# The lectin-like domain of thrombomodulin ameliorates diabetic glomerulopathy via complement inhibition

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# ErKlärung

Hiermit erkläre ich, dass ich die von mir eingereichte Dissertation zum dem Thema

## The lectin-like domain of thrombomodulin ameliorates diabetic glomerulopathy via complement inhibition

selbständig verfasst, nicht schon als Dissertation verwendet habe und die benutzten Hilfsmittel und Quellen vollständig angegeben wurden.

Weiterhin erkläre ich, dass ich weder diese noch eine andere Arbeit zur Erlangung des akademischen Grades doctor rerum naturalium (Dr. rer. nat.) an anderen Einrichtungen eingereicht habe.

Magdeburg, den 27.08.2013

Dr. med. Hongjie Wang

**Dedicated to my Parents**
**&**
**All my Teachers**

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## List of Abbreviations

| | |
|---|---|
| AA | amino acid |
| aPC | activated protein C |
| AGE | advanced glycation endproducts |
| aHUS | atypical hemolytic uremic syndrome |
| BAX | Bcl2 associated X protein |
| BCL2 | B cell lymphoma gene 2 |
| c | control |
| C1-9 | complement component 1-9 |
| CFB/D/H/I | complement factor B/D/H/I |
| d | day(s) |
| DMT1 | diabetes mellitus type 1 |
| DMT2 | diabetes mellitus type 2 |
| DMSO | dimethyl sulfoxide |
| DTT | dithiothreitol |
| EDTA | ethylenediamine tetraacetic acid |
| EGF-domain | epidermial growth factor-like domain |
| ELISA | enzyme- linked immunosorbent assay |
| Enox | enoxaparin |
| EPCR | endothelial protein C receptor |
| FBS | fetal bovine serum |
| FCS | fetal calf serum |
| Fonda | fondaparinux |
| FVL | factor V Leiden |
| GBM | glomerular basement membrane |
| GECs | glomerular endothelial cells |
| GLEPP1 | glomerular epithelial protein 1 |
| h | hour(s) |

| HBSS | hank's balanced salt solution |
| HMGB-1 | high mobility group protein B1 |
| ICAM-1 | intercellular adhesion molecular-1 |
| i.p. | intraperitoneally |
| LPS | lipopolysacharide |
| MAC | membrane attack complex |
| MAPK | mitogen activated protein kinase |
| MBL | mannose binding lectin |
| min | minute(s) |
| NFκB | Nuclear factor kappa B |
| P53 | protein 53 or tumour suppressor protein 53 |
| PAI-1 | plasminogen activator inhibitor-1 |
| PAR | protease-activated receptor |
| PAS | periodic acid/Schiff staining |
| PBS | phosphate buffered saline |
| PC | protein C |
| RAGE | receptor for advanced glycation endproducts |
| RT | room temperature |
| RT-PCR | reverse transcriptase polymerase chain reaction |
| s.c. | subcutaneously |
| sec | second(s) |
| SDS-PAGE | sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| SEM | standard error of the mean |
| STZ | streptozotocin |
| TAFI | thrombin-activatable fibrinolysis inhibitor |
| TAT | thrombin-antithrombin |
| TM | thrombomodulin |
| tPA | tissue plasminogen activator |
| TTP | thrombotic thrombocytopenia purpura |
| TUNEL | terminal deoxynucleotidyl transferase-mediated nick end labeling |

| | |
|---|---|
| uPA | urokinase plasminogen activator |
| VCAM-1 | vascular cell adhesion molecular-1 |
| vWF | von willebrand factor |
| wt | wild type |
| WT-1 | Wilm's tumour suppressor gene 1 |

# 1. Introduction

## 1.1 Thrombomodulin Protein C System

Thrombomodulin (TM), CD141 or BDCA-3, is an integral membrane protein expressed mostly on the surface of endothelial cells. TM has a key function in regulation of the coagulation system, not only by inhibiting thrombin, but also by activation of protein C (PC). In complex with TM thrombin can activate PC zymogen and produce activated PC (aPC), PC activation can be enhanced by approximately 20 folds when binding to Endothelial protein C receptor (EPCR). aPC then provides a feedback inhibition by inactivating factor Va and VIIIa. Besides providing a negative feedback mechanism of coagulation activation, aPC mediates anti-inflammatory and anti-apoptotic activity via protease activated receptor-1 (PAR-1, Figure 1.1).
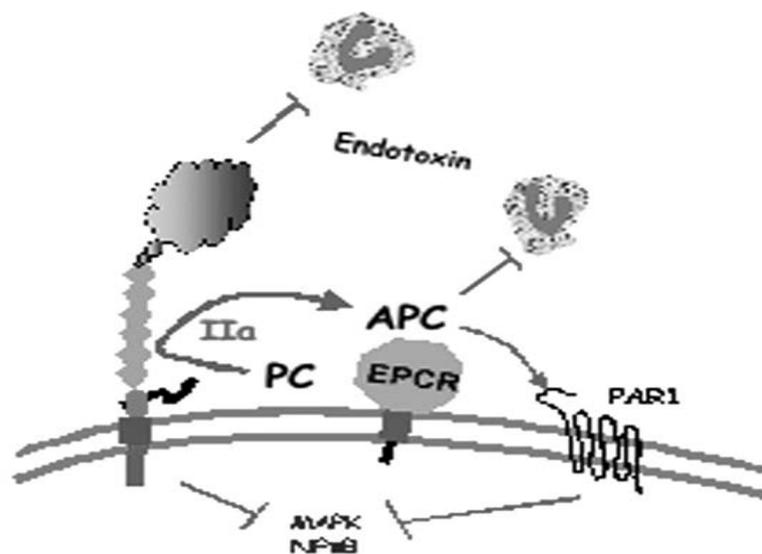


**de Wouwer, M. V. *ATVB* 2004**

**Figure 1.1 Overview of thrombomodulin Protein C System.**

A simplified model is presented to describe the possible functions of TM, PC/aPC, and EPCR. Thrombin (IIa) is primarily generated locally by the tissue factor pathway

(e.g. induced by endotoxin or following mechanical injury of the endothelial cell layer). Adequate vasculoprotection to prevent fulminant tissue damage can be provided by aPC, which prevents further thrombin generation, but also provides cytoprotection via receptor dependent mechanisms. The latter is typically achieved when aPC – in the presence of the co-receptor EPCR (endothelial protein C receptor) – binds to and proteolytically activates PAR-1 (protease activated receptor-1). This results, for example, in inhibition of MAPK signaling and NF-kB activation, which interferes with leukocyte activation/adhesion and cytokine production. The net effect is endothelial cell protection (1).

## 1.2 Structure and function of thrombomodulin

TM was first identified and isolated by Esmon and Owen owing to its anticoagulant properties (2; 3) more than 25 years ago. Since then, steady progress has been made in elucidating the molecular mechanisms by which this single molecule regulates coagulation, inflammation, fibrinolysis and cellular proliferation. Although originally described as vascular endothelial cell receptor, TM has since been detected in variety of cells and tissue in adults and during development, including astrocytes, keratinocytes, mesothelial cells, neutrophils, monocytes and platelets. Consequently, it is no surprise that it has functions beyond coagulation (1; 4).

### 1.2.1 Molecular structure of thrombomodulin

TM is a type 1 transmembrane glycoprotein (557 AA (amino acids)) consisting of 6

functional domains. A large, 154 AA N-terminal lectin like domain is followed by a

hydrophobic domain, 6 EGF-like domains, a Serine/Threonine rich domain, a single

transmembrane domain, and a short intracellular domain (Figure 1.2) (4).
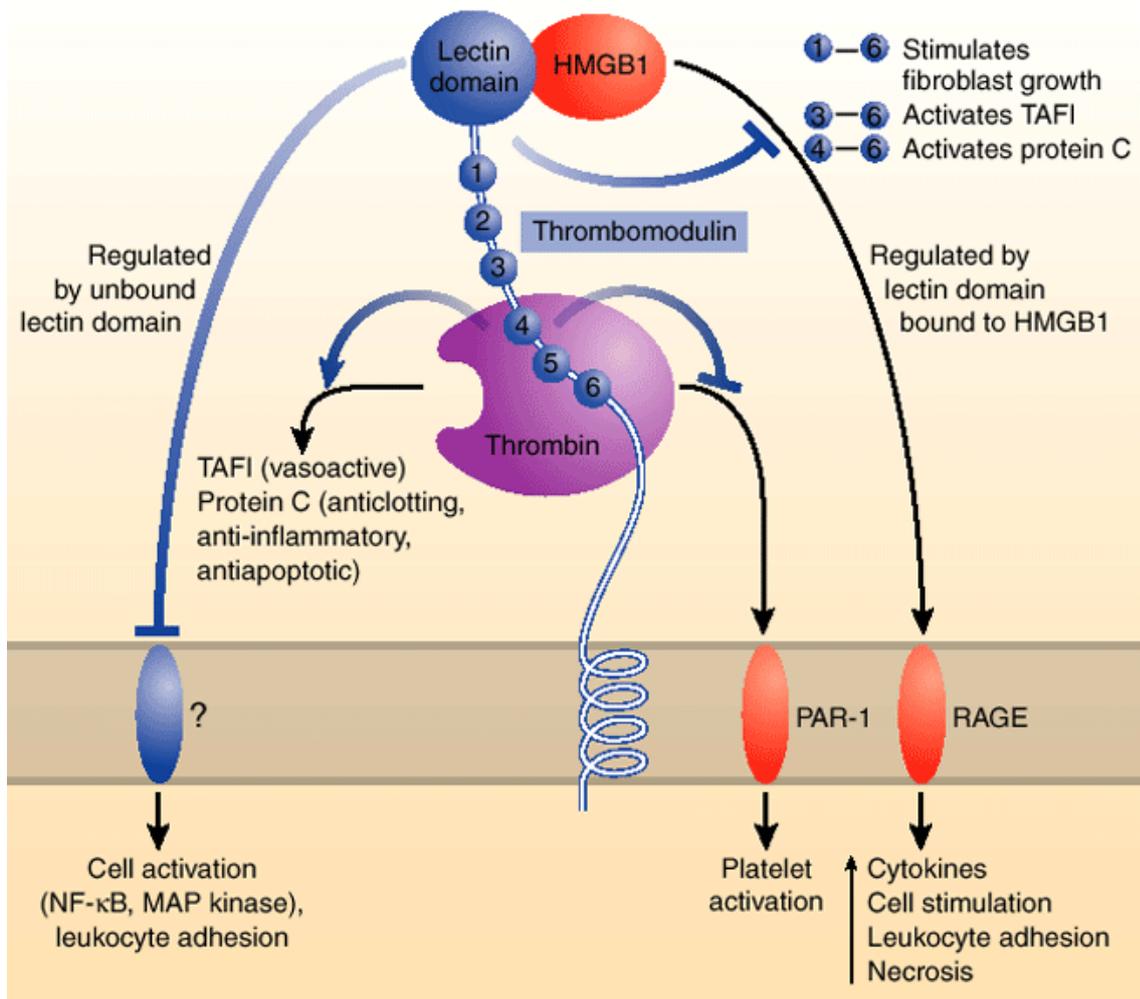
**Figure 1.2 Structure and function of Thrombomodulin.**

TM is a multi-domain proteoglycan found primarily on the endothelium. Addition, TM

can bind thrombin, the terminal enzyme of the blood clotting cascade. EGF like

repeats 4, 5, and 6 (EGF 4 to 6) have been studied in detail by several groups and are

essential in activation of protein C (Figure 1.2). EGF 3 to 6 are important for activation of thrombin activatable fibrinolysis inhibitor (TAFI) by thrombin (5). When TAFI is activated by proteolysis at residue Arginine 92 by the thrombin/thrombomodulin complex, it exhibits carboxypeptidase activity. Activated TAFI reduces fibrinolysis by removing the fibrin C-terminal residues that are important for the binding and activation of plasminogen(6; 7). Additional antifibrynolytic activity is supported by the EGF-like repeats of TM, because they also accelerate thrombin-mediated conversion of single-chain urokinase-type plasminogen activator (scu-PA) to thrombin-cleaved 2-chain urokinase-type plasminogen activator (tcu-PA/T), thereby interfering with the generation of plasmin(8; 9). The major functions of TM are described in details below.

### 1.2.2   Activated Protein C

Physiological proteolytic activation of protein C (PC) by thrombin occurs on the surface of the endothelial cell and involves the two membrane receptors, thrombomodulin (TM) and endothelial protein C receptor (EPCR, Figure 1.3)(1). Activation of PC by the TM-thrombin complex is augmented by localization of protein C on the endothelial surface by its binding to EPCR (Figure 1.3) (10) .

Activation of the blood coagulation system is triggered by various stimuli, which all lead to exposure of TF and direct contact of TF with soluble coagulation zymogens within the blood. TF then interacts with small amounts of fVIIa, activating factor X, which results in a short-lived generation of small thrombin levels. If the initial thrombin
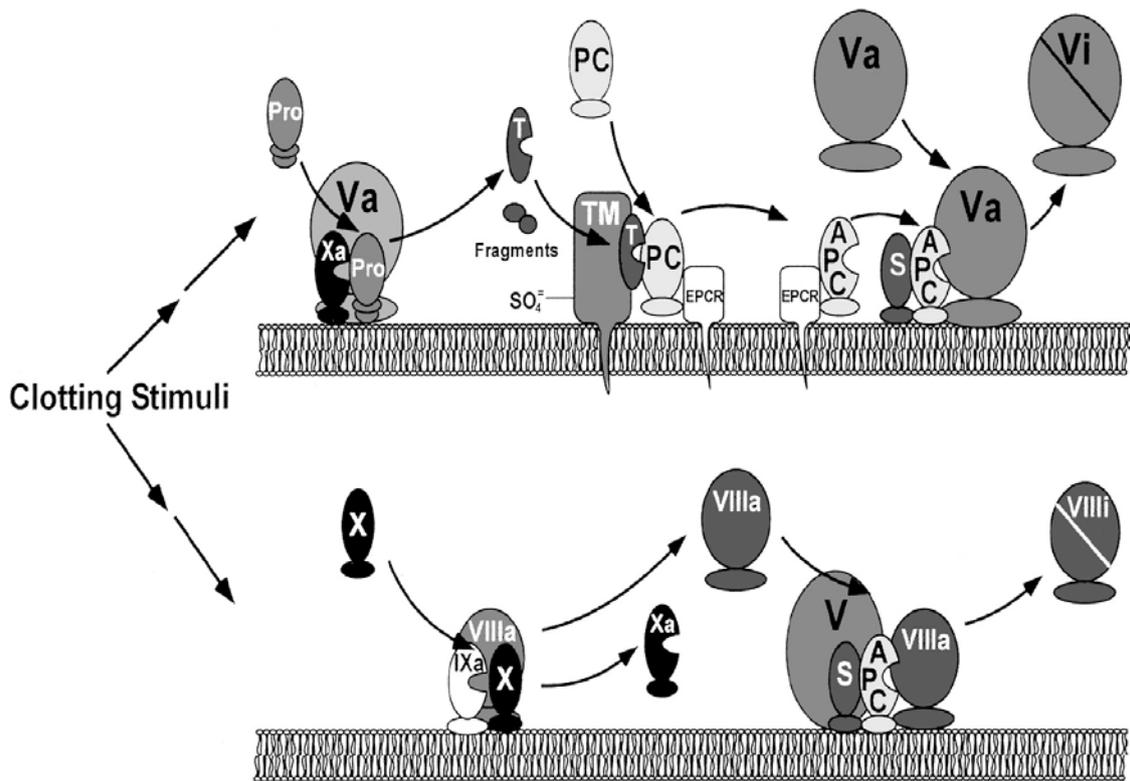
**Figure 1.3** Inactivation of factor Va and VIIIa by aPC generated on endothelium. TM, thrombomodulin; PC, protein C; APC: activated protein C; Pro, Prothrombin; T, thrombin; S, protein S; EPCR, endothelial protein C receptor, aPC, activated protein C; X, factor X; Xa, activated factor X; IXa, activated factor IX; V, factor V; Va, activated factor V; Vi, inactivated factor V; VIIIa, activated factor VIII; VIIIi, inactivated factor VIII;.

generated is not effectively inhibited it will initiated a feedback amplification, activating factor XI as well as the cofactors fVIII an fV. This mechanism allows for sustained and sufficient thrombin generation to occur. Thrombin activates fibrinogen, resulting in fibrin formation, and activates platelets, resulting in thrombus formation, which is required for vascular healing, but my also result in vascular thrombosis in the case of uncontrolled thrombin generation. One important feedback mechanism to prevent the latter is provided by thrombin binding to thrombomodulin (TM). When complexed with

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TM thrombin acquires a new substrate specificity, becoming a potent activator of protein C. Once aPC dissociates from EPCR, it binds to protein S, and this complex then inactivates factors Va and VIIIa. In the case of factor VIIIa, the process is enhanced further by factor V (in regard to factor VIIIa inactivation), high density lipoprotein, anionic phospholipids (e.g. phosphotidylserine, cardiolipin), and glycosphingolipids (e.g. glucosylceramide) (11).

aPC is a serine protease with well-characterized anticoagulant activity. Studies have demonstrated that aPC reduces organ damage and improves survival in animal model of sepsis (12) and in humans with severe sepsis (13), prevents apoptosis in cortical neurons and protects brain from ischemic injury (14). It also prevents p53-dependent apoptosis in hypoxic endothelium *in vitro* and *in vivo* (15). And most of the above mentioned cytoprotective effects are protease activated receptors dependent (12-16).

### 1.2.3  Protease activated receptors

Protease activated receptors (PARs) belong to the member of the 7 transmembrane domain G-protein coupled receptor family, and their activation requires cleavage at a specific site within their extracellular amino terminus (Figure 1.2). This cleavage produces a new aminoterminus, which then acts as a tethered ligand for the receptor. PAR-1 was first discovered as a human platelet thrombin receptor (17) and today four homologous PARs (PAR-1, 2, 3, and 4) are known in man and other species (18; 19).

PARs are activated by the action of serine proteases such as thrombin (acts on PARs 1, 3 and 4) and trypsin (PAR-2) (7). The tissue and cell specific expression of PARs, different activating proteases, and various co-receptors which have been discovered over the last years allow differential effects, surprisingly different concentration of thrombin has be shown to have not only disruptive but aslo protective effect on endothelial cells (20), we were also able to show low-dose thrombin (50pM) prevented, whereas high-dose thrombin (20nM) aggravated, glucose-induced apoptosis in cultured podocytes (21), this effect is PAR-3 dependent(unpublished data).

In 2007 we found out that within the renal glomeruli, aPC preserves endothelial cells via a PAR-1 and endothelial protein C receptor-dependent mechanism (16). Conversely, the signaling mechanism through which aPC protects podocytes remains unknown. While exploring the latter, we identified a novel aPC/PAR-dependent cytoprotective signaling mechanism. In podocytes, aPC inhibits apoptosis through proteolytic activation of PAR-3 independent of EPCR. PAR-3 is not signaling competent itself as it requires aPC-induced heterodimerization with PAR-2 (human podocytes) or PAR-1 (mouse podocytes). This cytoprotective signaling mechanism depends on caveolin-1 dephosphorylation (Figure 1.4). This novel, aPC-mediated interaction of PARs demonstrates the plasticity and cell-specificity of cytoprotective aPC signaling (22). The evidence of specific, dynamic signaling complexes underlying aPC-mediated cytoprotection may allow the design of cell type specific targeted therapies.
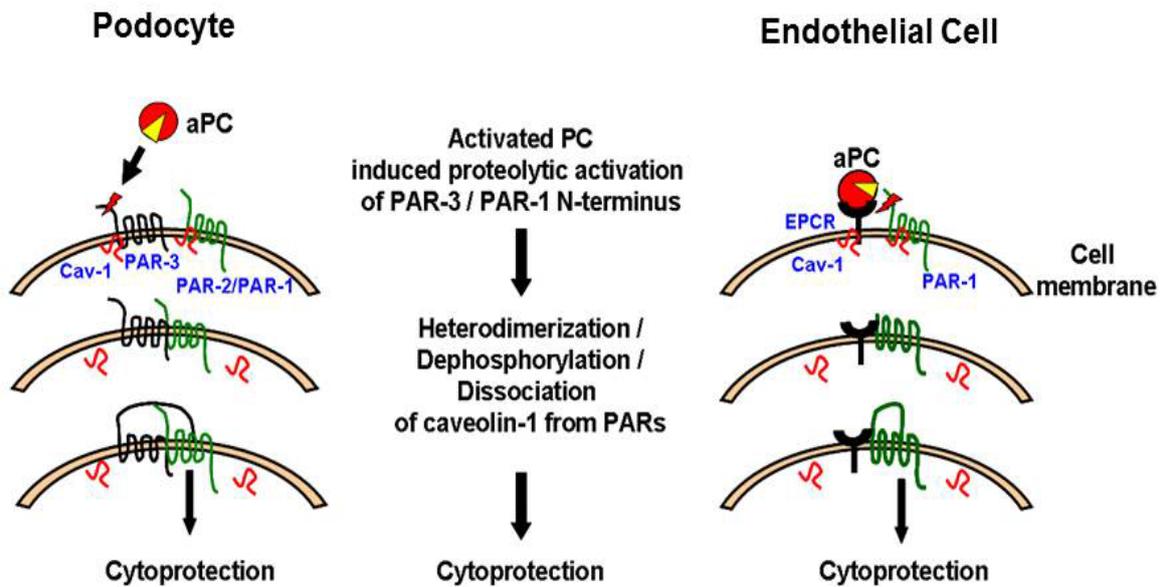
**Figure 1.4 Schematic representation of differential signaling of aPC in podocytes and endothelial cells.**

Madhusudhan T,   Wang H, Isermann B et al *Blood* 2012

aPC's receptor dependent, cytoprotective effects result in (1) alteration of gene expression profile; (2) anti-inflammatory activities; (3) anti-apoptotic activities; and (4) endothelial barrier stabilization. Although potentially inter related, each of these activities of aPC is distinct and may or may not involve same intracellular mechanisms with their particular characteristics, depending on a cell's receptor profile and on a particular cell's location.

## 1.2.4  Lectin like domain of thrombomodulin

In addition to TM´s activated PC-dependent cytoprotection, the function of its lectin-like domain has been broadly investigated. At the N-terminus of the molecule there is a 154-amino acid residue module with homology to other C-type lectins (23; 24). Electron microscopy and computer models indicate that the lectin-like domain of TM is globular and situated furthest away from the plasma membrane, because of its

unique structure and location it might effectively and easily interact with other molecules (25; 26). Study shows that the lectin-like domain confers protection from neutrophil-mediated tissue damage by suppressing adhesion molecule expression via nuclear factor kappaB and mitogen-activated protein kinase pathways (27). Abeyama *et al.* show that the lectin-like domain binds the proinflammatory molecule. HMGB1 blocks cell signaling mediated by RAGE and possibly other receptors (28). The lectin-like domain can also dampen cell activation signals by dampening the MAPK and NF-κB pathways, presumably through interaction with an as yet unidentified receptor. Another group in Japan shows the lectin-like domain dependent effects may be related to scavenging of HMGB-1, a proinflammatory mediator and inhibition of apoptosis (Figure 1.2) (29).

## 1.2.5  Thrombomodulin and complement

Besides the above mentioned diverse function, TM plays also very important roles in regulating complement system. Via its epidermal growth factor-like repeats, TM suppresses thrombin (IIa)-mediated activation of C5 to C5a. TM also accelerates thrombin-mediated activation of TAFI (thrombin activatable fibrinolysis inhibitor), and activated TAFI (TAFIa) in turn cleaves and inhibits C3a and C5a (30). In the complement alternative pathway TM binds directly to complement factor H (CFH) and C3b, and facilitates the cofactor activities of CFH and of C4bBP (C4b-binding protein) for CFI (complement factor I)-mediated inactivation of C3b (30). TM also limits the complement activating properties of CFB/CFD (complement factors B and D) (31; 32).

The binding site(s) on TM for CFH, C3b and C4b are not known. In the classical/lectin pathway TM is a receptor for C4b and augments the cofactor activity of C4bBP in CFI-mediated inactivation of C4b (Figure 1.4) (30)
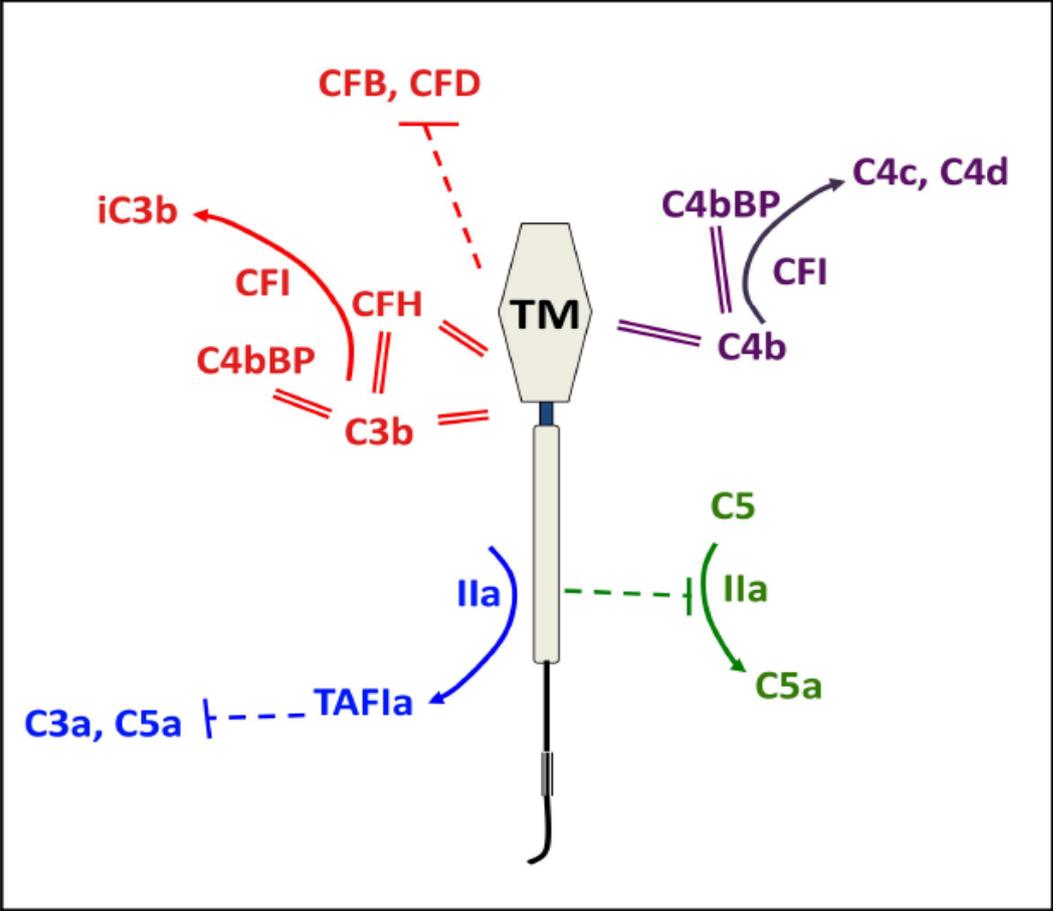


**Figure 1.5 Model representing mechanisms by which TM negatively regulates complement.**

Taken together each of these diverse functions is mediated by different domains of TM. Especially, the lectin-like domain of TM can mediates anti-inflammatory and cytoprotective effects *via* different pathways. Van de Wouwer *et al* shows that the lectin like domain can interfere with complement activation and protect against arthritis (33; 34). The relevance of TM-dependent complement inhibition in chronic vascular diseases, such as diabetic nephropathy, remains elusive.

## 1.3 Diabetic nephropathy

Diabetic nephropathy is the most frequent cause of end-stage renal failure in industrialized countries (35; 36). In addition to impairment of renal function, the manifestation of diabetic nephropathy is associated with a poor prognosis for affected patients, increasing the risk for cardiovascular complications and death.
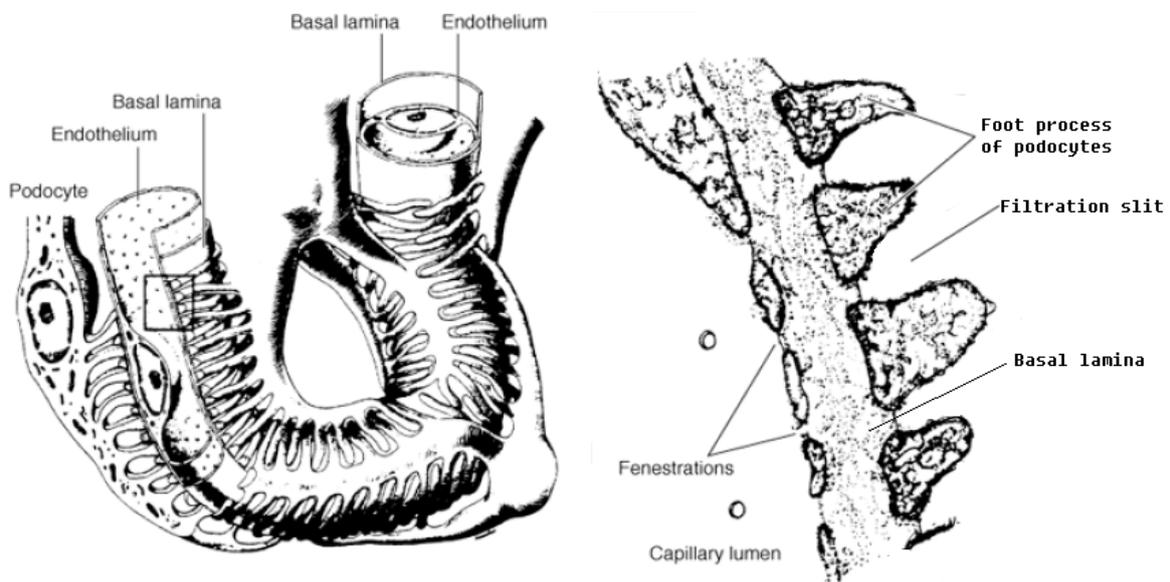


**Figure 1.6 Schematic representation of glomerular filtration barrier.** Overview (left) and detailed view (corresponding to the boxed area on the left) of the glomerular filtration barrier. Fenestrated endothelial cells, facing the capillary lumen, form the inner aspect, while podocytes and their foot processes form the outer aspect of the glomerular filtration barrier. Both cell layers are separated by a basal lamina (also known as the glomerular basement membrane). Spaces in between the foot processes form the filtration slit. (37).

Despite significant progress in our understanding of diabetic nephropathy, the translational efforts fell short their expectations (16). Thus, the delineation of new therapeutic approaches based on novel pathophysiological insights is needed.

## 1.3.1 Endothelial dysfunction in diabetic nephropathy

Diabetic vascular disease is a major clinical problem thought to be causative for diabetes related complications such as arteriosclerosis and nephropathy. Chronic exposure to hyperglycemia induces toxic metabolites like reactive oxygen species and advanced glycation end products in various cell types and this is thought to contribute to diabetic structural vascular alterations (38; 39). Studies in humans indicate that markers of endothelial dysfunction are associated with microangiopathy in type 1 and 2 diabetes. Since the endothelium is a key component of the glomerular filtration barrier (Figure 1.6), the association of markers of endothelial dysfunction with diabetic complications is particularly true in patients with type 1 diabetes who have either early (microalbuminuria) or late (macroalbuminuria) nephropathy. In these patients increased blood levels of von Willebrand factor (vWF), adhesion molecules (ICAM, VCAM), selectins, thrombomodulin, PAI-1, type IV collagen and tPA are considered markers of endothelial dysfunction (40; 41). In addition, circulating annexin V-positive endothelial microparticles are associated with vascular dysfunction in patients with end stage renal failure indicating endothelial cell apoptosis (42).

*In vitro* studies have demonstrated that hyperglycemia increases expression of pro-inflammatory and pro-apoptotic proteins that have been linked with endothelial dysfunction and apoptosis (43; 44). The presence of these markers of EC dysfunction before the development of the disease suggests endothelial dysfunction precedes

diabetes induced end organ failure (16; 40; 41). However the mechanism underlying endothelial cell dysfunction and apoptosis and their pathogenic role in development of diabetic nephropathy is not well understood.

**1.3.2 The role of podocyte in diabetic nephropathy**

The filtration barrier is a dynamic structure, in which all three components (endothelial cells, GBM and podocytes) play an integral functional role (Figure 1.6). There have been several studies of glomerular ultrastructure in patients with type 1 diabetes spanning the spectrum from normoalbuminuria through microalbuminuria to overt nephropathy. Using light microscopy, comparisons of renal ultrastructural changes in microalbuminuric patients and healthy controls have focused on basement membrane thickening and matrix/glomerular volume fraction. In some studies, microalbuminuric patients were shown to have significantly increased glomerular basement membrane thickness and matrix/glomerular volume fraction when compared with normoalbuminuric patients(45-47). Using electron microscopy, alterations in podocytes have been described even in normoalbuminuric patients (Tab. 1.1). In a mixed group of type 1 diabetic patients with microalbuminuria and overt nephropathy, the first observations of an altered glomerular epithelial cell structure, like process width, filtration slit length density and total filtration slit length, were reported in 1987 (48). This showed increased foot process width in patients with either microalbuminuria or overt nephropathy compared with control subjects. Widening of filtration slits did not correlate with increases in albuminuria, but a

decrease in filtration slit width was associated with a decrease in glomerular filtration rate (49).    A subsequent study by Steffes in 2001 found that podocyte density did not change with duration of diabetes or severity of diabetic nephropathy (49). However, decrease in podocyte number was found in patients with type 1 diabetes compared with controls, even in those with a relatively short duration of diabetes.

The main findings of studies examining podocyte dysfunction in type 2 diabetes are summarized in Table 1.2. The first two studies of podocyte ultrastructure in type 2 diabetes were performed in Pima Indians. Thus, these results, which may be confounded by the specific genetic background, cannot necessarily be extended to other groups. Anyway, in the first study, 51 patients with a range of albumin excretion rates from normo- to macroalbuminuria were studied (50). Microalbuminuria could not be distinguished from normoalbuminuria on structural grounds. Broadening of foot processes was associated with enlarged glomeruli and a decrease in podocyte density predicted progression of albumin excretion rate over 4 year (51).

**Table 1.1 Ultrastructure of Podocytes in type 1 Diabetes**

| *Study* | *Subjects* | *Stage of nephropathy* | *Findings* |
|---|---|---|---|
| Ellis (1987) | 28 diabetic<br>28 nondiabetic | 15 N<br>5 micro<br>8 overt | 1. FPW in micro equal to FPW in N<br>2. ↑ FPW in micro patients c/w control subjects<br>3. ↑ FPW with overt DN |
| Bjorn (1995) | 27 diabetic<br>11 nondiabetic | 9 N<br>9 micro<br>9 overt | 1. Overall trend to ↑ FPW with ↑ AER<br>2. Widening of filtration slits does not explain ↑ AER<br>3. Filtration slit width ↓ as GFR ↓ |
| Berg (1998) | 36 diabetic<br>(adolescents) | 36 N<br>(8 transient micro at<br>time of biopsy) | 1. Negative correlation between FPW and AER<br>2. Negative correlation between filtration slit length density and AER<br>3. ↑ FPW suggests glomeruli are enlarged and/or exposed<br>to ↑ intraglomerular pressure |
| Steffes (2001) | 46 diabetic<br>36 nondiabetic | Varied (AER data not shown) | 1. Podocyte density did not change with duration of diabetes or severity of DN<br>2. Podocyte number in diabetes c/w controls, even in short duration diabetes |
| White (2002)<br>Esprit Study | 50 diabetic<br>(all normotensive)<br>10 nondiabetic | 32 Micro<br>18 overt<br>Enal april vs nifedipine<br>vs placebo | 1. Overall no significant ↓ in podocyte number in normotensive patients with<br>raised AER<br>2. In subgroup with overt DN: podocyte number correlated negatively with ↑ AER<br>3. In placebo-treated patients: decrease in podocyte density over 3 yr correlated<br>with final AER<br>4. Association between podocyte loss and AER may be response to, cause of, or<br>concomitant with, |

DN, diabetic nephropathy; N, normoalbuminuria; micro, microalbuminuria; FPW, foot process width; AER, albumin excretion rate; c/w, compared with

**from: George Jerums, Podocytes and Diabetic Nephropathy, 2006**

**Table 1.2 Ultrastructure of Podocytes in type 2 Diabetes**

| Study | Subjects | Stage of nephropathy | Findings |
|---|---|---|---|
| Pagtalunan (1997) | 51 diabetic (Pimas) Eight nondiabetic | 10 N-early 12 N-late 17 micro 12 overt | 1. Micro could not be distinguished from N on structural grounds 2. Broadening of foot processes associated with large glomeruli and ↓ podocyte number in overt DN 3. Podocyte loss contributes to progression of DN |
| Meyer (1999) | 16 diabetic (Pimas) | micro | 1. Podocyte density predicts AER ↑ over 4 yr 2. Podocytes important in development and progression of DN |
| Dalla Vestra (2003) | 67 diabetic (Caucasian) 20 nondiabetic | 21 N 23 micro 23 overt | 1. Podocyte density ↓ in all diabetic c/w controls 2. Podocyte density and filtration slit length density ↓ as AER ↑ 3. Foot process width ↑ as AER ↑ 4. Podocyte density may be more relevant than absolute number |
| White (2004) | 16 diabetic (Caucasian) (hypertensive) 28 nondiabetic | 16 overt | 1. Podocyte number ↓ in diabetic c/w controls 2. Glomerular volume ↑ in diabetic c/w controls 3. Negative correlation between proteinuria and podocyte number and podocyte density per glomerulus 4. Longitudinal studies are required to determine sequence of events leading to podocyte loss |

DN, diabetic nephropathy; N, normoalbuminuria; Micro, microalbuminuria; AER, albumin excretion rate; c/w, compared with.

**from: George Jerums, Podocytes and Diabetic Nephropathy, 2006**

Subsequently, two studies were performed in Caucasian patients with type 2 diabetes. In the first, a group of 67 patients with an approximately equal proportion of normo-, micro-, and macroalbuminuric subjects was studied (52). Podocyte density was decreased in all diabetic patients regardless of albuminuria status compared with controls. Podocyte density and filtration slit length density decreased as albuminuria increased and foot process width increased as albuminuria increased. It was concluded that podocyte density may be more relevant to nephropathy than absolute podocyte number. A second study in 16 hypertensive Caucasian diabetic patients with overt nephropathy was performed recently (53). This showed that podocyte number was decreased whereas glomerular volume was increased in diabetic subjects compared with controls. There was a negative correlation between proteinuria and podocyte number and between proteinuria and podocyte density per glomerulus. As in their previous study in type 1 patients, these authors raised the possibility that podocyte loss may be part of the disease process rather than a causal factor. This would be consistent with the concept that albuminuria is a marker but not the culprit in the progression of renal disease (54). The mechanism underlying podocyte loss during disaese progression remain incomplelety understood, but may be linked to apoptosis or complement activation.

### 1.3.3 Complement and diabetic nephropathy

Recent evidence suggests that excess complement activation contributes to the establishment of diabetic nephropathy (55). The complement system is part of the innate immune system, consisting of an enzyme cascade that is activated by one of three different pathways (the classical complement, alternative, and mannose-binding lectin (MBL) pathways) (56; 57). These three pathways converge, forming the protease C3-convertase, which generates C3a and C3b and leads to the formation of the membrane attack complex (MAC). Owing to its pro-inflammatory and cell-death promoting properties the complement system requires tight regulation. Among the negative regulators of complement activation are three membrane proteins, CD46, CD55, and CD59, which protect self-cells from complement, either by inhibiting complement activation (CD46, CD55) or by regulating MAC assembly (CD59).

In patients with type 1 and type 2 diabetes mellitus high serum levels of MBL and high-coding MBL genotypes are associated with the presence and development of microalbumiuria and diabetic nephropathy (36; 58-60). Impairment of complement inhibitors may contribute to complement activation and renal damage in diabetes mellitus. *In vitro* high glucose concentrations induce loss of cell surface CD55 and CD59 and inactivation of CD59 *via* glycation (61-63). These data support a role of the complement system in diabetic nephropathy. Of note, CD55 and CD59 are expressed on endothelial cells and are thus directly exposed to high blood glucose concentrations.

Given the function of endothelial TM as a complement regulator and the potential role of complement in diabetic nephropathy we hypothesized that endothelial TM modulates diabetic nephropathy not only through generation of cytoprotective aPC, but in addition through complement regulation *via* its lectin like domain. To test this hypothesis we induced persistent hyperglycemia in mice lacking the lectin like domain of TM (TM$^{LeD/LeD}$) (27) and analyzed the extent of experimental diabetic nephropathy and potential mechanisms involved. We show that TM's lectin-like domain modulates diabetic nephropathy through complement regulation.

## 2. Material and Methods

## 2.1 Chemicals and Reagents

| | |
|---|---|
| Agarose, Ultra Pure | Invitrogen, Karlsruhe, Germany |
| Ammonium Hydroxide | Sigma-Aldrich, Taufkirchen, Germany |
| Antigen Unmasking Solution | Vector Labs, Burlingame, CA, USA |
| Aprotinin | Sigma-Aldrich, Taufkirchen, Germany |
| Benzamidine | Sigma-Aldrich, Taufkirchen, Germany |
| Bovine Serum Albumin (BSA) | Promega, Mannheim, Germany |
| Bovine Serum Albumin, fraction V | Sigma-Aldrich, Taufkirchen, Germany |
| Bradford reagent | Sigma-Aldrich, Taufkirchen, Germany |
| Chloroform | Sigma-Aldrich, Taufkirchen, Germany |
| Collagen | Sigma-Aldrich, Taufkirchen, Germany |
| 2´-Deoxynucleoside 5´-triphosphates (dNTPs) | Invitrogen, Karlsruhe, Germany |
| Diethylpyrocarbonate (DEPC) | Roth, Karlsruhe, Germany |
| Dimethyl Sulfoxide (DMSO) | Roth, Karlsruhe, Germany |
| EDTA | Sigma-Aldrich, Taufkirchen, Germany |
| Eosin | Roth, Karlsruhe, Germany |
| Ethanol | Sigma-Aldrich, Taufkirchen, Germany |
| Ethidium Bromide | Sigma-Aldrich, Taufkirchen, Germany |
| Formalin, 4% Buffered | Roth, Karlsruhe, Germany |
| Gelatin from Bovine Skin | Sigma-Aldrich, Taufkirchen, Germany |
| D-Glucose | Sigma-Aldrich, Taufkirchen, Germany |
| Glycerol | Sigma-Aldrich, Taufkirchen, Germany |

| | |
|---|---|
| Hematoxylin, Gill's Formula | Roth, Karlsruhe, Germany |
| HOECHST 33258 | Sigma-Aldrich, Taufkirchen, Germany |
| Hydrogen Peroxide | Sigma-Aldrich, Taufkirchen, Germany |
| Isoamylalcohol | Becton Dickinson, Heidelberg, Germany |
| Isopropanol | Becton Dickinson, Heidelberg, Germany |
| Kcl (potassium chloride) | Roth, Karlsruhe, Germany |
| Ladder, DNA, 100bp | Promega, Mannheim, Germany |
| Leupeptin | Sigma-Aldrich, Taufkirchen, Germany |
| Magnesium Chloride | Sigma-Aldrich, Taufkirchen, Germany |
| Mannitol | Sigma-Aldrich, Taufkirchen, Germany |
| MassRuler, DNA Ladder, Low Range | Fermentas, St. Leon-Rot, Germany |
| 2-Mercaptoethanol | Sigma-Aldrich, Taufkirchen, Germany |
| Methanol | Roth, Karlsruhe, Germany |
| Milk Powder | Roth, Karlsruhe, Germany |
| Nacl (sodium chloride) | Sigma-Aldrich, Taufkirchen, Germany |
| Na-deoxycholate | Sigma-Aldrich, Taufkirchen, Germany |
| Na-fluoride | Sigma-Aldrich, Taufkirchen, Germany |
| Na-orthovanidate | Sigma-Aldrich, Taufkirchen, Germany |
| Nonidet-p40 (NP-40) | Sigma-Aldrich, Taufkirchen, Germany |
| Protein Ladder (kDa) | Fermentas, St. Leon-Rot, Germany |
| Phenol | Roth, Karlsruhe, Germany |
| PMSF | Sigma-Aldrich, Taufkirchen, Germany |
| Proteinase K | Sigma-Aldrich, Taufkirchen, Germany |

| | |
|---|---|
| Protein A/G agarose beads | Santa Cruz Biotechnology, Heidelberg, Germany |
| Roti Histo-Kitt II | Roth, Karlsruhe, Germany |
| Schiff's reagent | Sigma-Aldrich, Taufkirchen, Germany |
| Sheath Fluid, BD FACS Flow | Becton Dickinson, Heidelberg, Germany |
| Sodium Acetate | Sigma-Aldrich, Taufkirchen, Germany |
| Sodium Chloride | Sigma-Aldrich, Taufkirchen, Germany |
| Sodium Dodecyl Sulfate (SDS) | Sigma-Aldrich, Taufkirchen, Germany |
| Streptozocin | Sigma-Aldrich, Taufkirchen, Germany |
| TEMED | Sigma-Aldrich, Taufkirchen, Germany |
| Tween-20 | Roth, Karlsruhe, Germany |
| Tissue Tek, O.C.T. Compound | Sakura, Zoeterwonde, The Netherlands |
| Tris-HCL | Sigma-Aldrich, Taufkirchen, Germany |
| TRIZOL | Invitrogen, Karlsruhe, Germany |
| Vectashield | Vector Labs, Burlingame, CA, USA |
| Xylol | Becton Dickinson, Heidelberg, Germany |

## 2.2 Proteins, inhibitors and Peptides

| | |
|---|---|
| aPC, Recombinant Human, Drotrecogin Alpha, Xigris | Eli Lilly Export S.A., Geneva, Switzerland |
| ARIXTRA,(fondaparinux sodium) Solution for Subcutaneous Injection | Organon, Sanofi-Synthelabo, USA |

| | | |
|---|---|---|
| Levolox (Enoxaparin), Low Molecular Weight Heparin, Unfractionated Porcine Heparin | Braun Melsungen AG, Melsungen, Germany | |
| Interferon gamma (IFγ) | Cell Sciences, Canton, MA | |
| Human Protein C | Calbiochem, Schwalbach, Germany | |
| Refludan (Hirudin) | Pharmion, Summit, NJ, USA | |
| Minocycline hydrochloride | Sigma-Aldrich, Taufkirchen, Germany | |
| (U0126) MEK1/2 inhibitor | New England biolabs, Frankfurt, Germany | |
| Thrombin, Human, Plasma Purified | Sigma-Aldrich, Taufkirchen, Germany | |
| Human PAR-1 Agonist TFLLRNPNDK-NH2 | Biosyntan, Berlin, Germany | |
| Human PAR-2 Agonist SLIGRL-NH2 | Biosyntan, Berlin, Germany | |
| Human PAR-3 Agonist TFRGAP-OH | Bachem, Weil am Rhein, Germany | |
| Human PAR-4 Agonist AYPGKF-NH2 | Sigma-Aldrich, Taufkirchen, Germany | |
| Control Peptide FSLLRN-NH2 (SCR) | Biosyntan, Berlin, Germany | |

## 2.3 Antibodies and Serum

| Antibody | Company | Dilution or Concentration |
|---|---|---|
| rabbit polyclonal antibody to Wilms' tumor-1 (C-19) | Santa Cruz Biotechnology, Heidelberg, Germany | 1:50 IHC |
| rabbit polyclonal antibody to | Santa Cruz | 1:50 IHC |

| | | |
|---|---|---|
| synaptopodin (H-140) | Biotechnology, Heidelberg, Germany | |
| rabbit polyclonal antibody to GLEPP1 (H-280) | Santa Cruz Biotechnology, Heidelberg, Germany | 1:50 IHC 1:400 WB |
| Mouse monoclonal anti-human PAR3 (blocking) | Santa Cruz Biotechnology, Heidelberg, Germany | 1:200 (20 µg/ml for blocking studies) |
| Rabbit polyclonal anti-mouse Bax | New England biolabs, Frankfurt, Germany | 1:1000 |
| Rabbit polyclonal anti-mouse Bcl2 | New England biolabs, Frankfurt, Germany | 1:1000 |
| Mouse monoclonal anti-human Bcl2 | Santa Cruz Biotechnology, Heidelberg, Germany | 1:200 |
| Mouse monoclonal anti-mouse P53 | New England biolabs, Frankfurt, Germany | 1:1000 |
| goat polyclonal antibody to complement factor C3 | Cappel, Ohio, USA | 1:50 IHC 1:400 WB |
| FITC labelled goat polyclonal antibody to complement factor C3 | Cappel, Ohio, USA | 1:50 IHC 1:100 FACS |
| Rabbit polyclonal beta-actin | New England biolabs, Frankfurt, Germany | 1:1000 |
| Rabbit monoclonal anti-mouse pERK1/2 | New England biolabs, Frankfurt, Germany | 1:2000 |

| | | |
|---|---|---|
| Rabbit polyclonal anti-mouse/human ERK1/2 | New England biolabs, Frankfurt, Germany | 1:1000 |
| mouse monoclonal antibody to TGFβ1(ab64715) | Abcam, Cambridgeshire, UK | 1:1000 |
| rabbit polyclonal antibody to VEGF(ab9953) | Abcam, Cambridgeshire, UK | 1:1000 |
| rabbit polyclonal antibody to HMGB1(ab18256) | Abcam, Cambridgeshire, UK | 1:1000 |
| Rabbit polyclonal anti-mouse WT1 | Santa Cruz Biotechnology, Heidelberg, Germany | 1:200 |
| Rabbit polyclonal anti-mouse Nephrin | Abcam, Cambridge, UK | 1:1000 |
| Phalloidin-FITC conjugated | Sigma-Aldrich, Taufkirchen, Germany | 50 µg/ml |
| Anti-mouse IgG- HRP | Abcam, Cambridgeshire, UK | 1:5000 |
| Ant-rabbit IgG- HRP | New England biolabs, Frankfurt, Germany | 1:2000 |
| Goat Anti-Rabbit IgG-HRP | Jackson Immuno Research, Suttolk, UK | 4 µg/ml |
| Fluorescein-goat Anti-mouse IgG | Vector Labs, Burlingame, CA, USA | 1:100 |
| Rabbit Serum, Normal | Vector Labs, Burlingame, CA, USA | 10% Blocking |

| | | |
|---|---|---|
| Goat Serum, Normal | Vector Labs, Burlingame, CA, USA | 10% Blocking |

## 2.4 Cells

| | |
|---|---|
| SVEC4-10 | ATCC, Manassas, VA, USA |
| Immortalized Human podocytes | Obtained from Dr. V. Schwenger (Nephrology center, University of Heidelberg) |
| Immortalized Mouse podocytes | Obtained from Dr.Jun Oh (Dept of Pediatric Nephrology, University of Heidelberg) |
| Mouse mesangial cells | ATCC, Manassas, VA, USA |

## 2.5 Media, Sera, Buffers and Supplements

| | |
|---|---|
| DMEM with L-glutamine | ATCC, Manassas, VA, USA |
| Growth Factors and Supplements (FCS, EGF, bFGF, Hydrocortisone, Gentamicin Sulphate, Amphotericin B) | PromoCell, Heidelberg, Germany |
| RMPI-1640 Medium | Sigma-Aldrich, Taufkirchen, Germany |
| Foetal Bovine Serum Standard Quality, EU approved | PAA laboratories, Pasching, Austria |
| Foetal calf Serum Standard Quality, EU approved | PAA laboratories, Pasching, Austria |

| ITS (insulin-transferin-selenium) | Sigma-Aldrich, Taufkirchen, Germany |
| 1x Dulbecco's PBS without Ca and Mg | PAA laboratories, Pasching, Austria |
| 1x HBSS without Ca and Mg | PAA laboratories, Pasching, Austria |
| 100x Penicillin / Streptomycin | PAA laboratories, Pasching, Austria |
| 0.05 % Trypsin and 0.02 % EDTA | PAA laboratories, Pasching, Austria |

## 2.6 Mouse Models

| C57BL/6 | Charles River Laboratories, Inc., Wilmington, MA, USA |
| TM$^{LeD/LeD}$ | Generous gift from Prof. Dr. EM. Conway, UBC Centre for Blood Research, Director, Life Sciences Centre Vancouver, BC, Canada |

## 2.7 Buffers and Solutions

### 2.7.1   Total cell lysate

**RIPA Buffer (final concentration) for whole cell and tissue lysates**

- Tris-HCl: 50 mM, pH 7.4
- NP-40: 1%
- Na-deoxycholate: 0.25%
- NaCl: 150 mM
- EDTA: 1 mM

- PMSF: 1 mM

- Aprotinin, Leupeptin, Benzamidine: 1 microgram/ml each

- $Na_3VO_4$: 1 mM

- NaF: 1 mM

**Note:** Use RIPA buffer with 0.5% Na-deoxycholate for isolation of proteins from tissue.

### 2.7.2   Nuclear Cell lysates

Buffer A: Cytoplasmic Fraction

KCl                  10mM

Hepes pH 7.6        10mM

$NaVO_3$                0.1mM

EDTA                0.1mM

EGTA                0.1mM

DTT                 0.1m

NP40                10%

Protease inhibitors (as mentioned above for RIPA buffer)

Buffer B: Nuclear Fraction

NaCl                   420mM

Hepes pH 7.6        20mM

Glycerol            25%

NaVO3               0.1mM

EDTA                0.1mM

EGTA                0.1mM

DTT                 0.1mM

Protease inhibitors (as mentioned above for RIPA buffer)

### 2.7.3 Buffers for SDS-PAGE

**Electrophoresis resolving-buffer:**

90.825 g (1.5 M) Tris-HCL

20 ml       (0.4 %) 10 % SDS

Adjust pH – 8.8

Make upto **500 ml** with $dH_2O$

**Stacking-buffer:**

30.275 g (1 M) Tris-HCL

8 ml         (0.4 %) SDS

Adjust pH – 6.8

Make upto **200 ml** with $dH_2O$

**Ammoniumpersulfate:**

10 % in $dH_2O$

0.1 g in 1 ml $dH_2O$

**1 x SDS Sample-buffer**

62.5 mM Tris-HCl (pH 6.8 at 25 $^{0}$C)

2 % w/v SDS

10 % Glycerol

50 mM DTT

0.01 % bromophenol blue

**5 x SDS Running-buffer:**

30.2 g (0.125 M) Tris

188 g (1.250 M) Glycine

100 ml 10 % SDS

Make upto 2 litres with     $dH_20$

**Blot-buffer:**

12.125 g (0.02 M) Tris

56.250 g (0.15 M) Glycine

1000 ml Methanol

Make upto 5 litres with $dH_20$

**Blocking buffer:**

**PBS-T and TBS-T**

5 g Non-fat dried milk powder

0.1 % or 0.05 % (v/v) Tween 20

100 ml 1x PBS or TBS

## 2.8 PCR Primer Pairs for RT-PCR

The following primers were purchased from Thermo Electron GmbH, Ulm, Germany

Annealing Temperature: 68°C; Cycles: 35

mTM-f                                    5' TGCTTCTGCTATGA GGCTATGAGTT 3'

mTM-r                                    5' GGGGTCACAGTCTTTGCTAATCTG A 3'

Annealing Temperature: 67°C; Cycles: 20

mβ-actin-f                              5' CCGTAAAGACCTCTATGCCAACA 3'

mβ-actin-r                              5' CGGACTCATCGTACTCCTGCT 3'

## 2.9 Kits

| | |
|---|---|
| Albumin ELISA kit | Bethyl Laboratories, Inc, Montgomery, Texas, USA) |
| BCA reagent | Pierce, Germany |
| DAB Substrate Kit for Peroxidase | Vector Labs, Burlingame, CA, USA |
| ECL reagent (enhanced chemiluminiscence) | Amersham Biosciences, Buckinghamshire, UK |
| *In Situ* Cell Death Detection Kit, Fluorescein and POD (TUNEL) | Roche Diagnostics, Mannheim, Germany |
| MOM kit | Vector Labs, Burlingame, CA, USA |
| TAT Elisa Kit | Simens Health Care |
| Superscript II Reverse Transcriptase Kit | Invitrogen, Karlsruhe, Germany |

## 2.10   Equipment

| | |
|---|---|
| ACCU-CHEK Glucose Sticks | Roche Diagnostics, Mannheim, Germany |
| Accutrent Glucometer | Roche Diagnostics, Mannheim, Germany |
| BD Chamber Slides for cell culture | Becton Dickinson, Heidelberg, Germany |
| BD FACS Scan flow cytometer | Becton Dickinson, Heidelberg, Germany |
| BioDoc Station | Biometra, Goettingen, Germany |
| Blot chamber | Trans-Blot Electrophoretic Transfer Cell, Biorad Laboratories, Germany |
| Cell culture flasks and dishes | Becton Dickinson, Heidelberg, Germany |

| | |
|---|---|
| Cell F Imaging Software | Olympus, Hamburg, Germany |
| Cover slips for cell culture | Paul Marienfeld GMBH & Co.KG, Lauda-Königshofen, Germany |
| Developer (for western blot) | CURIX 60, Agfa |
| Immobilon-P Transfer Membrane | Millipore Corporation, Billerica, MA |
| Power supply unit | Biorad Laboratories, Germany |
| Pipettes | Gilson Pipetman, Gilson, Germany |
| Pipettes, pipette tips and falcon tubes | Sarstedt, Germany |
| Spectrophotometer | BIO-TEK-Instruments, Inc, Vermont, USA |
| Spectrofluorimeter | BIO-TEK-Instruments, Inc, Vermont, USA |
| Olympus Ax70 Microscope | Olympus, Hamburg, Germany |
| T3000 Thermocycler | Biometra, Goettingen, Germany |
| Hettich-Table Centrifuge | GMI, Inc, Minnesota, USA |
| Centrifuge-labofuge-400R | Heraeus, M & S laborgeräte GMBH, Wiesloch, Germany |
| High speed ultracentrifuge | Beckman Coulter GMBH, Krefeld, Germany |
| X-ray film | Amersham Biosciences, UK |

## 2.11 Induction of diabetes using streptozotocin

Diabetes was induced by intraperitoneal administration of streptozotocin (STZ) at 60 mg/kg, freshly dissolved in 0.05 M sterile sodium citrate, pH 4.5, on 5 subsequent days in eight week old mice. Mice were considered diabetic if blood glucose levels

were larger 300 mg/dl 16–25 days after the last STZ injection. Blood glucose levels were determined in samples from the tail vein using ACCU-CHEK glucose sticks. In the first 2 weeks after onset of diabetes, blood glucose was measured daily. If glucose levels occasionally recovered, an additional STZ injection was given on days 25–27. On average 90% of mice became diabetic (blood glucose > 300 mg/dl) within the first 4 weeks and were used throughout the experiments. Mice not developing diabetes and maintaining blood glucose levels < 200 mg/dl were included in the control group. Mice with blood glucose levels between 200 and 300 mg/dl were excluded. Further control mice were injected with sodium citrate, pH 4.5 only. Mice displaying blood glucose levels above 500 mg/dl received individual supplementation with 1–2 U Insulin Semilente. After blood glucose levels had stabilized, blood glucose was determined at least once a week. Only mice with blood glucose levels consistently larger than 300 mg/dl were used in the diabetes groups. Additional groups of diabetic mice we treated with enoxaparin, a low molecular weight heparin , or fondaparinux , a FXa inhitor or minocycline, a semisynthetic tetracycline, inhibiting the release of cytochrome *c* from mitochondria and caspase-1, capase-3 and iNOS transcriptional up-regulation and activation. Blood samples and tissue samples were obtained after 26 weeks of persistent hyperglycemia in diabetic mice. Control samples were obtained from age matched littermates. At time of analysis the mouse weight and weight of organs was recorded.

## 2.12    Determination of Albuminuria

Shortly before sacrificing individual mice were placed in metabolic cages and

twenty-four hour urine samples were collected. Urine albumin was determined using the mouse albumin ELISA according to the manufacturer's instructions. Urine creatinine was determined using the Jaffé method.

## 2.13    Histology and immunohistochemistry

Animals were perfused with ice-cold PBS followed by perfusion with 4% buffered Para formaldehyde. Tissues were further fixed in 4% buffered Para formaldehyde for 2 days, paraffin embedded, and processed for sectioning. TUNEL assays on tissue sections were done according to the manufacturer's instructions. The frequency of apoptotic cells was determined by a blinded investigator by counting TUNEL positive cells and total cell number within a glomeruli and calculating the percentage of TUNEL positive cells. Podocyte numbers were determined by counting WT-1 positive cells within glomeruli, atleast 50 glomeruli were counted from each sample.   For morphological analyses at least 7 different mice per group with at least 50 glomeruli each were included.

## 2.14    RT-PCR

RNA was isolated using TRIZOL reagent from tissue samples stored in RNA-later immediately after prepping. cDNA was generated using 1 μg total RNA following treatment with DNAse in the concentration 5U/5μg RNA followed by reverse transcription using Superscript II. The PCR products were separated on a 1.8% agarose gels and visualized by ethidium bromide staining. Expression was

normalized to β-actin. Reactions lacking reverse transcriptase served as controls.

## 2.15    Cell culture

All experiments performed with mouse vascular endothelial cells (SVEC4-10) were done with passages 4 to 10. Cells were grown on 0.2% gelatin coated plates and maintained at 37 $^{o}$C in a humidified 5% CO2 incubator using endothelial growth medium in the presence of growth factors and supplements. Cells were subcultered at confluence by trypsinization with 0.05% trypsin and 0.02% EDTA and medium changed every other day. All *in vitro* experiments, except those where thrombin was added, were performed in the presence of hirudin (1µg/ml).

Conditionally immortalized mouse wild-type podocytes were cultures as described elsewhere(64; 65). In brief, podocytes were routinely grown on collagen type 1 at 33 $^{o}$C in the presence of interferon γ (10 U/ml) to enhance expression of a thermosensitive T antigen. Under these conditions, cells proliferate and are undifferentiated. To induce differentiation, podocytes were grown at 37 $^{o}$C in the absence of interferon γ for 14 days. We performed experiments 14 days after induction of differentiation. Differentiation was confirmed by determining expression of Synaptopodin and Wilms´ tumor-1 protein.

## 2.16    Protein C activation assay *in vivo*

Mice were anesthetized and human PC (20 µg in 100 µl 1xPBS) or 1xPBS (100 µl) per mouse were injected *via* the tail vein. After 10 min blood samples were collected from the vena cava into 0.38% sodium citrate and 50 mM benzamidine HCl (final

concentrations). Human aPC was captured from these plasma samples using an antibody highly specific for human aPC (HaPC 1555), and the activity of the captured human protein C was determined using the chromogenic substrate SPECTROZYME® PCa as described elsewhere (16).

## 2.17   Immunoblotting

Tissue homogenates were prepared using the RIPA buffer containing 50 mM Tris (pH7.4), 1% NP-40, 0.5% sodium-deoxycholate, 150 mM Nacl, 1 mM EDTA, 1 mM $Na_3VO_4$, 1 mM NaF supplemented with protease inhibitor cocktail (from Roche). Supernatants were quantified and equal amount of protein samples were electrophoretically separated on 10% or 12.5% SDS polyacrylamide gel, transferred to PVDF membranes (Millipore) and probed with desired primary antibodies. The membrane was then washed with TBST and incubated with α-mouse IgG (1:5000) or α-rabbit IgG (1:2000) horseradish peroxidase conjugated antibody. Blots were developed with the enhanced chemiluminiscence system (Amersham Piscataway, NJ, USA). To compare and quantify levels of proteins, the density of each band was measured by densitometer (Biorad). Equal loading was confirmed by staining with coomassie brilliant blue -R (CBB) and by actin-western blot.

## 2.18   Subcellular fractionation

For determination of Bax-translocation into mitochondria the cytosolic and

membranous fractions were isolated as described previously (67). Cells were washed in PBS and scraped into isotonic buffer (200 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM HEPES-NaOH (ph-7.4), 1 mM DTT, protease inhibitors (as mentioned above) and homogenized with a potter Elvehjem homogenizer. Nuclei and unbroken cells were removed by centrifugation at 500 g for 10 min at 4$^{o}$C. The supernatant was centrifuged again at 100,000 g for 60 min, and the supernatant was saved as the cytosolic fraction. The pellet was washed again in isotonic buffer (15000 x g, for 5 min at 4$^{o}$C), re-suspended in extraction buffer (20 mM HEPES; ph-7.4, 250 mM Nacl, 1 % NP-40, 1 mM DTT, 2 mM EDTA, 2 mM Sodium orthovanadate, Protease inhibitors (as mentioned above) and centrifuged at 20,000 g for 5 min to remove debris. Samples were subsequently used for Western Blotting as described.

For isolation of cytoplasmic and nuclear extracts from tissues, tissues were homogenized in buffer containing 10 mM HEPES, pH 7.9, 10 mM KCL, 1.5 mM MgCl2, and 0.5 mM dithiothreitol. After incubation at 4$^{o}$C for 15 min, the lysates were vortexed briefly, centrifuged for 30 s in a microcentrifuge, and the supernatant (cytoplasmic fraction) was removed and frozen. The pellet, which contained nuclei was resuspended in extraction buffer containing 100 µl of 20 mM HEPES, pH 7.9, 20% v/v glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethanesulfonyl fluoride, incubated at 4$^{o}$C for 30 min. The nuclear extracts were then centrifuged at 14000 rpm for 20 min at 4$^{o}$C. The supernatant was collected and stored at -80 $^{o}$C. Protein concentration was measured using Bradford reagent or BCA reagent (Pierce, Germany).

## 2.19　Isolation and culture of primary mouse podocytes

Mouse primary cultured podocytes from out growths of isolated glomeruli were prepared essentially as described previously (68). Briefly, glomeruli were isolated from wild type and $TM^{Led/Led}$ mice (male, 8 weeks old) under sterile conditions by differential sieving of cortical tissue with decreasing pore sizes (200 µM, 150 µM, 100 µM and 70 µM). After the final wash the isolated mouse glomeruli (purity >95%) were resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum and ITS solution (Sigma).　For outgrowth of podocytes, isolated whole glomeruli were placed on 0.2% collagen (Sigma) coated 6 well plates and maintained at 37 $^{o}$C in a humidified 5% $CO_2$ incubator.　When cells reached confluence, they were removed with 0.025% trypsin-0.5 mM EDTA and subcultured in collagen coated 6 well plates at 1:4 dilutions. The cells showed typical podocyte morphology and positive immunocytochemical staining of synaptopodin.

## 2.20　*In vitro* analyses of complement activation

For *in vitro* analyses of complement activation we used either SVEC4-10 cells, which do not endogenously express TM, or primary mouse podocytes isolated from $TM^{LeD/LeD}$ or $TM^{wt/wt}$ mice. Cells were cultured as described above, but mouse serum was used for the experiments. A subset of cells was incubated for 48 h with high glucose concentrations (30 mM final concentration), SVEC4-10 cells were trypsinized and resuspend in 1xPBS, Following 30 min incubation with 10% mouse (sources of complement) at 37 °C in the presence or absence enoxaparin (55 mg/ml), or

fondaparinux (12,5 mg/ml). Concentrations of anticoagulants for *in vitro* studies were based on calculated peak concentrations from mouse *in vivo* experiments. We incubated the cells with FITC-conjugated goat antibody to C3 (Cappel ICN) and analyzed the cells for the presence of surface C3 by flow cytometry (BD FACS scan), as described else where (69). Since the number of primary podocytes obtained was not sufficient for FACS analyses cells were grown in chamber slides, briefly fixed in 4% formaldehyde ), washed three times with PBS, incubated with the FITC labelled goat anti-C3 antibody (30 min), washed again, and then were counterstained with Hoechst 33258 (3.5 µg/ml). The overall C3 staining intensity was determined by a blinded investigator scoring C3 positive and total cell number in at least 20 random areas with the help of an image analysis software Imagine Pro 5.0. Experiments were performed in triplicates.

## 2.21   Red blood cell hemolysis assay

The red cell hemolysis assay was conducted employing a well-established protocol (70). Briefly, freshly collected and carefully washed (3 times) rabbit red blood cells (RBC) were incubated with goat anti-rabbit RBC antiserum (20 min, 37 °C, Cappel, Ohio, OH, USA). RBCs were then again washed (3 times) and resuspended at a concentration of 1.5 x 108 cells/ml. Human (a) or mouse (b) serum (300µl) with different dilution of anticoagulants were then added to 50 µl of antibody incubated RBCs in the presence of 0.15 mM $Ca^{2+}$ and 1 mM $Mg^{2+.}$ For positive control 300 µl water and for a negative control 300 µl assay buffer were added. Cells were

incubated at 37°C for 60 min, then centrifuged (1800 RPM for 10 minutes), and the absorbance (415 nm) in the supernatants was determined within 15 minutes after centrifugation. The hemolysis rate was calculated by comparing the sample OD with the positive control (100%) after subtracting the value of the negative control.

## 2.22    Immunoprecipitation

Immunoprecipitation of podocyte total cellular proteins was done as mentioned else where (71). Total cellular proteins were extracted with PBS containing 25 µM of proteosome inhibitor MG132 and complete protease inhibitor mixture and were sonicated (set at 10% of maximum speed) for 10 sec followed by 1-min incubation on ice. This process was repeated five times. Lysates were combined with 3 µg of specific antibody and incubated for 2 h at $4^0$C. Immunoprecipitates were collected with with protein A/G agarose beads and washed with PBS containing 25 µM MG132 and protease inhibitor mixture. Immunoprecipitates were fractionated by SDS-PAGE (10%), transferred to membranes, and subjected to Immunoblotting with appropriate primary and secondary antibodies.

## 2.23    Cloning of TM$^{wt}$ and TM$^{Led}$ expression constructs

The expression construct for wild type TM (TM$^{wt}$) was generated as follows: mouse genomic DNA was used as a template for PCR amplification. The whole intronless coding sequence was amplified using the primers mTM 1F (5'- GGC GCG CCG CCA CCA TGC TTG GGA TTT TCT TTC -3') and mTM 1R (5'- GAA TTC TCA GAA CTT

CTG CAG CGT CCG -3') using Phusion® High-Fidelity Tag Polymerase. Cycling parameters were as follows: 1 min at 98°C; followed by 35 cycles of 10 seconds at 98°C, 20 seconds at 68°C, 30 seconds at 72°C, and finally a 10 min extension step at 72°C. After amplification, 50 µL of the amplimer was electrophoretically separated on a 0.8% agarose gel containing 10 µg/ml ethidium bromide and visualized under UV transillumination, yielding single 1754bp amplimers. PCR fragments were gel purified and subcloned into the mammalian expression vector pcDNA™3.1/V5-His TOPO and sequenced on both strands to exclude mutations.

The lectin-like module within the N-terminal domain of TM was removed by PCR as follows: DNA fragments encoding the 1-45 AA peptide and 148-577AA peptide of mouse TM were amplified using primers mTM 1F (5'- GGC GCG CCG CCA CCA TGC TTG GGA TTT TCT TTC -3'), mTM 2R (5'-GGG CGC TGG AAG GAG GAA TTC-3' ) and mTM 2F (5'- GAA TTC CTC GGC CGG ACC CTT C-3'), mTM 3R (5'-CAC GTG TCA GAA CTT CTG CAG CGT CCG -3'), respectively. TM[wt] construct served as a template. Amplimers were purified and subcloned into the mammalian expression vector pcDNA™3.1/V5-His TOPO as described above, yielding the plasmid pT-TM[Led]-1 and pT-TM[Led]-2. The TM[Led] mutant was obtained by subcloning the HindIII/EcoRI fragment encoding the 1-45 AA peptide of mouse TM (isolated from pT-TM-1) into HindIII/EcoRI of pT-TM-2.

## 2.24 Generation of stably transfected TM<sup>Led</sup> and TM<sup>wt</sup> expressing SVEC4-10 cells

SVEC4-10 cells were cultured in DMEM supplemented with 10% FCS. For *in vitro* transfection cells were grown in 6 well plates and transfected with TM plasmids according to the manufacturer's instructions. Stably transfected cell lines were selected in the presence of 0.2 mg/ml G418. After 7–10 days of selection cells were subcloned by seeding individual clones into 96 well plates. About 30 clones each were isolated and clones with comparative TM expression were selected by characterizing protein C activation (a function of TM independent of the lectin like domain) (72).

## 2.25 Statistical analyses

The data are summarized as the mean ± SEM (standard error of the mean). Statistical analysis was performed using Student's *t* test or ANOVA (as indicated in the figure legend or the text). StatistiXL software (www.statistixl.com) was used for all statistical analyses. Statistical significance was accepted at the $P < 0.05$ level.

## 3. Results

Consistent with our observation in the past (16), aPC protects against diabetic nephropathy *via* PAR-1 and EPCR, later on we were able to show that low dose thrombin is also protective in diabetic nephropathy (21). So far we have addressed the role of aPC and thrombin in TM-PC system in diabetic nephropathy, the role of TM in diabetic nephropathy still remains largely unknown. In order to elucidate the role of TM in diabetic nephropathy, the next we applied a mutant mouse strain which lacks the N-terminal lectin like domain.

## 3.1 Diabetic nephropathy is enhanced in diabetic TM$^{LeD/LeD}$ mice

To evaluate the role of TM's lectin-like domain in diabetic nephropathy we induced persistent hyperglycemia in mice lacking the lectin-like domain (TM$^{LeD/LeD}$ mice) and in wild type littermates (TM$^{wt/wt}$). The mortality of mice following streptozotocin injections or during the 26 week follow up did not differ. Likewise, blood glucose levels did not differ between TM$^{wt/wt}$ and TM$^{LeD/LeD}$ mice (Figure 3.1a). After 26 weeks of hyperglycemia albuminuria was significantly increased in diabetic TM$^{LeD/LeD}$ mice (290 µg/mg vs. 166 µg/mg, $P$=0.03, Figure 3.1b), while no difference in albuminuria was observed in non-diabetic TM$^{LeD/LeD}$ and TM$^{wt/wt}$ mice (46 µg/mg vs. 49 µg/mg, $P$=0.51, Figure 3.1b).

Histological analyses of tissue sections obtained from perfusion fixed renal tissues showed enhanced accumulation of PAS-positive matrix in diabetic TM$^{LeD/LeD}$ mice (mesangial index 17.3% vs. 12.0%, $P$=0.03, Figure 3.1c-e). Of note, a significant increase of the mesangial index was already apparent in non-diabetic TM$^{LeD/LeD}$ mice (6.4% vs. 4.0%, $P$<0.001, Figure 3.1c-e). Similar results were obtained when analyzing Masson`s trichrome stained sections (Figure 3.1f-g). As noted above, the morphological changes in non-diabetic TM$^{LeD/LeD}$ mice were not associated with albuminuria. Furthermore, VEGF and TGFβ1 expression and Smad3 phosphorylation (a target of TGFβ1 signalling) did not differ between non-diabetic TM$^{LeD/LeD}$ and TM$^{wt/wt}$ mice  (Figure 3.1h-j). However, these markers of diabetic nephropathy were increased in diabetic TM$^{LeD/LeD}$ mice in comparison to diabetic TM$^{wt/wt}$ mice (VEGF:

239% vs. 179%, *P*=0.03; TGFβ1: 290% vs. 198%, *P*=0.01; Smad3 phosphorylation: 314% vs. 191%, *P*<0.001, Figure 3.1h-j), which is in agreement with the albuminuria data. Thus, the absence of TM's lectin-like domain aggravates nephropathy in diabetic mice.
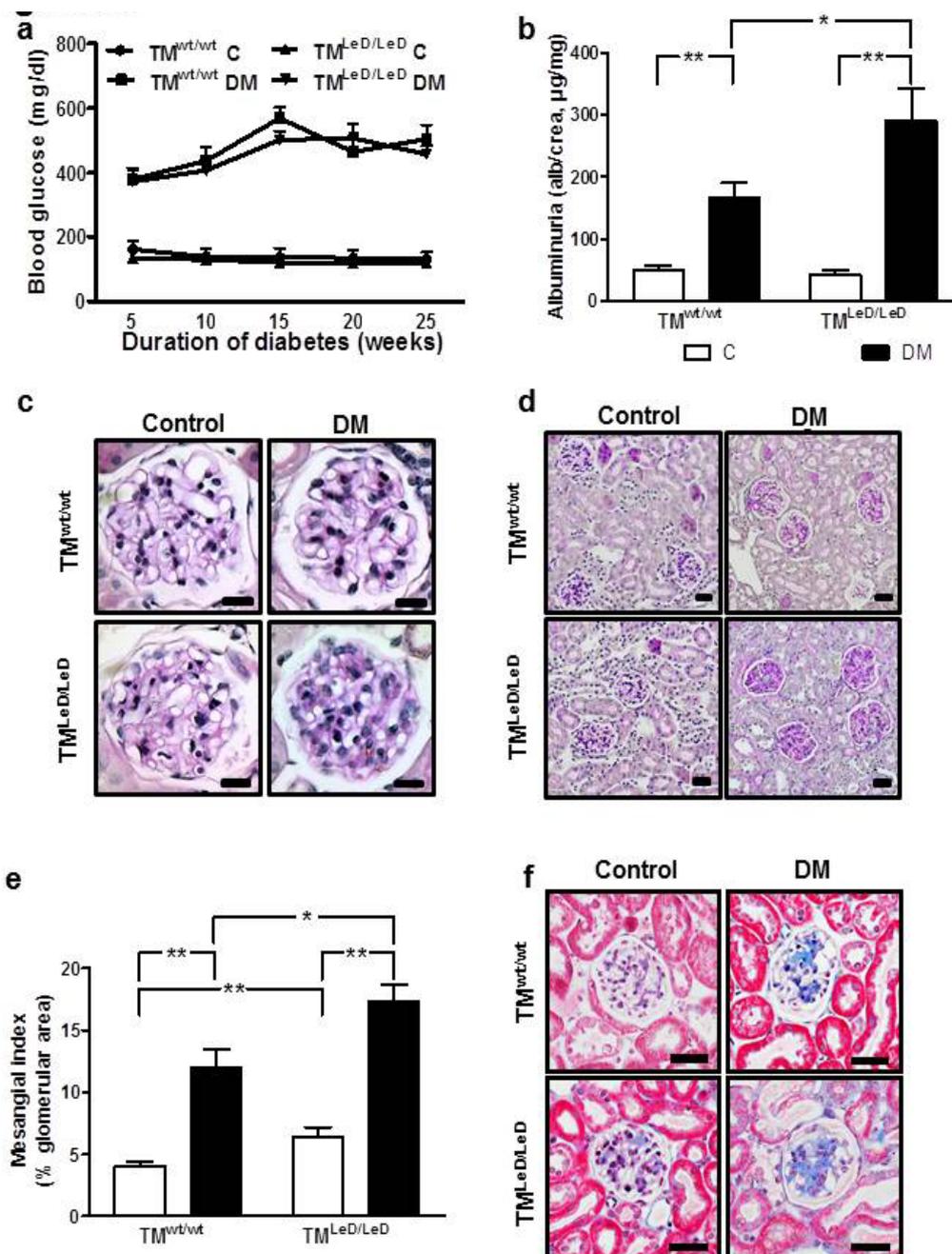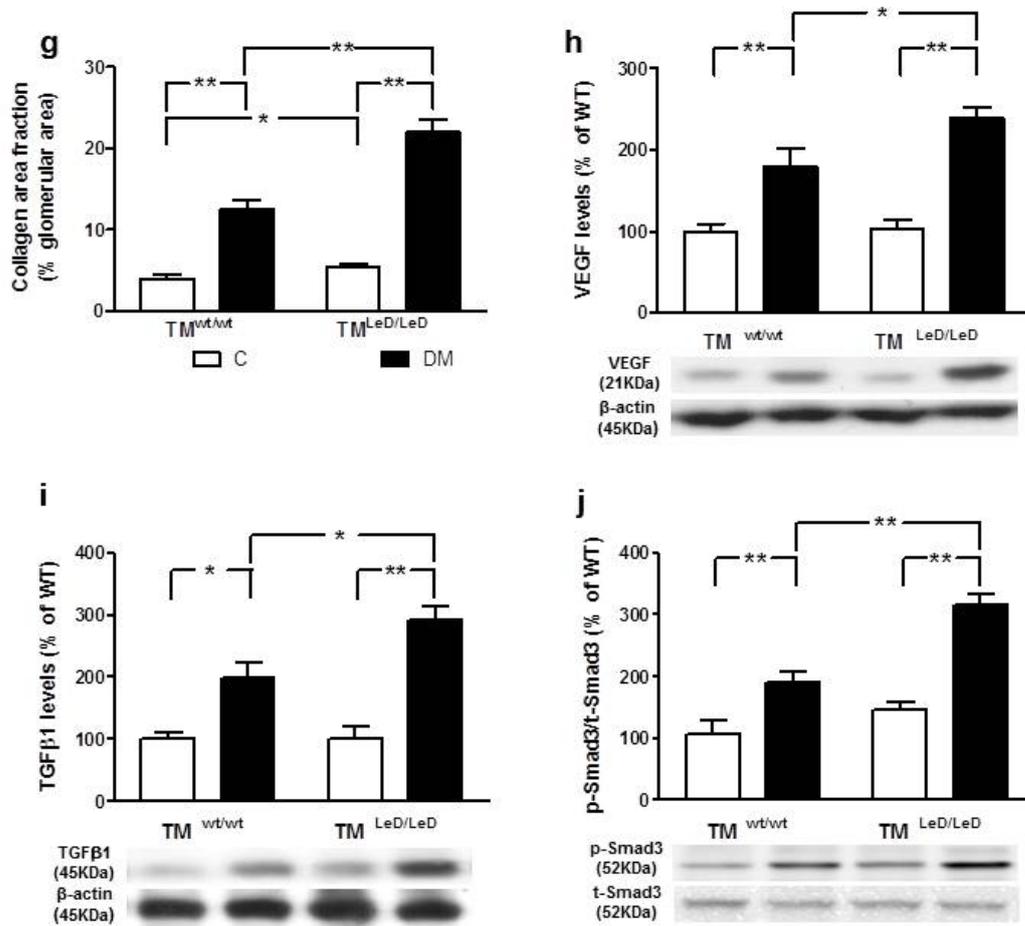
**Figure 3.1 Loss of TM's lectin-like domain aggravates experimental diabetic nephropathy**

a: Blood glucose concentrations in non-diabetic wild-type (TM^wt/wt C, n=15) and TM^LeD/LeD (TMLeD/LeD C, n=14) and diabetic wild-type (TM^wt/wt DM, n=12) and TM^LeD/LeD (TM^LeD/LeD DM, n=12) mice.

b-j: Albuminuria (b), the mesangial index (c,d,e), expression of VEGF (h) or TGFβ1 (i), and phosphorylated Smad3 (j) in renal cortex extracts of TMwt/wt or TM^LeD/LeD mice without or with diabetes (n≥10 for each group). These indices of diabetic nephropathy are significantly increased in diabetic TM^LeD/LeD as compared to diabetic TM^wt/wt mice. Representative images of histological sections (c,d,f), bar graph showing the mesangial index (d; ≥50 glomeruli of at least 7 different mice), images of immunoblots against VEGF, TGFβ1, and Smad3 (h-j; bottom), and bar graph summarizing results of immunoblots (h-j, top; n≥7 per group).

Scale bar: 20 μm (c, f) 40μm (d); C: non-diabetic control mice, open bars; DM: diabetic mice, black bars; mean value ± SEM; ★: P<0.05, ★★: P<0.01.

## 3.2 TM's lectin-like domain aggravates diabetic nephropathy independent of HMGB-1

The lectin-like domain of TM has been previously documented to mediate cytoprotection by scavenging HMGB-1 or by inhibiting apoptosis. Hence we evaluated whether the protective properties of the lectin-like domain of TM in diabetic nephropathy are related to these functions. Using immunohistochemistry we did not
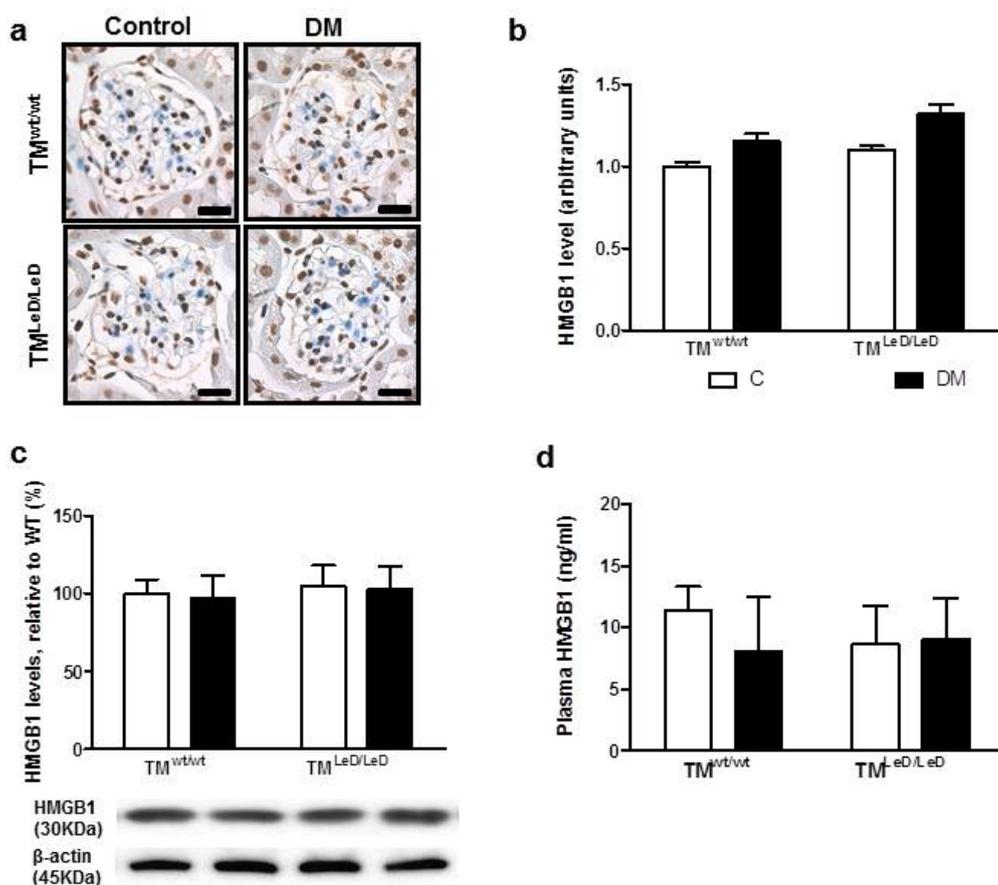


**Figure 3.2 TM's lectin-like domain regulates diabetic nephropathy independent of HMGB1**

**a,b:** Using immunohistochemical staining no difference of tissue HMGB1 is detected in TM$^{wt/wt}$ or TM$^{LeD/LeD}$ mice without or with diabetes. Representative images of immunohistochemical stains (a, HMGB1 detected by

observe increased staining for HMGB-1 in renal glomeruli of diabetic TM^LeD/LeD mice (score 1.32 vs. 1.15 in non-diabetic TM^wt/wt mice, *P*=0.17, Figure 3.2a, b). Likewise, no differences were observed in HMGB-1 protein levels in kidney lysates (Figure 3.2c) or in plasma (Figure 3.2d) irrespective of the genotype or the presence or absence of diabetes. Furthermore, treatment of diabetic TM^LeD/LeD mice with transhinone IIA, which attenuates cellular HMGB-1 release and is used in patients with cardiovascular diseases (73), had no effect on albuminuria or histological indices of diabetic nephropathy in diabetic control or TM^LeD/LeD mice (data not shown).

Taken together, TM's lectin-like domain modulates diabetic nephropathy independent of its scavenger function in regard to HMGB-1 and independent of its antiapoptotic effect.

## 3.3 TM's lectin-like domain aggravates diabetic nephropathy independent of apoptosis

We have previously shown that experimental diabetic nephropathy in mice can be ameliorated by inhibiting glomerular apoptosis (16). Since TM's lectin-like domain mediates antiapoptotic effects (1) we next evaluated whether apoptosis contributes further to the diabetic nephropathy in diabetic TM^LeD/LeD mice. Consistent with

previous reports glomerular apoptosis was enhanced in diabetic mice (16), both in
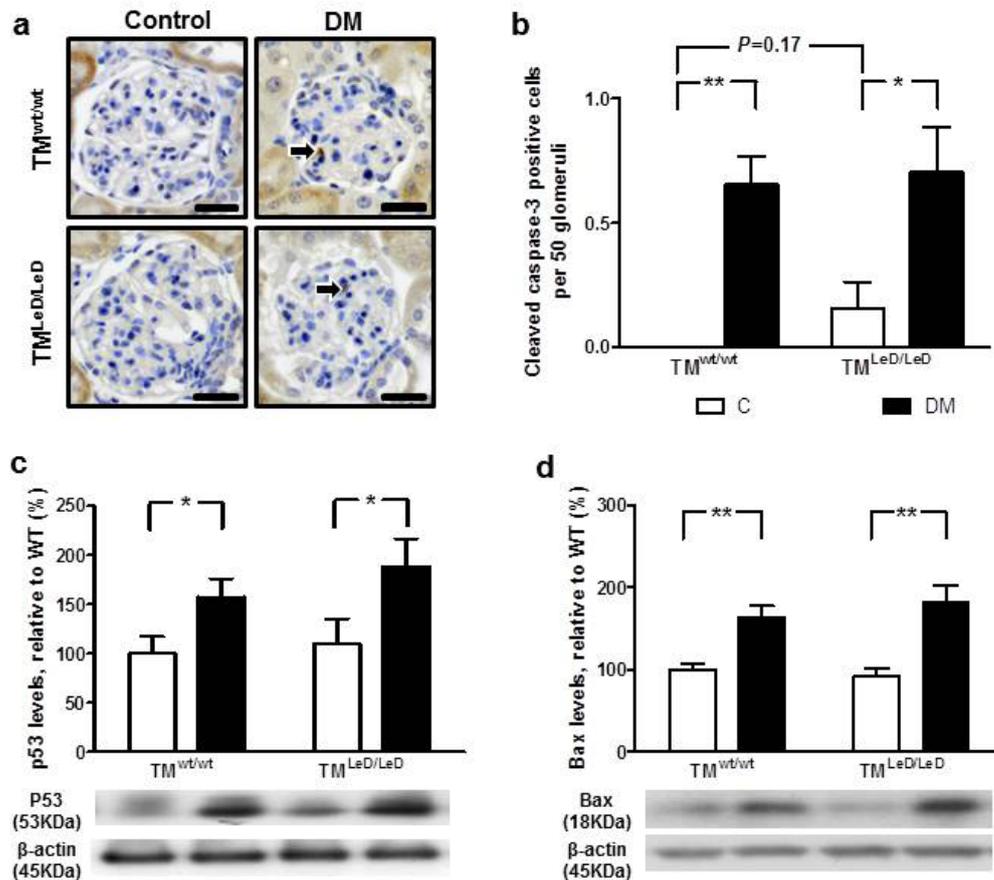


**Figure 3.3 TM's lectin-like domain regulates diabetic nephropathy independent of apoptosis**

a,b: Cleaved caspase-3, immunohistochemically detected, is increased to a similar extent in diabetic TM[wt/wt] and diabetic TM[LeD/LeD] mice. Representative images (a, cleaved caspase-3 detected by HRP-DAB reaction, brown; hematoxylin counterstain, blue). The frequency of cleaved caspase-3-positive cells per 50 glomerular profiles was determined (b, n≥10 for each group).

c,d: Expression of apoptosis regulators in renal cortex extracts. Bar graph (top) and representative immunoblot (bottom) showing p53 (c) and Bax (d) expression in renal cortex tissue samples (n≥7 for each group).

Scale bar: 20 µm (a); C: non-diabetic control mice, open bars; DM: diabetic mice, black bars; mean value ± SEM; ★: $P<0.05$, ★★: $P<0.01$.

diabetic TM[wt/wt] mice and in diabetic TM[LeD/LeD] mice (Figure 3.3a,b). However, no difference in apoptosis was apparent when comparing diabetic TM[wt/wt] with diabetic TM[LeD/LeD] mice (3.86% vs. 4.34%, $P=0.13$, Figure 3.3a, b). Likewise, expression of p53 or Bax did not differ between diabetic TM[wt/wt] or TM[LeD/LeD] mice (188% vs. 157%

for p53, *P*=0.36, 159% vs. 142% for Bax, *P*=0.41, Figure 3.3c, d). Consistent with these findings, a tetracycline derivate (minocycline) which prevents glomerular apoptosis in diabetic mice (16) had no effect on indices of diabetic nephropathy in diabetic TM^LeD/LeD mice (data not shown).


## 3.4 Enhanced glomerular complement deposition in diabetic TM^LeD/LeD mice


To explore whether the role of TM's lectin-like domain in experimental diabetic nephropathy may be related to complement activation we determined renal C3 complement deposition. C3 complement deposition did not differ between non-diabetic TM^wt/wt mice and TM^LeD/LeD mice (1.0 vs. 1.5, *P*=0.75, Figure 3.4a-c). In diabetic mice C3 deposition was only occasional observed outside of glomeruli and did not differ between genotypes (Figure 3.4a). Conversely, glomerular C3 deposition was increased in diabetic TM^wt/wt and to an even larger extent in diabetic TM^LeD/LeD mice (5.2 vs. 2.6 in diabetic TM^wt/wt mice, *P*=0.03, Figure 3.4b,c). Tissue levels of the C3 40 kDa α-chain fragment, which reflects complement activation, were increased in diabetic TM^wt/wt mice (165% vs. 100%, *P*=0.02) and were further elevated in diabetic TM^LeD/LeD mice (242% vs. 165% in diabetic TM^wt/wt mice, *P*<0.001, Figure 3.4d,e). Likewise, C5b-9 formation was augmented in diabetic TM^wt/wt mice (161% vs. 100%, *P*=0.01) and to a larger extend in diabetic TM^LeD/LeD mice (216% vs. 161% in diabetic TM^wt/wt mice, *P*=0.01, Figure 3.4f).
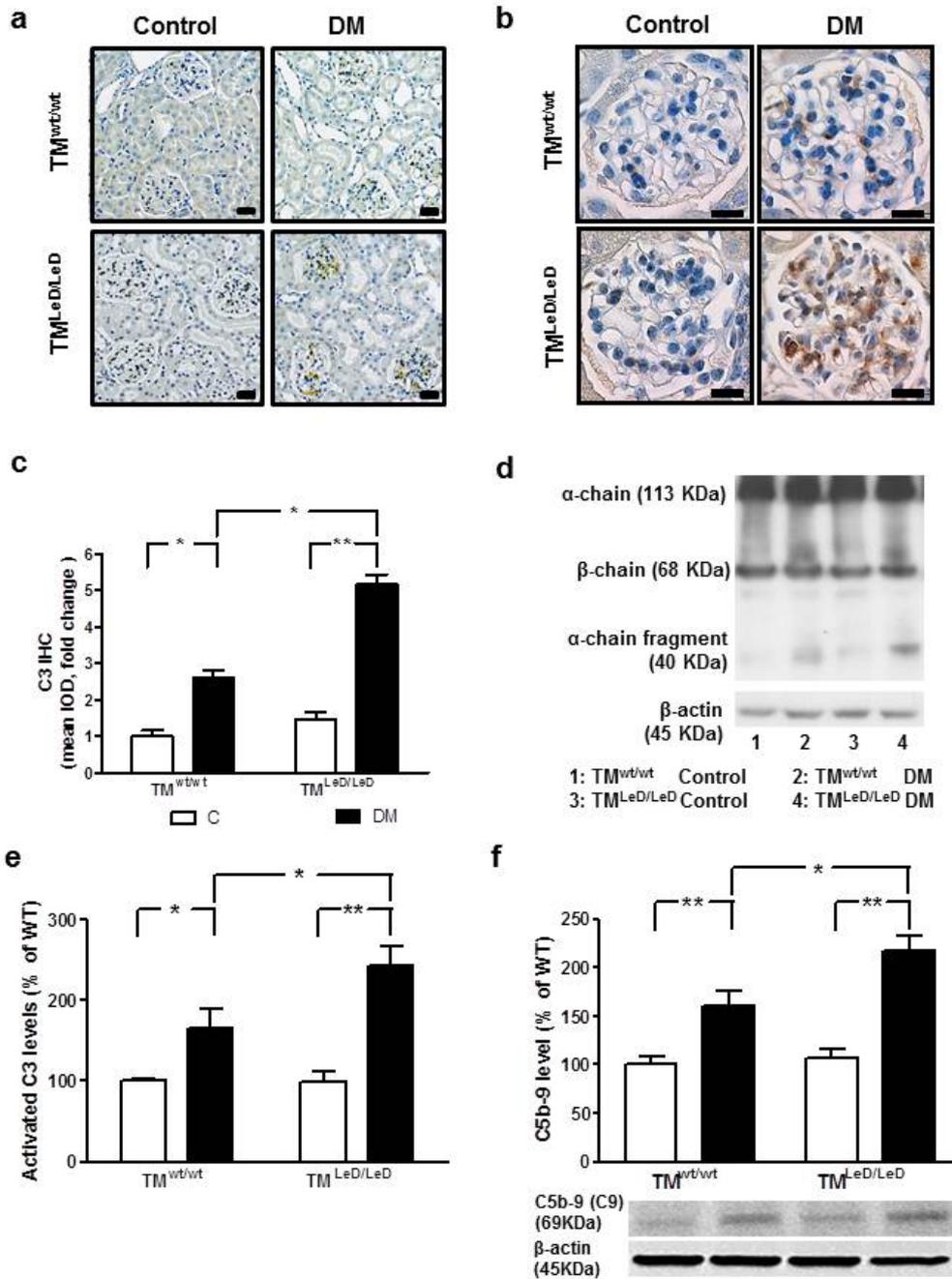
**Figure 3.4 Enhanced glomerular C3 deposition in diabetic TM$^{LeD/LeD}$ mice**

a-c: Immunohistochemical detection of glomerular C3 deposition in TM$^{wt/wt}$ or TM$^{LeD/LeD}$ mice without or with diabetes. Representative images (a and b, C3 detected by HRP-DAB reaction, brown; haematoxylin counterstain, blue) and bar graph reflecting glomerular C3 deposition (c), n≥10 for each group.

d,e: Increased levels of the 40 kDa C3 α-chain fragment in renal cortex extracts of diabetic TM$^{LeD/LeD}$ as compared to diabetic TM$^{wt/wt}$ mice. Representative immunoblot (d) and bar graph summarizing results (e; n≥7 for each group).

f: C5b-9 (MAC) deposition is increased in diabetic TM$^{LeD/LeD}$ compared to diabetic TM$^{wt/wt}$ mice; representative immunoblot and bar graph summarizing results (n≥10 for each group).

Scale bar: 40 μm (a), 20 μm (b); C: non-diabetic control mice, open bars; DM: diabetic mice, black bars; mean value ± SEM; ✶: P<0.05.

Contrasting the differential effect of TM`s lectin like domain on complement activation in renal glomeruli of diabetic mice no differences in glomerular apoptosis, as reflected by cleaved caspase-3 or expression of apoptosis regulators in renal cortex extracts, were observed between non-diabetic or diabetic groups, respectively (Figure 3.3). In addition, no differences in HMGB-1 levels were observed (Figure 3.4). Taken together, these data suggest that TM's lectin-like domain regulates diabetic nephropathy by modulating complement activity in hyperglycemic mice.

## 3.5 Effect of enoxaparin and fondaparinux on complement activation
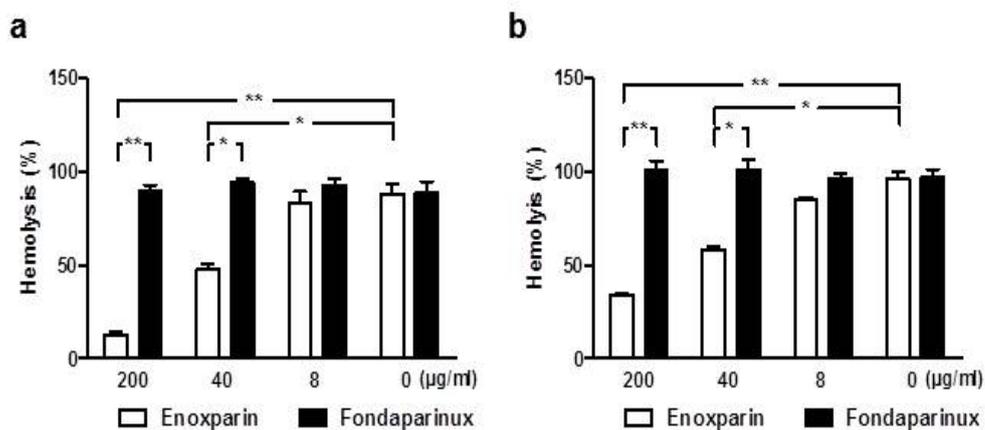


**3.5 Effect of enoxaparin and fondaparinux on complement activation**
Enoxaparin (open bars), but not fondaparinux (black bars) reduces complement activation in human (a) and background matched mouse (b) serum samples, as determined by the red cell hemolysis assay.

With human serum samples as complement source, Enoxaparin (open bars) at

concentration of 40µg/ml can supress the hemolysis by about 50%, at 200µg/ml even further to about 80%, but not fondaparinux (black bars), at concentration of 200µg/ml can only suppress about 5% of the hemolysis, which is not significant in compare with control group(Figure 3.5a) and by applying background matched mouse serum samples we could get the same tendency as determined by the red cell hemolysis assay (Figure 3.5b).

## 3.6 Enoxaparin, but not Fondaparinux, reduces glomerular complement C3 deposition in diabetic TM$^{LeD/LeD}$ mice

Complement inhibitors specifically for mice, in particular for long term *in vivo* intervention studies, are yet not readily available. To this end we used enoxaparin and fondaparinux, two factor Xa inhibitors which differ in their ability to inhibit complement (69). As described in the last paragraph, employing the red cell hemolysis assay we first confirmed that enoxaparin (at 40 µg/ml or higher), but not fondaparinux (up to 200 µg/ml), inhibited complement activation (Figure 3.5).

Treatment of diabetic TM$^{LeD/LeD}$ mice with enoxaparin or fondaparinux provided comparable anticoagulation (Figure 3.6a). Both treatments had no effect on blood glucose levels (Figure 3.6b). Yet, only enoxaparin significantly reduced glomerular C3 (3.1 vs. 5.1, *P*=0.003, Figure 3.6c-e) and C5b-9 (150% vs. 242%, *P*<0.001, Figure 3.6f) deposition.
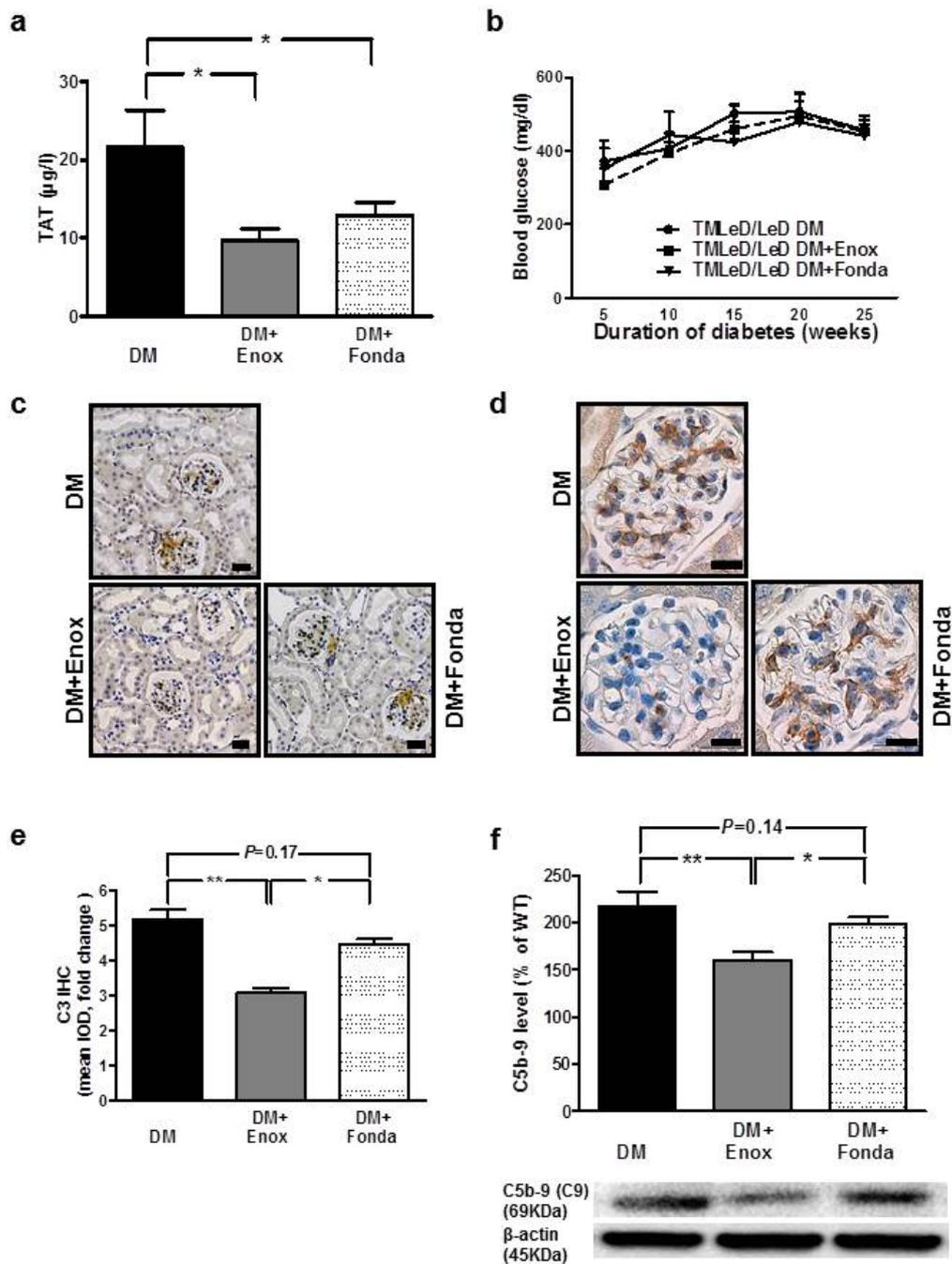
**Figure 3.6 Treatment with enoxaparin, but not with fondaparinux, efficiently prevents glomerular complement deposition**

**a:** Plasma levels of thrombin-anti-thrombin (TAT) complexes, a marker of thrombin activation. Enoxaparin and fondaparinux both reduce TAT levels in diabetic TM$^{LeD/LeD}$ mice.

**b:** Blood glucose concentrations in diabetic TM$^{LeD/LeD}$ mice without (TM$^{LeD/LeD}$ DM, n=12), with enoxaparin (TM$^{LeD/LeD}$ DM+Enox, n=10), or with fondaparinux (TM$^{LeD/LeD}$ DM+Fonda, n=10) treatment.

**c-f:** Only enoxaparin efficiently reduces glomerular C3 and C5b-9 (MAC) deposition in diabetic TM$^{LeD/LeD}$ mice. Representative images of immunohistochemical staining of C3 (c,d, C3 detected by HRP-DAB reaction, brown; haematoxylin counterstain, blue) and of an immunoblot for C5b-9 (MAC, f); bar graphs summarizing results (e,f, n≥7 for each group).

Scale bar: 40 µm (c) 20 µm (d); DM, black bars: diabetic TM$^{LeD/LeD}$ mice; DM + Enox, grey bars: diabetic TM$^{LeD/LeD}$ mice treated with enoxaparin; DM + Fonda, speckled bars: diabetic TM$^{LeD/LeD}$ mice treated with fondaparinux; mean value ± SEM; ★: $P<0.05$, ★★: $P<0.01$.

## 3.7 Enoxaparin, but not fondaparinux, ameliorates nephropathy in diabetic TM$^{LeD/LeD}$ mice

Consistent with the complement deposition data show above (Figure 3.6), Enoxaparin, but not fondaparinux markedly reduced the severity of nephropathy in diabetic TM$^{LeD/LeD}$ mice.

Albuminuria in (85 µg/mg vs. 249 µg/mg, $P<0.001$, Figure 3.7a), mesangial index (10.4% vs. 14.6%, $P=0.002$, Figure 3.7 b,c,g), renal cortex expression of VEGF (139% vs. 198%, $P<0.001$, Figure 3.7d) or TGFβ1 (131% vs. 266%, $P=0.003$, Figure 3.7e), and Smad3 phosphorylation (225% vs. 286%, $P=0.04$, Figure 3.7f) were significantly reduced in enoxaparin as compared to fondaparinux-treated diabetic TM$^{LeD/LeD}$ mice. Similar results were obtained when analyzing Masson`s trichrome stained sections (Figure 3.7h-i). These data provide experimental evidence that enhanced complement activation aggravates nephropathy in diabetic TM$^{LeD/LeD}$ mice.
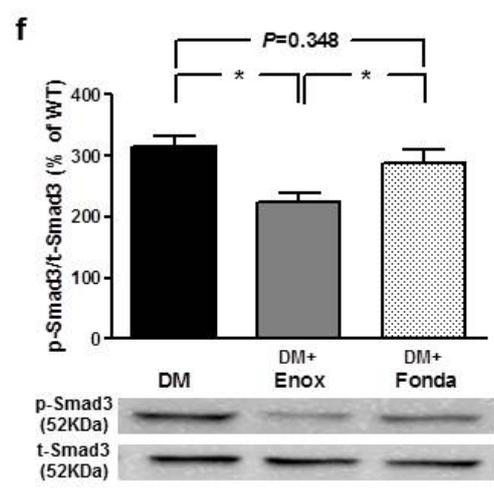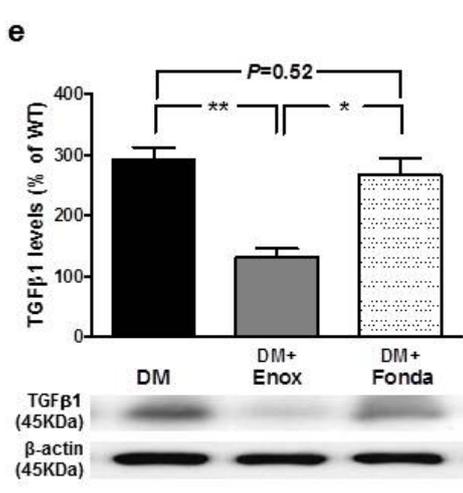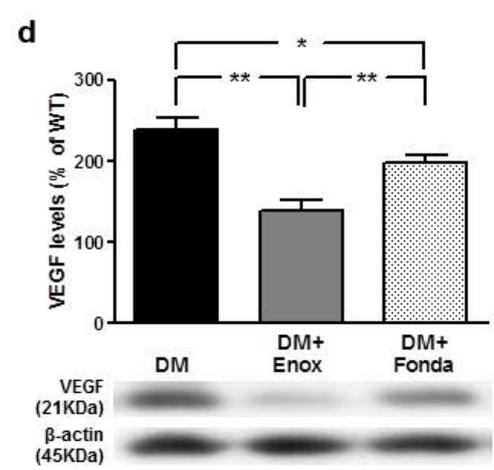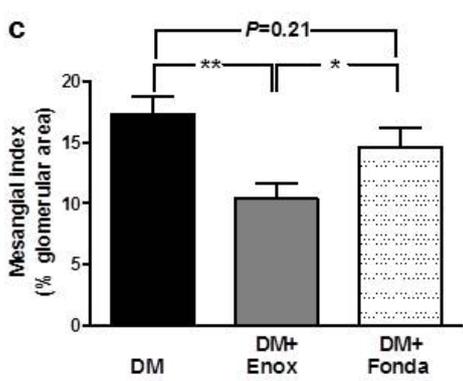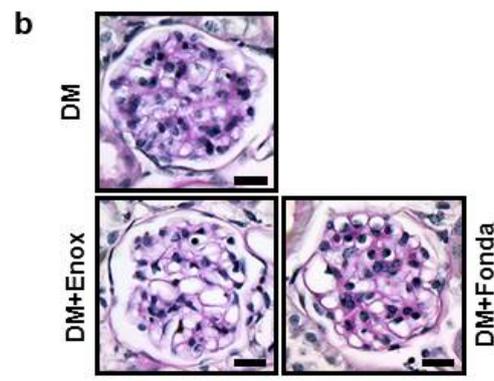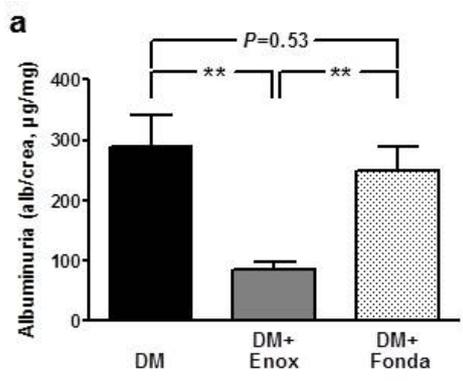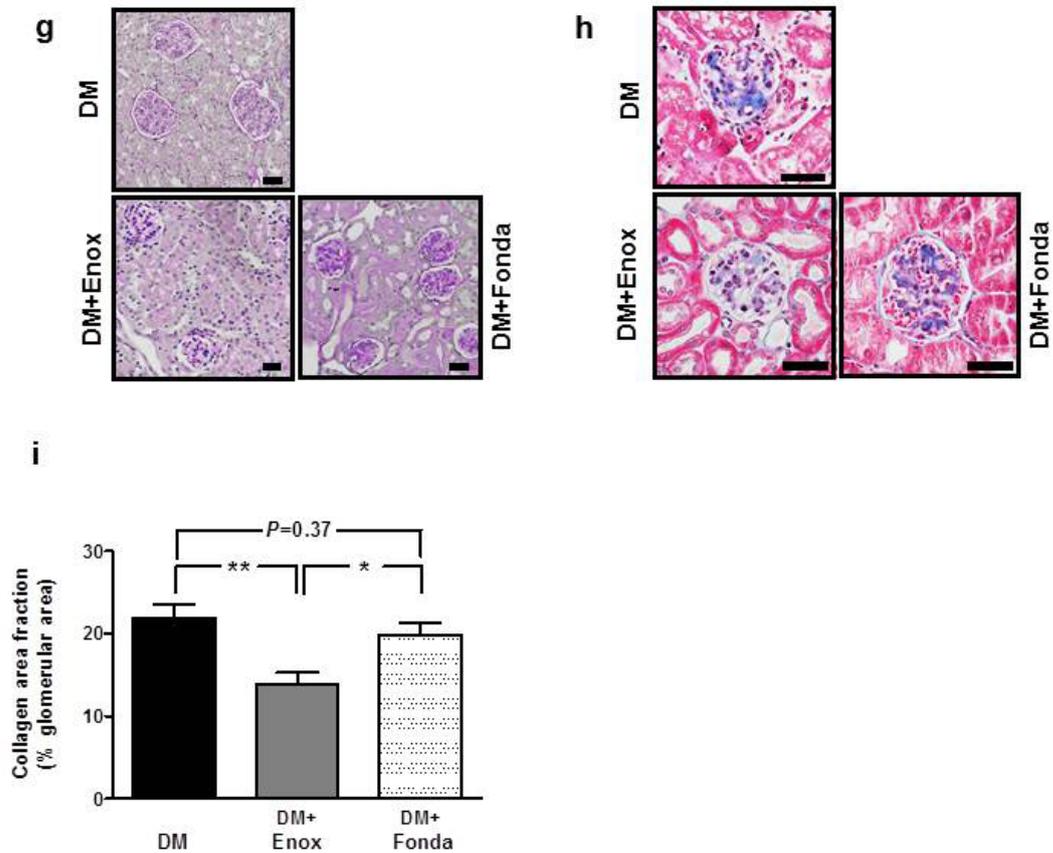
**Figure 3.7 Enoxaparin, but not fondaparinux, ameliorates nephropathy in diabetic TM$^{LeD/LeD}$ mice**

**a:** Enoxaparin, but not fondaparinux, reduces albuminuria in diabetic TM$^{LeD/LeD}$ mice.

**b-i:** Enoxaparin, but not fondaparinux, reduces indices of glomerular injury in diabetic TM$^{LeD/LeD}$ mice. Exemplary images of PAS stained renal sections (b) and bar graph reflecting mesangial index (c), immunoblots for VEGF (d, bottom), TGFβ1 (e, bottom), and phosphorylated Smad3 (f, bottom), and bar graph summarizing results (d-f, top; n≥10 for each group).Lower magnification representative renal PAS staining pictures(g), Exemplary images of Masson`s trichrome stained sections(h) and bar graph summarizing results reflecting collagen index (i).

Scale bar: 15 µm (b); DM: diabetic TM$^{LeD/LeD}$ mice, black bars; DM + Enox: diabetic TM$^{LeD/LeD}$ mice treated with enoxaparin, grey bars; DM + Fonda: diabetic TM$^{LeD/LeD}$ mice treated with fondaparinux, speckled bars; mean value ± SEM; ★: $P<0.05$, ★★: $P<0.01$.

## 3.8 The lectin-like domain of thrombomodulin prevents glucose-induced C3 deposition on endothelial cells and podocytes *in vitro*

The glomerular filtration barrier consists of endothelial cells and podocytes, which both express TM (16) (Figure 3.8a and data not shown). To evaluate the role of TM's lectin-like domain for complement deposition on endothelial cells we used murine endothelial cells (SVEC4-10), which lack endogenous TM (74). SVEC4-10 cells stably expressing wild-type ($TM^{wt}$) or lectin-deficient ($TM^{LeD}$) murine TM were cultured in the presence of murine serum and low (5 mM, control) or high (30 mM, HG) glucose concentrations. In $TM^{wt}$ SVEC cells incubation with HG had only a minor effect on cellular C3 deposition (Figure 3.8b), whereas C5b-9 deposition was slightly, but significantly increased (1.7 vs. 1.0 in control $TM^{wt}$ cells, $P$=0.035, Figure 3.8c). Conversely, both C3 (180% vs. 100%, $P$<0.001, Figure 3.8b) and C5b-9 (3.4 vs. 1.0, $P$<0.001, Figure 3.8c) deposition were markedly increased in HG $TM^{LeD}$ SVEC4-10 cells. Co-incubation of HG treated $TM^{LeD}$ cells with enoxaparin markedly reduced C3 (55% vs. 180%, $P$<0.001, Figure 3.8b) and C5b-9 (1.9 vs. 3.4, $P$<0.001, Figure 3.8c) deposition. Fondaparinux likewise reduced glucose-induced C3 and C5b-9 deposition on $TM^{LeD}$ endothelial cells, but – in agreement with previous reports (69; 75) – was less efficient than enoxaparin (Figure 3.8b-c).
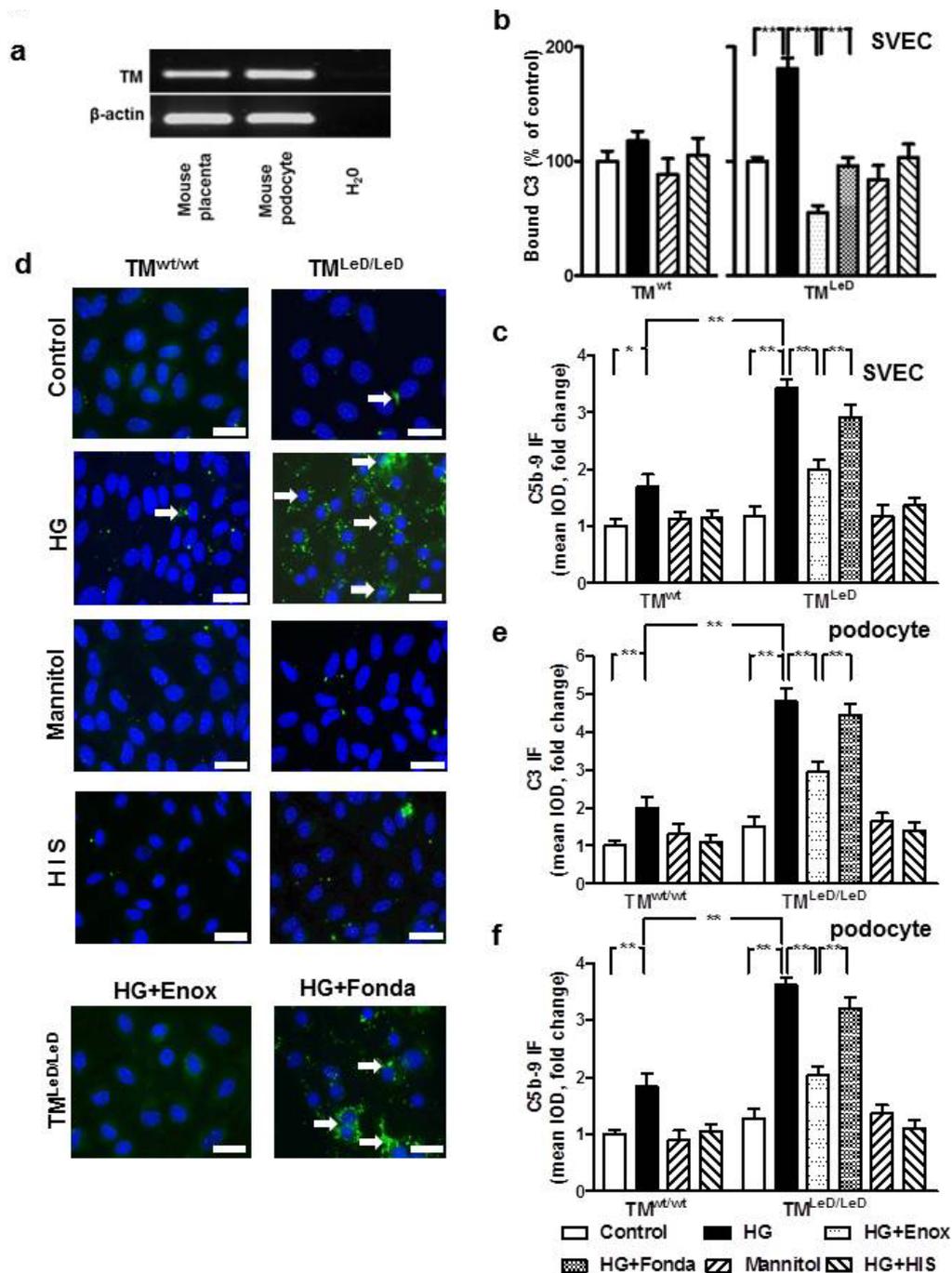
**Figure 3.8 TM's lectin-like domain prevents C3 deposition on endothelial cells and podocytes *in vitro***

a: Mouse podocytes express TM; representative RT-PCR image (positive control: mouse placenta).

b,c: Complement deposition (C3, b, and C5b-9, c) on SVEC4-10 cells expressing either wild type TM (TM^wt) or a TM mutant lacking the lectin-like domain (TM^LeD). In TM^wt transfected SVEC4-10 cells cultured using mouse serum and treated with high glucose concentrations (30 mM, HG) no increase in C3 and only a minor increase of C5b-9 deposition is observed, while HG markedly increases C3 and C5b-9 deposition on TM^LeD SVEC4-10 cells. No C3 or C5b-9 deposition is observed in cells exposed to mannitol (30 mM) or to glucose (30 mM) in the presence of heat inactivated mouse serum (HG+HIS). Enoxaparin (HG+Enox) more efficiently reduces HG-induced C3 or C5b-9 deposition as compared to fondaparinux (HG+Fonda); bar graphs summarizing results of FACS (b) or immunohistochemical (c) analyses.

d-f: C3 or C5b-9 deposition on primary murine podocytes obtained from TM^wt/wt and TM^LeD/LeD mice. C3 and C5b-9

deposition is increased in glucose treated (30 mM, HG) TM$^{wt/wt}$ podocytes, but the increase of C3 and C5b-9 is larger in HG TM$^{LeD/LeD}$ podocytes. No C3 or C5b-9 deposition is observed in cells exposed to mannitol (30 mM) or to glucose (30 mM) in the presence of heat inactivated mouse serum (HG + HIS). Co-incubation with enoxaparin (HG+Enox) reduces C3 and C5b-9 deposition, while fondaparinux (HG+Fonda) has no effect. Immunofluorescent analyses (d) using a primary FITC-labelled anti-C3 antibody (green); Hoechst 33258 nuclear counter stain (blue); bar graph summarizing scoring results for C3 (e) and for C5b-9 (f).

Scale bar: 20 µm (d); HG: high glucose; Enox: enoxaparin; Fonda: fondaparinux; TM$^{wt}$: cells transfected with TM$^{wt}$ construct; TM$^{LeD}$: cells transfected with a TM mutant lacking the lectin-like domain; TM$^{wt/wt}$: podocytes isolated from wild type control mice; TM$^{LeD/LeD}$: podocytes isolated from TM$^{LeD/LeD}$ mice; ✶: P<0.05, ✶✶: P<0.01.

We next determined whether TM's lectin-like domain modulates complement deposition on podocytes. We isolated primary podocytes from TM$^{wt/wt}$ and TM$^{LeD/LeD}$ mice. In TM$^{wt/wt}$ podocytes HG induced C3 (2.0 vs. 1.0, $P$=0.005, Figure 3.8d-e) and C5b-9 (1.8 vs. 1.0, $P$<0.001, Figure 3.8f) deposition. Again, C3 (4.8 vs. 1.0, $P$<0.001, Figure 3.8d-e) and C5b-9 (3.6vs. 1.0, $P$<0.001, Figure 3.8f) deposition were much more pronounced in HG TM$^{LeD/LeD}$ podocytes. Glucose-induced C3 and C5b-9 deposition on podocytes were markedly reduced following co-incubation with enoxaparin (2.9 vs. 4.8, $P$<0.001, and 2.0 vs. 3.6, $P$<0.001, respectively, Figure 3.8d-f). Conversely, fondaparinux failed to reduce C3 (4.4 vs. 4.8, $P$=0.41, Figure 3.8 c,d) or C5b-9 (3.2 vs. 3.6, $P$=0.12, Figure 3.8f) deposition on HG TM$^{LeD/LeD}$ podocytes. Taken together, TM's lectin-like domain cell-autonomously inhibits complement activation on endothelial cells and podocytes.

## 3.9 The lectin-like domain of TM constrains hyperglycemia induced podocyte injury

Considering the efficacy of enoxaparin in preventing complement deposition on podocytes *in vitro* we hypothesized that TM's lectin-like domain protects against complement-dependent podocyte injury in diabetic mice. To test this we evaluated the extent of podocyte injury in diabetic TM$^{LeD/LeD}$ mice using podocyte specific markers. A slightly, but significantly reduced frequency of glomerular WT-1 (Wilm's tumor-1) positive cells was already apparent in non-diabetic TM$^{LeD/LeD}$ mice (7.7 vs. 8.6 in non-diabetic TM$^{wt/wt}$ mice, $P$=0.002, Figure 3.9 a-b). In diabetic TM$^{LeD/LeD}$ mice the frequency of WT-1 positive glomerular cells was markedly reduced (4.8 vs. 7.8 in non-diabetic TM$^{LeD/LeD}$ mice, $P$<0.001, and vs. 6.23 in diabetic TM$^{wt/wt}$ mice, $P$<0.001, Figure 3.9a-b). Likewise, loss of GLEPP1 expression, a podocyte specific protein, was more pronounced in diabetic TM$^{LeD/LeD}$ mice as compared to diabetic TM$^{wt/wt}$ mice (55% vs. 79%, $P$=0.005, Figure 3.9c-d). Since C3 deposition partially co-localized with podocytes (Figure 3.9e) we determined next whether inhibition of complement activation prevented podocyte injury. Indeed, following treatment with enoxaparin WT-1 (6.7 vs. 5.3, P<0.001, Figure 3.9a-b) and GLEPP1 (84% vs. 55%, $P$=0.003, $P$=0.003, Figure 3.9c-d) expression were better preserved than following treatment with fondaparinux.
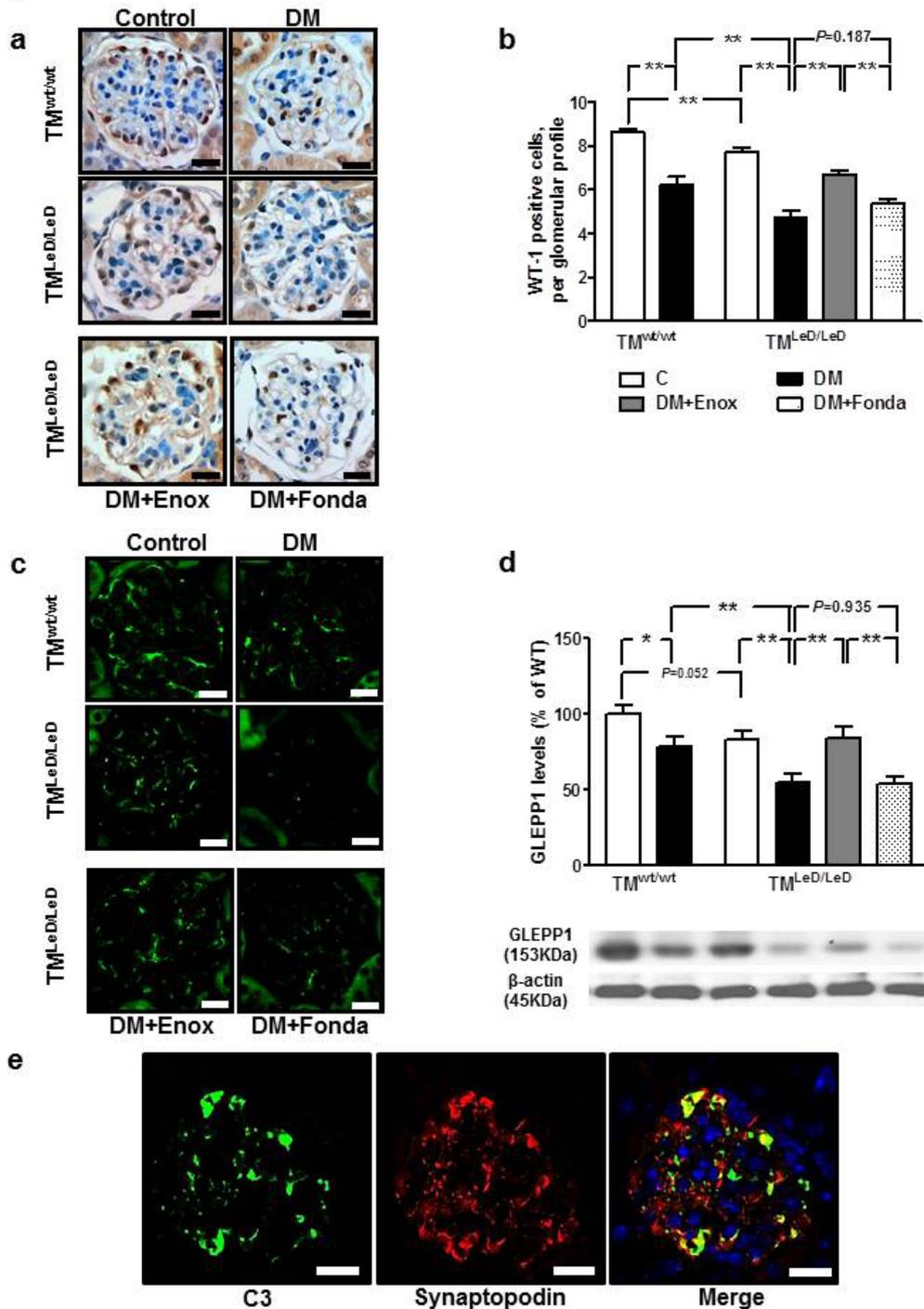
**Figure 3.9 TM's lectin-like domain protects against complement mediated podocyte injury in diabetic mice**

**a,b:** Podocyte frequency, as determined by WT-1immunohistochemical staining, is reduced in diabetic TM^wt/wt mice and to a larger extent in diabetic TM^LeD/LeD mice. Treatment with enoxaparin, but not with fondaparinux, reduces podocyte loss. Representative immunohistochemical images (a, WT-1 detected by HRP-DAB reaction, brown; hematoxylin counterstain, blue) and bar graph summarizing results (b, n≥10 for each group).

**c,d:** Expression of GLEPP1 is reduced in diabetic TM^LeD/LeD mice compared to diabetic TM^wt/wt mice, as determined by immunofluorescence staining (c) and immunoblotting (d). Enoxaparin, but not fondaparinux, prevents loss of GLEPP-1 expression. Representative immunohistochemical images (c) and immunoblots (d, bottom) and bar graph summarizing results (d, top, n≥10 for each group). Anti-GLEPP1 primary antibody and a FITC-labelled secondary antibody (c, green).

**e:** Immunofluorescent images showing partial co-localization (yellow) of C3 (green) with the podocyte specific marker synaptopodin (red) in diabetic TM$^{LeD/LeD}$ kidney samples; conventional fluorescence microscopy on paraffin section, Hoechst 33258 nuclear counter stain (blue).

Scale bar: 15 μm (a,c,e); C: non-diabetic control mice, open bars; DM: diabetic mice, black bars; mean value ± SEM; ★: *P*<0.05, ★★: *P*<0.01 (ANOVA).

Taken together, TM's lectin-like domain ameliorates glomerular and podocyte injuries in experimental diabetic nephropathy by regulating complement activation.

## 4. Discussion

A potential pathophysiological role of the complement system in diabetic nephropathy is supported by studies demonstrating an association between diabetic nephropathy and MBL (mannose-binding lectin, a complement activator) in type 1 diabetic patients (58; 60) and enhanced immunohistochemical MAC-staining in glomeruli of diabetic patients (62; 76). These clinical studies are further supported by experimental work in rodent diabetes models (77-80). In the current study we identify a novel mechanism regulating complement activation and glomerular damage in experimental diabetic nephropathy. Loss of the lectin-like domain of TM results in glomerular complement deposition associated with increased indices of experimental diabetic nephropathy. Notably, TM, which is primarily known for its expression on and function in endothelial cells, is expressed in podocytes, where it cell-autonomously regulates glucose-induced complement activation. Hence, the current study does not only identify a new function of endothelial TM in regulating complement activation in diabetes, but also reveals a novel cytoprotective function of TM in podocytes.

Previously we demonstrated that TM-dependent PC activation protects glomerular cells in hyperglycemic mice (16). To evaluate the role of TM-dependent PC activation we used a mouse model with a genetically superimposed PC-activation defect (secondary to the TM$^{Pro}$-mutation, Glu404Pro) (81). Based on in vitro

structure-function analyses this mutation is expected to similarly impair activation of both PC and TAFI (thrombin activatable fibrinolysis inhibitor, also known as plasma procarboxypeptidase B), although this has formally not been tested in vivo (5) . Activated TAFI catalyzes the removal of the C-terminal basic amino acid residues Lysine and Arginine, thus inhibiting fibrinolysis, but also modifies the complement factors C3a and C5a (82). However, the aggravation of experimental diabetic nephropathy in TM$^{Pro/Pro}$ mice must be independent of TAFI-dependent complement regulation, as concomitantly increasing circulating levels of aPC is fully sufficient to protect TM$^{Pro/Pro}$ mice from experimental diabetic nephropathy (16). Conversely, the mechanism identified in the current study must be independent of PC-activation, since activation of PC is not impaired in TM$^{LeD/LeD}$ mice (27) (and data not shown). Taken together, our previous and the current results establish that TM prevents diabetic nephropathy and podocyte injury in experimental diabetic nephropathy through two independent mechanisms, i.e. activation of PC and inhibition of complement.

TM is increasingly being recognized as a regulator of complement activation and TM's importance as a complement regulator has recently been demonstrated in patients with atypical hemolytic-uremic syndrome (aHUS) (30). aHUS is one of the thrombotic microangiopathies, along with thrombotic thrombocytopenia purpura (TTP) and preeclampsia. Of note, all three conditions (aHUS, TTP, preeclampsia) are acute and potentially life threatening diseases associated with endothelial dysfunction and

renal failure. The current study establishes that – at least in mice – TM´s lectin-like domain regulates via complement activation diabetic nephropathy, a chronic vascular complication frequently leading to renal failure. Thus, impaired TM-dependent complement inhibition does not only contribute to acute and potentially catastrophic vascular diseases and renal failure, but is in addition mechanistically linked to chronic nephropathy, such as occurs in diabetes.

The current in vitro finding that TM's lectin-like domain prevents complement mediated podocyte injury in a cell-autonomous fashion suggests that TM may not only protect against endothelial dysfunction, e.g. in the before mentioned microangiopathies or in diabetic nephropathy, but directly protects podocyte function via inhibition of complement. This raises the question as to whether expression of TM on podocytes and hence outside the vascular compartment regulates complement activation – and thus podocyte damage – locally. Some fragments of activated complement factors are small enough to cross the glomerular filtration barrier (e.g. C3a, C3c, or C3dg) and may then interact with podocyte TM. In this case endothelial TM may primarily regulate complement activation within the vascular compartment and smaller complement fragments crossing the glomerular filtration barrier may be subject to regulation by TM expressed by the podocyte. Inactive complement factors are generally too large (e.g. complement factor C5: ~177 kDa, or C6: ~93 kDa) and regulation of these complement factors by podocytes may only be relevant following an initial injury of the glomerular filtration barrier. The expression of some complement

factors by podocytes or mesangial cells raises yet another possibility: podocyte TM may lessen glomerular injury by regulation these locally synthesized complement factors (83). Interestingly, the current finding that TM is expressed on podocytes raises the same question in regard to PC activation: Is PC activation on endothelial cells or on podocytes required for podocyte protection? Or is PC expressed by podocytes? These mechanistic questions can now be addressed using cell-type specific deletion of TM's lectin-like domain or of TM specifically in podocytes or endothelial cells.

Normally, the complement system, part of the innate immune system, is tightly regulated to avoid collateral tissue damage. The mechanism of complement activation in diabetic patients remains incompletely understood. Hyperglycemia and the associated metabolic changes appear to be causally related to complement activation, considering the reversibility of glomerular C3 deposition in rodents following normalization of blood glucose levels (84). High concentrations of glucose decrease endothelial expression of CD59, an endogenous inhibitor of complement activation in vitro. Glycation of CD59 attenuates its capacity to inhibit MAC formation on host cells (61; 85) and renders erythrocytes from diabetic patients more susceptible to MAC-mediated lysis (62; 63).

However, murine CD59 lacks the preferential glycation motif, indicating that murine CD59 cannot be inactivated by glycation (61). The current and previous observation

of enhanced glomerular complement deposition in diabetic mice appear therefore to be independent of CD59, pointing towards additional mechanisms. Hyperglycemia induced loss of endothelial TM (16; 86; 87), impairing complement inhibition through TM's lectin-like domain, constitutes such an alternative mechanism. Impairment of TM-dependent complement regulation may be of broader relevance for diabetes mellitus and associated metabolic alterations, considering (A) that enhanced C5b-9 deposition has been demonstrated not only in the kidney, but in all principal target tissues of diabetic complications (62; 76; 88; 89), and given (B) the role of the complement system in modulating obesity, lipids, and energy expenditure (90; 91). We acknowledge that the current findings need to be evaluated in future studies, including studies of human diabetic nephropathy, given the limitations of murine models of diabetic nephropathy. Nevertheless, the therapeutic potential arising from the current results, i.e. therapeutic intervention with soluble TM (sTM) or small molecules targeting complement activation, may constitute a promising approach for the treatment of diabetic complications.

## 5. Conclusion

This study establishes that thrombomodulin ameliorates experimental diabetic nephropathy not only via aPC, but in addition by dampening complement inhibition via its lectin like domain. Thrombomodulin expressed on endothelial cells and podocytes controls glucose induced complement activation. This indicates a new function of TM outside the vasculature. Thus, these results identify a new function of thrombomodulin in chronic vascular disease such as diabetic microangiopathy and a novel pathway regulating complement activation in diabetes mellitus.

## 6. Summary

Recent evidence supports a pathogenic role of the complement system in diabetic nephropathy. The mechanism through which complement activation is regulated and whether complement activation mediates podocyte loss in diabetic nephropathy remains unknown. Hyperglycemia reduces expression of thrombomodulin (TM), a transmembrane molecule with anticoagulant and cytoprotective functions. TM's cytoprotective effect depends in part on its lectin like domain, which scavenges HMGB-1, a RAGE ligand, and inhibits apoptosis and complement. Here we determined the role of TM's lectin like domain in diabetic nephropathy.

Hyperglycemia was induced in mice lacking TM's lectin like domain (TM$^{LeD/LeD}$ mice) using streptozotocin. Following persistent hyperglycemia for 26 weeks albuminuria and markers of diabetic nephropathy were analyzed. The role of apoptosis, HMGB-1, or complement activation was determined by immunohistochemical analyses, immunoblotting, and intervention studies. *In vitro* experiments were performed to characterize complement regulation through TM's lectin like domain on endothelial cells and podocytes.

Albuminuria and indices of diabetic nephropathy were aggravated in diabetic

TM^LeD/LeD mice, but this was independent of HMGB-1 or apoptosis. Conversely, C3 deposition is markedly increased in glomeruli of diabetic TM^LeD/LeD mice. Inhibition of complement activation (using the low molecular heparin enoxaparin) ameliorated indices of diabetic nephropathy. *In vitro* TM's lectin like domain cell-autonomously prevented glucose induced complement activation on endothelial cells and podocytes, the two cellular components of the glomerular basal membrane. Consistently, podocyte loss, which was enhanced in diabetic TM^LeD/LeD mice, was reduced following enoxaparin dependent complement inhibition.

The current study identifies a novel mechanism regulating complement activation on endothelial cells and podocytes in diabetic nephropathy. Loss of TM's lectin like domain enhances complement activation, aggravating albuminuria, glomerular damage, and podocyte loss.

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## 8. Curriculum vitae

**Personal data:**

| | |
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## 9. List of publications

**Original research communication published in English in international peer-reviewed journals**

Bock F, Shahzad K, **Wang H**, Stoyanov S, Wolter J, Dong W, Pelicci PG, Kashif M, Ranjan S, Schmidt S, Ritzel R, Schwenger V, Reymann KG, Esmon CT, Madhusudhan T, Nawroth PP, Isermann B.: Activated protein C ameliorates diabetic nephropathy by epigenetically inhibiting the redox enzyme p66Shc**. Proc Natl Acad Sci U S A**. 2013 Jan 8;110(2):648-53.                                                    9.681

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