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CHAPTER 5

MicroRNA-126 contributes to renal microvascular heterogeneity of VCAM-1 protein expression in acute inflammation

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Abstract

Endothelial cells in different microvascular segments of the kidney have diverse functions and exhibit differential responsiveness to disease stimuli. The responsible molecular mechanisms are largely unknown. We previously showed that during hemorrhagic shock VCAM-1 protein was expressed primarily in extra-glomerular compartments of the kidney, while E-selectin protein was highly induced in glomeruli only. Here, we investigated the molecular control of expression of these endothelial cell adhesion molecules in mouse models of renal inflammation. Microvascular-segment specific responses to the induction of anti-glomerular basement membrane (anti-GBM) glomerulonephritis and systemic TNFα treatment showed that E-selectin expression was transcriptionally regulated, with high E-selectin mRNA and protein levels preferentially expressed in the glomerular compartment. In contrast, VCAM-1 mRNA expression was increased in both arterioles and glomeruli, while VCAM-1 protein expression was limited in the glomeruli. These high VCAM-1 mRNA/low VCAM-1 protein levels were accompanied by high local microRNA (miR)-126 and Egfl7 levels, as well as higher Ets1 levels compared to arteriolar expression levels. Using miR-reporter constructs functional activity of miR-126 in glomerular endothelial cells could be demonstrated. Moreover, *in vivo* knock-down of miR-126 function unleashed VCAM-1 protein expression in the glomeruli upon inflammatory challenge. These data imply that miR-126 has a major role in the segmental, heterogenic response of renal microvascular endothelial cells to systemic inflammatory stimuli.

Introduction

It is well recognized that endothelial cells in different vascular segments of the kidney differ in function [21]. Renal arteries branch repeatedly into small-diameter afferent arterioles in which the endothelial cells are covered by smooth muscle cells, forming the primary site of vascular resistance in the kidney. Endothelial cells lining the glomerular capillaries are fenestrated and together with podocytes, mesangial cells, and the glomerular basal membrane, regulate the glomerular filtration of water and small molecules into the urine, while preventing loss of large serum proteins. After glomerular filtration, blood leaves the glomerular capillary networks by efferent arterioles to peritubular capillaries and postcapillary venules [20].

In spite of the broad knowledge of structural and functional heterogeneity of endothelial cells in the kidney, the underlying molecular basis for microvascular endothelial heterogeneity is largely unknown. Also in acute and chronic renal diseases such as glomerulonephritis, vasculitis, and ischemia related acute renal failure, knowledge on how distinct microvascular endothelial cells subsets

respond at the molecular level to disease stimuli is almost non-existent. Molecular heterogeneity of endothelial cells can be controlled at multiple levels, ranging from the heterogenic expression of transmembrane signaling receptors and local concentrations of their ligands, to differentially activated signal transduction cascades and differentially controlled ubiquitinylation-dependent protein degradation pathways [18].

In recent years, the importance of post-transcriptional regulation of inflammation by microRNAs (miRs) has become increasingly apparent [28]. Mature miRs are short non-coding RNAs that bind to (partially) complementary sequences, most commonly found in the 3'UTR (untranslated region) of target mRNAs, which results in inhibition of protein synthesis by degradation or translational repression of the target mRNA. MicroRNAs play a role in endothelial biology [29, 38], and also have been associated with pathogenesis and progression of various kidney diseases [16, 31, 37, 39]. Harris et al, and more recently Salvucci et al. demonstrated a link between miR expression in endothelial cells and inflammation, by showing that high miR-126 levels related to low VCAM-1 protein expression in human umbilical vein endothelial cells (HUVEC) *in vitro* [10, 26]. MiR-126 is highly enriched in the endothelium [5], exerts a regulatory function in vascular integrity and vascular pathology [9, 30, 34, 35, 40], and its expression was shown to be partly driven by vascular associated Ets transcription factors [11, 23].

The current study describes two distinct molecular mechanisms for two eminent pro-inflammatory endothelial adhesion molecules, namely post-transcriptional, miR-126 controlled expression of VCAM-1, and transcriptional regulation of E-selectin, that contribute to heterogeneity in renal microvascular endothelial engagement in response to an inflammatory challenge. Previously, we reported that exposure of mice to hemorrhagic shock resulted in a highly compartmentalized microvascular segment restricted expression of adhesion molecules in the kidney. While E-selectin protein was primarily expressed in the glomerular compartment, VCAM-1 protein was predominantly expressed in the arteriolar and peritubular endothelial cells [33]. A similar microvascular segment restricted VCAM-1 protein expression pattern was previously reported in a rat renal allograft model [36]. Based on the *in vitro* results reported by Harris et al [10]. we hypothesized that differential expression of miR-126 in microvascular segments of the kidney might be involved in the heterogenic expression of VCAM-1 protein in the kidney. As different microvascular segments engage in different (renal) diseases [19], understanding the contribution of transcriptional respectively posttranscriptional miR control will provide a basis for further studies into their role in heterogenic microvascular responsiveness in disease development.

Materials and methods

Cell cultures

The conditionally immortalized human glomerular endothelial cell line ciGEnC and the glomerular podocyte cell line AB8/13 were cultured at 33°C for propagation of cells. Unless otherwise stated, 5 and 14 days prior to experiments, respectively, ciGEnC and AB8/13 cells were transferred to 37°C to inactivate the SV40 T antigen and allow the cells to differentiate [24, 27]. Human glomerular mesangial cells were cultured at 37°C as described previously [4].

Animal models of acute inflammation

All animal experiments were performed according to national guidelines and upon approval of the local Animal Care and Use Committees. C57bl/6 mice (8–10 weeks) were purchased from Harlan (Zeist, The Netherlands). Animals were maintained on mouse chow and tap water *ad libitum* in a temperature-controlled chamber at 24°C with a 12-hr light/dark cycle. Induction of anti-GBM glomerulonephritis was performed as described previously [2]. Mice were sacrificed at 2 h. Alternatively, inflammation was systemically induced by i.v. injection of recombinant mouse TNFα (BioSource Europe, Nivelles, Belgium) at 200 ng dose. Mice were sacrificed 2h after TNFα administration. To induce hemorrhagic shock, mice under anesthesia, breathing spontaneously and on a temperature controlled surgical pad (37-38°C), were subjected to blood withdrawal until a reduction of the MAP to 30 mmHg within 15-30 minutes as described previously [33]. Mice were sacrificed at 90 minutes after MAP of 30 mmHg was achieved. Organs from all treatment groups were harvested, snap-frozen in liquid nitrogen and stored at -80°C prior to RNA isolation, laser microdissection, and/or immunohistochemical staining.

Antagomir-126 effects on protein expression in vivo

Five days prior to TNFα challenge, C57bl/6 mice were injected in the orbital plexus with 200 µL antagomir-126 or scramblemir in saline at 1.0 mg/animal, or saline, 2 mice per group [34]. At the day of sacrifice, mice were injected in the orbital plexus with 70 µL TNFα at 200 ng per animal in saline/BSA, or with vehicle only. After 2 hours, mice were sacrificed and relevant organs were taken out, snap frozen on liquid nitrogen, and stored at -80°C for further analysis.

Immunohistochemical staining

Five-µm cryosections were fixed in acetone for 10 min and incubated for 45 min with monoclonal antibodies: rat anti-mouse CD31 (PECAM-1; BD Pharmingen, Alphen a/d Rijn, the Netherlands), rat anti-mouse E-selectin (MES-1; kindly provided by Dr. D. Brown, UCB Celltech, Slough, UK) and rat anti-mouse VCAM-1 (CD106, BD Biosciences, Breda, The Netherlands). Antibody dilution and

washing steps were performed with PBS/5% fetal calf serum (FCS). Staining was carried out with Envision+ system-HRP kit (DakoCytomation, Denmark), according to manufacturer's protocol, including a blocking step for endogenous peroxidase activity. Sections were incubated for 45 min with rabbit anti-rat antibodies (AI-4001, Vector Laboratories Inc., Burlingame, CA, USA; diluted in PBS/5% FCS/5% normal mouse serum/5% normal sheep serum) followed by 30 min incubation with Envision+ system-HRP anti-rabbit polymer. Detection was performed with 3-amino-9-ethylcarbazole (AEC) and sections were counter-stained with Mayer's hematoxylin. Between all incubation steps sections were washed extensively with PBS. Isotype matched controls were consistently found to be devoid of staining.

Quantification of VCAM-1 protein levels by ELISA

To quantify VCAM-1 protein, snap frozen 10 µm cryostat cut kidney and liver sections were homogenized in ice cold RIPA-buffer (150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 0.5% sodium deoxycholate and 0.1% SDS) containing protease inhibitors (Complete Mini, Roche Applied Science, Mannheim, Germany), phosphatase inhibitors (PhosSTOP, Roche Applied Science) and 1 mM sodium orthovanadate. The homogenates were sonicated and centrifuged at 13,000g for 10 minutes at 4°C. Total protein was determined by DC Protein Assay (Bio-Rad Laboratories, Hercules, USA), before quantification of VCAM-1 by ELISA (mouse sVCAM-1/CD106 (MVC00), R&D Systems Inc. Minneapolis, USA) according to the manufacturer's instructions. VCAM-1 levels were normalised to total protein concentrations in the tissue homogenate and expressed as pg VCAM-1 per µg total protein.

Laser microdissection of renal microvascular segments

Seven hundred glomeruli (area ~3*106 µm2) and arteriolar vascular segments (area ~6*105 µm2) were laser microdissected from 9-µm hematoxylin stained cryosections using the Leica Microbeam System. Glomeruli were dissected through the Bowmans capsule in order to only obtain cells within the glomeruli. Arterioles were identified based on their morphology, the inner layer of endothelial cells was laser microdissected from the tissue [3].

RNA isolation and quantitative RT-PCR

Total RNA (18 nt and larger) was isolated by miRNeasy Mini kit RNA and included a DNase treatment on the column (Qiagen Benelux B.V., Venlo, The Netherlands). RNA integrity for high RNA yield samples was determined by 28S/18S ratio detection on an agarose gel. For laser microdissected samples, RNA integrity was determined on an Experion Automated Electroforeses System using RNA HighSens Chips (Bio-Rad Laboratories, Hercules CA, USA). For gene expression analysis RNA was reverse transcribed using Superscript III

reverse transcriptase (Invitrogen, Breda, The Netherlands) and random hexamer primers (Promega, Leiden, The Netherlands). For expression analysis of miRs, RNA was reverse transcribed using Taqman miRNA reverse transcription kit (Applied Biosystems, Applera Nederland, Nieuwerkerk a/d IJssel, The Netherlands) in the presence of specific miR reverse transcription primers.

Quantitative PCR (qPCR) amplifications of each sample were performed in duplicate or triplicate according to manufacturer's protocol on an ABI Prism 7900HT Sequence Detection System with gene- and miR-specific Taqman primers/probe from Applied Biosystems. Gene expression of E-selectin (Mm00441278_m1 and Hs00174057_m1), VCAM-1 (Mm00449197_m1 and Hs00365486_m1), CD31 (Mm00476702_m1 and Hs00169777_m1), Egfl7 (Mm00618004_m1 and Hs00211952_m1), and Ets-1 (Mm00468970_m1 and Hs00428293_m1) was determined as relative to the house keeping gene GAPDH (Mm99999915_g1 and Hs99999905_m1) based on the comparative Ct method. Similarly, the expression of miR-126 (assay 002228) and miR-31 (assays 000185 and 002279) was related to the expression of sno202 (assay 001232) for mouse samples, and of RNU48 (assay 001006) for human samples. The expression of the pan-endothelial marker gene CD31 was used to correct for the relative content of endothelial cells in laser microdissected arterioles and glomeruli in the case of analysis of endothelial restricted miR and genes.

miR-126 reporter assay

The human VCAM-1 3'UTR was amplified by PCR using the following primers: VCAM-1 (sense): TATAAAACTAGTCAAGCCATGCATTCAGACTTC and VCAM-1 (antisense): TATAAAAAGCTTGATGACTATGCTAGGCTCCTG.

These primers were designed to generate two flanking enzyme restriction sites (SpeI and HindIII) that enabled direct cloning into the pMIR-reportTM Expression Reporter Vector System (Applied Biosystems), which contains an experimental firefly luciferase reporter gene. After cloning, the plasmids were sequenced to evaluate their fidelity. To determine the regulatory capacity of miR-126 in ciGEnC on the 3'UTR of VCAM-1, ciGEnC were seeded at 5 x 10^5 cells per well in 6-wells plates at 33 °C. Five µg/mL cholesterol-conjugated antagomir-126 (5'-gscsauuau-uacucacgguascsgsas-Chol-3') or control (scramblemir), that was selected to lack complementarily to the human and mouse transcriptome (5-asusgacuaucgcua-uucgcsasgs-Chol-3') were added to the cells 16 hours prior to transfection. Transfection was performed by electroporation in serum free medium (Optimem; Gibco/Invitrogen) as previously described [34]. A Renilla luciferase expressing plasmid (pRL-SV40, Promega, Leiden, The Netherlands) served as control for the efficiency of electroporation. In short, 1.5 µg pMIR- VCAM-1 3'UTR or pMIR-Report control plasmid was mixed with 150 ng pRL-SV40 and added to the cells. The cell suspension was incubated for 10 min at 4°C and electroporated with Gene Pulser II (Bio-Rad Laboratories, Veenendaal, The

Netherlands). Cells were recovered for 10 min at room temperature, plated in triplicate at 1.5 x 10^5 cells per well in 24-wells plate and incubated for 24 h at 33°C. Firefly luciferase and Renilla luciferase signals were measured using Dual-Luciferase Assay Reporter System (Promega) and Lumat LB9507 (EG&G Berthold, Bundoora, Australia). The ratio of firefly/Renilla luciferase signals was calculated. To evaluate the relative luciferase signals, ciGEnC that were incubated with antagomir-126 prior to transfection were arbitrarily set at 100%.

Statistical analysis

Statistical significance of differences in gene and miR expression was performed by means of two-sided Student's t-test, assuming equal variances. Differences were considered to be significant when $p<0.05$.

Results

Expression of cell adhesion molecules in response to disease induction

Induction of anti-GBM glomerulonephritis in mice as well as *in vivo* exposure to i.v. TNFα resulted in a rapid activation of endothelial cells, as represented by a strong upregulation of E-selectin and VCAM-1 mRNA expression in the kidney (Fig. 1A – anti-GBM, Fig. 1B – TNFα). In both models, E-selectin protein was predominantly expressed in glomerular endothelial cells, while expression of VCAM-1 protein was highest in arterioles, and expressed to a limited extent in the glomeruli (Fig. 1C). Also in a third inflammation model, i.c., induced by hemorrhagic shock, a similar pattern of restricted expression of E-selectin in glomerular capillaries and high VCAM-1 protein expression in arterioles with concomitant low expression in glomeruli was observed (Fig. 1C). This implies that heterogenic endothelial responsiveness to acute inflammatory stimuli in different renal microvascular segments is a general response irrespective the nature of the stimulus.

E-selectin and VCAM-1 gene expression in renal microvascular segments

To analyze the vascular segment restricted effects of inflammatory stimuli on gene expression, we isolated glomeruli and arterioles from the kidney by laser microdissection prior to qRT-PCR analyses. This protocol combines preservation of RNA levels with providing information on the original location of the vascular segments in the kidney. Using this, we could show high and preferential up regulation of E-selectin mRNA in glomerular capillaries, both in the anti-GBM model (Fig. 2A) and in the i.v. TNFα challenge model (Fig. 2B). Together with the protein data from Figure 1, this indicates regulation of E-selectin expression in the glomerular compartment at the transcriptional level. Surprisingly, VCAM-1

mRNA levels were upregulated to a similar extent in both glomeruli and arterioles in both models (Fig. 2A and 2B) revealing a marked discordance in VCAM-1 gene versus protein expression in the glomeruli that suggested a marked regulation of VCAM-1 expression at the post-transcriptional level.

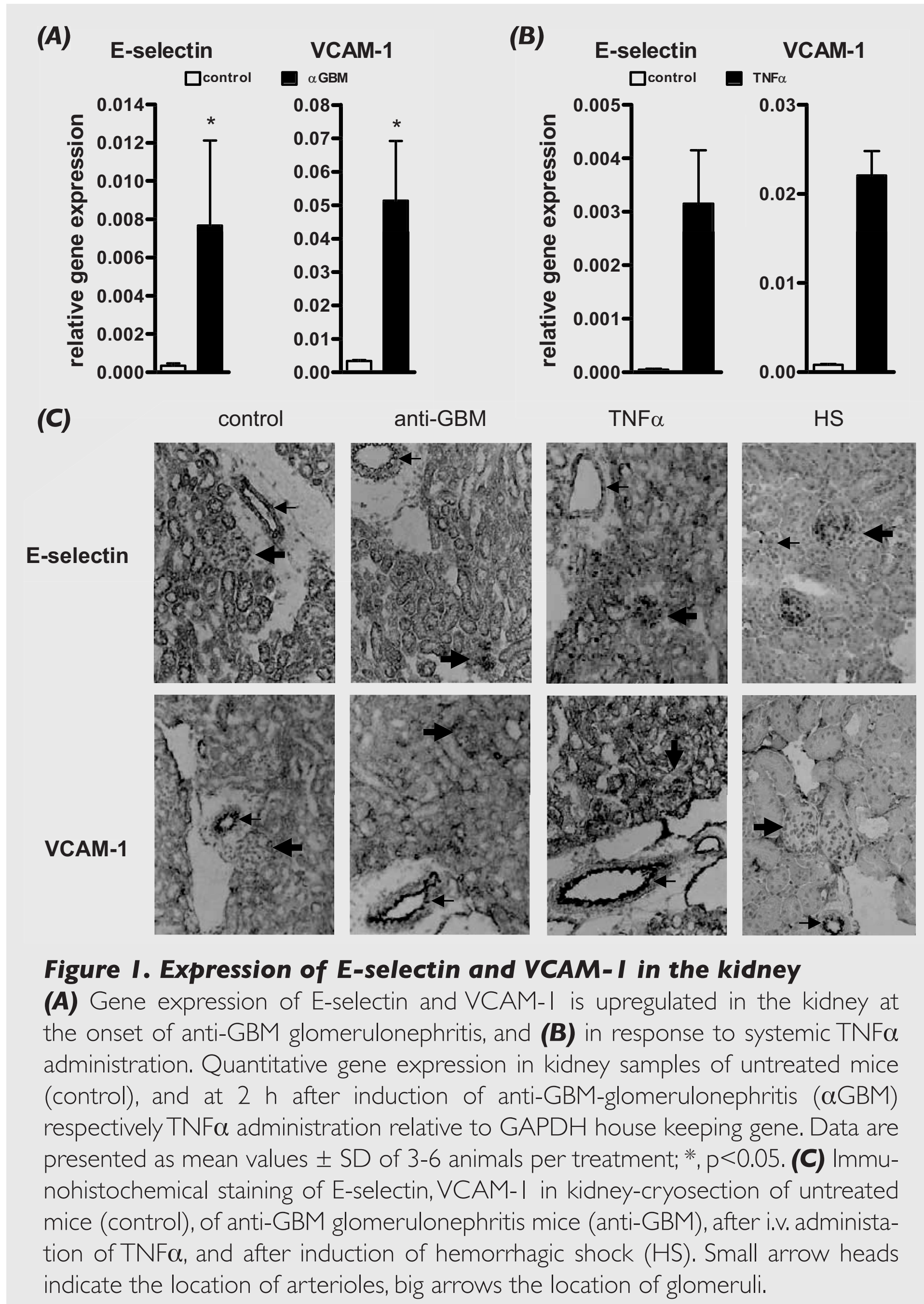


Figure 1. Expression of E-selectin and VCAM-1 in the kidney
(A) Gene expression of E-selectin and VCAM-1 is upregulated in the kidney at the onset of anti-GBM glomerulonephritis, and ***(B)*** in response to systemic TNFα administration. Quantitative gene expression in kidney samples of untreated mice (control), and at 2 h after induction of anti-GBM-glomerulonephritis (αGBM) respectively TNFα administration relative to GAPDH house keeping gene. Data are presented as mean values ± SD of 3-6 animals per treatment; *, $p<0.05$. ***(C)*** Immunohistochemical staining of E-selectin, VCAM-1 in kidney-cryosection of untreated mice (control), of anti-GBM glomerulonephritis mice (anti-GBM), after i.v. administation of TNFα, and after induction of hemorrhagic shock (HS). Small arrow heads indicate the location of arterioles, big arrows the location of glomeruli.

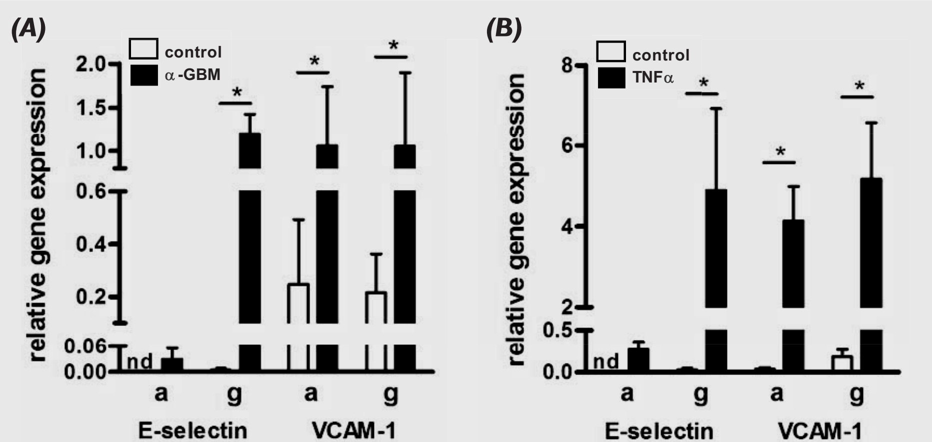


Figure 2. E-selectin and VCAM-1 gene expression in vivo are compartmentalized in different microvascular segments in the kidney

Both in anti-GBM glomerulonephritis ***(A)*** and in response to systemic TNFα treatment ***(B)***, gene expression of E-selectin was highly and specifically upregulated in glomerular endothelial cells (g) while hardly affected in the arterioles (a), while VCAM-1 gene expression was induced to a similar extent in arterioles (a) and in glomeruli (g). Mice were sacrificed at 2h after disease induction and vascular segments were laser microdissected prior to qRT-PCR analysis as described in M&M. Data are presented as mean values ± SD of 3-6 animals per treatment; *, gene expression in mice subjected to disease stimulus was significantly different compared to untreated control mice, $p<0.05$; nd - not detected.

miR-126 levels of expression follow the expression patterns of Egfl7 and Ets1

Based on the recent data published by Harris et al. [10] regarding the role of miR-126 in posttranslational regulation of VCAM-1 expression, we hypothesized that in acute inflammation, the observed limited glomerular VCAM-1 protein was related to a high expression of miR-126. To test this hypothesis, we analyzed miR-126 levels *in vivo* in arterioles and glomeruli obtained by laser microdissection, in which the endothelial cell input in each sample was normalized using the pan-endothelial marker CD31. miR-126 expression was significantly higher in the glomerular segments than in the arteriolar segments (Fig. 3A, white bars). These levels did not change in response to the induction of acute inflammation (Fig. 3A, black bars). In contrast, the non-endothelial restricted microRNA miR-31 was expressed at similar levels in the two renal microvascular segments both in quiescent and in inflammatory conditions (Fig. 3B).

Because miR-126 is an intronic product of the gene Egfl7 (epidermal growth factor-like domain 7, also known as vascular endothelial statin [22]), and miR-126 was previously reported to follow endothelial restricted expression patterns of

Egfl7[22, 38] we also analyzed Egfl7 expression in the two microvascular beds. This analysis revealed a similarly higher expression of Egfl7 in the glomerular compartment compared to its expression in the arteriolar compartment, both in control conditions and in response to the inflammatory challenge (Fig. 3C). In contrast to the others, Egfl7 expression was modestly downregulated in both compartments under acute inflammatory conditions.

While performing our study, Harris et al. reported that Ets1 is one of the main transcription factors in control of miR-126 expression in endothelial cells *in vitro* [11]. Analyzing Ets1 expression levels in the two vascular compartments under study revealed a significant, 6-fold higher expression level of this transcription factor in the glomeruli as compared to the arterioles, both of which were not affected by the inflammatory process (Fig. 3D).

miR-126 expression is only expressed in the endothelial cells of glomeruli

Since laser microdissected glomeruli consist of more cell types besides

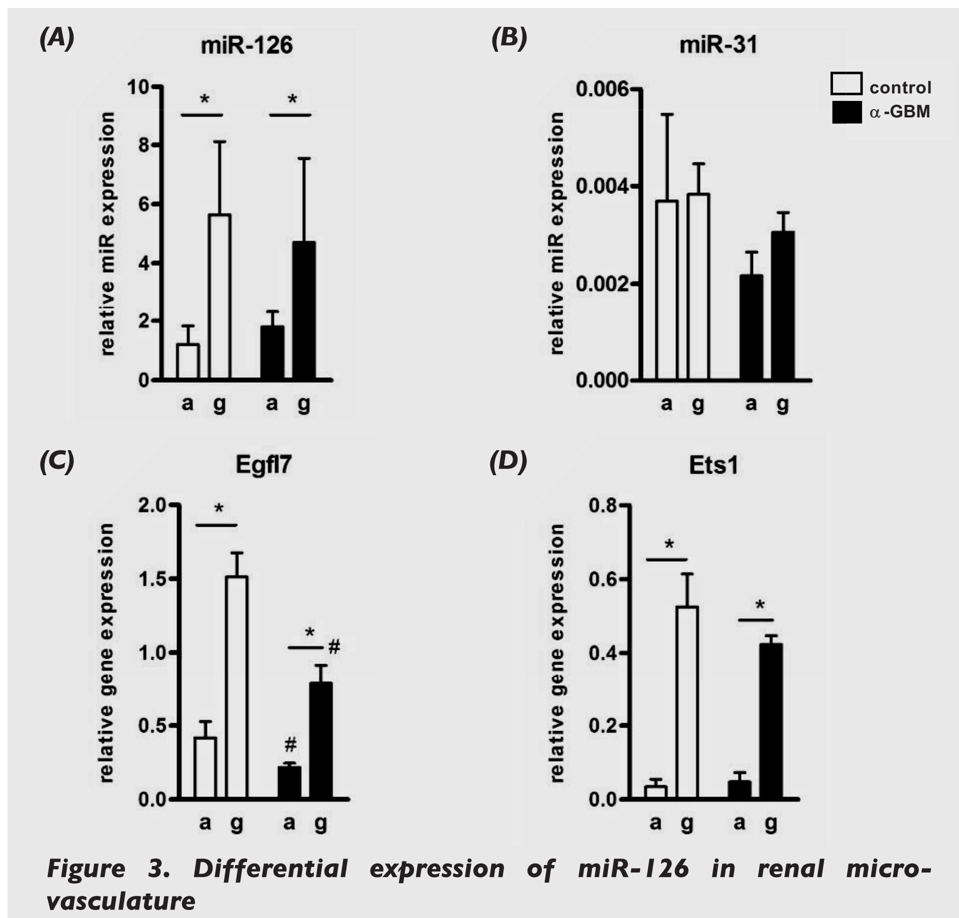


Figure 3. Differential expression of miR-126 in renal microvasculature

Figure 3. Differential expression of miR-126 in renal micro-vasculature
MicroRNA respectively mRNA expression levels in arterioles (a) and glomerular (g) compartments, in control mice (white bars) and mice subjected to the anti-GBM glomerulonephritis model (black bars). Levels were quantified after laser micro-dissection of the respective microvascular beds from frozen mouse kidney biopsies as described in *Materials and methods.* The level of expression of miR-126 ***(A)*** was significantly higher in the glomerular compartment than in the arteriolar compartment, and not affected by the inflammatory process, while the non-endothelial restricted miR-31 was expressed in both compartments to a similar extent and also not affected by the inflammation ***(B)***. The expression patterns of Egfl7 ***(C)***, the host gene of miR-126, as well as of Ets1 ***(D)***, a transcription factor shown to control miR-126 expression, followed the same spatial expression pattern as observed for miR-126. While Egfl7 was modestly decreased by the inflammation induced, Ets1 was not affected. Data are presented as mean values ± SD of 3-6 animals per group; *, $p<0.05$ arterioles vs. glomeruli, #, $p<0.05$ control vs. anti-GBM.

endothelial cells, we analyzed the expression of miR-126, miR-31, Egfl7, and Ets1 in cultured glomerular endothelial cells, mesangial cells and podocytes. The data presented in Figure 4A support the notion that miR-126 expression is endothelial cell restricted, with detectable but very low levels in mesangial cells and podocytes, while the non-endothelial restricted miR-31 was found to be expressed at similar levels in all three glomerular cell types. Also Egfl7 exhibited an endothelial restricted expression profile, while Ets1 was expressed to a similar extent in all three cell types (Fig. 4A). TNFα mediated regulation of the miRs and genes in glomerular endothelial cells was only observed for Egfl7 and Ets1. Egfl7 was downregulated to 50% of control levels at a later stage of TNFα mediated activation (24h), while Ets1 expression increased approx. 4.5 fold versus control within 4h after start of TNFα exposure (Fig. 4B).

miR-126 is functionally active in glomerular endothelial cells

To investigate whether miR-126 can be functionally active in the glomerular endothelial cells, we used reporter analysis to validate binding to the 3'UTR region of VCAM-1 (Fig. 5A). Prior to the transfection experiments, glomerular endothelial cells were pre-cultured overnight with a 21 nucleotide cholesterol-conjugated random RNA sequence (scramblemir) or with identically sized and modified RNA complementary to mature miR-126 (antagomir-126). While in scramblemir treated cells the relative firefly luciferase expression of the reporter plasmid harboring the 3'UTR region of VCAM-1 (pMIR-VCAM-1 3'UTR) was low compared to a control reporter plasmid lacking these sequences (pMIR-report; Fig. 5B), incubation with antagomir-126 restored luciferase levels of pMIR-VCAM-1 3'UTR without affecting luciferase levels of the pMIR-reporter. These results imply that miR-126 is functional in glomerular endothelial cells as

a negative regulator of VCAM-1 expression.

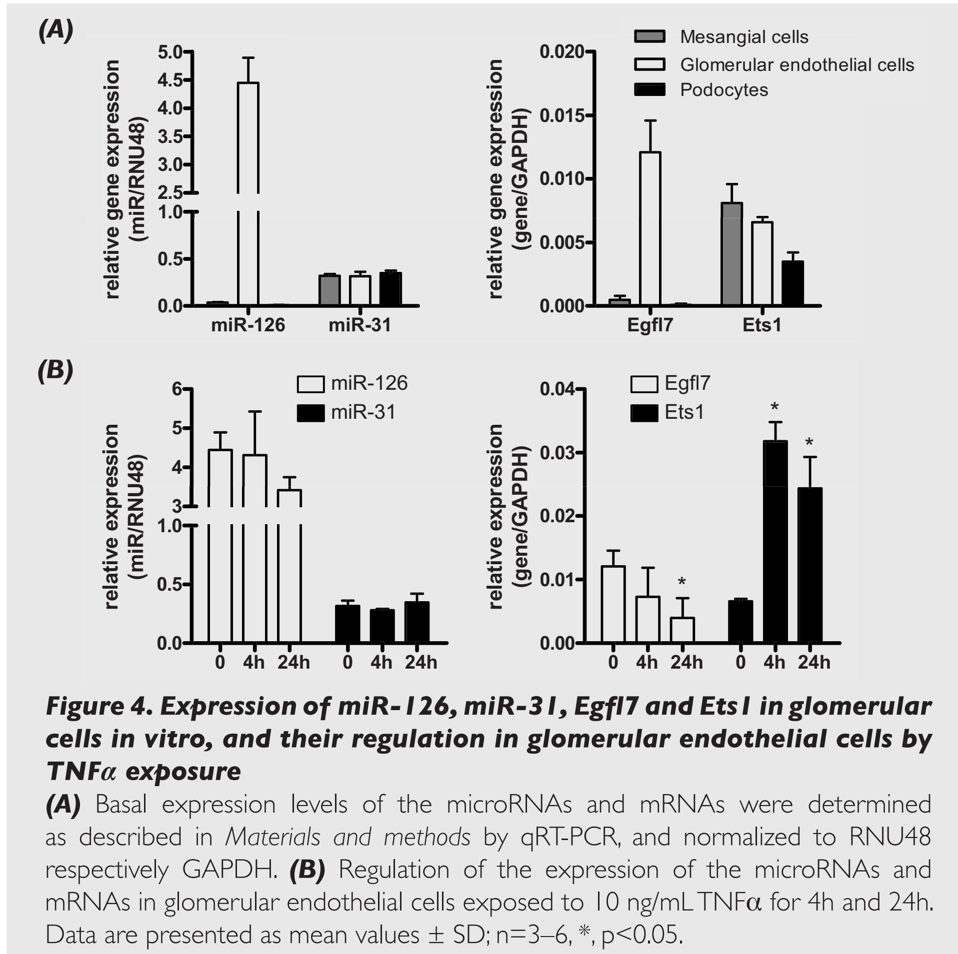


Figure 4. Expression of miR-126, miR-31, Egfl7 and Ets1 in glomerular cells in vitro, and their regulation in glomerular endothelial cells by TNFα exposure

(A) Basal expression levels of the microRNAs and mRNAs were determined as described in *Materials and methods* by qRT-PCR, and normalized to RNU48 respectively GAPDH. ***(B)*** Regulation of the expression of the microRNAs and mRNAs in glomerular endothelial cells exposed to 10 ng/mL TNFα for 4h and 24h. Data are presented as mean values ± SD; n=3–6, *, $p<0.05$.

In vivo silencing of miR-126 by antagomir-126 resulted in increased VCAM-1 protein expression upon TNFα challenge

The above data suggested that miR-126 can play a role in repressing glomerular VCAM-1 protein expression upon an inflammatory challenge and that this post-transcriptional role by miR-126 could explain the observed heterogenic VCAM-1 protein expression patterns in the microvasculature of the kidney. To study whether such a relation exists, we treated mice with antagomir-126 and scramblemir control oligonucleotides prior to an acute challenge by i.v. administration of TNFα. As a control for proper induction of inflammation, we followed the expression of E-selectin protein by immunohistochemistry (Fig. 6A) and observed that, as expected, the expression of this protein was not affected by antagomir-126 or scramblemir treatment. In contrast, we observed a marked increased expression of VCAM-1 protein in the glomerular compartment of

the mice treated with the antagomir-126 (Fig. 6). Moreover, non-glomerular segments also displayed an antagomir-126 treatment related increased expression of VCAM-1 protein, which is most likely due to the presence of functional, albeit lower levels, of mir-126 in the other microvascular segments, as we previously showed qualitatively by *in situ* hybridization [34]. Scramblemir treatment did not affect VCAM-1 protein expression levels in any of the vascular segments. Quantification of VCAM-1 protein levels in whole kidney protein isolates by ELISA also showed that VCAM-1 protein levels were increased by the antagomir-126 treatment, while scramblemir treatment did not affect the levels (Fig. 6B).

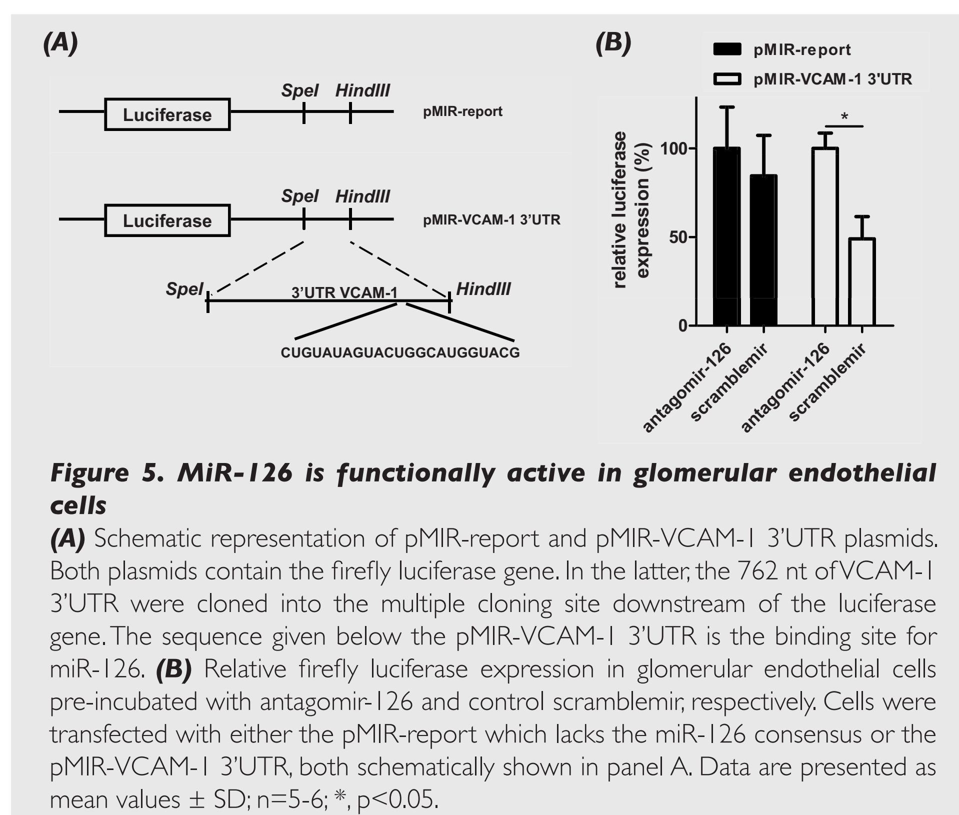


Figure 5. MiR-126 is functionally active in glomerular endothelial cells

(A) Schematic representation of pMIR-report and pMIR-VCAM-1 3'UTR plasmids. Both plasmids contain the firefly luciferase gene. In the latter, the 762 nt of VCAM-1 3'UTR were cloned into the multiple cloning site downstream of the luciferase gene. The sequence given below the pMIR-VCAM-1 3'UTR is the binding site for miR-126. ***(B)*** Relative firefly luciferase expression in glomerular endothelial cells pre-incubated with antagomir-126 and control scramblemir, respectively. Cells were transfected with either the pMIR-report which lacks the miR-126 consensus or the pMIR-VCAM-1 3'UTR, both schematically shown in panel A. Data are presented as mean values ± SD; n=5-6; *, $p<0.05$.

In parallel, we analyzed the microvascular responsiveness to antagomir-126 administration in the liver, where we also observed prominently enhanced VCAM-1 protein expression in the microvascular sinusoidal endothelial cells, which was further substantiated by ELISA quantification of VCAM-1 protein content of the liver (Fig. 7). In contrast, the capillaries of the lungs and heart were devoid of such a response (data not shown). This implies that not only in the kidney, but also in other organs, yet not in all, miR-126 exerts a role in VCAM-1 protein expression control in response to an acute inflammatory stimulus.

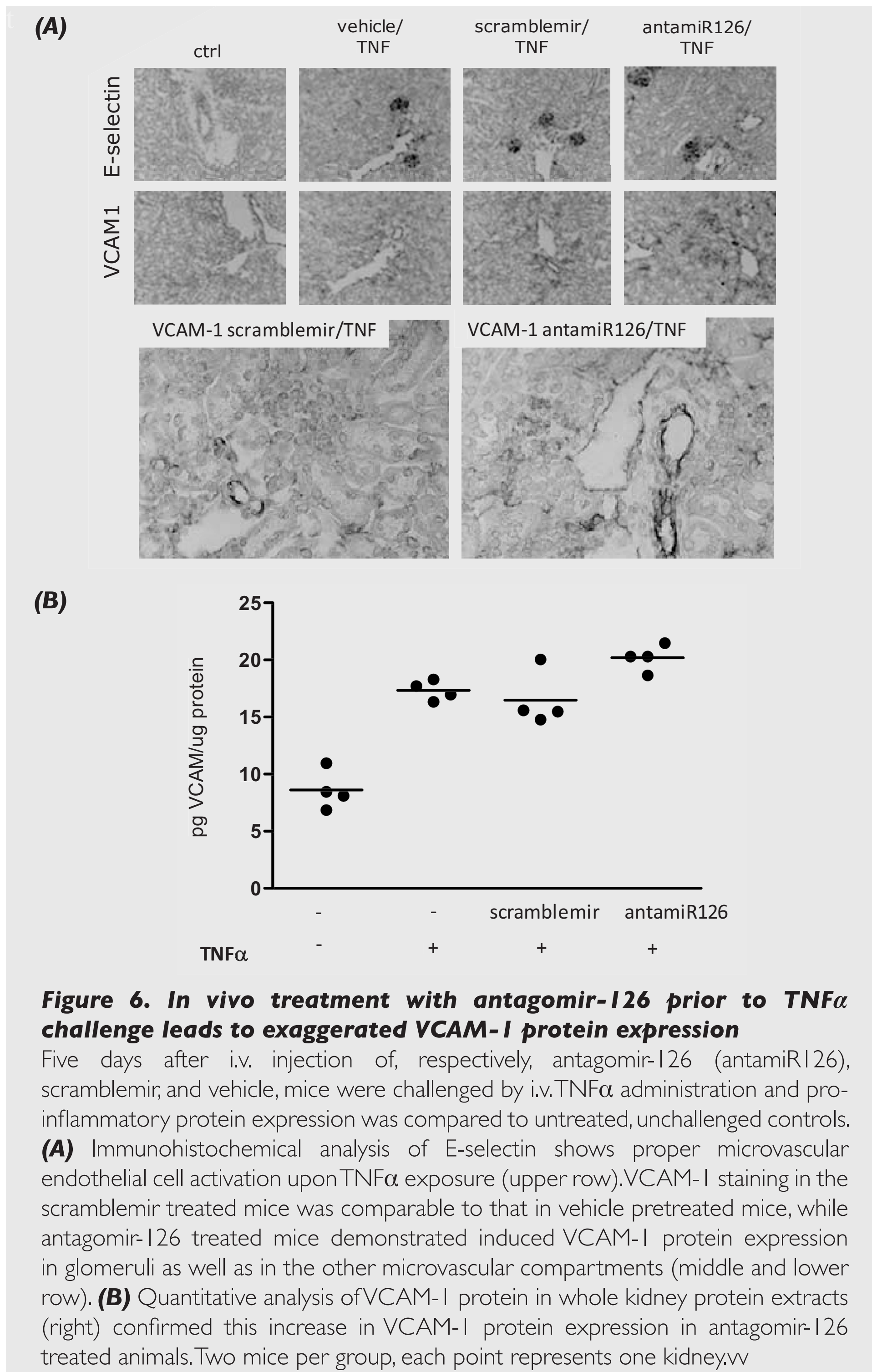
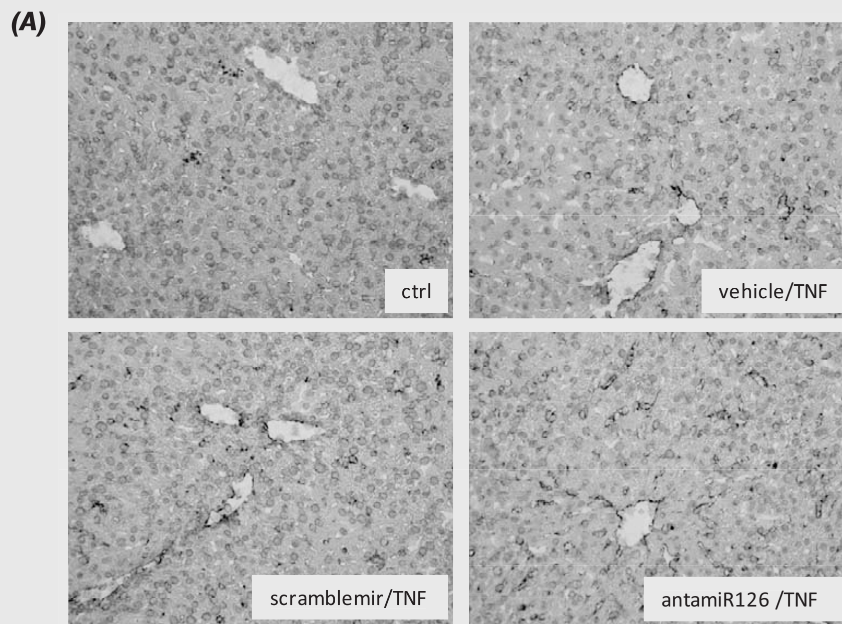


Figure 6. In vivo treatment with antagomir-126 prior to TNFα challenge leads to exaggerated VCAM-1 protein expression

Five days after i.v. injection of, respectively, antagomir-126 (antamiR126), scramblemir, and vehicle, mice were challenged by i.v. TNFα administration and pro-inflammatory protein expression was compared to untreated, unchallenged controls. ***(A)*** Immunohistochemical analysis of E-selectin shows proper microvascular endothelial cell activation upon TNFα exposure (upper row). VCAM-1 staining in the scramblemir treated mice was comparable to that in vehicle pretreated mice, while antagomir-126 treated mice demonstrated induced VCAM-1 protein expression in glomeruli as well as in the other microvascular compartments (middle and lower row). ***(B)*** Quantitative analysis of VCAM-1 protein in whole kidney protein extracts (right) confirmed this increase in VCAM-1 protein expression in antagomir-126 treated animals. Two mice per group, each point represents one kidney.vv

(A)



(B)

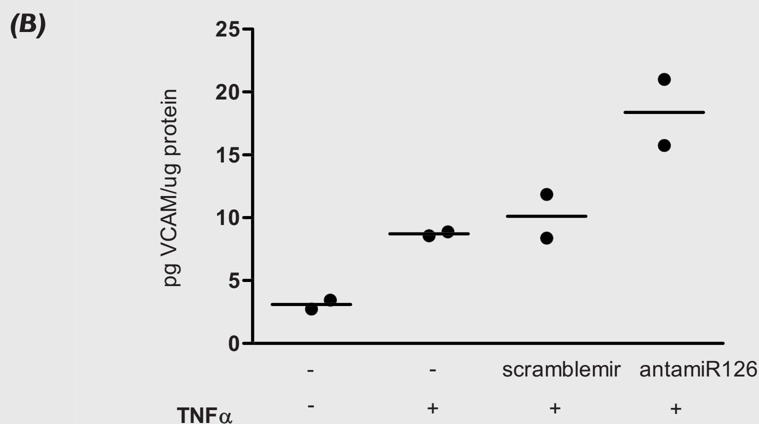


Figure 7. In vivo treatment with antagomir-126 prior to TNFα challenge leads to exaggerated VCAM-1 protein expression in the liver microvasculature

Five days after i.v. injection of, respectively, antagomir-126 (antamiR126), scramblemir, and vehicle, mice were challenged by i.v. TNFα administration and pro-inflammatory protein expression was compared to untreated, unchallenged controls. ***(A)*** Immunohistochemical analysis of VCAM-1 expression in the scramblemir treated mice was comparable to that in vehicle pretreated mice, while antagomir-126 treated mice demonstrated induced VCAM-1 protein expression upon TNFα challenge. ***(B)*** Quantitative analysis of VCAM-1 protein in whole liver protein extracts confirmed this increase in VCAM-1 protein expression in antagomir-126 treated animals. Two mice per group, each point represents one liver.

Discussion

The molecular basis for endothelial heterogeneity in renal microvascular segments is rather elusive, though knowledge thereof forms an essential fundament for understanding segmental involvement in disease and (lack of) responsiveness to drug intervention strategies [14, 18]. In the current study we show that expression of miR-126 in the glomerular microvascular compartment is a governing factor in the control of VCAM-1 protein expression in response to acute inflammation. High miR-126 levels in the glomerular compartment coincided with low VCAM-1 protein expression, while mRNA levels were highly induced. In contrast, in arterioles low miR-126 levels were associated with high VCAM-1 protein levels (see Figure 8 for a schematic presentation of this molecular concept). This posttranscriptional control mechanism is clearly distinct from the transcriptional control of E-selectin.

This is one of the first studies that show the validity of the concept of an inverse, causal relation between miR-126 expression in endothelial cells and VCAM-1 protein expression in an *in vivo* setting. Upon inhibiting miR-126 function using antagomir-126, we observed increased VCAM-1 protein expression in response to TNFα challenge in glomeruli and other renal microvascular segments as well as in the liver microvasculature (Fig. 6 and Fig. 7, respectively). This is in line with studies by Krutzfeldt and colleagues who

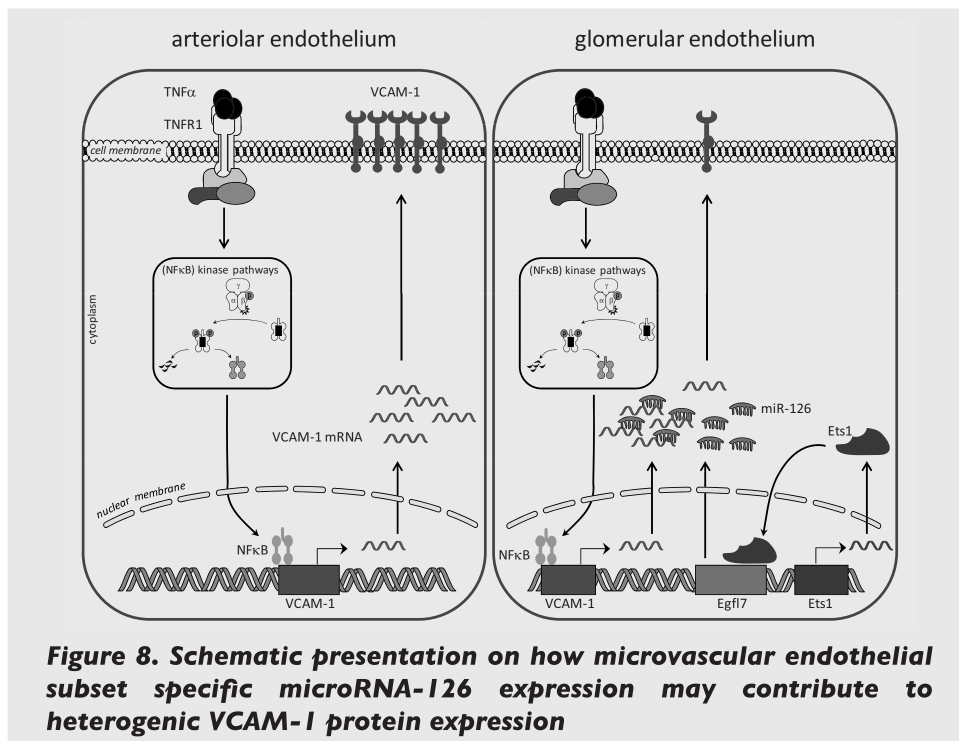


Figure 8. Schematic presentation on how microvascular endothelial subset specific microRNA-126 expression may contribute to heterogenic VCAM-1 protein expression

Figure 8. Schematic presentation on how microvascular endothelial subset specific microRNA-126 expression may contribute to heterogenic VCAM-1 protein expression

The expression of microRNA-126 (miR-126), an intronic product of the endothelial restricted Egfl7 gene, is substantially higher in glomerular endothelium than in arteriolar endothelium in the kidneys of C57bl/6 mice, as shown in this study. One consequence of this differential expression is that one of its target genes, the pro-inflammatory adhesion molecule VCAM-1, is translationally repressed primarily in the glomerular compartment. In both microvascular compartments, inflammatory activation, e.g., via TNFα binding to its receptor TNFR1, leads to activation of kinase signaling pathways which includes Nuclear Factor κ-B (NFκB) activation. NFκB mediated VCAM-1 transcription is initiated in both endothelial subsets upon glomerulonephritis induction or TNFα administration, but in the glomerular endothelial cells the higher miR-126 levels represses VCAM-1 protein transcription. As a consequence, in reaction to an inflammatory stimulus, less VCAM-1 protein is being produced in the glomerular compartment.

reported access of the inhibitory nucleotides to all tissues due to the cholesterol derivatization [13], although proper *in vivo* biodistribution studies to substantiate their accumulation in all microvascular beds are at present lacking. Ideally, endothelial specific miR-126 inhibitory nucleotides or transgenic mice should be used to prove this causal relation, to exclude indirect extra-endothelial effects being the cause of the observations reported here. Knowledge regarding genes that are restrictedly expressed in the endothelial subsets under study to identify useful promoters to make these transgenic mice is, however, missing. Although we previously showed that endothelial subset specific drug delivery systems can be designed and applied successfully to interfere with endothelial cells in glomerulonephritis [2], systems specific for quiescent endothelial subsets have not been reported yet [12]. Still, in our study set-up, the molecular target of the inhibitory nucleotides, miR-126, creates a certain level of specificity as miR-126 is restrictedly expressed in the endothelium [5, 9, 34].

Intuitively, one would expect miR-126 levels to drop after antagomir treatment. We, however, did not observe this, either in arteriolar or in glomerular segments of antagomir-126 treated mice (data not shown). Quantifying miRs after antagomir approaches by PCR-based methods is dependent on the timing of analysis after administration and the nature of the tissue analyzed. Moreover, after the antagomir inhibits its miR target by forming a duplex structure, the exact fate of miR is unknown. Torres et al. [32] recently suggested that upon binding the miR:antagomir-duplex is secluded, but not degraded. Furthermore, the fate of the duplex may depend on its binding chemistry, and it is not unlikely that different pathways prevail in different cell types. Technical issues may complicate this matter when using quantitative RT-PCR for miR detection, which includes several heating steps that can melt the duplex. By this means, the silenced miR may be

unleashed and can bind to complementary primers during the PCR reaction. In addition, Davis et al. [7] reported that currently available detection techniques of inhibited miR can be non-informative, since non-complexed antagomirs can interfere with miR levels. Therefore, the identification of a secondary end-point, the miR target gene, in our case VCAM-1, is absolutely critical for the interpretation of a miR-inhibition study.

Within the broader concept of endothelial heterogeneity [1], it is of interest to note that in the other three organs examined – liver, lungs and heart – only in the liver induced expression of VCAM-1 protein was observed after antagomir-126/TNFα treatment. This implies that not in every organ in the microvasculature miR-126 is in control of VCAM-1 protein expression and that expression control at other molecular levels is plausible. Competing endogenous RNAs, including other microRNAs and long non-coding RNAs, may contribute to a more complex control of VCAM-1 protein expression [25]. The observed divergence between Ets1 and Egfl7/miR-126 expression in the glomerular cells, in addition, points to another level of complexity of (endothelial subset restricted) gene expression control, that in the case of Efgl7 may involve transcription factors other than Ets1, such as Erg, GATA-2 [15] and Ets2 [11].

The observed glomerular specific induction of E-selectin, both at the mRNA and protein level, also instigates the question of how this highly compartmentalized expression is controlled, as all models applied have a systemic inflammation component. Negative regulation of E-selectin expression by miR-31 was recently described by Suarez and colleagues [30]. As similar levels of miR-31 were detected in glomeruli and arterioles (Fig. 3), we could not directly associate differential expression levels of miR-31 with preferential E-selectin expression in the glomeruli. Alternatively, restricted glomerular expression of E-selectin is associated with specific modifications of the chromatin architecture of the E-selectin promoter [8]. Which mechanisms are exactly involved in the distinct microvascular segment-specific, inflammation induced E-selectin expression *in vivo* remains, for now, unknown.

It needs to be established which other targets are directly or indirectly affected in time by antagomir treatment, to better understand the role of miR-126 in the molecular complexity of the changes that occur and the functional consequences thereof. To address this, less complex models such as cell culture systems are first choice, for both analytical and experimental reasons. We were, however, not able in endothelial cell cultures to show a miR-126 / VCAM-1 relation in a direct fashion: pre-incubation of glomerular endothelial cells and HUVEC with antagomir-126 followed by TNFα activation did not consistently result in induced VCAM-1 protein expression (data not shown). Both in our studies as well as in that of Harris and others [9, 10] molecular tools including pre-miR-126 expression plasmids and VCAM-1 reporter plasmids were employed to demonstrate a relation. Taking endothelial cells from an organ into a culture

system leads to a major drift of genes [6], and major changes in responsiveness to e.g., proinflammatory stimuli [17], which may underlie the fact that we cannot directly recapitulate the *in vivo* observations in an *in vitro* context. This may also play a role in the observed differences between Egfl7 expression control under inflammatory conditions in the cells in culture and *in vivo*, and emphasizes the importance of studying both molecular processes and the functional consequences in an *in vivo* context.

In summary, we here showed that glomerular expression of E-selectin in response to an acute inflammatory stimulus is transcriptionally controlled, while the contained, limited expression of VCAM-1 in this microvascular compartment is posttranscriptionally controlled. Understanding the more detailed microvascular segment specific mechanisms of control and functional consequences for endothelial engagement in disease is a prerequisite to identify whether these processes will herald new venues for renal microvascular segment targeted therapeutic intervention strategies.

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