting natriuresis and diuresis, and renal adaptation to dietary salt intake [23]. There are three different isoforms of NOS: endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) enzymes, and the three are present in the kidneys [20, 21]. The eNOS isoform was shown to be the major NOS enzyme in renal vasculature, nNOS is more abundant in the macula densa whereas iNOS is weakly expressed in the kidney [23, 24].

The eNOS enzyme is only fully functional in a dimeric form and the functional activity of the dimer depends on the level of tetrahydrobiopterin (BH4), a cofactor for NOS enzyme [17, 21-25]. Under physiological conditions eNOS exists as a dimer and produces NO but the enzyme reduces oxygen to superoxide anion when there is decreased availability of BH4. Additionally, the uncoupling of eNOS induced by oxidation of the enzyme zinc-thiolate cluster by small amounts of $ONOO^-$ decreases NO synthesis and increases superoxide anion production by the enzyme [17]. Diabetes is associated with eNOS uncoupling and decreased BH4 levels due to its oxidation by ROS. This condition promotes further oxidative stress and decreased NO bioavailability [17, 25]. Indeed, uncoupling of eNOS is one of the pathways identified as potentially major contributors of the pathogenesis of diabetic kidney disease [15].

Dysregulation of NO has been described in patients with DN, including increased eNOS activity early after the onset of diabetes, with augmented basal NO release which might explain intrarenal vasodilation and hyperfiltration seen in this first stage. Prolonged diabetes is associated with downregulation of glomerular or renal eNOS expression which leads to NO deficiency that may facilitate the progression of DN [20, 21, 26]. The other isoforms nNOS and iNOS are expressed variably in diabetic kidneys [24].

Additionally, in vitro studies have evidenced that hyperglycemia reduces eNOS activity and/or NO bioavailability in endothelial cells leading to decreased endothelial cell survival and endothelial dysfunction [11, 19]. Also, the combination of eNOS deficiency and

hyperglycemia leads to podocyte injury [26]. In fact, eNOS deficiency by itself provokes profound effects on podocyte integrity and leads to albuminuria [12, 26].

Increased evidence shows the cross-talk between glomerular endothelial cells and podocytes through the secretion of cytokines and growth factors. This cross-talk can influence the behaviour of the glomerular filtration membrane and the susceptibility for the development and progression of kidney disease [27]. Indeed, there is evidence that the severity of DN lesions and renal function in type 1 diabetic patients is associated with reduction of glomerular endothelial cells fenestration and increase in podocyte detachment [28].

Accordingly, the understanding of the mechanisms and mediators involved in the pathophysiology of DN including the communication between the cells in the kidney is an important action to tackle the development of new potential therapies in order to prevent early complications.

1.5 Therapeutic strategies in DN

The major therapeutic strategies involved in the management and prevention of DN have included intensified glycaemic control and antihypertensive agents particularly those which interrupt the renin-angiotensin system such as angiotensin receptor blockers (losartan or irbesartan) [12, 13]. Recently, novel strategies have been under clinical investigations and include inhibitors of AGEs, inhibitors of metabolic pathways such as specific PKC isoforms or the enzyme aldose reductase, or inhibitors of vasoactive factors [29]. All of these targeted pathways seem to have a common mechanism as they are triggered by oxidative stress, and some contribute to enhance the oxidative stress inside the cells. It is predicted that multiple therapies will be required to reduce the progression of DN.

Considering that hyperglycemia-induced oxidative stress plays such an important role in the pathogenesis of diabetic complications, the investigation of new molecules with preventative effects or protective action against oxidative stress may be a wise adjuvant therapy required to further optimize renoprotection in diabetes. Some molecules that

have conferred protection to the cells from oxidative damage are the acidic and basic fibroblast growth factors (aFGF, bFGF).

1.6 The acidic and basic Fibroblast Growth Factors

The aFGF (or FGF1) and bFGF (or FGF2) belong to a large family of heparin-binding growth factors (polypeptides) that has 23 members to date. Both are considered prototype members of this family and are closely related molecules: they have 53 % sequence homology, interact with the same receptor and posses mitogenic properties toward fibroblasts and endothelial cells [30, 31, 32]. They can affect differentiation, migration and survival of many cell types, playing an important role in the control of nervous system, wound healing and in tumor angiogenesis. FGFs mediate their cellular responses by binding to and activating a family of 4 receptor tyrosine kinases. These peptides are located in the ECM, bind to heparin or heparan-sulfate proteoglycans which act as an accessory molecule that regulate FGF-binding and the activation of the occupied signaling receptors [30-33].

The four isoforms of the receptors (FGFR1-4), encoded in four different genes, are generated by alternative splicing of FGFR transcripts [33]. The activation of these receptors initiates further downstream signaling by mitogen-activated protein kinase (MAPK), phosphatidilinositol-3 kinase (PI-3K) and phospholipase C [31-33]. This signalling is mediated via direct recruitment of signalling proteins that bind to tyrosine auto-phosphorylation sites on the activated receptor and, via closely linked docking proteins (that become tyrosine phosphorylated in response to FGF-stimulation), form a complex with additional complement of signaling proteins [33] (Figure 1.2). The receptors seem to mediate activation of the same targets and differ only in the strength of their tyrosine kinase activity. Furthermore, they have different abilities to mediate the translocation of external FGF to the cytoplasm and nucleus, which appears to be due to variation in their C-terminal tail. As a result, FGFR1 and FGFR4 are able to mediate translocation whereas FGFR2 and FGFR3 lack this ability [34]. It has been shown that this translocation to the

nucleus determines the mitogenic activity of FGFs since it is a necessary condition to activate DNA synthesis and proliferation [35, 36] (Figure1.2).
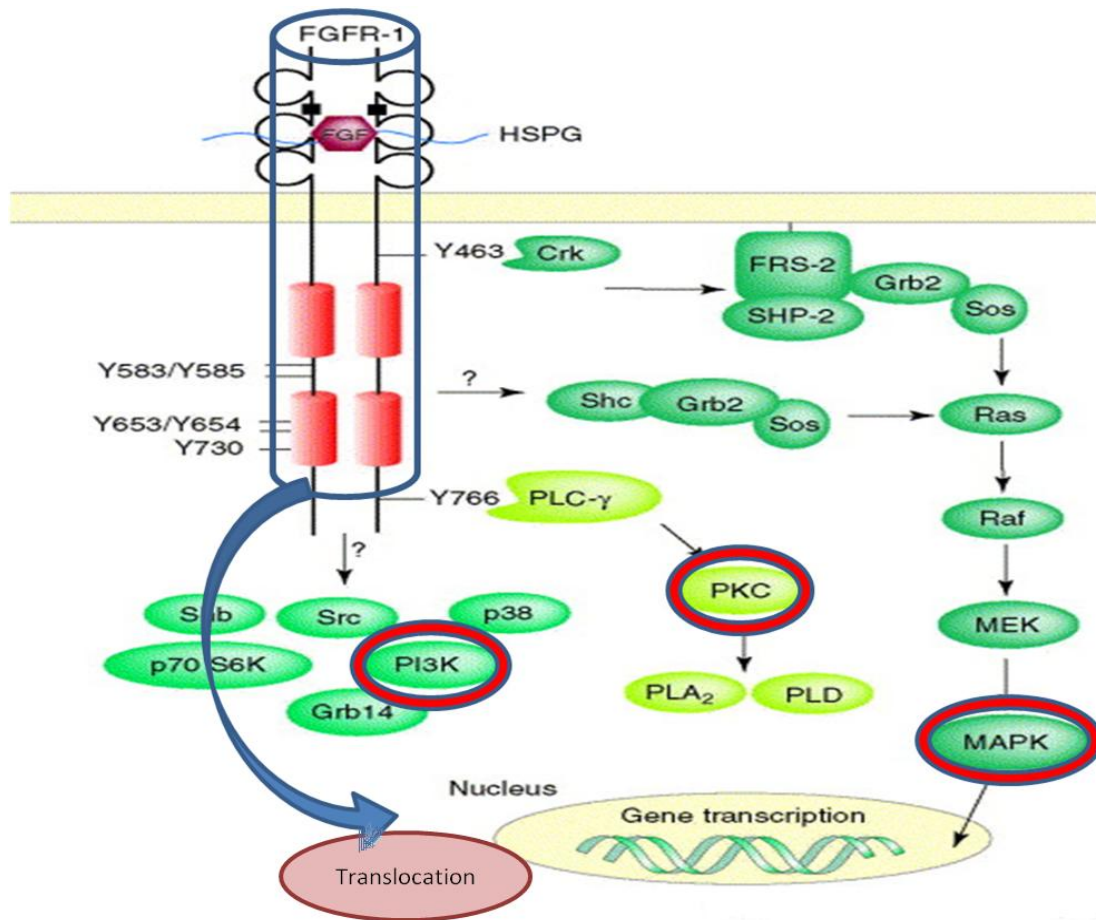
Figure 1.2 Diagram depicting signaling pathways downstream of FGFR-1 upon tyrosine phosphorylation. Besides the activation of the downstream signalling by MAPK (mitogen-activated protein kinase), PI-3K (phosphatidilinositide-3 kinase) and PKC (protein kinase C), the receptor complex is translocated to the nuclei for activation of DNA synthesis. This is thought to be a necessary condition to induce proliferation (Figure adapted from reference # [37]).

Some studies show that FGFs interact with the FGFRs based on its tridimensional structure. The FGF residues interacting with the specific tyrosine kinases cluster in two separate patches of its three dimensional structure: recognition site 1 (RS1) which seems to be important for mitogenesis and recognition site 2 (RS2) that seems to be involved in vasodilatory and ischemic protective actions and have a less important rate in the mitogenic activity of the protein [38].

FGF has been found to be in an inactive form in the cytoplasm and is activated by cell injury [33]. It is produced by endothelial cells, fibroblasts, macrophages, smooth muscle cells, myoblasts, lens epithelial cells and osteoblasts [30, 33].

1.6.1 The aFGF and bFGF as non-mitogenic and pleiotropic peptides

FGFs are implicated in a wide range of pathological conditions such as tumorigenesis and metastasis, mainly due to its potent angiogenic activity. In the context of diabetic complications, bFGF has been reported to contribute to the glucose-induced vascular dysfunction [39], and is upregulated in diabetic retinopathy [40, 41, 42, 43, 44]. Particularly in DN, there are reports of upregulated expression of bFGF with a good correlation to the degree of renal injury [32]. Furthermore, it is known that bFGF is induced in human renal fibrosis (characteristic of DN) and induces proliferation in human renal fibroblasts after activation by TGF-β1 [39].

On the other hand, there is evidence of the survival effects of FGFs in the context of oxidative stress. As these peptides are multifunctional, multiple studies are based on the nonmitogenic properties of FGFs in differentiated cells. In the retina, basic FGF has been found to augment endothelial cells' resistance to oxidative stress [45] and improves recovery from ischemia and reperfusion injury in brain and kidney [46,47]. In the heart, aFGF has attenuated tissue damage after myocardial ischemia followed by reperfusion [48], and has shown protection on ischemic kidney, liver and gut injuries [49].

The FGFs can also produce vasoactive responses as confirmed in some studies where aFGF and bFGF were found to induce vasodilation in microvessels indicating that FGFs are able to stimulate endothelial NOS expression and subsequent synthesis of NO [50, 51, 52]. These results are evident not only in endothelial cells but in smooth muscle cells and some types of cancer cells, and may be associated with the survival actions of FGFs [51, 52, 52, 53, 54].

However such potential therapeutic applications have been limited due to the presence of the mitogenic property of these molecules. Hence, the use of a non-mitogenic form of FGF would potentially avoid undesirable proliferative effects observed with the native mitogenic proteins.

1.6.2 The modified human aFGF

Considering the various functions of FGFs, some modifications have been done to the native FGF molecule to uncouple the mitogenic activity from the vasodilatory and antiapoptotic actions [38, 47, 55, 56, 57, 58, 59, 60, 61]. Although the primary translation product for aFGF is a 155 aminoacids protein, it could result in polypeptides of 134, 140 or 154 residues due to specific proteolytic cleavages [62, 63]. Several studies have also been made with non mitogenic human acidic fibroblast growth factor (nhaFGF; nm-aFGF), recombinant human acidic FGF (FGF28-154) or modified human acidic FGF (maFGF), which are devoid of mitogenic activity and show potent antiapoptotic actions [38, 55, 57-61].

The mutated aFGF has been generated by deleting the sequence of the first 27 aminoacids near the NH2-terminus (FGF28-154) [57]. This modification suggests that the lack of mitogenic activity of this polypeptide derives from the alteration of the RS1 tridimensional structure. Moreover, this change is considered to confer instability to the maFGF to successfully interact with the receptor. This mutated domain has the role to maintain the structural integrity of aFGF required for optimal binding to and activation of the transmembrane receptor complex [38, 64]. The truncated protein does not induce cell division even though it is recognized by the cell membrane receptor, triggers the

early mitogenic events, and retains the neuromodulatory, vasoactive, and cardio- and neuroprotective properties of the native full-length molecule [38].

Recent studies using maFGF have shown cardiovascular protection from oxidative stress in vitro and in vivo [58] and protection from the cytotoxic effects of hydrogen peroxide treatment in cultured cardiomyocytes [59]. The effects of ischemia/reperfusion- induced cardiac dysfunction and tissue damage were significantly attenuated in rats with in-vivo-administered maFGF [48]. Similar results were obtained in cerebral ischemia/reperfusion injury which was prevented by the same treatment [59]. Oxygen free radicals generation is one of the mechanisms responsible for ischemia-reperfusion damage. Moreover, some of these studies reveal that the protective effect of the maFGF is associated with increase in cellular antioxidant activity [59, 60]. Additionally, the maFGF treatment has shown prevention of diabetic cardiomyopathy [61]. These latter results suggest protection through attenuation of cardiac oxidative stress, hypertrophy, and fibrosis.

Since hyperglycemia-induced oxidative stress plays an important role in the pathogenesis of DN, we hypothesize that maFGF prevents kidney damage in DN.

1.7 Rationale

Metabolic changes in DN induce oxidative stress that triggers the activation of pathological mechanisms which lead to dysfunction of endothelial cells and glomerular visceral epithelial cells and eventually apoptosis. Since modified aFGF is a non-mitogenic form of FGF that has antiapoptotic and protective actions from oxidative damage in tissues, it is possible that it could preserve the functionality of these cells and prevent or attenuate DN. The role of FGFs in diabetic nephropathy is not very well defined; hence the study of the maFGF could shed light to the possible ways that the native FGF exerts its actions in the metabolic changes in DN, granting further importance to this study.

1.8 Hypothesis

The study is based on the hypothesis that modified aFGF treatment has a protective effect in diabetic nephropathy

1.9 Specific Aims

In order to test this hypothesis we formulated two specific aims.

Aim 1: To investigate the effects of the maFGF on the biochemical, functional, and structural changes in the kidneys of type 1 diabetic mice.

Aim 2: To investigate the effects of the maFGF action in preventing glucose induced alterations in vitro.

Chapter 2: Materials and Methods

2.1 In vivo studies

Groups of male FVB mice 8-10 weeks of age (Jackson Laboratory, Bar Harbor, Maine) received intraperitoneal injections of streptozotocin (STZ) (Sigma-Aldrich, St Louis, MO) dissolved in 0.1M sodium citrate (PH 4.5) at 50 mg/Kg body weight daily on 5 consecutive days. Control mice received multiple injections of the same volume of sodium citrate buffer. Five days after the last injection of STZ, the blood glucose was measured. Mice with hyperglycemia (blood glucose levels 250 mM/dL) were defined as diabetic. Groups of control and diabetic mice were also subjected to treatment (intraperitoneal (IP) injections) with or without maFGF (produced by our collaborators using gene engineering) (10μg/Kg) every day for 1 month or for 6 months. Animals were monitored for body weight and blood glucose weekly and urine was collected before the end of the experiment. After each time point, animals were sacrificed and plasma, serum and kidney tissues were collected.

Production of maFGF:

Generation of the maFGF (aFGF28-154) has been previously described [57]. Briefly, it was generated by amplification of appropriate DNA fragments followed by subcloning the products into pET vectors. The vectors were expressed in BL21 (DE3) cells (strain of E. coli) and purified on an M2 agarose affinity column. The powder form was then reconstituted in PBS and stored in -80°C until needed.

2.2 In vitro studies

2.2.1 Human microvascular endothelial cells (HMVEC):

Dermal-derived human microvascular endothelial cells (HMVECs) (Lonza Walkersville, MD) were cultured in EBM-2 media containing 5mmol/L D-Glucose, 10% fetal bovine serum (FBS) (Sigma-Aldrich, Oakville, ON) and necessary supplements provided in EBM2 single kits. Cells were grown and maintained in a humidified atmosphere containing 5% CO_2 at 37 °C. At 80% confluence, cells were arrested in serum-free media with no growth factors overnight, before incubation with glucose (25mmol/L). The experiments were

performed after 24 or 48 hours of glucose incubation with or without maFGF 1 hour pre-treatment, at the dose 200ug/ml or aFGF (200ug/ml) as positive control. These doses were chosen based on previous experiments performed in our lab with HMVEC. For some experiments, 500 µmol/L of *N*-nitro-L-arginine methyl ester hydrochloride (L-NAME) (Sigma-Aldrich), an inhibitor of NOS, was added to the serum-free media when cells were arrested. Osmotic controls were also used where necessary (20mmol/L L-glucose + 5mmol/L D-glucose). All experiments were performed with 3 to 5 replicates.

2.2.2 Podocytes (E11 cell line)

The E11 cell line, conditionally immortalized mouse podocytes were obtained (CLS Eppleheim Germany), and was handled as described [42]. The growing cells were maintained under permissible conditions: in RPMI 1640 media (Life technologies) supplemented with 50 units/ml mouse recombinant- interferon ɣ (Prospec Protein Specialists), 100 units/ml of penicillin/streptomycin and 100 units/ml of antimicotic in collagen-coated flasks at 33°C. When the cells reached 60% confluence, they were passage and allowed differentiating under non permissive conditions before any experimental manipulation: at 38°C for 14 days without interferon ɣ in RPMI 1640 media. Podocyte differentiation was confirmed by expression of Synaptopodin (mRNA expression) (Sigma-Aldrich). When confluent, cells were arrested overnight in RPMI-1640 serum-free media containing 0.2% BSA before incubation with glucose (30mmol/L). The experiments were performed similar to HMVEC. The dose used for maFGF and the native aFGF treatment was 20ug/ml, chosen after dose-response experiments conducted with 5ug/ml, 10ug/ml and 20ug/ml.

2.3 RNA extraction and real-time PCR

RNA isolation was done using the Trizol reagent (Invitrogen Burlington, Canada) following the protocol established in our lab. RNA was extracted with 0.2 ml chloroform added to the trizol. Following centrifugation at 15000 x g for 15 mins at 4°C, the aqueous phase was separated from the organic phase and placed in a fresh tube. RNA was

recovered from the aqueous phase by isopropyl alcohol precipitation using equal volume of the aqueous phase. The samples were vortexed and centrifuged at 15000 x g for 15 mins at 4°C and the pellet was washed with 75% ethanol in diethylpyrocarbonate (DEPC) -treated water and dried. The total RNA was suspended in 15 ul DEPC water and the concentration was quantified with spectrophotometry at 260 nm (Gene Quant, Pharmacia Biotech, USA). Total RNA (2 ug) was used for cDNA synthesis with high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, USA). Real-time RT-PCR was performed by using the LightCycler 96 (Roche Diagnostics Canada, Laval QC) and SYBR Green detection (Clontech, Mountain View, USA). According to the manufacturer's protocol, for a final reaction volume of 20 uL the following reagents were added: 10 uL SYBR Advantage qPCR Premix, 1 uL each of forward and reverse primers (10 mmol/L), 7 uL H_2O, and 1 uL cDNA template. Primers for mouse or human β-actin, ANG, TGFβ-1, eNOS, iNOS, FN, Col4α1, VEGF, Bcl-2 and Bak-1 are listed in table 2.1 (Sigma-Aldrich) and HO1 were obtained (Qiagen, Germantown, MD). The mRNA levels were quantified by using the standard curve method. Standard curves were constructed by using a serially diluted standard template. The data was normalized to β-actin ribosomal RNA to account for differences in reverse transcription efficiencies and the amount of template in the reaction mixtures.

Table 2.1 Oligonucleotide sequences RT-PCR

Gene	Primer sequences (5'-3'): Forward Reverse
β-actin (mouse/human)	CCTCTATGCCAACACAGTGC CATCGTACTCCTGCTTGCTG
VEGF (mouse/human)	GGCCTCCGAAACCATGAACTTTCTGCT GCATGCCCTCCTGCCCGGCTCACCGC
Col4α1 (mouse)	ACAGGCACAAGTTAGGAAA ATCTCCTTTCTCTCCCAAG
FN (mouse)	CGGTGTTTTTTACGGGAGTTTC GCGCACACACTCGCACACACG
TGFβ-1 (mouse)	GGACTCTCCACCTGCAAGAC GACTGGCGAGCCTTAGTTTG
iNOS (mouse)	CTGGCTCGCTTTGCCACGGA GCTGCGACAGCAGGAAGGCA
eNOS (mouse)	TTGGCGGCGCAAGAGGAAGG TCTCGGGCAGCAGCTTTGGC
ANG (mouse)	CACCCCTGCTACAGTCCATT GTCTGTACTGACCCCCTCCA
Bak1 (mouse)	CATCCACCACCATGAATCCAC CTCCAGATGTGGGATGGCTC
Bcl-2 (mouse)	CCTGTGGATGACTGAGTACC GAGACAGCCAGGAGAAATCA
Synaptopodin (mouse)	TTGCTTAGGGCGCTGAGTGG CTCCTTCAGGTCGTTTTCGGT
Col4α1 (human)	CAAGAGGATTTCCAGGTCCA TCATTGCCTTGCACGTAGAG
FN (human)	GATAAATCAACAGTGGGAGC CCCAGATCATGGAGTCTTTA
TGFβ-1 (human)	CAACAATTCCTGGCGATACC CGAAAGCCCTGTATTCCGTCTCC
eNOS (human)	TCGACCCAGGATGGGCCCTG CGTCACGGGGTTTCTGGGGC
ANG (human)	GCAGATAACAACCCCGGACA TGCAGGCTTCTACTGCTCAC
Bak1 (human)	GGGGTGTAGATGGGGGAACT GGGTGAGGGGATTGCACAGTTTA
Bcl-2 (human)	TTGCTTTACGTGGCCTGTTTC GAAGACCCTGAAGGACAGCCAT

2.4 Measurement of renal parameters

The albumin excretion was measured after 1 month and 6 months of the onset of type 1 diabetes and normalized in relation to creatinine in order to measure renal function. Urinary albumin was measured using the murine microalbuminuria ELISA kit (Albuwell Philadelphia, PA) following the manufacturer's protocol. Mouse urine was collected by placing the animals in a metabolic cage for 24 hours before the animals were euthanized. The collected urine was stored at -80°C for future use. Urine concentration for each sample was diluted relative to the standards before performing the assay. Duplicate samples, controls and standards were incubated with Rabbit Anti-murine Albumin Antibody for 30 minutes (primary incubation). After 10 wash cycles with wash buffer (provided by the kit), the test plate was incubated with Anti-rabbit HRP conjugate for 30 minutes (secondary incubation). The color development was obtained after washing the plate and adding the chromogenic substrate. After 5-10 minutes, the color stopper was added to each well to terminate the color reaction. The plate was then examined in a Multiskan FC Microplate Photometer (Thermo Scientific, Finland) to determine the absorbance at 450nm.

The determination of creatinine was done using a Creatinine Companion kit (Albuwell Philadelphia, PA) to normalize albumin in relation to creatinine. The same specimens used for the albumin quantification were used for creatinine determination following the manufacturer's protocol. The procedure uses an adaptation of the alkaline picrate method and requires determination of the differential absorbance in a sample before and after the addition of acid to correct for color generation due to interfering substances. Hence, the absorbance was determined on the plate reader at 500 nm in two different time points: 10 minutes after adding the picrate working solution and 5 minutes after adding the acid reagent.

2.5 Protein extraction and ELISA

Kidney tissues were washed with cold phosphate buffered saline (PBS) and homogenized in 0.4 ml RIPA lysis buffer (Upstate, Temecula CA) with a micro tissue grinder on ice. Homogenates were centrifuged for 5 minutes at 5000 rpm and the supernatant was aliquot and stored or used for the assay. The total protein concentration was measured using BCA^{TM} protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. The concentrations of all samples were adjusted before performing ELISA. We performed FN (Kamiya Biomedicals, WA, USA) and Col1 α(IV) (Cloud- Clone Corp, TX USA) protein measurement following the manufacturer's protocols.

2.6 Cellular reactive oxygen species levels

Intracellular ROS generation was assessed using a dichlorofluorescein diacetate (DCFDA) Cellular ROS Detection Assay kit (Abcam Toronto, ON) according to the manufacturer's instructions. Podocytes were seeded 2.5x10^4 cells per well on a dark 96 well microplate, they were allowed to adhere overnight. The next day, cells were starved overnight by replacing the media with serum-free media. On the following morning, cells were incubated with high glucose (25mmol/L) for 48 or 72 hours with or without maFGF one hour pre-treatment at the dose 20μ/ml or aFGF (20μg/ml) used as positive control. At both time points, the cells were incubated with DCFDA solution for 30 minutes at 37°C in the dark. Plate was read in a Gemini Fluorescence Microplate Reader with excitation wavelength at 485 nm and emission wavelength at 535 nm. The data generated as ratio of the relative fluorescence intensity of control and treated wells to the relative fluorescence intensity of the blank wells. Data were normalized respective to percentage of number of cells.

2.7 Antioxidant enzymes activity

Catalase and Glutathione Peroxidase activities were assessed through a Catalase assay kit and a Glutathione Peroxidase assay kit (Cayman Chemical Ann Arbor, MI) following the manufacturer's instructions. Tissue was homogenized on ice in 5 ml of cold buffer

consisting in 50mM potassium phosphate, ph 7.0, containing 1mM EDTA. The homogenates were centrifuged for 15 minutes at 8000 rpm at 4°C. The supernatant was aliquot and used for the assays on the same day or stored at -80°C. Catalase activity was expressed as nmol of formaldehyde produced per minute in the presence of H_2O_2. Glutathione peroxidase activity was expressed as the amount of enzyme that caused the oxidation of 0.1 nmol of NADPH to NADP$^+$ per minute. Both enzymes activity were reported respective to ug of proteins.

2.8 NO Assay

Total Nitric Oxide production was measured in kidney tissue homogenates and cell lysates using a fluorometric nitric oxide assay (Abcam, Toronto Ontario). The total concentration of nitrite and nitrate was used as a quantitative measure of NO production. The enzyme cofactor working solution for Nitrate Reductase was added to the wells followed by the addition of Nitrate Reductase. In this step nitrate is converted to nitrite. The plate was incubated at room temperature for 2 hours. Afterwards, enhancer was added and incubated for 30 minutes to quench interfering compounds. The fluorescent probe DAN (2, 3-diaminonaphthalene) that reacts with nitrite, was added and incubated for 10 minutes followed by the addition of NaOH for 10 minutes to increase the fluorescent field. The plate was read in a Gemini Fluorescence Microplate Reader with excitation wavelength at 360 nm and emission wavelength at 450 nm.

2.9 TUNEL Assay

Apoptotic assays were performed by Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) reaction using In Situ Apoptosis Detection Kit (Takara Bio Inc). Podocytes were cultured on eight-chamber tissue culture slides and incubated for 48 hours with the presence of glucose (25mmol/L) and maFGF (20μg/ml), aFGF (20μg/ml) was used as positive control. The cells were counterstained with DAPI for nuclear staining.

For tissues embedded in paraffin, deparaffinization and rehydration (using xylene and ethanol dilutions) was performed. The sections were treated with proteinase K (20 mg/L) for 15 min and then incubated with the TUNEL reaction mixture containing terminal deoxynucleotidyl transferase (TdT) and digoxigenin-11-dUTP for 1 h. The TdT reaction was carried out in a humidified chamber at 37°C. For the negative control, TdT was omitted from the reaction mixture. Apoptotic cell death was quantitatively analyzed by counting the TUNEL-positive cells selected randomly from 10 fields. Microscopic observation was performed by an examiner unaware of the identity of the sample, using a Zeiss LSM 410 inverted laser scan microscope equipped with fluorescein, rhodamine, and DAPI filters (Carl Zeiss)

2.10 Western Blotting

Kidney tissues or cell extracts were sonicated and lysed in 0.4 ml or 0.1 ml RIPA (Upstate, Temecula CA) lysis buffer respectively. The tissue and cell extracts were centrifuged at 3000 rpm at 4°C for 30 minutes to remove cell debris. The total protein concentration was measured using BCA^{TM} protein assay kit (Pierce, Rockford, IL, USA) using BSA as protein standard. Proteins were electrophoresed through a 10% SDS-PAGE gel before transferring to a PVDF membrane. After blocking for 30 minutes at 4°C in blocking buffer (3% BSA in PBS with 0.1% Tween 20), the membrane was incubated overnight with mouse anti-eNOS (1:1000) or mouse anti-peNOS (1:1000) (Santa Cruz Biotechnology, Inc). The membrane was washed and incubated for 30 minutes at room temperature with a goat anti-mouse antibody conjugated with HRP. After further washing, the membrane was detected with ECL kit (Amersham Pharmacia Biotech, Arlington, IL, USA). β-actin was used as an internal controls and detected by rabbit anti- β-actin antibody conjugated with HRP. Western blotting images were captured by Kodak 4000 mm and density of the bands was quantitated by using ImageJ

2.11 Cell Viability Assay

To determine the effects of maFGF treatment in the proliferation of HMVEC, the WST-1 Cell Viability Assay (Roche) was used. Cells were seeded (aprox. 5×10^4/well) onto a 96-well plate and were allowed to attach overnight. Starting on the following day, cells were starved overnight by replacing the media with serum-free media. The next morning, maFGF (200ug/ml) was added to the wells for 6, 20, 24 or 48 hours. After each time point, 10μL of WST-1 reagent was added to each well and the plates were incubated for 1 hour at 37°C to produce a colour reaction. Absorbance was measured in a Multiskan FC Microplate Photometer (Thermo Scientific, Finland) at 450nm with a reference wavelength of 690nm.

2.12 Immunofluorescence

HMVECs cells were plated on eight-chamber tissue culture slides and incubated for 48 hours with the presence of glucose (5 mmol/L, 25mmol/L) and maFGF (200μg/ml) or aFGF (200μg/ml), then these cells were fixed with ethanol for staining with 8-OHdG antibody (Santa Cruz Biotehnology, Dallas). Goat IgG labeled with FITC (Vector Laboratories, Burlingame, CA) was used for detection of the fluorescence. Slides were mounted in Vectashield fluorescence mounting medium with 4, 6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame) for nuclear staining. Microscopic observation was performed by an examiner unaware of the identity of the sample, using a Zeiss LSM 410 inverted laser scan microscope equipped with fluorescein and DAPI filters (Carl Zeiss)

2.13 Immunohistochemistry

Formalin-fixed tissues embedded in paraffin were sectioned at 5 μm thickness on positively charged slides. The kidney tissues were analyzed for 8-hydroxy-2-deoxyguanosine (8-OHdG) (Chemicon International Inc. CA, USA). The chromogen 3,3-diamino benzine (Sigma-Aldrich) was used for detection. Non-immune horse serum was used as a negative control. 8-OHdG immunoreactivity was assessed by the presence of

positively stained nuclei in the glomeruli with a dark brown color. The dark brown-stained nuclei were counted as a measurement of 8-OHdG immunoreactivity and the results were expressed as percentage of positive nuclei respective to the total number of nuclei in each glomerulus. The measurement was done in 15 glomeruli for each sample (n=2).

2.14 Histological Analysis

Formalin-fixed tissues embedded in paraffin were sectioned at 5 µm thickness on positively charged slides. The sections were stained with hematoxylin and eosin and periodic acid-schiff stain (PAS). The PAS stain is used to detect extracellular matrix proteins deposition and it gives a purple magenta color as indication of the presence of glycogen, proteoglycans, glycoproteins and glycolipids.

2.15 Statistical analysis

Data is presented as mean ± standard error. Statistical significance of differences between groups was tested with Student's T-test for two groups or one-way ANOVA with multiple comparisons (post hoc analysis) for multiple groups. A p-value of 0.05 or less was considered to be statistically significant. All calculations were performed with GraphPad Prism version 5.03 software.

Chapter 3: Results

3.1 MaFGF treatment did not affect body weight and blood glucose but decreased renal hypertrophy following long term diabetes.

After inducing type 1 diabetes in specific groups of mice, diabetic and age-matched non diabetic mice were divided into groups with and without treatment with maFGF (10μg/Kg daily) for 1 month and 6 months. The body and kidney weights and blood glucose of each group of mice were evaluated after each time point. The body weights were not significant different among the groups at 1 month after the treatment. However the diabetic animals showed a significantly decreased in body weight at 6 months compared to non-diabetic groups. At the same time, the blood glucose was significantly increased in diabetic animals with or without treatment with maFGF both at 1 month and 6 months. Similarly, the ratio kidney weight- tibia length was significantly increased in the diabetic groups at both time points which is probably result of the hypertrophy and increased filtration area of the glomeruli as result of diabetes.

MaFGF treatment didn't show any effects in the body weight and blood glucose compared to the untreated diabetic group after 1 month or 6 months (Fig 3.1-A). However, the kidney weight- tibia length ratio was significantly reduced in the diabetic group after treatment for 6 months (Fig 3.1-B) which indicates prevention of renal hypertrophy.
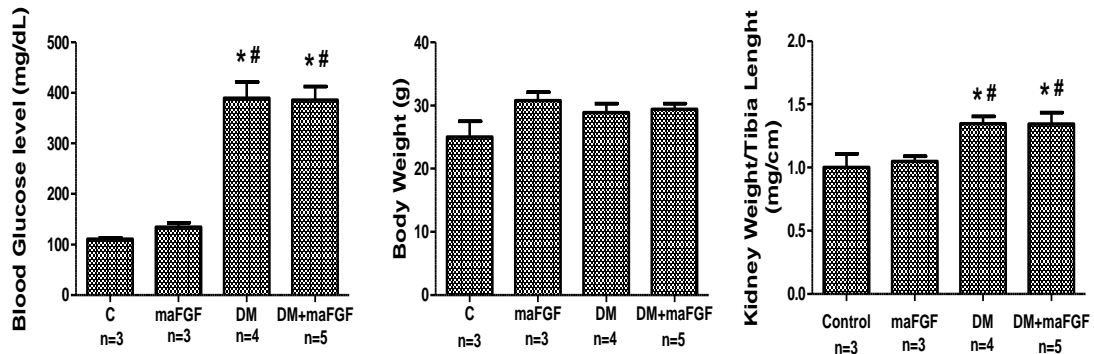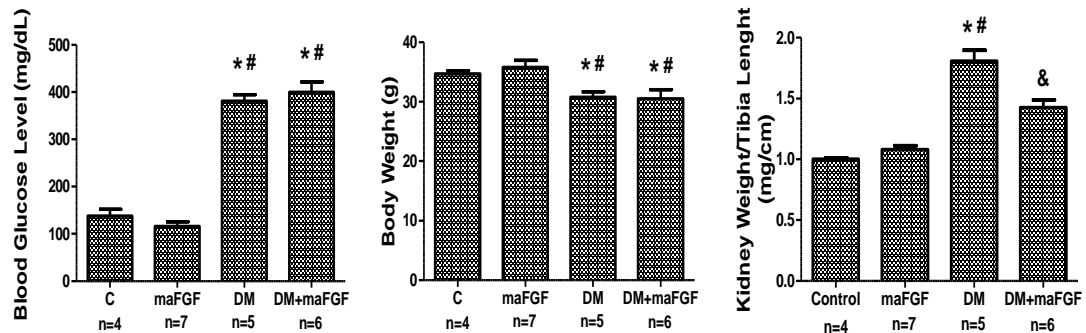
Figure 3.1 Effects of maFGF on body weight, blood glucose levels and kidney weight/tibia length ratio in non-diabetic and diabetic mice after (A) 1 month and (B) 6 months of treatment with maFGF. MaFGF had no effect on body weight or blood glucose levels. However it prevented renal hypertrophy after 6 months of follow up. C: control, maFGF: modified aFGF, DM: diabetes mellitus, DM+maFGF: diabetic treated with maFGF.*p<0.05 vs control, # p<0.05 vs. maFGF, & p<0.05 vs. DM group.

3.2 MaFGF treatment prevented functional damage

Microalbuminuria is an established marker for the renal dysfunction in DN. Following onset of type 1 diabetes the glomerular filtration rate increases due to increased renal blood flow, glomerular capillary hypertension and increased filtration surface. Interestingly, increased excretion of albumin happens and persists along with the declining of GFR which is the result of progressive reduction of the filtration surface possibly due to mesangial expansion [9]. We measured the albumin-to-creatinine ratio (µg albumin/mg creatinine) which is an alternative to 24 urine collection in the detection of microalbuminuria to assess nephropathic change. The results showed that the albumin-to-creatinine ratio significantly increased in the diabetic groups. MaFGF treatment prevented renal functional alterations in diabetes at 1 and 6 months as shown in figure 3.2.

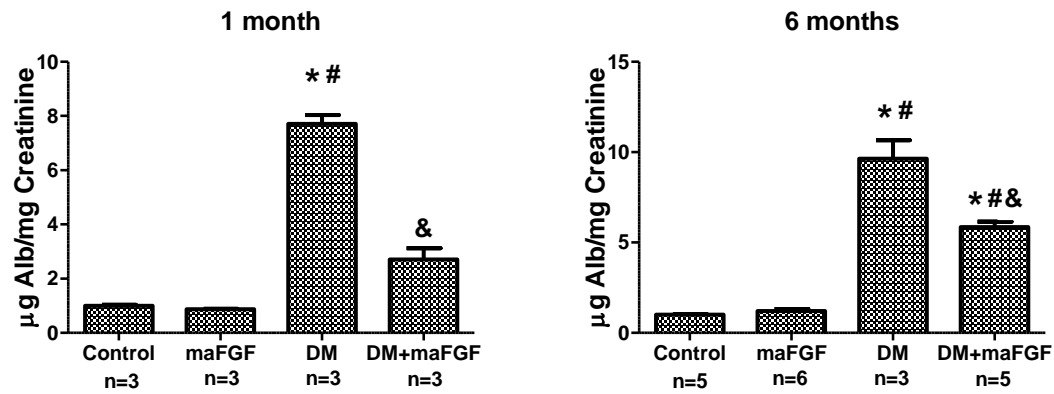Figure 3.2 Effects of maFGF on albuminuria in Diabetic Nephropathy. Urinary albumin-to-creatinine ratio with or without maFGF treatment at 1 or 6 months in diabetic and non-diabetic mice. maFGF prevented diabetes induced albuminuria at both time points. maFGF: modified aFGF, DM: diabetes mellitus, DM+maFGF: diabetic treated with maFGF.*p<0.05 vs control, # p<0.05 vs. maFGF, & p<0.05 vs. DM group.

3.3 MaFGF treatment prevented vasoactive factors alterations in DN.

Current evidence suggests the elevation of several vasoactive factors in early DN such as angiotensinogen (AGT), Nitric Oxide and vascular endothelial growth factor (VEGF). Various of these factors are also associated with elevation of systemic and intraglomerular pressure and increased permeability [8, 9, 11]. With progression of DN, the levels of some of these factors (NO, VEGF) fall possibly due to continue endothelial dysfunction leading to the arrest of endothelial growth and apoptosis. Results show that maFGF treatment prevented diabetes induced alteration of the vasoactive factors transcripts AGT, eNOS, iNOS and VEGF compared to diabetic groups (Figure 3.3). The changes were pronounced after 6 months and maFGF treatment prevented such changes.

The control group for the maFGF treatment didn't show significant difference respective to the control group (non diabetic mice) in the analysis of the transcripts or subsequent analysis performed; therefore it was omitted from the figures.

A
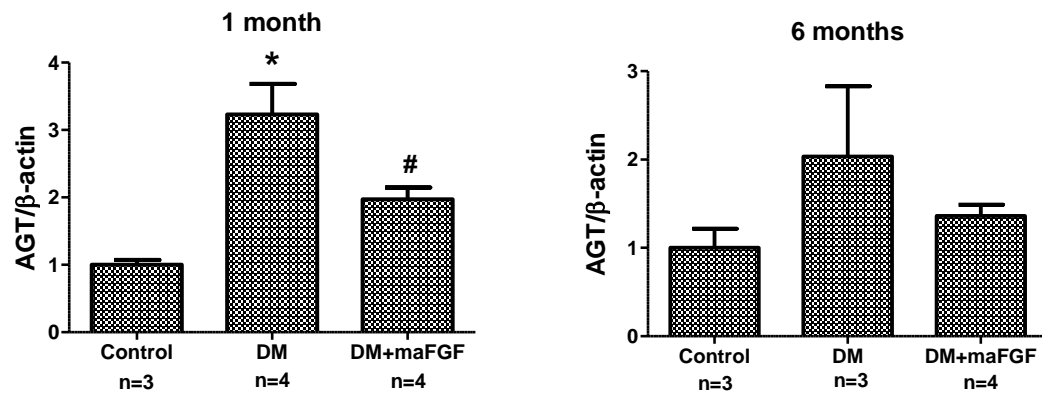
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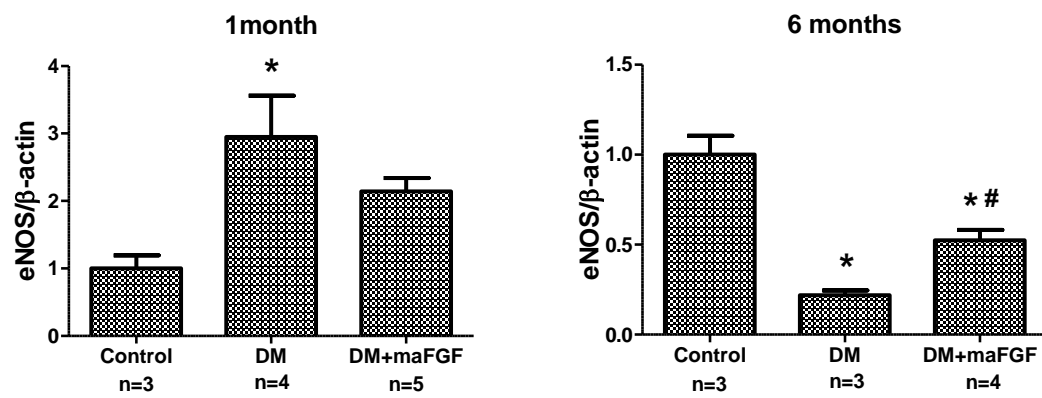
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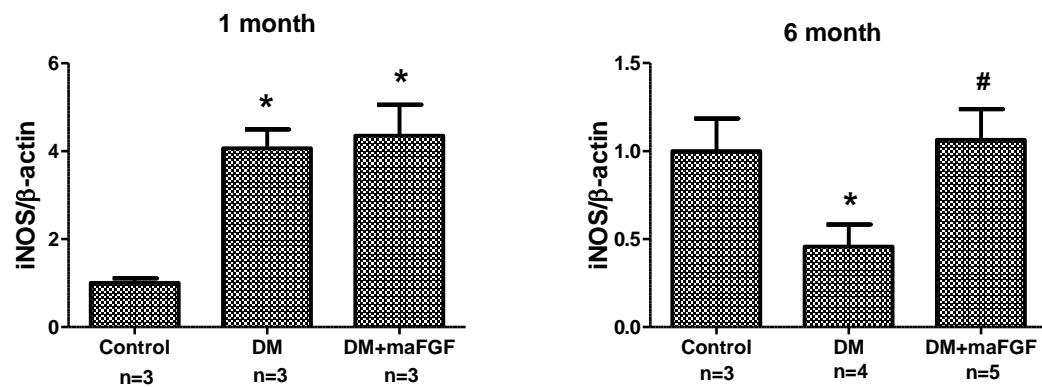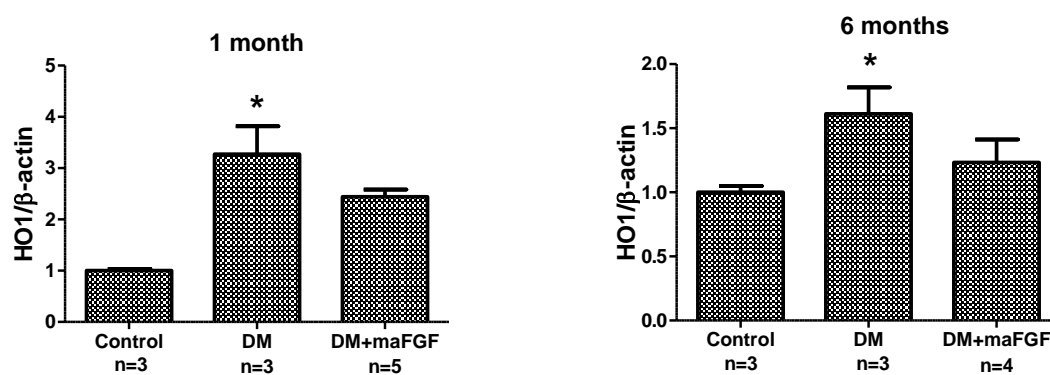
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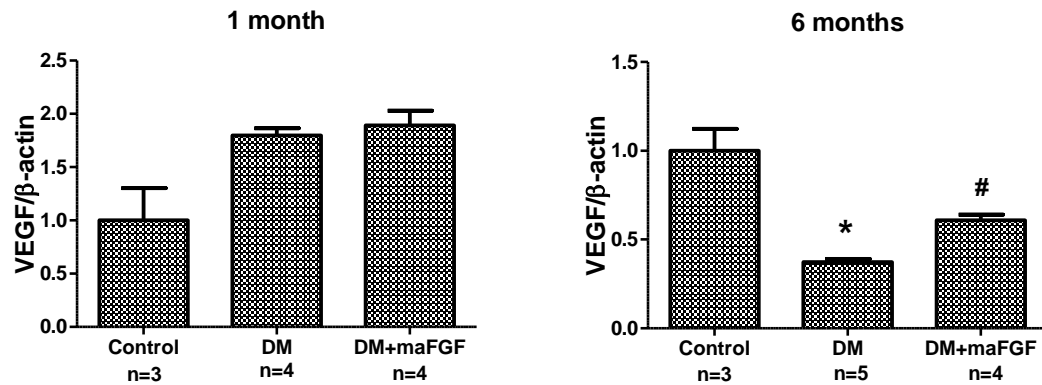
Figure 3.3 Effects of maFGF treatment on vasoactive factor regulation in Diabetic Nephropathy. Quantitative RT-PCR analysis of diabetes induced mRNA expression in mouse kidneys after 1 month and 6 months with maFGF treatment. The treatment prevented increased production of (A) Angiotensinogen (AGT) and significantly prevented down-regulation of (B) endothelial Nitric Oxide Synthase (eNOS), (C) inducible Nitric Oxide Synthase (iNOS) and (D) Vascular Endothelial Growth Factor (VEGF) at the later time point (6 months). DM: diabetes mellitus, DM+maFGF: diabetic treated with maFGF.*$p < 0.05$ vs control, # $p < 0.05$ vs. DM group.

3.4 MaFGF treatment decreased diabetes-induced oxidative stress and DNA damage in the kidney

Development of DN is related to the induction of renal oxidative stress. We examined the effects of diabetes-induced oxidative stress through various methods in kidney tissue of type 1 diabetic mice. Diabetic animals showed increased HO1 transcript levels, a marker of oxidative stress, at 1 month and 6 months after the onset of diabetes (Figure 3.4-A). Treatment with maFGF reduced the upregulation of this enzyme at both time points. We further assessed the activity of enzymes that are activated by oxidative stress such as Catalase (Figure 3.4-B) and Glutathione peroxidase (Figure 3.4-C). Such activity was increased in diabetes and reduced with the maFGF treatment after 1 month and 6 months with significant difference in the case of Catalase activity. In addition, DNA damage of kidney tissues showed increased immunohistochemical staining for 8-OHdG (Figure 3.4-D) in the nuclei of glomeruli cells of diabetic animals and the treatment with maFGF reduced the number of positive cells.

A

B
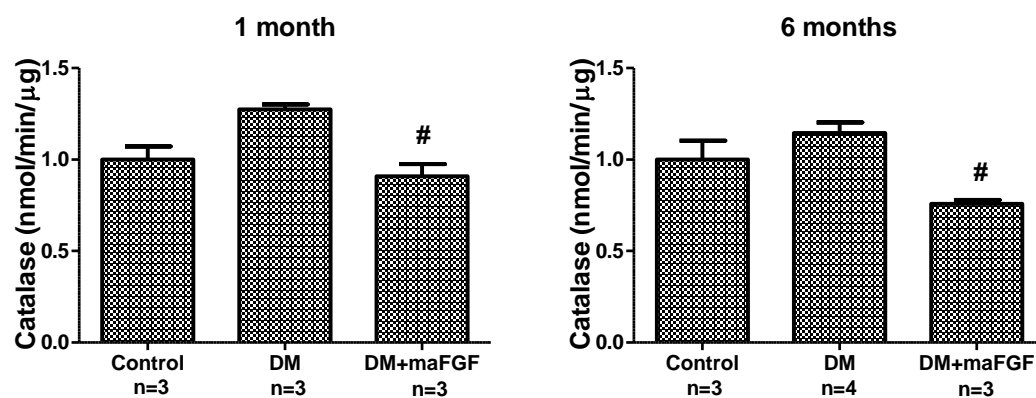
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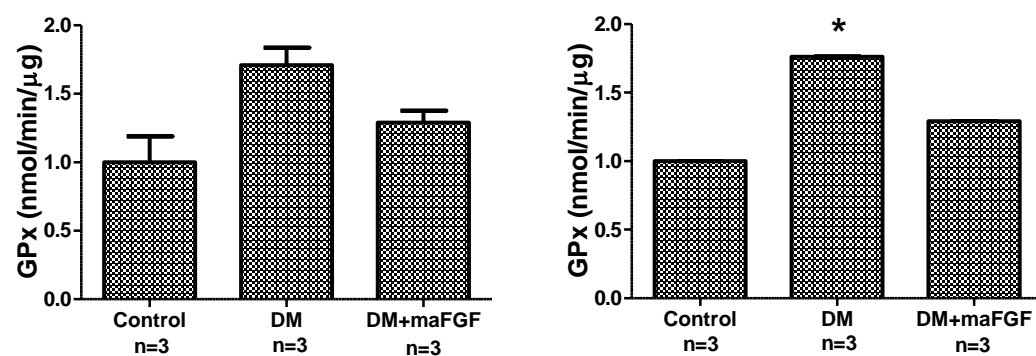
Figure 3.4 Effects of maFGF treatment on diabetes induced oxidative stress. RT-PCR analysis of diabetes induced mRNA expression for the enzyme (A) Heme Oxygenase 1 (HO1), a marker for oxidative stress, in mouse kidneys after 1 month or 6 months with maFGF treatment. (B) Catalase (Cat) activity (expressed as nmol of formaldehyde produced/min in the presence of H_2O_2 respective to µg of protein) and (C) Glutathione peroxidase (GPx) activity (expressed as the amount of enzyme that caused the oxidation of 0.1 nmol of NADPH to $NADP^+$/min respected to µg of proteins) indicating that diabetes-induced Cat and GPx activities were reduced by maFGF treatment at both time points. DM: diabetes mellitus, DM+maFGF: diabetic treated with maFGF.*$p < 0.05$ vs control, # $p < 0.05$ vs. DM group.

D

Figure 3.4- (D) Effect of maFGF treatment on diabetes induced oxidative DNA damage. 8-OHdG shows diabetes-induced increased positivity in the glomeruli cells and the preventative effects of maFGF treatment versus diabetic. 8-OHdG: 8-hydroxy-2-deoxyguanosine. Magnifications 60X, arrows indicate positive nuclei. n=2x15 glomeruli. DM: diabetes mellitus, DM+maFGF: diabetic treated with maFGF.*p<0.05 vs control, # p<0.05 vs. DM group.

3.5 MaFGF partially prevented fibrotic response in DN

Fibrosis is one of the hallmarks of DN. The increased deposition of extracellular matrix proteins (ECM) leads to diffuse thickening of the glomerular and tubular basement membranes and mesangial matrix expansion. We investigated the effects of maFGF treatment on the diabetes induced alteration of the fibrogenic cytokine TGF-β1 and extracellular matrix proteins FN and Col 1α (IV).

The results showed that the induced fibrogenic cytokine TGF-β1 was not prevented by maFGF treatment, as shown in the mRNA expression. However, the maFGF treatment showed some preventive effects on alterations of the ECM protein FN and Col 1α (IV) mRNA (Figure 3.5) and protein expression (Figure 3.6), especially when the maFGF was administered for longer period. Histological analysis of the extracellular matrix in the kidney through acid-Schiff stain (PAS) was performed to conclude whether these molecular alterations produced structural changes in the organ (Figure 3.7). These stains confirmed deposition of extracellular matrix proteins in diabetes suggestive of mesangial expansion, as it was previously indicated by upregulated transcripts and protein expression of Col 1α (IV). The maFGF treatment failed to prevent the fibrogenic factor TGF-β1, although partially prevented diabetes-induced upregulation of extracellular matrix proteins.
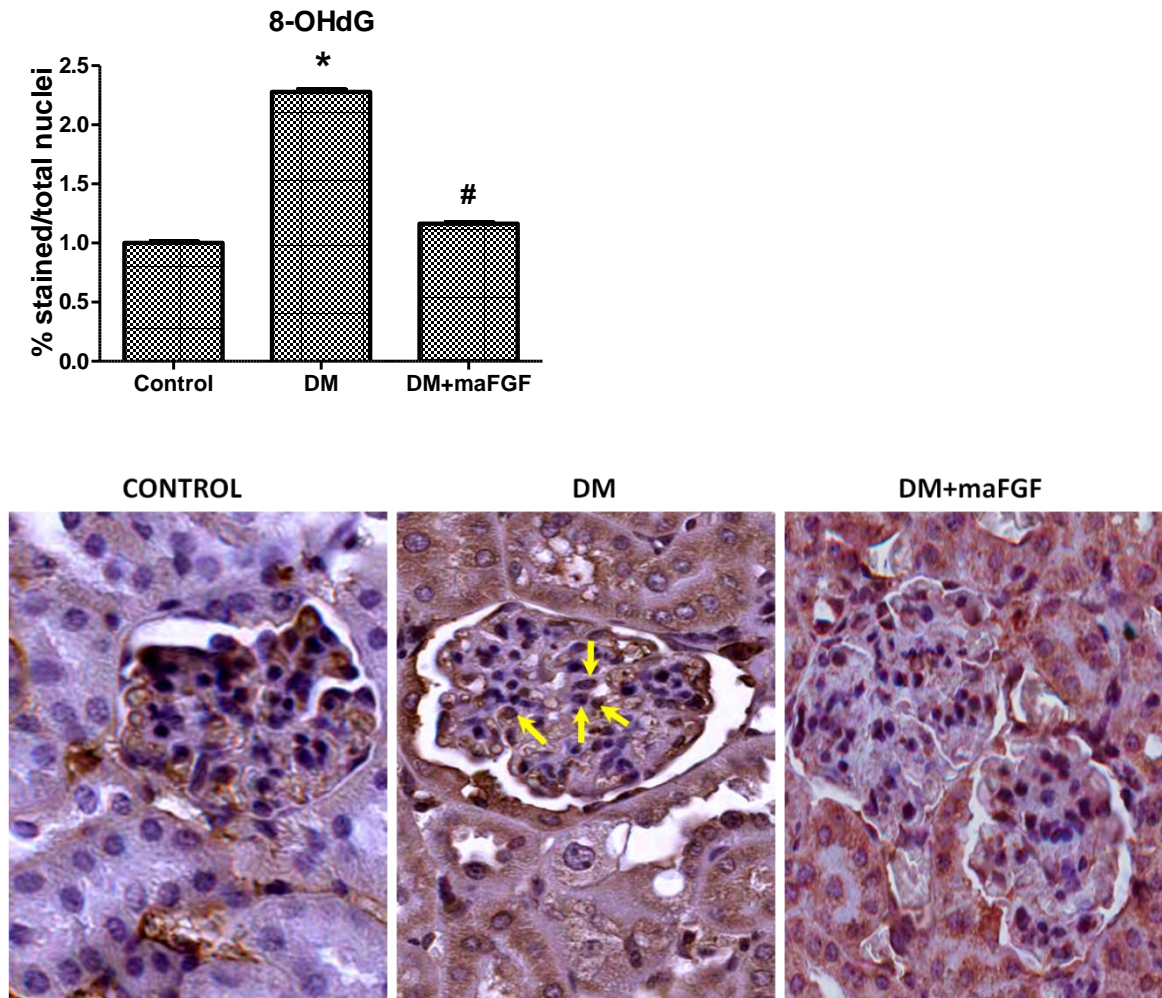
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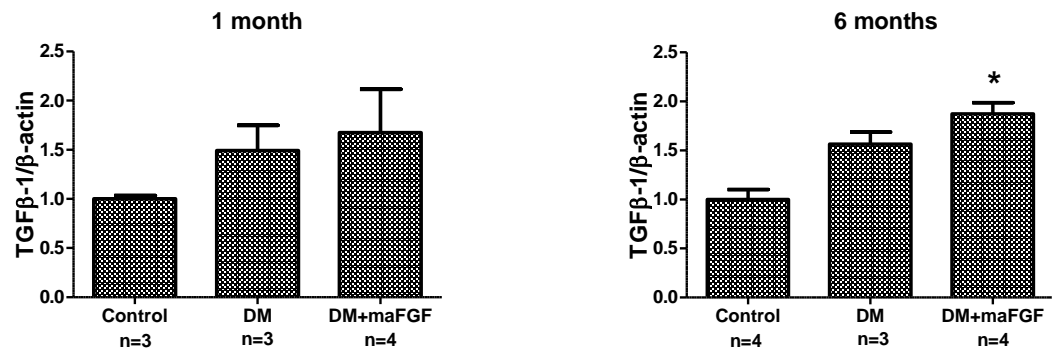
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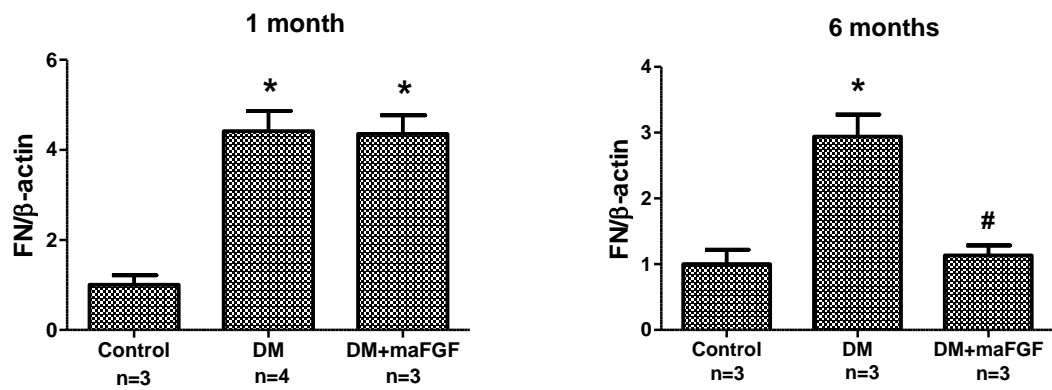
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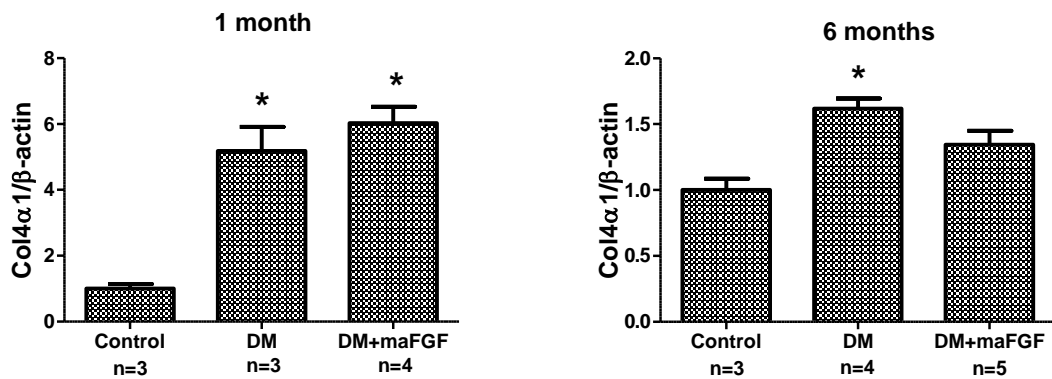
Figure 3.5 Effects of maFGF treatment on diabetes induced alterations of fibrosis-related genes. Quantitative RT-PCR analysis of diabetes induced mRNA expression for (A) the fibrogenic cytokine TGFβ1 and extracellular matrix proteins (B) FN and (C) Col 1 α (IV) in mouse kidneys after 1 month or 6 months with maFGF treatment. maFGF treatment failed to prevent the fibrogenic factor TGF-β1, although partially prevented diabetes-induced upregulation of extracellular matrix proteins after 6 months. Con: control, DM: diabetes mellitus, DM+maFGF: diabetic treated with maFGF.*p<0.05 vs control, # p<0.05 vs. DM group.

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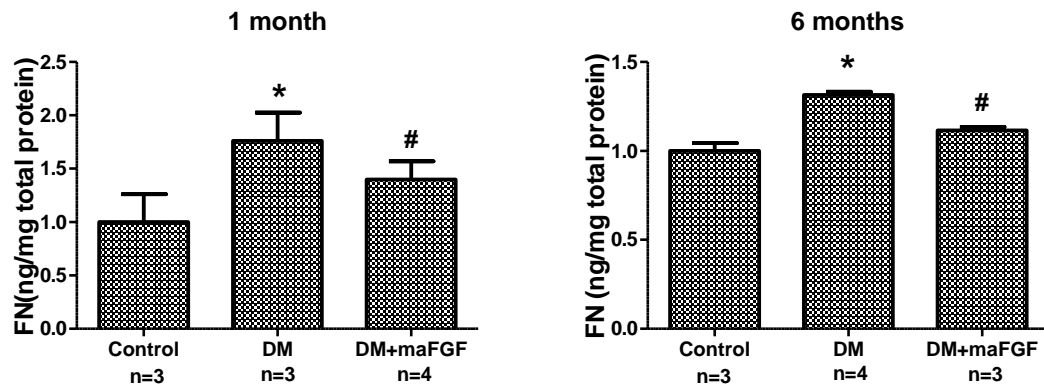
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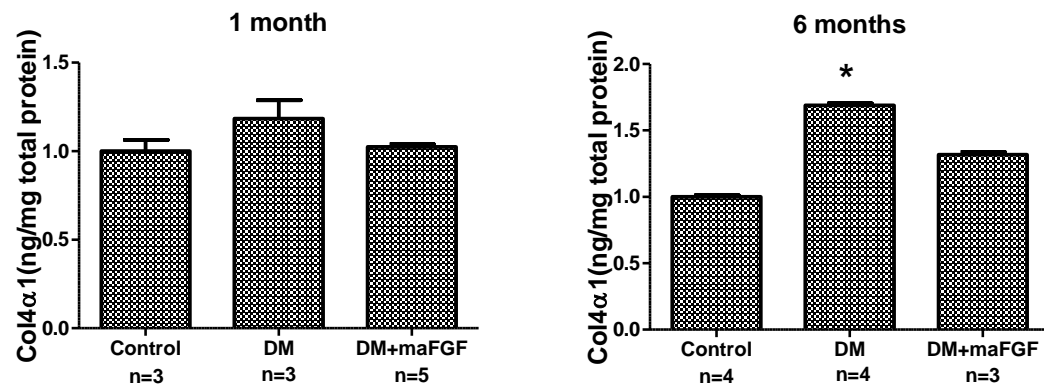
Figure 3.6 Effects of maFGF treatment on ECM proteins expression. (A) fibronectin and (B) collagen 1 α (IV) protein expression in non- diabetic and diabetic mouse kidneys after 1 month or 6 months with maFGF treatment. Con: control, DM: diabetes mellitus, DM+maFGF: diabetic treated with maFGF.*$p<0.05$ vs control, # $p<0.05$ vs. DM group.

| Control | DM | DM+maFGF |
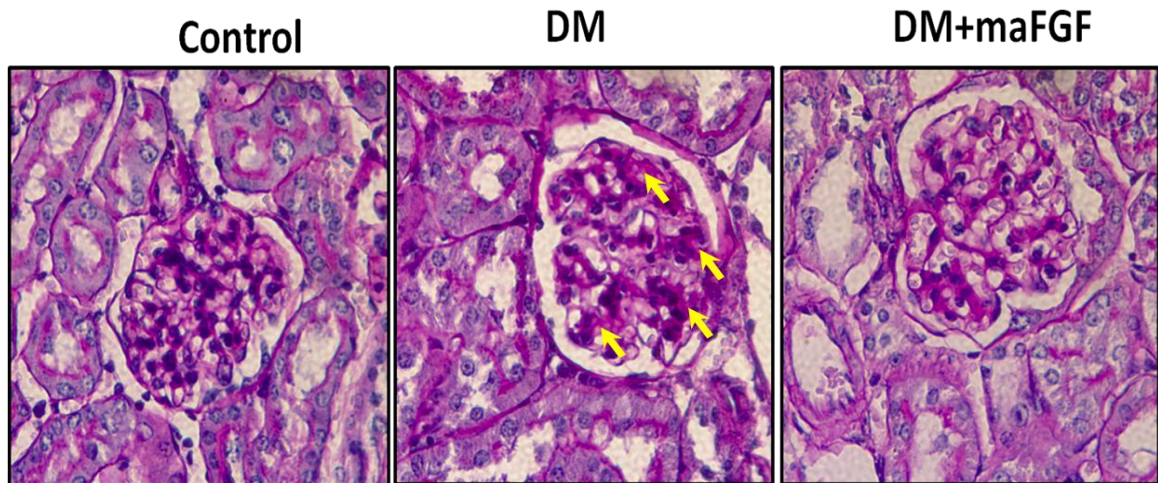
Figure 3.7 Histologic analysis using PAS stain confirmed the molecular results indicating that the maFGF treatment partially prevented diabetes-induced mesangial expansion versus diabetes. Magnifications 60X, arrows indicate increased extracellular matrix proteins deposition represented by areas stained purple magenta. 6 months samples DM-Diabetes Mellitus, DM+maFGF-Diabetic treated with maFGF.

3.6 MaFGF treatment prevented high-glucose induced oxidative stress and damage

Our in vivo study showed that maFGF treatment significantly prevented functional damage in the kidney at 1 month and 6 months and partially prevented structural changes. We further assessed the effects of maFGF on oxidative stress as a key mechanism causing DN; the results showed that maFGF treatment prevented diabetes induced upregulation of the oxidative stress marker Heme Oxygenase (HO1). Moreover, maFGF prevented diabetes-induced increased activity of the antioxidant enzymes catalase and glutathione peroxidase and oxidative DNA damage in the glomerulus of diabetic mice.

We further investigated how the maFGF treatment affects two of the various cellular elements of the kidney that are targets of high glucose injury. We intended to explore the effects of maFGF treatment in high glucose induced alteration in microvascular endothelial cells and mouse glomerular epithelial cells (podocytes). Microvascular endothelial cells play an important role in the pathophysiology of DN and have been used to reproduce diabetic vascular complications in our laboratory. Podocytes are specialized visceral epithelial cells acting as the final barrier to molecular flowing during urine filtration. Furthermore, podocytes are closely related and in proximity with endothelial cells in the glomeruli comprising the glomerular filtration barrier in a way that the action of one type of cell may influence the function of the other.

Hence, we examined various parameters, consistent with those considered in vivo, in dermal- human derived microvascular endothelial cells and podocytes. In previous studies in our lab, it was shown that the treatment of HMVEC with maFGF prevented high glucose-induced increase of cellular ROS levels [61]. We confirmed these results investigating oxidative DNA damage in HMVEC using the sensitive immunofluorescent staining of 8-OHdG. The positive staining increased in HMVECs after 48-hour of high glucose treatment (Figure 3.8). The increased 8-OHdG stain in the nucleus induced by exposure to high glucose was significantly attenuated by treatment with both maFGF

(200µg/ml) and the native aFGF (200µg/ml) used as positive control. The 8-OHdG staining shown in green was co-localized with the blue of nuclear dye DAPI.

Our experiments with podocytes showed similar results for cellular ROS levels under the same experimental conditions. After 1 hour pre-treatment of differentiated podocytes with maFGF (20µg/ml) or aFGF (20 µg/ml) followed by 48 and 72 hours treatment with high glucose, the upregulation of cellular ROS was significantly prevented (Figure 3.9). Additionally, we detected mRNA expression of the enzyme HO1 in HMVEC and podocytes. The results showed high glucose-induced significant upregulation of this transcript which is prevented by the treatment with both maFGF and the native aFGF after 24 and 48 hours of treatment with high glucose (Figure 3.10-A, 3.10-B).

| | **8-OHdG** | **DAPI** | **Merge** |

NG

HG

HG+aFGF
(200 μg/ml)

HG+maFGF
(200 μg/ml)
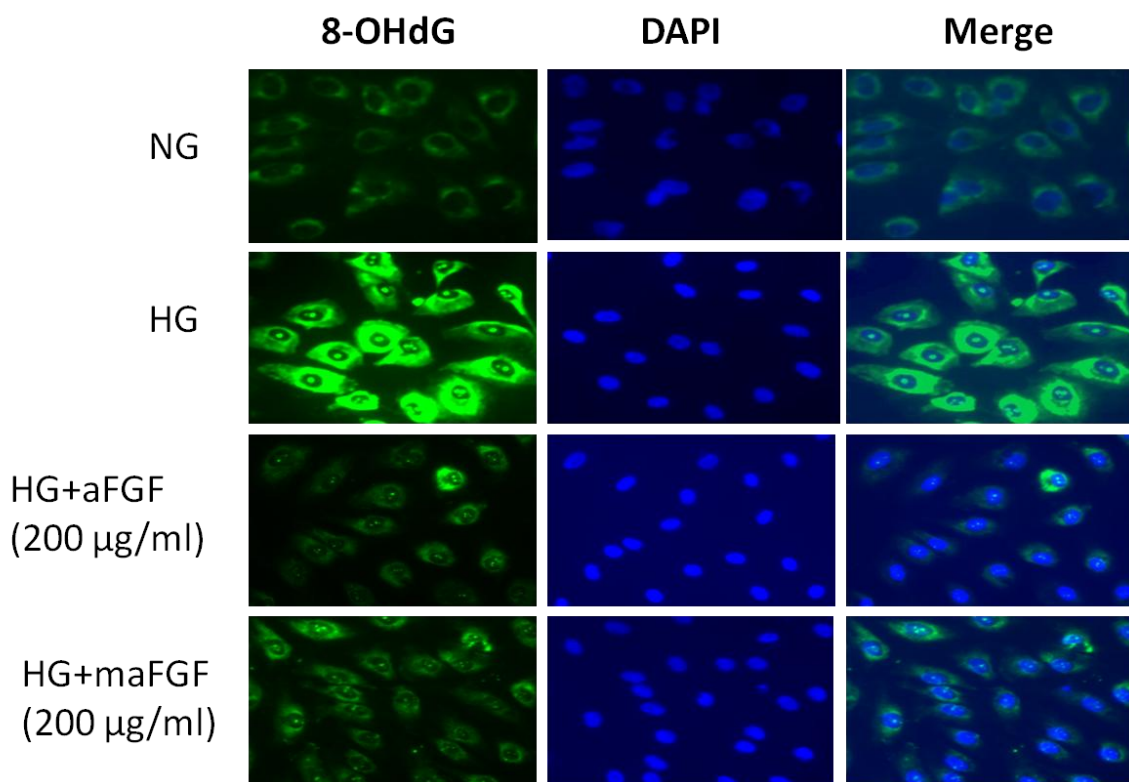
Figure 3.8 Effects of maFGF treatment on high glucose induced DNA oxidative damage in HMVEC. Cells were incubated with high glucose (25 mmol/L) and maFGF (200μg/ml) or aFGF (200μg/ml) for 48 hours. Immunocytochemistry analysis of 8-OHdG and DAPI (for nuclear morphology) was performed using fluorescence microscopy. 8-OHdG is shown in green (left column) and DAPI staining in blue (middle column). Images were merged (right column). The increased 8-OHdG stain in the nucleus induced by exposure to high glucose was significantly attenuated by treatment with both maFGF and the native aFGF. Magnifications 60X. 8-OHdG: 8-hydroxy-2-deoxyguanosine, DAPI: 4, 6-diamidino-2-phenylindole. NG: Normal Glucose, HG: High Glucose
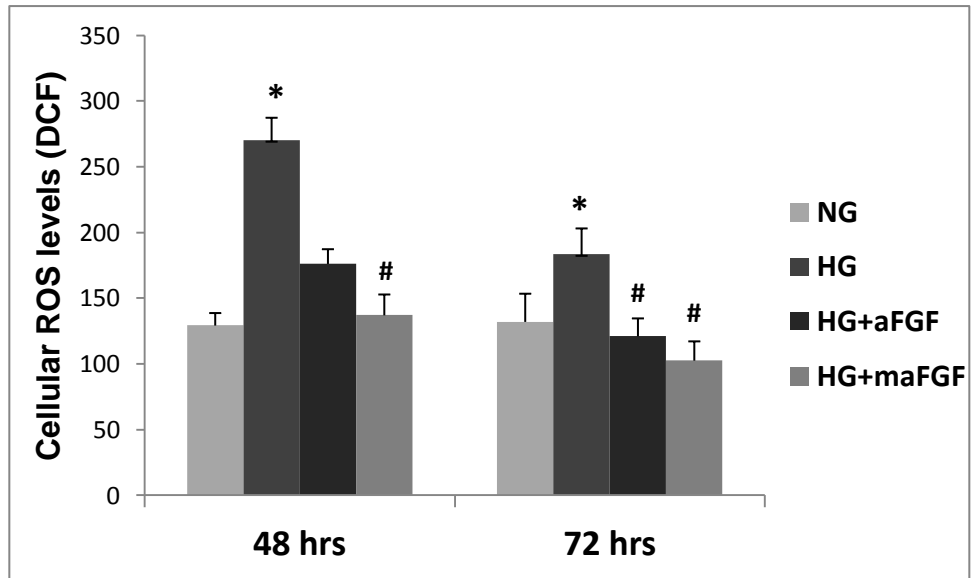
Figure 3.9 Effects of maFGF (20μg/ml) and the native aFGF (20μg/ml) treatment on high glucose- induced oxidative stress in podocytes. Both native and maFGF prevented high glucose induced increased production of cellular ROS levels. Data presented respective to percentage of number of cells. n=6. NG: Normal Glucose. *p<0.05 vs. control (NG), # p<0.05 vs. high glucose (HG)
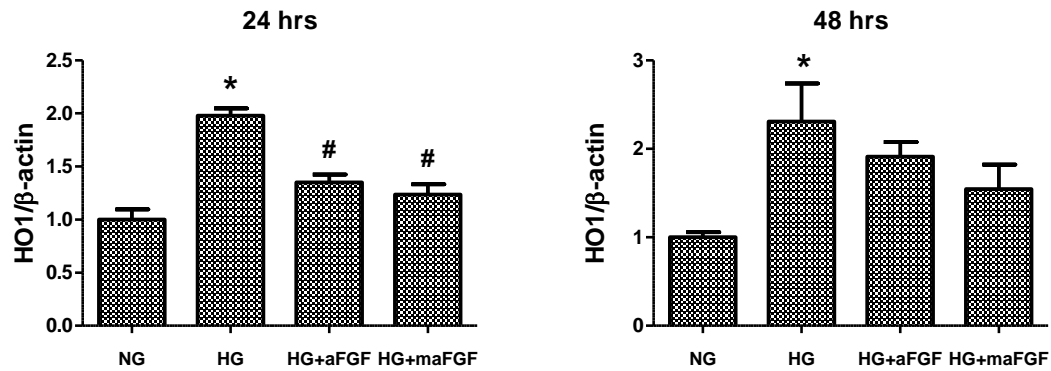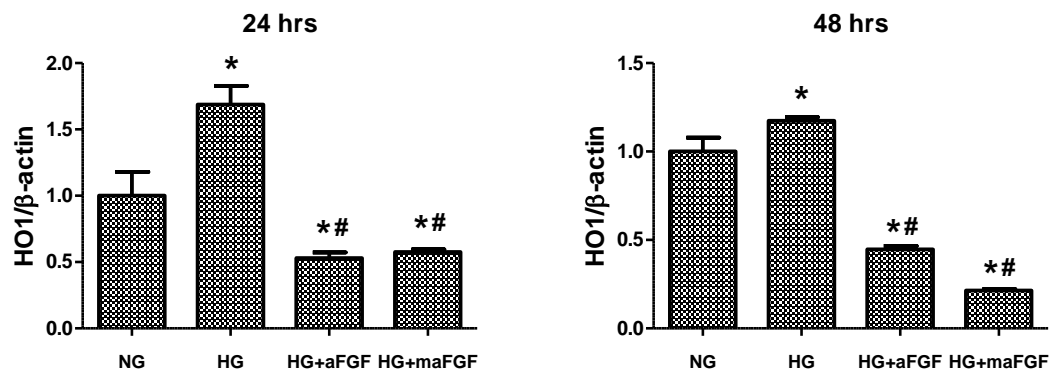
A

B

Figure 3.10 Effects of maFGF or native aFGF on high glucose induced oxidative stress in (A) HMVEC and (B) podocytes- RT-PCR analysis of mRNA expression for the enzyme Heme Oxigenase 1 (HO1). Pre-treatment for 1 hour with maFGF or native aFGF followed by 24 and 48 hours treatment with high glucose. Both native aFGF and maFGF prevented high glucose induced up-regulation of HO1 mRNA expression in both cell types at both time points. All in vitro data was obtained from at least 3 independent experiments. NG: Normal Glucose. *p<0.05 vs. control (NG), # p<0.05 vs. high glucose (HG)

3.7 MaFGF treatment prevented high glucose induced vasoactive factors alteration

Results in our in vivo study showed that maFGF treatment prevented diabetes induced upregulation of AGT and downregulation of eNOS in the context of progressive diabetic nephropathy. Therefore, we investigated the effects of maFGF which is known by its vasodilator properties, in the expression of these factors in endothelial cells and podocytes.

We found that podocytes express eNOS and this expression is affected by high glucose after exposure for 48 hours similar to the high glucose induced effects in endothelial cells eNOS expression (Figure 3.11-A). The eNOS mRNA expression is suppressed under high glucose condition and the maFGF treatment significantly prevented high glucose induced down-regulation of this transcript. We also tested the effects of maFGF in high glucose induced upregulation of AGT mRNA expression in HMVEC and podocytes at 48 hours. maFGF treatment significantly prevented this alteration in both cell types (Figure 3.11-B).
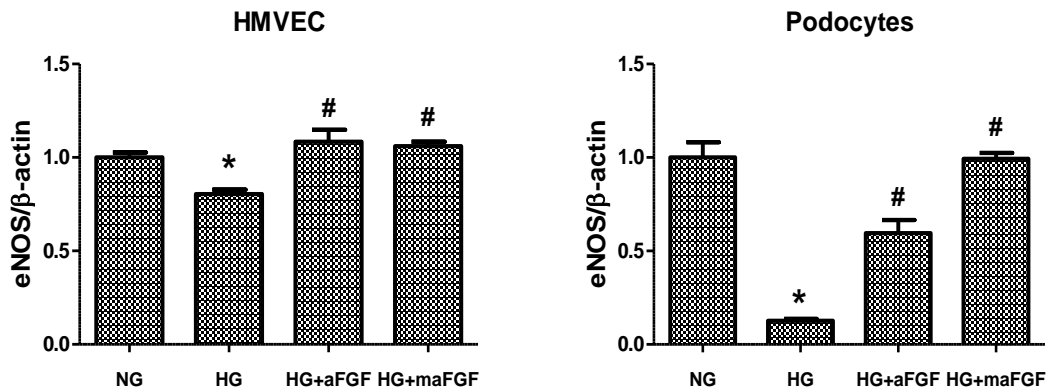
A

B
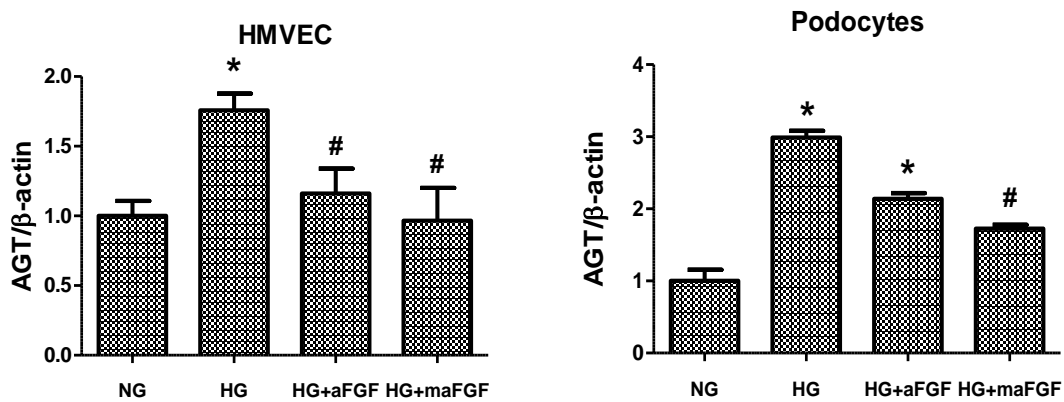
Figure 3.11 Effects of maFGF or native aFGF treatment on high glucose induced alteration of vasoactive factors- Quantitative RT-PCR analysis of high glucose-induced mRNA expression of (A) endothelial Nitric Oxide Synthase (eNOS) and (B) Angiotensinogen (AGT) in HMVEC and podocytes after 1 hour pre-treatment with maFGF or native aFGF followed by 48 hours treatment with high glucose. Both native aFGF and maFGF prevented high glucose induced alteration of AGT and eNOS mRNA expression in both cell types. NG: Normal Glucose. *p<0.05 vs. control (NG), # p<0.05 vs. high glucose (HG)

3.8 MaFGF partially prevented high glucose induced fibrotic response

MaFGF partially prevented fibrosis in our in vivo study. Hence, we examined the expression of the fibrosis-related genes TGFβ1, FN and Col 1α (IV), under high glucose condition in HMVEC and podocytes. The results showed that mRNA expression of these transcripts was significantly upregulated after 48 hours (Figure 3.12). The maFGF significantly prevented the high glucose induced upregulation of the extracellular matrix protein transcripts FN and Col 1α (IV) in podocytes but failed to prevent the aforementioned up-regulation in HMVEC (Figure 3.12-B, 3.12-C). Additionally, it failed to prevent the high glucose induced up-regulation of the fibrogenic cytokine TGFβ1 in both cell types (Figure 3.12-A).
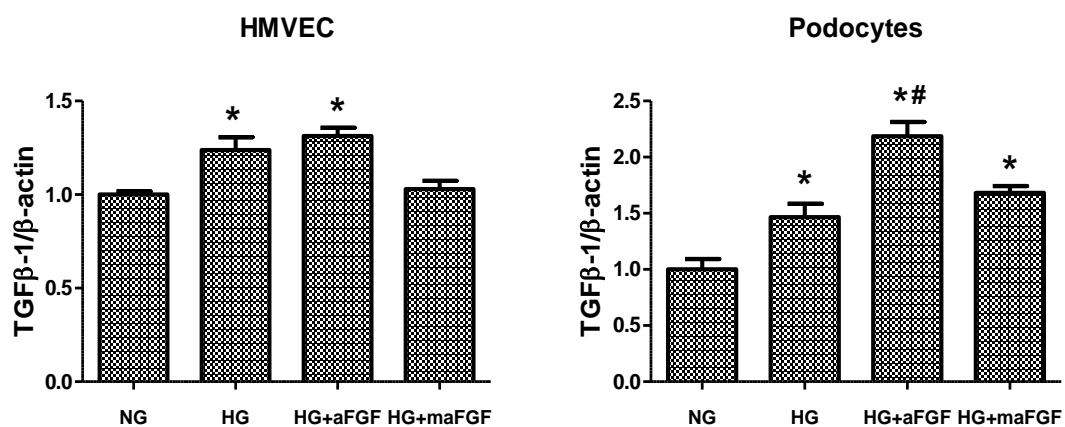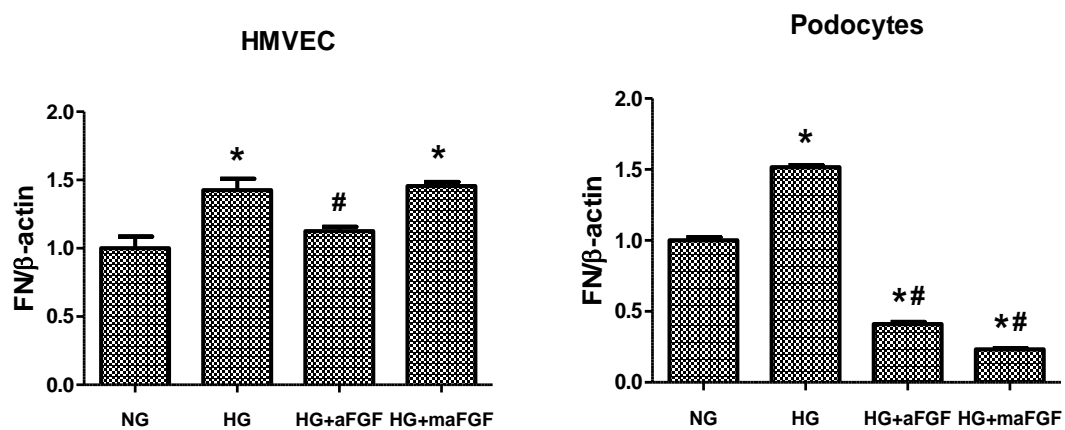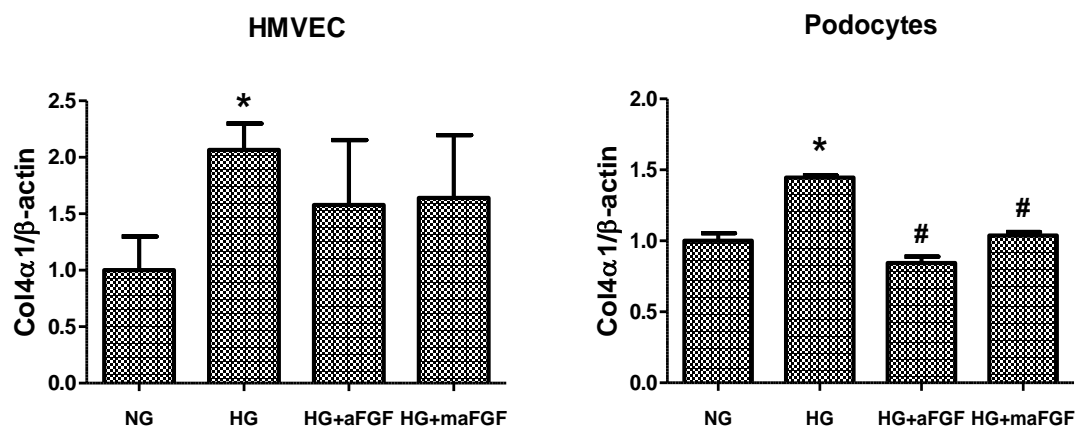
A

B

C

Figure 3.12 Effects of maFGF or native aFGF treatment on high glucose induced up-regulation of fibrosis-related genes - Quantitative RT-PCR analysis of high glucose-induced mRNA expression of (A) Transforming Growth Factor β1 (TGFβ1), (B) Fibronectin (FN) and (C) Collagen 1α (IV) (Col 1α(IV)) in HMVEC and podocytes after 1 hour pre-treatment with maFGF or native aFGF followed by 48 hours treatment with high glucose. maFGF or native aFGF failed to prevent glucose-induced upregulation of the fibrogenic factor TGFβ1 expression in both cells types and FN and Col 1α (IV) in HMVEC; whereas it prevented upregulation of the later in the podocytes. NG: Normal Glucose. *$p<0.05$ vs control (NG), # $p<0.05$ vs. high glucose (HG).

3.9 MaFGF treatment activated eNOS enzyme and enhanced production of Nitric Oxide in the kidney

Evidence reveals that FGFs stimulate the expression of eNOS and the production of NO [50-54], which is known to play a key role in vascular homeostasis. Our study shows that maFGF treatment stimulates the expression of eNOS mRNA in the kidney in the context of diabetes induced suppression of eNOS expression. Moreover, we found similar results in high glucose induced alterations in HMVEC and podocytes after 48 hours.

On the other hand, uncoupling of nitric oxide synthase is one of the known pathways that generate ROS in the kidney and has been identified as potentially major contributor to the pathogenesis of diabetic kidney disease [15, 20-23]. eNOS uncoupling leads to production of superoxide anion rather than NO. Our in vitro study and current studies in our lab showed that maFGF treatment prevented high glucose-induced oxidative stress in glomerular epithelial cells and microvascular endothelial cells. maFGF also showed prevention of oxidative DNA damage in endothelial cells. Simultaneously, our in vivo study illustrated that maFGF treatment significantly prevented functional damage in the kidney and had protective effects against diabetes induced oxidative stress.

To further explore the possible mechanism by which maFGF treatment exerts the aforementioned effects, we intended to check the levels of activated eNOS protein expression and NO production in the kidney as an indication of eNOS activity in a coupled state. The results showed that the upregulation of eNOS mRNA in diabetic animals treated with the maFGF was accompanied by increase levels of the eNOS protein (Figure 3.13-A) and the 1177-serine phosphorylation status of eNOS (Figure 3.13-B). The levels of NO production, which indicate the function of eNOS enzyme in a coupled state, were slightly downregulated in diabetic compared to the basal levels of control. The treatment of diabetic animals with maFGF increased significantly NO production (Figure 3.14). Furthermore, similar results were obtained in our in vitro study which showed that under high glucose conditions for 48 hours the production of NO is reduced significantly in both cell types: microvascular endothelial cells and podocytes

(Figure 3.15). The treatment with maFGF restored NO production with significant difference in the case of HMVEC.

We also examined the effects of maFGF treatment in the activation of the enzyme producing NO by incubating the cells with L-NAME, a non specific NOS inhibitor. Under normal conditions, the incubation of the cells with L-NAME decreased significantly the production of NO and the treatment with maFGF restored NO production comparable to the control group (figure 3.16)

Figure 3.13 Western Blot analysis for total protein expression of (A) endothelial Nitric Oxide Synthase (eNOS) or (B) serine (1177) phosphorylated eNOS in kidney tissues in diabetic animals after 6 months of treatment with maFGF. With maFGF treatment the levels of eNOS protein and the serine phosphorylation status of eNOS, which indicates activation, increased in the context of diabetes. maFGF: modified aFGF, DM: diabetes mellitus, DM+maFGF: diabetic treated with maFGF.*$p<0.05$ vs control, # $p<0.05$ vs. DM.
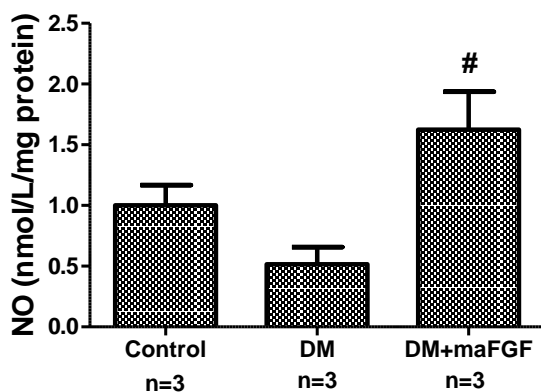
Figure 3.14 Effects of maFGF treatment in total Nitric Oxide (NO) production in kidney tissues of diabetic animals after maFGF treatment for 6 months. The production of NO was increased by the treatment with maFGF. maFGF: modified aFGF, DM: diabetes mellitus, DM+maFGF: diabetic treated with maFGF. #$p < 0.05$ vs. DM group
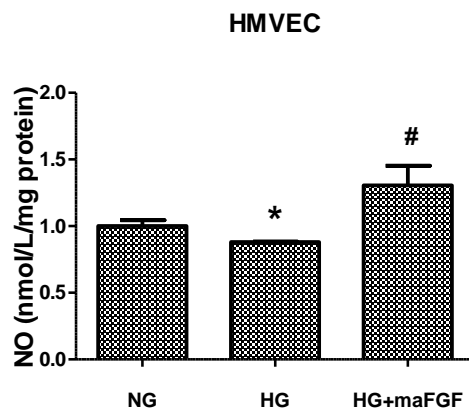
A   **B**
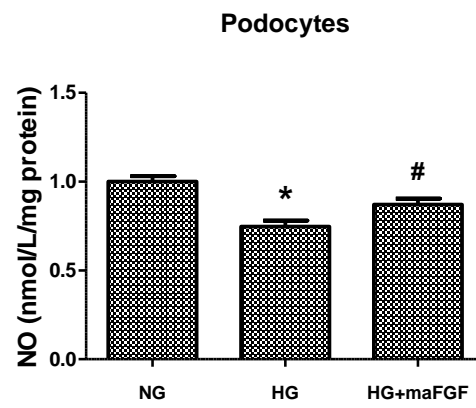
Figure 3.15 Effects of maFGF treatment in total Nitric Oxide (NO) production in high glucose induced alterations in (A) HMVEC and (B) podocytes. Under high glucose conditions for 48 hours the production of NO is significantly reduced in both cell types and treatment with maFGF restored such reduction. NG: Normal Glucose. *p<0.05 vs. control (NG), # p<0.05 vs. high glucose (HG)

A   **B**
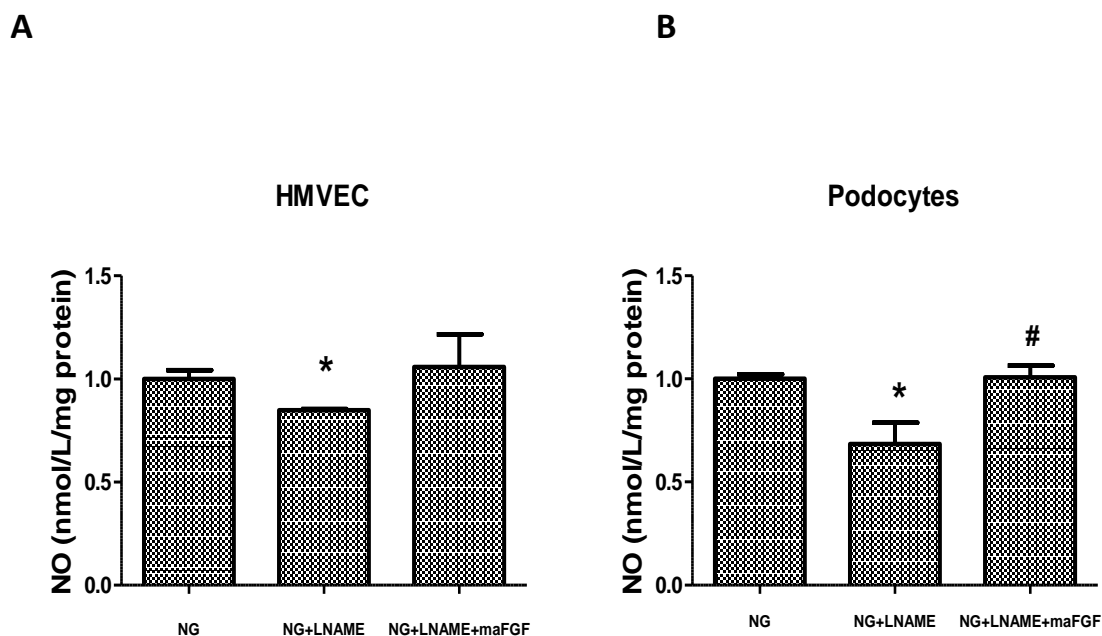
HMVEC   **Podocytes**

Figure 3.16 Effects of maFGF treatment in total Nitric Oxide (NO) production after incubation with L-NAME (500 μmol/L) (an inhibitor of Nitirc Oxide Synthase enzymes) for 48 hours in (A) HMVEC and (B) podocytes. Incubation of cells with L-NAME significantly decreased the production of NO and the treatment with m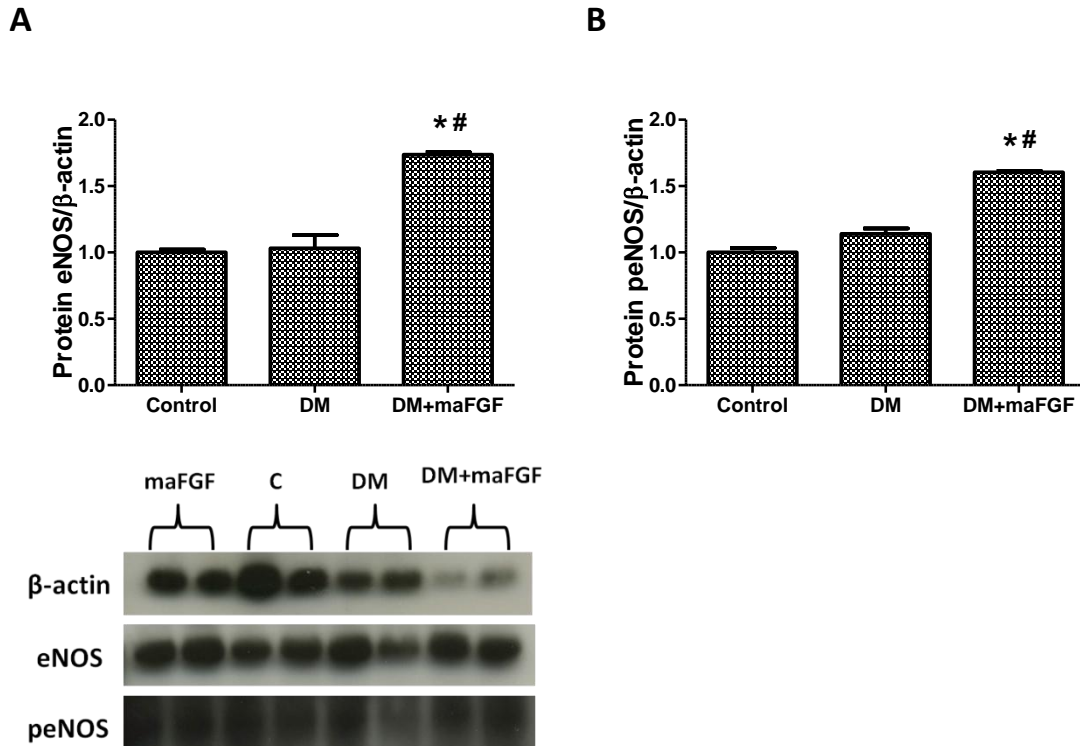aFGF restored NO production comparable to the control group. NG: Normal Glucose, L-NAME: *N*-nitro-L-arginine methyl ester hydrochloride.*p<0.05 vs. control (NG), # p<0.05 vs. high glucose (HG).

3.10 MaFGF treatment inhibits apoptosis in diabetes

Albuminuria is often the first evidence of kidney injury in diabetes. Results from our in vivo study illustrated that maFGF prevented functional damage in kidneys of diabetic mice by preventing increased albumin to creatinine ratio. Urinary albumin content is considered to reflect the underlying pathology of endothelial dysfunction and damage to glomerular podocytes [65]. Indeed, the progression of DN correlates with podocyte loss due to apoptosis, and hyperglycemia leads to decreased endothelial cell survival. In view of this evidence and the prevention shown by maFGF treatment against oxidative stress and oxidative DNA damage, we tested the possible effect that maFGF could trigger in the apoptosis of HMVEC and podocytes under high glucose conditions. We evaluated apoptosis through the mRNA expression of antiapopototic (B-cl2) - proapototic (Bak-1) ratio. Results showed that, in both cell types, the ratio Bcl-2/Bak-1 was significantly reduced under high glucose conditions (Figure 3.17). The treatment with maFGF restored this reduction in both cases with significant difference in HMVEC (Figure 17-A). Additionally, TUNEL assay after 6 months showed increased TUNEL positive cells (Figure 3.18). maFGF treatment of diabetic mice prevented such positivity.

A

B
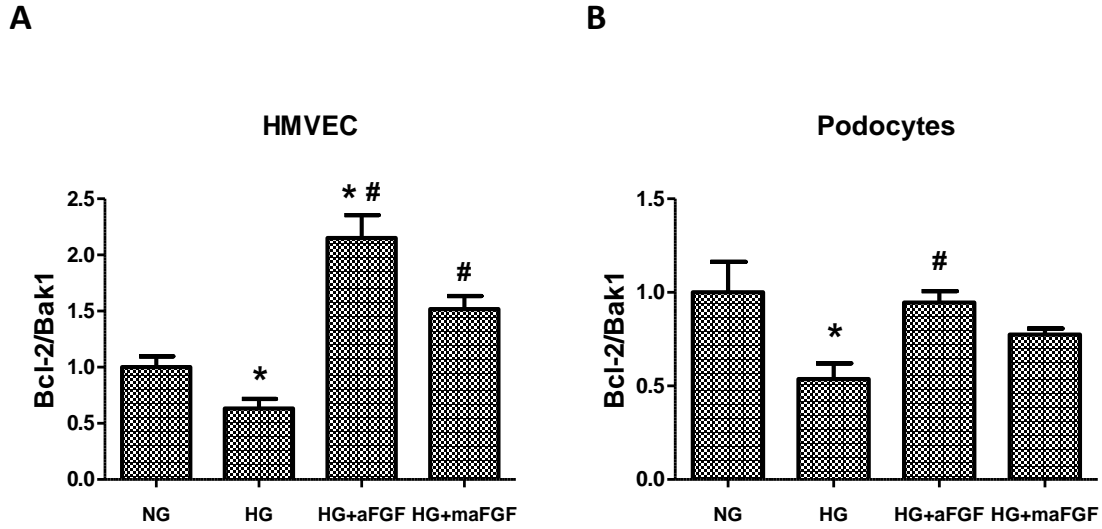
Figure 3.17 Effects of maFGF treatment on apoptosis. Quantitative RT-PCR analysis of high glucose-induced mRNA expression of Bcl-2 (antiapoptotic)- Bak-1 (pro-apoptotic) ratio in (A) HMVEC and (B) podocytes after 1 hour pre- treatment with maFGF or native aFGF followed by 48 hours treatment with high glucose. maFGF or native aFGF treatment increased the ratio Bcl-2/Bak-1 transcripts under high glucose conditions. NG: Normal Glucose. *p<0.05 vs. control (NG), # p<0.05 vs. high glucose (HG)
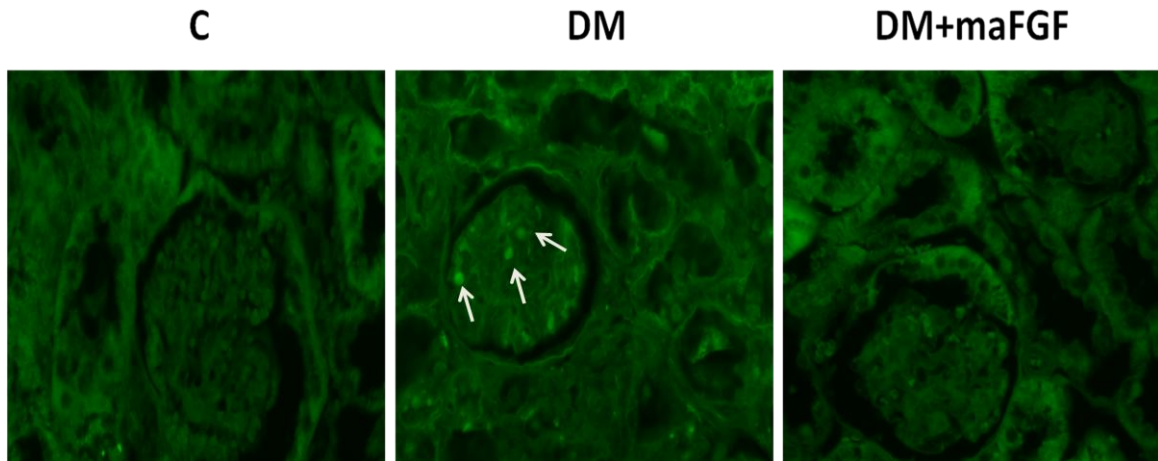
Figure 3.18 Effect of maFGF treatment in apoptosis in vivo. TUNEL assay immunofluorescence in kidney tissue of non-diabletic and diabetic animals after treatment with maFGF for 6 months. Diabetes-induced glomerular TUNEL positivity (indicated with arrows) was prevented by maFGF treatment. Magnifications 40X. C: Control, DM: Diabetes Mellitus, DM+maFGF: diabetic treated with maFGF.

Chapter 4: Conclusions

4.1 Discussion

4.1.1 maFGF as a potential approach to prevent Diabetic Nephropathy

Diabetes and its complications are devastating medical problems. Worldwide, there is increased incidence and prevalence of diabetic complications. DN, one of the very debilitating diabetic problems, commonly progresses to end stage renal disease unless preventive strategies are implemented in the initial stages. It has been proposed that oxidative stress is a key component in the development of DN leading to pathological changes such as endothelial dysfunction, decreased cell survival, podocyte loss and eventually, glomerulosclerosis and interstitial fibrosis. In view of this fact, the search of new insights into the understanding of the pathophysiology of this disease and new therapeutic concepts with reasonably designed antioxidant approaches is a necessity.

The protective effects of FGFs in the context of oxidative stress have been well documented [45-49, 58-60]. On the other hand, under pathological conditions such as DN, there are reports of upregulation of bFGF [32]. Concomitantly, some studies have shown that aFGF is upregulated by oxidative stress [66]. This evidence suggests that the increase in expression of FGFs could be an adaptive response to protect the tissues against oxidative stress. Alternatively, FGFs are also implicated in pathological conditions such as tumorigenesis and metastasis due to their potent mitogenic activity. However, the use of modified forms of FGFs, including maFGF in order to annul its mitogenic function have been tested in several studies that demonstrate they can keep its vasodilator and survival actions [38, 55, 57-61].

In the context of diabetes, studies done by us and others have revealed protective roles of bFGF and aFGF in the hearts of type 1 diabetic mouse models [61, 67]. Moreover, recent work has demonstrated that both aFGF and a non mitogenic form of aFGF (maFGF) that lacked oncogenicity, showed similar protective effects on diabetic cardiomyopathy [61]. This protection is thought to be mediated by suppression of oxidative stress and damage. With this perspective, we examined if maFGF may induce protection in DN.

Thus, we investigated such effects using a variety of approaches ranging from biochemical to structural and functional studies. We employed a type 1 diabetes mouse model and cell lines exposed to high glucose for the identification of possible mechanisms.

4.1.2 Prevention of DN by maFGF is probably mediated by attenuation of oxidative stress.

In the present study, the DN was established in low-dose STZ-induced type 1 diabetic mouse models, which were examined at 1 month and 6 months. The diabetic mice showed significant renal dysfunction as evidence by increased albuminuria. We showed for the first time that maFGF treatment significantly prevented renal dysfunction at both time points.

DN is considered to be in large part a glomerular disease that is identified by the development of functional and morphologic abnormalities secondary to hyperglycemia [67]. It is postulated that oxidative stress plays a key role in the sequence of events accompanying development of glomerular and renal injury [15, 16]. Hyperglycemia triggers increased production of ROS in renal cells that surpasses the local antioxidant capacity, which in normal conditions would keep the oxidative balance. This imbalance between ROS and antioxidants results in harmful effects in renal cells, causing changes in cellular function and eventually cell death.

Here, we demonstrated diabetes- induced upregulation of HO1 mRNA, a marker of oxidative stress, and increased activity of the antioxidant enzymes Catalase and Glutathione peroxidase. All such abnormalities were prevented by maFGF treatment.

Our results are consistent with studies that have shown that Glutathione peroxidase and Catalase mRNA expression and activities are increased under conditions of high glucose and in the renal cortex of diabetic rats [68, 69], corresponding with increased oxidative stress. However, decreases in expression and sometimes in the activities of antioxidants enzymes such as superoxide dismutase (SOD) have been reported in DN [15, 70]. Reduced

antioxidant capacity is also one of the causes that lead to increased oxidative stress in diabetic conditions. Although we did not assess the activity of SOD enzymes in this study maFGF was shown to exert increased activity of superoxide dismutase (SOD) in a model of ischemia- reperfusion injury in which maFGF showed protective effects in the brain [60]). However maFGF did seem to have a direct effect in the activity of the antioxidant enzymes assessed in this study. Our findings suggest that some of the observed effects of maFGF are possibly mediated through a mechanism related to decreasing oxidative stress.

Since hyperglycemia is thought to be the main cause that produces alterations in diabetic complications, we performed mechanistic studies exposing HMVEC and podocytes to high glucose for mimicking diabetes like conditions in vitro. Podocytes and endothelial cells are closely related in the glomerular filtration barrier. They affect the behaviour of the glomerular filtration membrane.

Oxidative stress may cause podocyte damage and loss. This represents an early change in DN related to microalbuminuria and progressive proteinuria; leading to glomerulosclerosis. Concurrently, endothelial dysfunction occurs and also leads to glomerulosclerosis [71]. For these studies, we added native or modified aFGF into the medium of podocytes or HMVEC cell culture to investigate whether aFGF can induce anti-oxidative effects in these cells under high glucose conditions. The level of ROS and HO1 were examined in podocytes. We found that both ROS and HO1 mRNA levels were increased under high glucose conditions and were significantly attenuated by either native aFGF or maFGF. This implies that anti-oxidative function of aFGF might be the mechanism to prevent podocyte injury and kidney dysfunction in the context of diabetes. Similar results were obtained when we assessed the possible protection by maFGF on endothelial cells. maFGF prevented high glucose-induced upregulation of HO1 expression. This is in keeping with other studies done in our lab demonstrating attenuation of high glucose induced ROS levels in this cell type [61].

Increased levels of ROS in the cells initiate an array of damaging reactions which can damage lipids, proteins, and DNA [15]. Therefore, in the present study we also tried to determine whether maFGF treatment could protect oxidative DNA damage in kidney tissues of diabetic animals and endothelial cells exposed to high glucose. We demonstrated increased positive 8-OHdG staining, a marker of oxidative DNA damage, in the nuclei of glomeruli cells after 6 months of onset diabetes. The treatment with maFGF reduced the number of positive cells, suggesting that prevention from oxidative stress prevents subsequent DNA damage. Similarly, after incubation of HMVEC in high glucose, increase 8-OHdG staining was evident compared to those under normal conditions. Nonetheless, maFGF or aFGF prevented such damage indicating that maFGF protects high glucose-induced oxidative DNA damage in HMVEC. It is to be noted that the high glucose exposed cells had also higher 8-OHdG stain in the cytoplasm. Exact reason is not clear. However, possibility that this may represent damaged mitochondrial DNA cannot be excluded.

4.1.3 Protection of DN by maFGF may be in part through mechanisms involving prevention of AGT and eNOS alteration which prevent cellular dysfunction.

Under high glucose ambience, a cross talk between metabolic and hemodynamic factors operates in the kidney. Like the ROS that amplify hyperglycemia injury, increased glomerular capillary pressure also contributes to the acceleration of diabetes- related alterations [8, 9, 11-13]. High glucose sensitizes the kidney to pressure-induced damage which is central to early hyperplasia and late hypertrophy of the renal cells [9]. This is enhanced by ROS generation with upregulation of various vasoactive factors and cytokines, which is followed by ECM accumulation [72]. Therefore, we tried to identify whether this protection could have effects in mRNA expression of vasoactive factors and cytokines in vivo.

We demonstrated that some vasoactive factors such as AGT, VEGF, NO producing enzymes eNOS and iNOS were significantly upregulated in diabetic animals after 1 month of onset of diabetes. These factors are shown to be associated with

intraglomerular pressure and increased permeability in the first stages of diabetic nephropathy [8]. The treatment with maFGF prevented upregulation of eNOS and AGT but failed to prevent upregulation of iNOS and VEGF in this first stage. However, after 6 months of administration of maFGF treatment, prevention of diabetes- induced mRNA upregulation of the hypertrophic marker AGT was sustained, indicating prevention of hypertrophy from an early stage. These results were supported by significant prevention of diabetic-induced increase of kidney weight - tibia length ratio by maFGF treatment after 6 months. Additionally, after 6 months of administration, maFGF also prevented diabetes- induced downregulation of VEGF and NO producing enzymes eNOS and iNOS which are features of advanced DN. Exact reason for such attenuation is not known. It is possible that in these later stages the levels of such factors decrease, possibly due to endothelial dysfunction causing the arrest of endothelial growth and inducing apoptosis [8, 9]. Treatment with maFGF could partially protect diabetes-induced molecular levels of renal damage in the first stages and prevent alteration of these molecular levels at later stages.

These in vivo findings were confirmed by in vitro studies: both, HMVEC and podocytes showed significant upregulation in the expression of AGT under high glucose condition, which was prevented by maFGF treatment at 48 hours. Some evidence revealed that exposing glomerular endothelial cells and podocytes in culture to high glucose activates production of AGT [73, 74]. AGT is the precursor of angiotensin II (ANG II) and is one of the components of the renin-angiotensin system (RAS) which could be locally activated in podocytes and endothelial cells by high glucose. The activation of intracellular RAS system in these cells is thought to be involved in progression of DN [74]. Parallel to this, we found that maFGF treatment significantly prevented high glucose- induced down regulation of eNOS mRNA expression in both cell types at 48 hours. In vitro studies done by others have evidenced that hyperglycemia- induced ROS production reduces eNOS expression and activity in endothelial cells leading to decreased endothelial cell survival and endothelial dysfunction [11, 19]. Similarly, eNOS deficiency provokes profound effects on podocyte integrity and leads to albuminuria [12, 26]. These results suggest that

treatment with maFGF could protect cellular dysfunction and damage induced by high glucose in part through mechanisms involving prevention of AGT and eNOS alteration, although further studies need to be done to elucidate whether this prevention is secondary of the prevention of ROS production.

4.1.4 The prevention of functional, biochemical and structural changes in DN by maFGF seems to work through a TGFβ-1 independent pathway

High glucose enhances cellular ROS generation exerting upregulation of various vasoactive factors and cytokines, which is followed by ECM accumulation [72]. We detected the mRNA expression of fibrosis markers TGFβ-1, FN and Col 1α (IV) in both in vivo diabetic mouse model after 1 month and 6 months after onset of diabetes, and in vitro cultured cell lines exposed to high glucose. We demonstrated up-regulation of mRNA expression of these fibrosis-related genes in diabetic animals and in both cell types HMVEC and podocytes exposed to high glucose. During the in vivo study maFGF showed some preventive effects on alteration of extracellular matrix proteins FN and Col 1α (IV) exhibited by mRNA and protein expression and confirmed by PAS staining, especially after longer administration. However, the treatment failed to prevent the induced fibrogenic cytokine TGF-β1. Surprisingly, the in vitro studies showed similar results with respect to maFGF failing to prevent the fibrogenic cytokine TGF-β1 in both cell types. Additionally, it failed to prevent the high glucose- induced upregulation of the ECM proteins FN and Col 1α (IV) in HMVEC, but not in podocytes in which showed significant prevention. According to this, maFGF treatment failed to prevent the fibrogenic factor TGF-β1, although partially prevented diabetes-induced upregulation of extracellular matrix proteins. TGF-β1 is known to be the main mediator of the abnormalities of DN, as it is a key regulator of ECM protein synthesis and responsible for apoptosis in podocytes and fibroblasts proliferation [75, 76, 77]. The results obtained in this study show that diabetes- induced damage and biochemical and structural changes in kidneys are attenuated by maFGF and that such process may work through a TGF- β1 independent pathway [20, 46, 50, 54, 78]. Current studies in our lab have shown the

involvement of other upstream mediators of FN expression in diabetes [79, 80, 81]. The vasoactive factor endothelin 1 (ET-1), which is upregulated in DN, mediates expression of ECM proteins such as FN via NF-κB and AP-1 (activating protein 1) activation[79]. Furthermore, the transcriptional coactivator p300, which is induced by diabetes in target organs including the kidneys, is involved in the regulation of gene expression of vasoactive factors and ECM proteins in DN [80, 81]. This transcription factor also interacts with NF-κB in mediating FN expression. The fact that TGF-β1 mRNA upregulation was not prevented by the maFGF treatment but ECM expression was partially prevented mainly through prevention of FN protein expression suggest that maFGF may exert its prevention through interacting with transcription factors such as the aforementioned ones. Further studies to elucidate this mechanism are needed.

4.1.5 The preventative effect of maFGF treatment in DN probably involves stimulation of eNOS activity towards production of NO and stimulation of antiapoptotic genes expression such as Bcl-2.

The protective effects of maFGF treatment in DN, as comparable to native aFGF, may be the result of vasodilation, antioxidant, anti-inflammatory and antiapoptotic functions of FGFs. Several studies, including ours, reveal protection of FGFs against oxidative stress in different models including diabetic complications [61, 67, 78], ischemia- reperfusion injury [56, 60] or direct induction of oxidative stress [58, 59]. Some mechanisms have been proposed to elucidate these pleiotropic effects including the activation of PI3 K/Akt and ERK1/2 pathways [20, 46], or the implication of FGFs on lipids metabolism [54, 78]. With the same perspective, some studies reveal the activation of eNOS enzyme and the production of NO by FGFs [50-54], linking this effect with the vasodilator and antiapoptotic functions.

Alternatively, evidence shows that production of superoxide may cause uncoupling of eNOS leading to production of superoxide anion rather than NO [15, 20-23] which is known to play a key role in vascular homeostasis. Our results demonstrated that maFGF causes prevention of oxidative stress in vivo and in vitro, as well as corrects the deficit of eNOS

enzyme expression. Therefore, we tried to identify whether maFGF has any effects in NO production in vivo and in vitro as indication of eNOS activation in a coupled state.

We demonstrated that diabetic-induced decrease of NO production was concomitant with the down regulation of eNOS enzyme in later stages of DN. The treatment with maFGF significantly increased NO production. These findings were confirmed in vitro showing that in both cell types HMVEC and podocytes, maFGF significantly restored the high-glucose induced decrease production of NO. Furthermore, similar results were obtained when these cells were treated with L-NAME, an eNOS inhibitor, showing that maFGF similar to aFGF, exerts activation of eNOS in a coupled state. In spite of using L-NAME NO levels were not completely abolished. Exact reason is not evident. Potential explanation may be in the specific system used in this study which showed higher basal levels. These results may suggest that some of the beneficial effects of maFGF in DN could be through activation of eNOS in a coupled state with subsequent production of NO that restores the deficit of NO and therefore preventing cell dysfunction. Moreover, maFGF would prevent uncoupled eNOS- induced ROS production (Figure 4.1).

Interestingly, evidence in support of the importance of high glucose induced- ROS and oxidative DNA damage in mediating apoptosis is reflected in our in vitro studies that show reduced antiapoptotic- proapoptotic Bcl-2/Bak-1 ratio mRNA expression in HMVEC and podocytes exposed to high glucose. Numerous studies show that aFGF and maFGF plays a key role in the prevention of cell death [48, 49, 55, 56]. The treatment with maFGF significantly restored the reduction of Bcl-2/Bak-1 ratio comparable with aFGF. These results were confirmed with TUNEL assay in kidney tissues that showed maFGF prevention of increased TUNEL positive cells observed in diabetic animals. Further correlation studies could establish an association between the ability of maFGF to activate the activity of eNOS towards the production of NO and the preventative effects of maFGF against oxidative stress, apoptosis, and subsequent biochemical and structural changes, and functional damage prevention. In addition, assessment of effects of maFGF

treatment in nitrosative stress is essential since $ONOO^-$ formation is potentially a major pathway governing NO reactivity [17, 82].
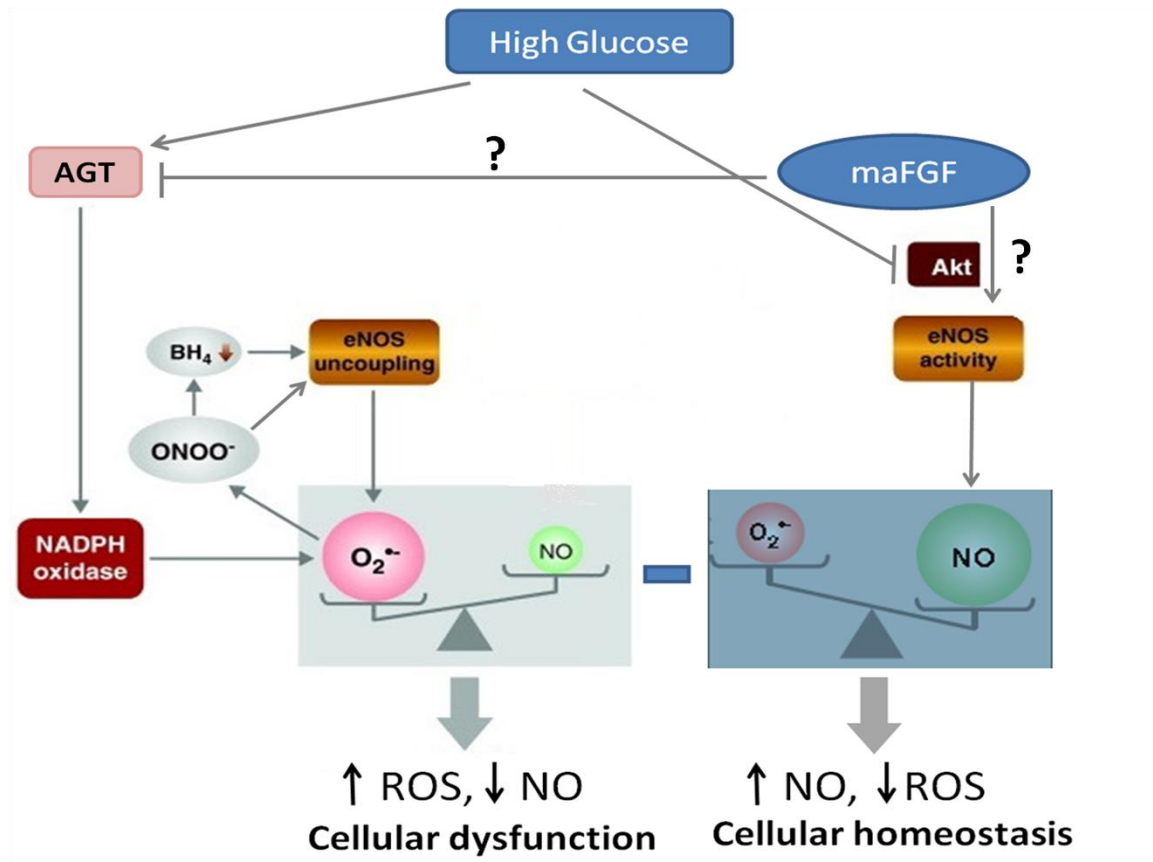
Figure 4.1 Scheme depicting proposed mechanism underlying prevention of DN by maFGF treatment. High glucose induces increase production of ROS which reacts with NO to form the potent oxidizing species peroxynitrite (ONOO$^-$). The oxidation of eNOS cofactor BH_4 decreases availability which, in addition to the oxidation of eNOS zinc-thiolate cluster, leads to eNOS uncoupling which produce ROS rather than NO. The activation of eNOS in a coupled state by maFGF prevents production of ROS and restores NO deficiency. The questions marks indicate further studies that need to be done to elucidate the exact mechanism of eNOS activation by maFGF. Other potential pathways that may additionally contribute to attenuation of ROS such as prevention of AGT mRNA expression need further clarification (Figure adapted from reference # [83]).

Finally, our findings show that maFGF prevents 1) functional damage and biochemical and structural changes in DN, 2) oxidative stress and oxidative DNA damage in DN, 3) diabetic-induced apoptosis in kidneys and high glucose-induced apoptosis in HMVEC and podocytes, 4) deficient NO production in kidney tissues of diabetic animals and in MHVEC and podocytes under high glucose conditions. These results suggest that the preventative effect of maFGF treatment in DN is probably mediated by attenuation of renal oxidative stress, stimulation of eNOS expression and activity in a coupled state that leads to increased levels of NO production and stimulation of expression of antiapoptotic genes such as Bcl-2. It is important to emphasize that, in these studies, maFGF showed a similar anti-oxidative and antiapoptotic capacity to native aFGF underscoring the possible advantage of maFGF for clinical implications. maFGF does not have mitogenic action as demonstrated before [55, 57] and therefore would lack oncogenic effect compared to the native aFGF. These results are also concomitant with previous studies done with maFGF and its effects in diabetic cardiomyopathy [61]. This indicates a potential therapeutic role of maFGF in DN.

4.2 Future directions

We have shown the protective effects of maFGF in DN in a type 1 diabetic mouse model and in high glucose-induced changes of two cells types present in the kidneys, namely: HMVEC and podocytes, which are part of the integrative filtration barrier. To further establish the protective effects of maFGF in DN, the assessment of the role of maFGF in inflammation and lipid metabolism in DN would be suggested. Similarly, the use of a type 2 diabetic mouse model could be of interest to study the involvement of FGF in DN- induced changes in the context of insulin resistance. Some studies have previously shown that FGF21 and FGF1 play an important role in glucose homeostasis as insulin sensitizers [75, 78]. Hence, enhancing its benefits as a potential therapy to avoid diabetic complications would be useful.

In vitro studies with other cell types present in the kidneys could also be considered. Advance identification of the effects of maFGF in the pathophysiology of diabetic glomerulopathy would require studies with mesangial cells which are also part of the filtration barrier [79]. Additionally, the same approach for the study of epithelial tubular cells and fibroblasts is recommended since high glucose- induced signalling abnormalities in these cells probably contributes to progressive fibrosis and can induce epithelial- to- mesenchymal transition in DN [12].

FGFs are pleiotropic peptides and can exert a variety of actions in the cells. Identification of other activated downstream pathways of maFGF or interaction with other cellular molecules in the regulation of high-glucose induced cellular alterations would be necessary to uncover new mechanisms. Finally, in an attempt to further understand the involvement of native aFGF, mechanisms regulating its production may further need to be clarified. In any chronic diseases, possible impact of epigenetic mechanisms, which may regulate such expressions, may need further experiments. It is important to point out that in some recent studies intricate relationship of FGF with microRNAs and epigenetic mechanisms have been demonstrated [84, 85, 86, 87]. A better understanding of such mechanisms will help open up the possibility of using maFGF in DN or other chronic diabetic complications.

Chapter 5: References

5.1 Reference List

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Abstracts

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