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

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Ásgeirsdóttir SA, van Solingen C, Kurniati NF, Zwiers PJ, Heeringa P, van Meurs M, Satchell SC, Saleem MA, Mathieson PW, Banas B, Kamps JA, Rabelink TJ, van Zonneveld AJ, Molema G. MicroRNA-126 contributes to renal microvascular heterogeneity of VCAM-1 protein expression in acute inflammation. *Am J Physiol Renal Physiol* 302: F1630–F1639, 2012. First published March 14, 2012; doi:10.1152/ajprenal.00400.2011.—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 extraglomerular compartments of the kidney, while E-selectin protein was highly induced in glomeruli only (van Meurs M, Wulfert FM, Knol AJ, de Haes A, Houwertjes M, Aarts LPHJ, Molema G. *Shock* 29: 291–299, 2008). 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 TNF- $\alpha$  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 Egr1 levels, as well as higher Ets1 levels compared with arteriolar expression levels. Using miR-reporter constructs, the functional activity of miR-126 in glomerular endothelial cells could be demonstrated. Moreover, in vivo knockdown 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.

E-selectin; VCAM-1; glomeruli; arterioles; (post)transcriptional control

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).

Despite 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 of how distinct microvascular endothelial cell subsets respond at the molecular level to disease stimuli is almost nonexistent. 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 posttranscriptional regulation of inflammation by microRNAs (miRs) has become increasingly apparent (28). Mature miRs are short noncoding 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. miRs play a role in endothelial biology (29, 38) and also have been associated with pathogenesis and the progression of various kidney diseases (16, 31, 37, 39). Harris et al. (10) and more recently Salvucci et al. (26) 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. 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 proinflammatory endothelial adhesion molecules, namely, posttranscriptional miR-126-controlled ex-

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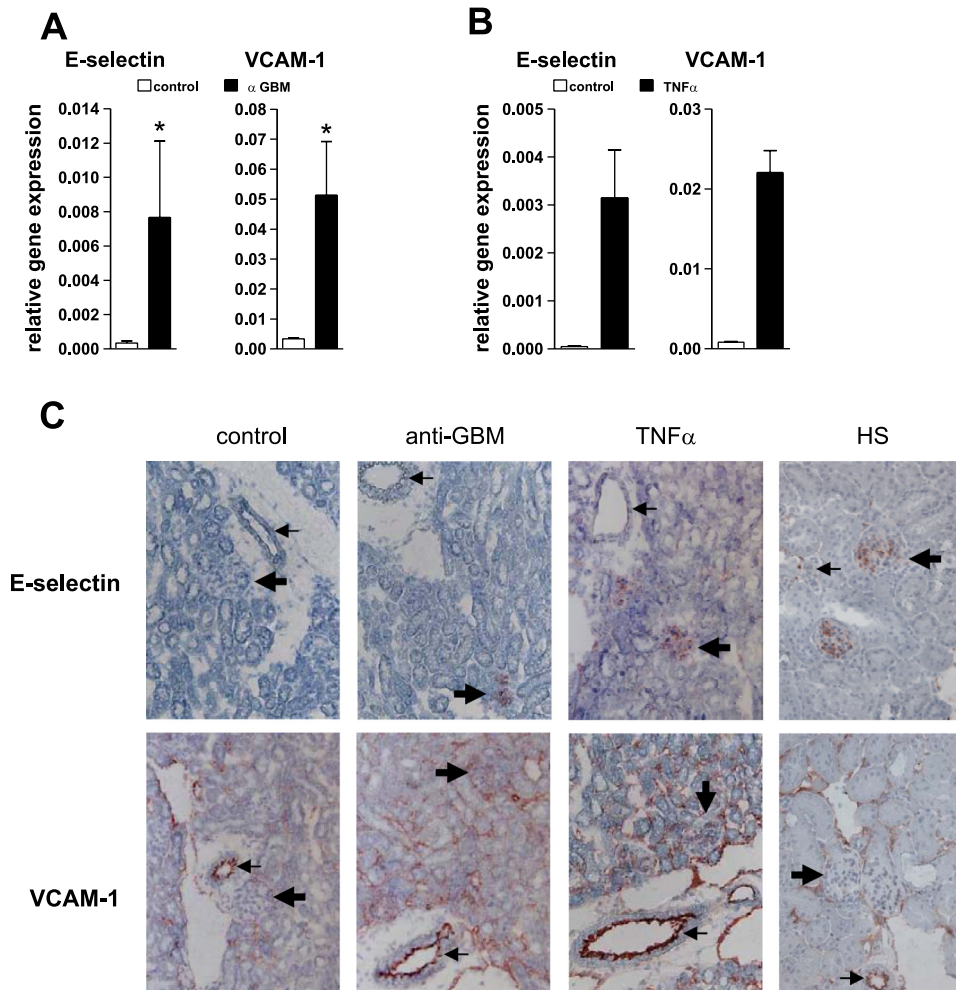


Fig. 1. Expression of E-selectin and VCAM-1 in the kidney. Gene expression of E-selectin and VCAM-1 is upregulated in the kidney at the onset of anti-glomerular basement membrane (GBM) glomerulonephritis (A) and in response to systemic TNF- $\alpha$  administration (B). Shown is quantitative gene expression in kidney samples of untreated mice (control) and at 2 h after induction of anti-GBM-glomerulonephritis (anti-GBM), respectively, TNF- $\alpha$  administration relative to GAPDH, a housekeeping gene. Values are means  $\pm$  SD of 3–6 animals/treatment. \* $P < 0.05$ . C: immunohistochemical staining of E-selectin and VCAM-1 in kidney cryosections of untreated mice (control), anti-GBM glomerulonephritis mice (anti-GBM), after intravenous (iv) administration of TNF- $\alpha$ , and after induction of hemorrhagic shock (HS). Thin arrowheads indicate the location of arterioles, thick arrowheads the location of glomeruli.

pression 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 ex-

pressed 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 kid-

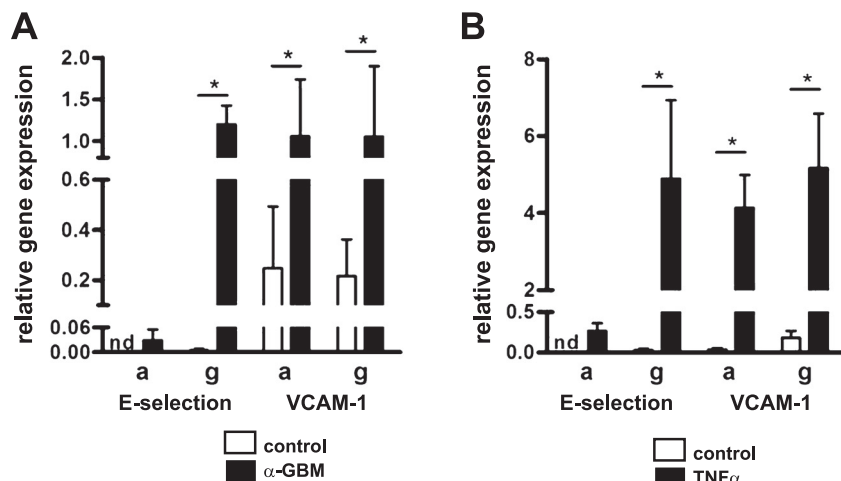


Fig. 2. E-selectin and VCAM-1 gene expression *in vivo* are compartmentalized in different microvascular segments in the kidney. Both in anti-GBM glomerulonephritis (A) and in response to systemic TNF- $\alpha$  treatment (B), gene expression of E-selectin was highly and specifically upregulated in glomerular endothelial cells (g) while hardly affected in the arterioles (a), while VCAM-1 gene expression was induced to a similar extent in arterioles and in glomeruli. Mice were euthanized at 2 h after disease induction, and vascular segments were laser microdissected before quantitative (q) RT-PCR analysis as described in MATERIALS AND METHODS. Values are means  $\pm$  SD of 3–6 animals/treatment. nd, Not detected. \* $P < 0.05$ , significant difference in gene expression in mice subjected to disease stimulus compared with untreated control mice.



ney 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 and 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 before 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 wk old) 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 12:12-h light-dark cycle. Induction of anti-glomerular basement membrane (GBM) glomerulonephritis was performed as described previously (2). Mice were euthanized at 2 h. Alternatively, inflammation was systemically induced by intravenous injection of recombinant mouse TNF- $\alpha$  (BioSource Europe, Nivelles, Belgium) at a 200-ng dose. Mice were euthanized 2 h after TNF- $\alpha$  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 mean arterial pressure (MAP) to 30 mmHg within 15–30 min as described previously (33). Mice were euthanized at 90 min after a MAP of 30 mmHg was achieved. Organs from all treatment groups were harvested, snap-frozen in liquid nitrogen, and stored at -80°C before RNA isolation, laser microdissection, and/or immunohistochemical staining.

**Antagomir-126 effects on protein expression in vivo.** Five days before TNF- $\alpha$  challenge, C57BL/6 mice were injected in the orbital plexus with 200  $\mu$ l antagomir-126 or scramblemir in saline at 1.0 mg/animal, or saline (2 mice/group) (34). At the day of death, mice were injected in the orbital plexus with 70  $\mu$ l TNF- $\alpha$  at 200 ng/animal in saline/BSA, or with vehicle only. After 2 h, mice were euthanized and relevant organs were taken out, snap frozen in liquid nitrogen, and stored at -80°C for further analysis.

**Immunohistochemical staining.** Five-micrometer 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 aan den 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% FCS. Staining was carried out with an Envision+ system-HRP kit (DakoCytomation), according to the 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, Burlingame, CA) 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 counterstained 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- $\mu$ m 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 13,000 g for 10 min at 4°C. Total protein was determined by DC Protein Assay (Bio-Rad Laboratories, Hercules, CA) before quantification of VCAM-1 by ELISA [mouse sVCAM-1/CD106 (MVC00), R&D Systems Minneapolis, MN] according to the manufacturer's instructions. VCAM-1 levels were normalized to total protein concentrations in the tissue homogenate and expressed as picograms VCAM-1 per microgram total protein.

**Laser microdissection of renal microvascular segments.** Seven hundred glomeruli (area  $\sim 3 \times 10^6 \mu\text{m}^2$ ) and arteriolar vascular segments (area  $\sim 6 \times 10^5 \mu\text{m}^2$ ) were laser microdissected from 9- $\mu$ m

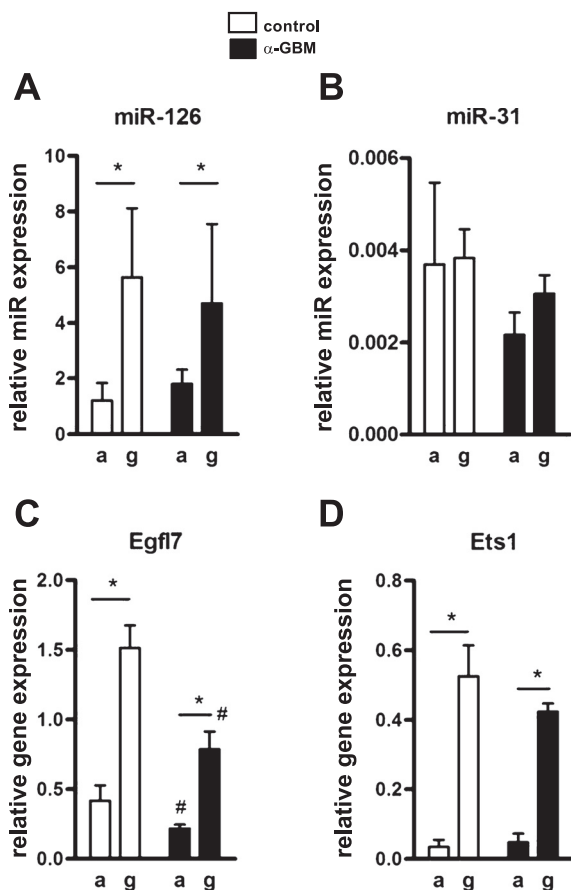


Fig. 3. miR-26 is differentially expressed in renal microvasculature. Shown are miRs, respectively, mRNA expression levels in arterioles and glomerular compartments in control mice (open bars) and mice subjected to the anti-GBM glomerulonephritis model (filled bars). Levels were quantified after laser microdissection 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. Values are means  $\pm$  SD of 3–6 animals/group. \* $P < 0.05$  arterioles vs. glomeruli. # $P < 0.05$  control vs. anti-GBM.

hematoxylin-stained cryosections using the Leica Microbeam System. Glomeruli were dissected through Bowman's capsule to only obtain cells within the glomeruli. Arterioles were identified based on their morphology, and 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 with an miRNeasy Mini kit RNA and included DNase treatment on the column (Qiagen Benelux, 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 Electrophoresis System using RNA HighSens Chips (Bio-Rad Laboratories). 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 a Taqman miRNA reverse transcription kit (Applied Biosystems, Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) in the presence of specific miR RT primers.

Quantitative PCR (qPCR) amplifications of each sample were performed in duplicate or triplicate according to the manufacturer's protocol on an ABI Prism 7900HT Sequence Detection System with gene- and miR-specific Taqman primers/probes 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 Ets1 (Mm00468970\_m1 and Hs00428293\_m1) was determined as relative to the housekeeping gene GAPDH (Mm99999915\_g1 and Hs99999905\_m1) based on the comparative  $C_t$  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) TATAAACTAGTCAAGCCATGCATTGACTTTC and VCAM-1 (anti-sense) TATAAAAAGCTTGATGACTATGCTAGGCTCCTG. These primers were designed to generate two flanking enzyme restriction sites (*SpeI* and *HindIII*) that enabled direct cloning into the pMIR-report 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  $5 \times 10^5$  cells/well in six-well plates at 33°C. Cholesterol-conjugated antagomir-126 (5  $\mu$ g/ml; 5'-g<sub>s</sub>c<sub>s</sub>auuauuacucacggua<sub>s</sub>c<sub>s</sub>g<sub>s</sub>a<sub>s</sub>-Chol-3') or control (scramblemir), which was selected to lack complementarity to the human and mouse transcriptome (5'-a<sub>s</sub>u<sub>s</sub>gacuaucgcuauucgc<sub>s</sub>a<sub>s</sub>g<sub>s</sub>-Chol-3'), was added to the cells 16 h before 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) served as a control for the efficiency of electroporation. In short, 1.5  $\mu$ g 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  $1.5 \times 10^5$  cells/well in 24-well plates, and incubated for 24 h at 33°C. Firefly luciferase and *Renilla* luciferase signals were measured using the 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,

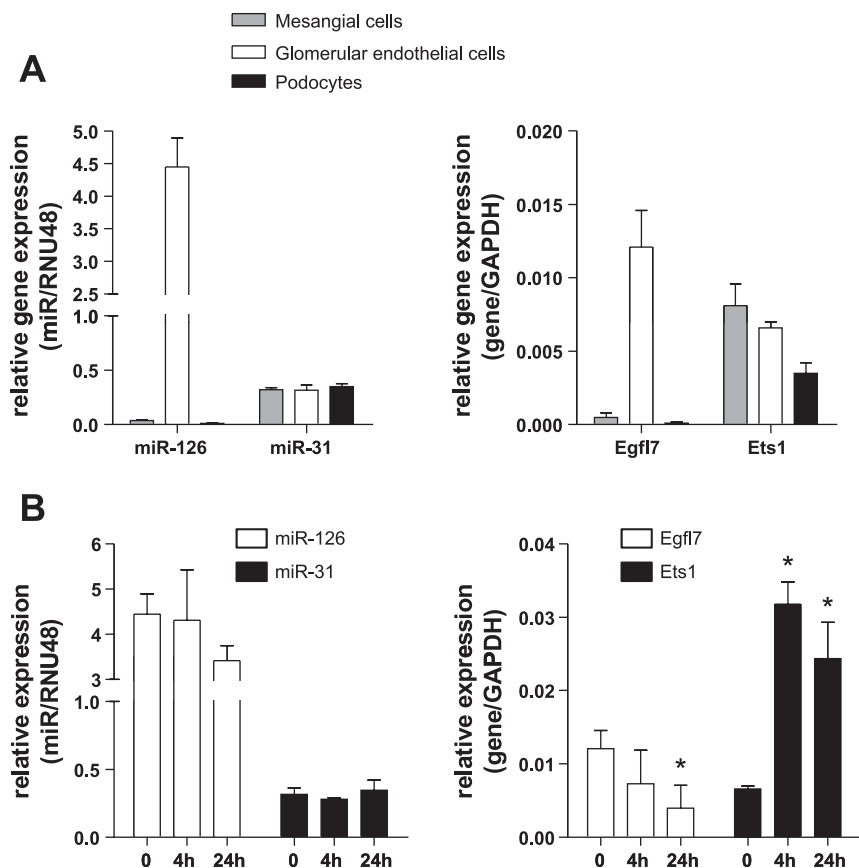


Fig. 4. Expression of miR-126, miR-31, Egfl7, and Ets1 in glomerular cells in vitro and their regulation in glomerular endothelial cells by TNF- $\alpha$  exposure. **A:** basal expression levels of miRs and mRNAs were determined as described in MATERIALS AND METHODS by qRT-PCR and normalized to RNU48 and GAPDH, respectively. **B:** regulation of the expression of the miRs and mRNAs in glomerular endothelial cells exposed to 10 ng/ml TNF- $\alpha$  for 4 and 24 h. Values are means  $\pm$  SD;  $n = 3$ –6. \* $P < 0.05$ .

ciGENc cells that were incubated with antagomir-126 before transfection were arbitrarily set at 100%.

**Statistical analysis.** Statistical significance of differences in gene and miR expression was performed by means of a 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 intravenous (iv) TNF- $\alpha$  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. 1, A (anti-GBM) and B (TNF- $\alpha$ )]. 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 glomeruli (Fig. 1C). Also, in a third inflammation model, i.e., 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 before 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 upregulation of E-selectin mRNA in glomerular capillaries, both in the anti-GBM model (Fig. 2A) and in the iv TNF- $\alpha$  challenge model (Fig. 2B). Together with the protein data from Fig. 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. 2, A and B), revealing a marked discordance in VCAM-1 gene vs. protein expression in the glomeruli that suggested a marked regulation of VCAM-1 expression at the posttranscriptional level.

**miR-126 levels of expression follow the expression patterns of Egf7 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, open bars). These levels did not change in response to the induction of acute inflammation (Fig. 3A, filled bars). In contrast, the non-endothelial-restricted microRNA miR-31 was expressed at similar levels in the two renal microvascular segments in both quiescent and inflammatory conditions (Fig. 3B).

Because miR-126 is an intronic product of the gene *Egf7* (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 *Egf7* (22, 38), we also analyzed *Egf7* expression in the two microvascular beds. This analysis revealed a similarly higher expression of *Egf7* in the glomerular compartment compared with its expression in the arteriolar compartment, both in control conditions and in response to the inflammatory challenge (Fig. 3C). In contrast to the others, *Egf7* expression was modestly downregulated in both compartments under acute inflammatory conditions.

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

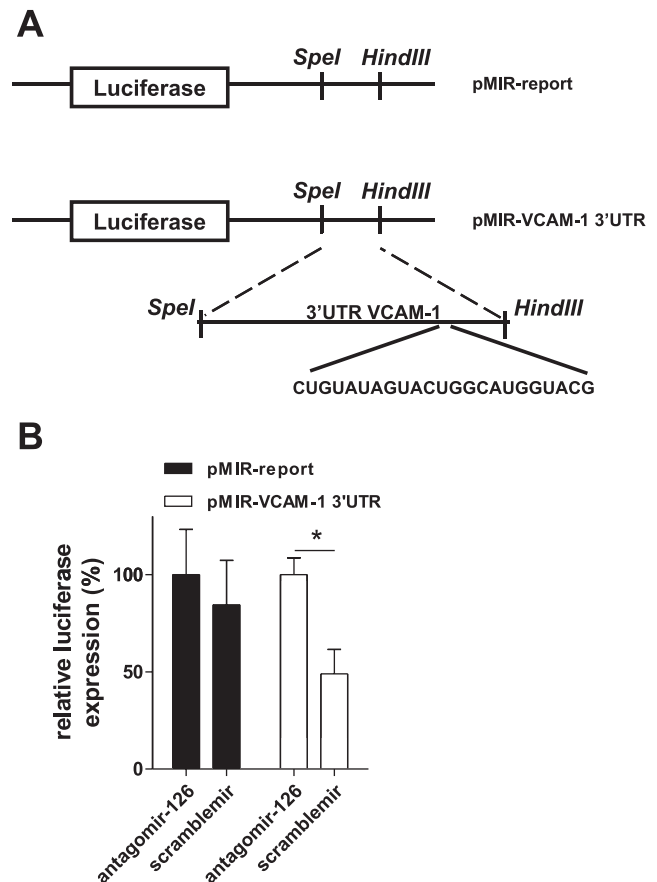


Fig. 5. miR-126 is functionally active in glomerular endothelial cells. **A**: schematic representation of pMIR-report and pMIR-VCAM-1 3'UTR plasmids. Both plasmids contain the firefly luciferase gene. In the latter, the 762 nucleotides of VCAM-1 3'UTR were cloned into the multiple cloning site downstream 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 preincubated with antagomir-126 and control scramble, 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 **A**. Values are means  $\pm$  SD;  $n = 5-6$ . \* $P < 0.05$ .



*miR-126 is only expressed in the endothelial cells of glomeruli.* Since laser-microdissected glomeruli consist of more cell types besides 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 Fig. 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). TNF- $\alpha$ -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 TNF- $\alpha$ -mediated activation (24 h), while Ets1 expression increased  $\sim$ 4.5-fold vs. control within 4 h after the start of TNF- $\alpha$  exposure (Fig. 4B).

*miR-126 is functionally active in glomerular endothelial cells.* To investigate whether miR-126 can be functionally active in glomerular endothelial cells, we used reporter analysis to validate binding to the 3'UTR region of VCAM-1 (Fig. 5A). Before the transfection experiments, glomerular endothelial cells were precultured 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 with 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.

*In vivo silencing of miR-126 by antagomir-126 resulted in increased VCAM-1 protein expression upon TNF- $\alpha$  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 posttranscriptional 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 relationship exists, we treated mice with antagomir-126 and scramblemir control oligonucleotides before an acute challenge by iv administration of TNF- $\alpha$ . 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, nonglomerular 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

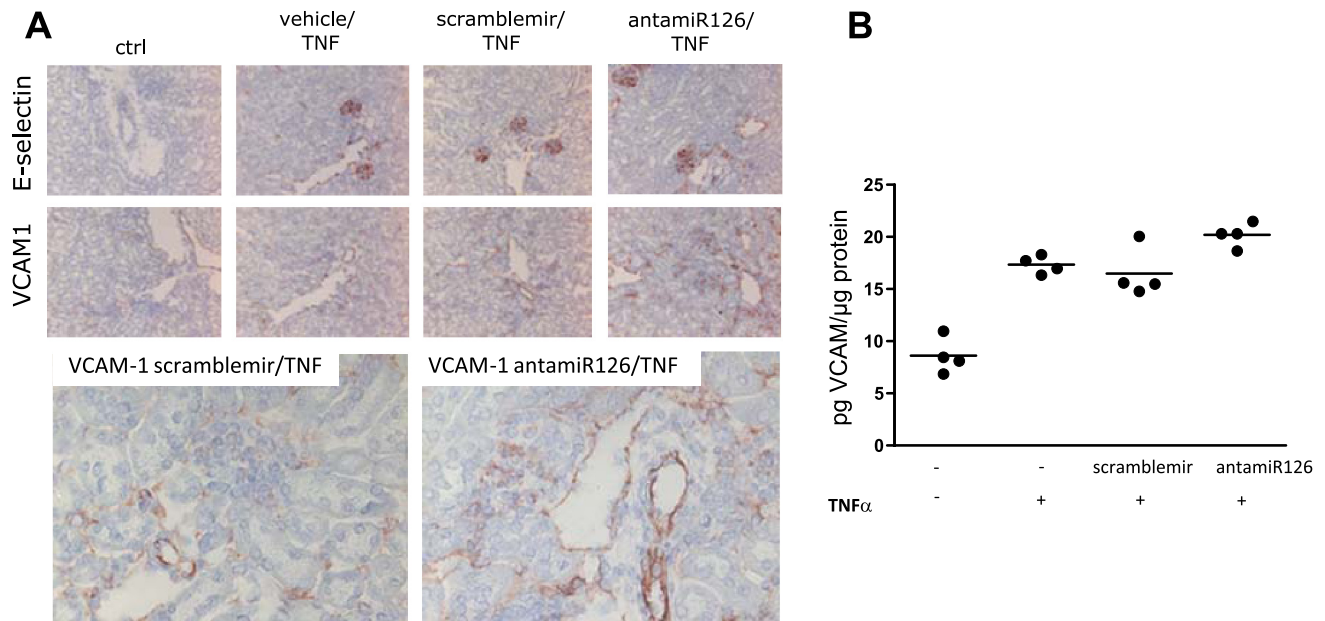


Fig. 6. In vivo treatment with antagomir-126 before TNF- $\alpha$  challenge leads to exaggerated VCAM-1 protein expression. Five days after iv injection of antagomir-126 (antamiR126), scramblemir, and vehicle, mice were challenged by iv TNF- $\alpha$  administration, and proinflammatory protein expression was compared with untreated, unchallenged controls. *A*: immunohistochemical analysis of E-selectin shows proper microvascular endothelial cell activation upon TNF- $\alpha$  exposure (*top 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 bottom rows*). *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;  $n = 2$  mice/group; each point represents 1 kidney.

treatment, while scramble-mir treatment did not affect the levels (Fig. 6B).

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 (although not in all) miR-126 exerts a role in VCAM-1 protein expression control in response to an acute inflammatory stimulus.

## DISCUSSION

The molecular basis for endothelial heterogeneity in renal microvascular segments is rather elusive, although knowledge

thereof is essential 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 Fig. 8 for a schematic presentation of this molecular concept). This posttranscriptional control mechanism is clearly distinct from the transcriptional control of E-selectin.

Ours is one of the first studies that show the validity of the concept of an inverse, causal relationship 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 a TNF- $\alpha$  challenge in glomeruli and other renal microvascular segments as well as in the liver microvasculature (Fig. 6, respectively, Fig. 7). This is in line with studies by Krutzfeldt and colleagues (13), who reported access of the inhibitory nucleotides to all tissues due to the cholesterol derivatization, 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 relationship, to exclude indirect extraendothelial effects being the cause of the observations reported here. However, knowledge regarding genes that are restrictively expressed in the endothelial subsets under study to identify useful promoters in developing these transgenic mice is 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 as yet (12). Still, in our study setup, the molecular target of the inhibitory nucleotides, miR-126, creates a certain level of specificity as miR-126 is restrictively expressed in the endothelium (5, 9, 34).

Intuitively, one would expect miR-126 levels to drop after antagomir treatment. However, we 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 sequestered, 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 qRT-PCR is used 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 PCR. In addition, Davis et al. (7) reported that currently available detection techniques of inhibited miR can be noninformative, since noncomplexed antagomirs can interfere with miR levels. Therefore, the identification of a secondary end point, the miR target gene, in our

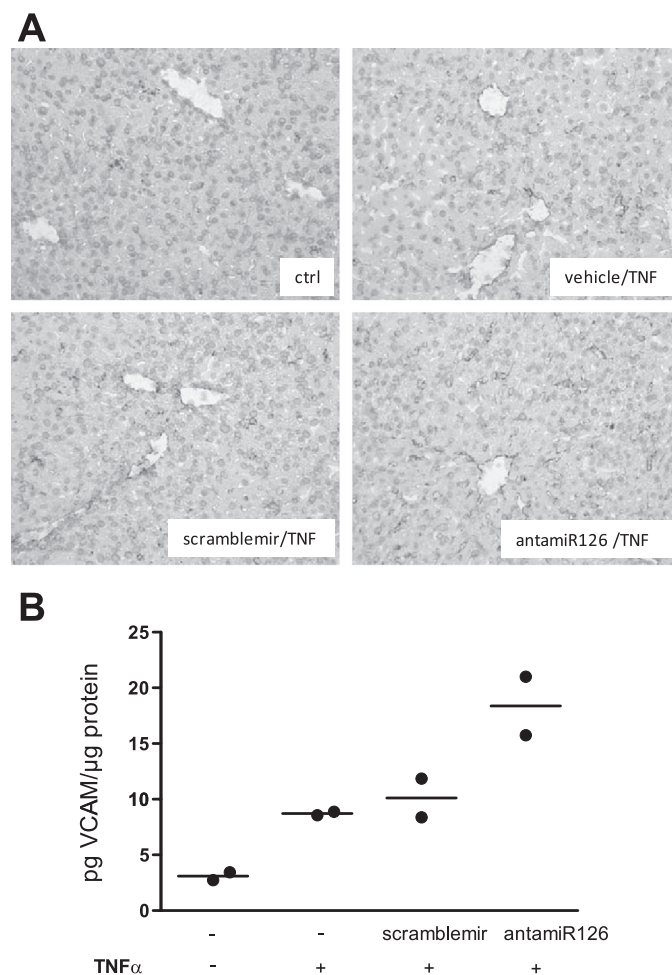


Fig. 7. *In vivo* treatment with antagomir-126 before TNF- $\alpha$  challenge leads to exaggerated VCAM-1 protein expression in the liver microvasculature. Five days after *iv* injection of, respectively, antagomir-126 (antamiR126), scramble-mir, and vehicle, mice were challenged by *iv* TNF- $\alpha$  administration, and proinflammatory protein expression was compared with untreated, unchallenged controls. *A*: immunohistochemical analysis of VCAM-1 expression in the scramble-mir-treated mice was comparable to that in vehicle-pretreated mice, while antagomir-126-treated mice demonstrated induced VCAM-1 protein expression upon TNF- $\alpha$  challenge. *B*: quantitative analysis of VCAM-1 protein expression in whole liver protein extracts confirmed this increase in VCAM-1 protein expression in antagomir-126-treated animals. ;  $n = 2$  mice/group; each point represents 1 liver.



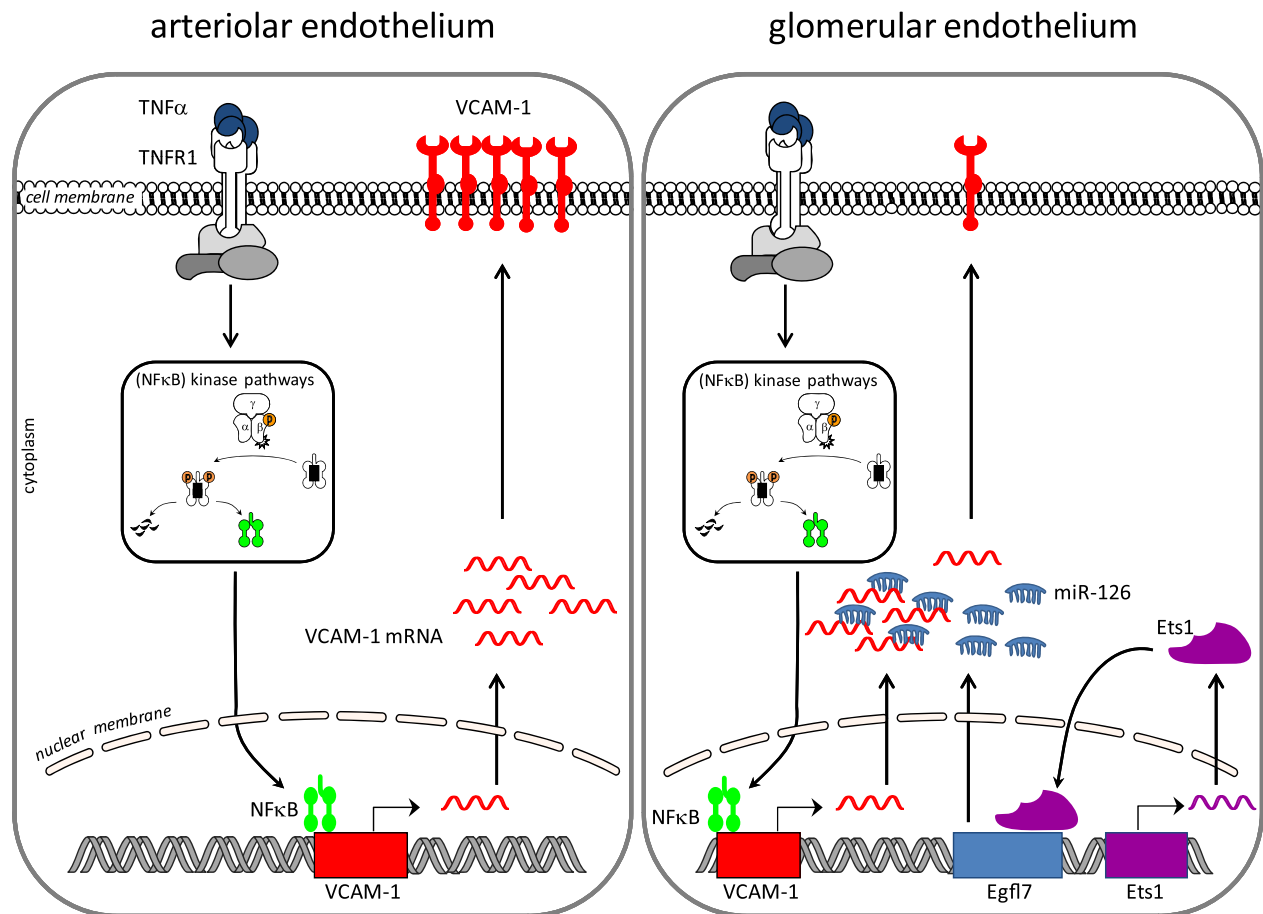


Fig. 8. Schematic presentation of how microvascular endothelial subset-specific miR-126 expression may contribute to heterogenic VCAM-1 protein expression. The expression of miR-126, an intronic product of the endothelial-restricted Egfl7 gene, is substantially higher in glomerular endothelium than in 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 proinflammatory adhesion molecule VCAM-1, is translationally repressed primarily in the glomerular compartment. In both microvascular compartments, inflammatory activation, e.g., via TNF- $\alpha$  binding to its receptor TNFR1, leads to activation of kinase-signaling pathways, which include NF- $\kappa$ B activation. NF- $\kappa$ B-mediated VCAM-1 transcription is initiated in both endothelial subsets upon glomerulonephritis induction or TNF- $\alpha$  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.

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, the liver, lungs, and heart, only in the liver induced expression of VCAM-1 protein was observed after antagomir-126/TNF- $\alpha$  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 miRs and long noncoding 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, which in the case of Efl17 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 raises 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). Exactly which mechanisms are 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 the first choice, for both analytic and experimental reasons. However, we were not able in endothelial cell cultures to show a miR-126/VCAM-1 relationship in a direct fashion: preincubation of glomerular endothelial cells

and HUVEC with antagomir-126 followed by TNF- $\alpha$  activation did not consistently result in induced VCAM-1 protein expression (data not shown). Both in our studies as well as in that of others (9, 10), molecular tools including pre-miR-126 expression plasmids and VCAM-1 reporter plasmids were employed to demonstrate a relationship. 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 Eglf7 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 for identifying whether these processes will herald new venues for renal microvascular segment-targeted therapeutic intervention strategies.

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#### DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

#### AUTHOR CONTRIBUTIONS

Author contributions: S.A.A., C.v.S., S.C.S., M.A.S., P.W.M., B.B., J.A.K., T.J.R., A.J.v.Z., and G.M. provided conception and design of research; S.A.A., C.v.S., N.F.K., P.J.Z., and M.v.M. performed experiments; S.A.A., C.v.S., N.F.K., P.J.Z., A.J.v.Z., and G.M. analyzed data; S.A.A., C.v.S., N.F.K., P.H., M.v.M., J.A.K., T.J.R., A.J.v.Z., and G.M. interpreted results of experiments; S.A.A., C.v.S., and G.M. prepared figures; S.A.A., C.v.S., and A.J.v.Z. drafted manuscript; S.A.A., C.v.S., N.F.K., P.J.Z., P.H., M.v.M., J.A.K., T.J.R., A.J.v.Z., and G.M. edited and revised manuscript; S.A.A., C.v.S., N.F.K., P.J.Z., P.H., M.v.M., S.C.S., M.A.S., P.W.M., B.B., J.A.K., T.J.R., A.J.v.Z., and G.M. approved final version of manuscript.

#### ENDNOTE

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