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Targeted transfection increases siRNA uptake and gene silencing of primary endothelial cells *in vitro* — A quantitative study

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ABSTRACT

Applications of small-interfering RNA (siRNA) call for specific and efficient delivery of siRNA into particular cell types. We developed a novel, non-viral targeting system to deliver siRNA specifically into inflammation-activated endothelial cells. This was achieved by conjugating the cationic amphiphilic lipid SAINT to antibodies recognizing the inflammatory cell adhesion molecule E-selectin. These anti-E-selectin–SAINT lipoplexes (SAINTarg) maintained antigen recognition capacity of the parental antibody *in vitro*, and *ex vivo* in human kidney tissue slices subjected to inflammatory conditions. Regular SAINT mediated transfection resulted in efficient gene silencing in human microvascular endothelial cells (HMEC-1) and conditionally immortalized glomerular endothelial cells (ciGEnC). However, primary human umbilical vein endothelial cells (HUVEC) transfected poorly, a phenomenon that we could quantitatively correlate with a cell-type specific capacity to facilitate siRNA uptake. Importantly, SAINTarg increased siRNA uptake and transfection specificity for activated endothelial cells. Transfection with SAINTarg delivered significantly more siRNA into activated HUVEC, compared to transfection with non-targeted SAINT. The enhanced uptake of siRNA was corroborated by improved silencing of both gene- and protein expression of VE-cadherin in activated HUVEC, indicating that SAINTarg delivered functionally active siRNA into endothelial cells. The obtained results demonstrate a successful design of a small nucleotide carrier system with improved and specific siRNA delivery into otherwise difficult-to-transfect primary endothelial cells, which in addition reduced considerably the amount of siRNA needed for gene silencing.

1. Introduction

Sequence-specific gene silencing with small-interfering RNA (siRNA) is a powerful and extensively explored technique with an enormous potential for pharmacological application to silence disease-causing genes [1]. The intracellular processing of synthetically produced double-stranded siRNA, which involves interaction with an RNA-induced silencing complex and which leads to posttranscriptional gene silencing, is well understood and documented [2]. The capability of siRNA to suppress gene expression can last from several days up to several weeks, depending on the stability and synthesis rate of the target RNA, as well as the division rate of a particular cell type [3].

Successful gene silencing by siRNA requires successful delivery of siRNA into the cytoplasm of the cell [4]. To make the highly anionic and relatively large (~13 kDa) siRNA accessible at its intracellular site of action, siRNA needs a delivery system for transport across the cell membrane. This requirement can be met by enclosing siRNA in viral or non-viral carriers. Non-viral transfection methods include complexation of siRNA with polymers and cationic lipid formulations [5]. The non-viral systems are less costly because of their relative simplicity, and theoretically will cause less safety problems than viral systems when applied in the clinic.

By nature non-viral transfection systems lack specificity in delivering siRNA into an appropriate cell type. In recent years, our laboratory demonstrated the feasibility of specific targeting to and intracellular delivery into activated endothelial cells of pharmacological active entities. Interfering with cellular processes in activated endothelial cells is an important approach for anti-inflammatory therapy because of the pivotal role of endothelial cells in leukocyte recruitment into the underlying tissue during the inflammatory

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processes [6]. In our previous studies, we employed immunoconjugates and immunoliposomes containing antibodies specific for the cell adhesion molecule E-selectin, which is exclusively expressed on the surface of endothelial cells in inflamed tissues. Specific intracellular delivery of drugs via receptor mediated endocytosis resulted in pharmacological effects that silenced endothelial cell activation *in vitro*, and inhibited inflammatory kidney disease progression *in vivo* [7–10].

In the current study a novel non-viral targeting device was developed to selectively deliver siRNA into activated endothelial cells. To achieve this, monoclonal anti-E-selectin antibody based cell specificity was combined with the transfection ability of the cationic amphiphilic lipid SAINT (1-methyl-4-(cis-9-dioleyl)methyl-pyridinium-chloride). This construct is hereafter referred to as SAINTarg. SAINT is a well established potent delivery agent of nucleotides and proteins [11,12]. Transfection efficiency of SAINT, as well as other cationic lipids is cell-type dependent [13,14]. Although it remains unclear whether specific cellular molecules are involved in the transfection processes, and what type of molecular pathways are responsible for the cell-type dependency, recent work has emphasized the importance of endocytotic pathways for intracellular delivery by SAINT above simple fusion events with the cell membrane [13,14]. In general, tumor cell lines are easily transfected by cationic lipid transfection agents, whereas transfection of *e.g.*, primary endothelial cells remains more challenging [15]. Transfection of human umbilical vein endothelial cells (HUVEC) with Oligofectamine-complexed siRNA specific for glyceraldehyde phosphate dehydrogenase (GAPDH) did not result in significant knock-down of GAPDH [16], while several papers have reported successful siRNA transfection of HUVEC using the transfection agent Lipofectamine 2000 [17–20]. However, the transfection experiments with lipofectamine were performed in the absence of serum which limits the applicability of the transfection protocol for *in vivo* studies. A more promising strategy for *in vivo* transfection was demonstrated by Kaufmann and co-workers, using cationic delivery systems [21–23]. Others have developed electrophoretic techniques to improve transfection efficiency in primary endothelial cells [24,25]. The success rate of delivery of siRNA via lipoplexes into primary endothelial cells will improve with more efficient transfection procedures and the introduction of cell specificity. The aim of the current study was to develop and evaluate an efficient system for delivery of siRNA into activated endothelial cells by using anti-E-selectin antibodies as specific targeting ligands for the cells and SAINT lipid as a nucleotide delivery vehicle. The selectivity and delivery features of the designed systems were explored by qualitative and quantitative analyses. We quantitated siRNA delivery using radiolabeled siRNA and related this to the efficacy of gene knock-down in endothelial cells.

2. Materials and methods

2.1. Endothelial cells and culture conditions

Human umbilical vein endothelial cells (HUVEC) were obtained from the Endothelial Cell Facility UMCG (Groningen, The Netherlands) and isolated from two umbilical cords to circumvent donor bias. Primary isolates were cultured on 1% gelatin-precoated plastic tissue culture plates or flasks (Costar Europe, Badhoevedorp, The Netherlands) at 37 °C under 5% CO_2/95% air. The culture medium consisted of RPMI 1640 (Lonza, Verviers, Belgium) supplemented with 20% (v/v) heat-inactivated fetal calf serum (FCS, Hyclone, Logan, Ut. USA), 2 mM L-glutamine (GIBCO-BRL), 5 U/ml heparin (Leo Pharma, Breda, The Netherlands), 100 U/ml penicillin (Yamanouchi Pharma, Leidendorp, The Netherlands), 100 µg/ml streptomycin (Radiumfarma-Fisiopharma, Milano, Italy), and 20 µg/ml endothelial cell growth factor extracted from bovine brain as described previously [26]. After attaining confluence, cells were detached from the surface by trypsin/EDTA (0.5/0.2 mg/ml in PBS) and split at a 1:3 ratio. For the experiments presented here HUVEC were used up to passage three.

The human dermal microvascular endothelial cell line HMEC-1 [27], a kind gift from Dr. E. W. Ades, Centers for Disease Control, Atlanta, GA, USA, was cultured at 37 °C under 5% CO_2/95% air in medium-199 with Hepes (#BE12-117F; Lonza, Verviers, Belgium), supplemented with 10% (v/v) heat-inactivated FCS, 10% (v/v) pooled human serum (Sanquin, Groningen, The Netherlands), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. The human conditionally immortalized glomerular endothelial cell line ciGEnC was cultured at 33 °C for propagation of cells in EGM2-MV (endothelial growth medium 2 — microvascular; Cambrex-Lonza, Breda, The Netherlands), containing 5% (v/v) heat-inactivated FCS and growth factors as supplied, excepting VEGF [28]. Five days prior to transfection experiments, cells were transferred to 37 °C.

2.2. Preparation and characterization of Ab_{Esel}SAINT conjugate

H18/7 mouse anti-human E-selectin monoclonal antibody-producing hybridoma (Ab_{Esel}) was kindly provided by Dr. M. Gimbrone Jr (Boston, Ma., USA). Ab_{Esel} was purified by HiTrap Sephadex-Protein G column (GE Healthcare Europe GmbH, Diegem, Belgium). Free sulfhydryl groups were introduced in Ab_{Esel} by N-succinimidyl-S-acetylthioacetate (SATA, Pierce, Rockford, Il., USA; molar ratio Ab_{Esel}:SATA, 1:10) [29]. Excess SATA was removed by gel permeability chromatography (HiTrap Desalting column; GE Healthcare) and acetylthioacetate-Ab_{Esel} was deacetylated by incubation at room temperature for 2 h with freshly prepared 0.5 M hydroxylamine pH 8 (Pierce). Determination of free sulfhydryl groups was performed according to Ellman [30] and demonstrated 6 SH groups per Ab_{Esel} molecule. SAINT-aminolinker (4-[(10*Z*)-1-(4-aminobutyl)octadec-10-en-1-yl]-1-methyl-pyridinium chloride) was reacted with an equimolar amount of 2-bromo-*N*-{3-[(2,5-dioxopyrrolidin-1-yl)oxy]-3-oxopropyl}acetamide (SBAP, Pierce). SAINT-aminolinker-SBAP was added to Ab_{Esel}-SH (molar ratio 15:1), incubated at room temperature for 40 min and excess SAINT-aminolinker-SBAP was removed by HiTrap gel permeability chromatography. Protein concentration of SAINT-aminolinker-Ab_{Esel} was determined by bicinchoninic acid protein assay (Pierce) and the number of SAINT-aminolinkers attached to the Ab_{Esel} monoclonal antibody was measured by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry on a Voyager-DE PRO Biospectrometry Workstation (Applied Biosystems, Nieuwekerk a/d IJssel, The Netherlands).

2.3. Particle size and zeta-potential measurement

Particle size and zeta-potential of SAINT and SAINTarg were measured by laser light scattering using a Nicomp submicron particle analyzer (Nicomp 380/ZLS, Santa Barbara, CA, USA). For particle sizing SAINT (15 nmol/ml) and SAINTarg (15 nmol/ml lipid; variable protein contents: 30, 7.5, and 1.88 pmol/ml Ab_{Esel}), were vortexed just prior to measurement. When appropriate, siRNA (concentration 60 and 0.6 pmol/ml, respectively) was added to SAINT and SAINTarg. Mean diameter was obtained from the volume distribution curves produced by the particle analyzer. For zeta-potential measurements, solutions were diluted 12 times with water.

2.4. Cell binding studies of Ab_{Esel}SAINT conjugate by flow cytometric analysis

HUVEC were activated with 10 ng/ml TNFα (Roche Diagnostics, Almere, The Netherlands) for 4 h, detached from tissue culture plates by short treatment with trypsin and washed with ice-cold PBS/5% FCS. Ab_{Esel}SAINT conjugate and parental anti-E-selectin antibody (H18/7) were added to the cells at indicated concentrations and incubated for 45 min at 4 °C. In parallel experiments HUVEC were

incubated with monoclonal mouse anti-human antibodies recognizing the endothelial marker CD31 (clone JC/70A; DakoCytomation, Glostrup, Denmark), or with monoclonal mouse anti-human ICAM-1 antibodies (clone hu5/3-2.1; kindly provided by Dr. M. Gimbrone Jr), the latter to demonstrate endothelial activation. After incubation with primary antibodies or Ab_{Esel}SAINT conjugate, cells were washed with ice-cold PBS/5%FCS, followed by incubation of fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse antibody (DakoCytomation) for 45 min on ice and analysis on an Epics-Elite Flow cytometer (Coulter Electronics, Mijdrecht, The Netherlands). Non-specific staining was assessed by incubation of cells with mouse isotype control monoclonal antibodies (Santa Cruz Inc., Santa Cruz, CA). A total of 5000 events were analyzed per sample.

2.5. siRNA, target sequences and modifications

Predesigned gene-specific siRNAs for VE-cadherin (Hs_CDH5_2_HP siRNA; target sequence ACGTATTATCACAATAACGAA), CD31 (Hs_PECAM1_1_HP siRNA; target sequence CCCAATACACTTCACAATTGA) and TNFR2 (Hs_TNFRSF1B_2HP siRNA; target sequence CCGGGAAGCGATGAATTTGGA) were purchased from Qiagen (Benelux, Venlo, The Netherlands). Non-silencing siRNA with no homology to any known mammalian gene (AllStars negative control, Qiagen) was used as a control. The same control siRNA but tagged with, respectively, $Alexa_{488}$ and $Alexa_{546}$, was purchased from Qiagen and applied for transfection efficiency studies. For quantitative uptake studies a 19 base pair scrambled negative control # 1 (Ambion/Applied Biosystems) was 5′ end labeled with [γ-^{32}P]ATP using KinaseMax 5′ End-Labeling Kit (Ambion/Applied Biosystems) and analyzed for quantification of uptake of siRNA into endothelial cells. In short, 2 pmol siRNA, 5 pmol [γ-^{32}P]ATP and T4 polynucleotide kinase were incubated at 37 °C for 60 min in 20 µl kinase buffer. Free nucleotides were removed by spin-column chromatography (NucAway spin column, Ambion/Applied Biosystems) and the amount of incorporated radioactivity was measured by Packard Tri-Carb 2500 TR liquid scintillation analyzer (PerkinElmer Life and Analytical Sciences, Inc., Waltham, Ma, USA). For transfection experiments trace amount of radioactive siRNA (0.15 pmol, ~8000 Bq) was mixed with non-radioactive siRNA to a total amount of 60 pmol/ml as described below.

2.6. Cell transfection with SAINT and SAINTarg

2.6.1. Standard transfection with SAINT

To obtain identical cell density at the start of the experiments HUVEC and HMEC-1 were seeded at 1.5×10^4 cells/cm^2, respectively, 2.5×10^4 cells/cm^2, at 37 °C one day prior to transfection experiment in tissue plates (Costar, San Diego, CA) or Lab-Tek Chamber Slides (Nunc, Rochester, NY). ciGEnC were seeded at 3.0×10^4/cm^2, cultured for 1 day at 33 °C and 5 days at 37 °C. SAINT siRNA delivery system (product name SAINT-RED, Synvolux Therapeutics) consisted of a 1:1 mixture of the cationic SAINT lipid and the neutral helper-lipid DOPE (dioleoyl-phosphatidylethanolamine) [12]. Where appropriate, cells were activated with 10 ng/ml TNFα 1 h prior to transfection. Standard transfection in a 6-wells plate was carried out by complexing 60 pmol siRNA with 15 nmol SAINT according to manufacturer's protocol. The complex was freshly made and mixed with culture medium in a total volume of 1 ml and added to the cells within 15 min. In the case of HMEC-1, serum-free medium was used, while HUVEC and ciGEnC were transfected in serum-containing medium, as these experimental conditions created the most efficient transfection conditions for each cell type. Cells were transfected for 4 h and harvested for flow cytometric analysis, fluorescence microscopy and quantitative siRNA uptake studies, as described below. For gene expression analysis and Western blot analysis 2 ml serum-containing medium was added 4 h after the start of transfection and incubation was continued at 37 °C for 2 days (gene expression analysis) and 4 days (Western blot analysis). When transfection experiments were carried out in 24-well plates and Lab-Tek chambers, the total volume and the amount of siRNA and SAINT were adjusted according to well surface, maintaining final concentration of all components as indicated above.

2.6.2. Targeted transfection with SAINTarg

Targeted transfection with freshly formulated SAINTarg was carried out as described above. Formulation of SAINTarg was performed by mixing Ab_{Esel}SAINT conjugate with SAINT in a fixed molar ratio. Unless otherwise stated, antibody:SAINT ratio was 1:2000 with a final lipid concentration of 15 nmol/ml. Just prior to transfection SAINTarg was vortexed for 30 s and complexed with siRNA.

2.7. Flow cytometric and microscopic analysis of Alexa-tagged siRNA uptake in HUVEC

Resting and TNFα-activated HUVEC were transfected for 4 h with $Alexa_{488}$-tagged siRNA (Qiagen) using SAINT and SAINTarg, respectively, detached as described above, washed and measured directly by flow cytometry. In competition experiments, transfection was performed in the presence of 750 pmol/ml H18/7 anti-E-selectin antibody or irrelevant control antibody. A total of 5000 events were analyzed per sample.

For fluoromicroscopic analysis TNFα-activated HUVEC were transfected with $Alexa_{546}$-tagged siRNA (Qiagen) in Lab-Tek Chambers using SAINT and SAINTarg, respectively, washed twice with ice-cold PBS, fixed at room temperature for 10 min in 4% paraformaldehyde and nuclei were stained with 4′,6-Diamidine-2′-Phenylindole Dihydrochloride (DAPI; Roche Diagnostics). Sections were mounted with citifluor (Agar Scientific, Stansted, UK) and analyzed by fluorescence microscope (DM RXA, Leica Microsystems AG, Wetzlar, Germany) and Leica Q600 Qwin software V01.06. Spinning disk confocal live cell microscopy was performed with living cells in Lab-Tek chambers after transfection as described above, on a Leica DM IRE2 Inverted microscope controlled by InVivo acquisition software (Media Cybernetics, Bethesda, MD). Series of images were acquired in successive 1 µm *z*-axis displacements with a Stanford Photonics XR/Mega-10 I-CCD camera. Pictures were processed by Imaris X64 6.2.1. software and edited by Adobe Photoshop CS3 (Adobe Systems Inc., San Jose, CA, USA).

2.8. Quantitative analysis of uptake of radioactive siRNA

Cells were seeded as described above and transfected with [γ-^{32}P] 5′ end labeled siRNA using SAINT and SAINTarg, respectively. Trace amount of radioactive siRNA (0.15 pmol, ~8000 Bq) was mixed with non-radioactive siRNA to a total amount of 60 pmol/ml. At $t=4$ h after transfection culture medium was removed, cells were washed 5 times with ice-cold PBS and lyzed in 0.1 M NaOH. Cell associated ^{32}P-radioactivity was measured by a Packard Tri-Carb 2500 TR liquid scintillation analyzer (PerkinElmer).

2.9. Preparation and incubation of human kidney slices

Human kidney tissue was obtained as tumor free surgical waste from patients subjected to carcinoma surgery. Kidney donors ($n=3$) were all male, age between 60 and 66 years, with normal kidney function. Tissue was transported in UW organ preservation solution (DuPont Pharmaceuticals, Waukegan, IL, USA) on ice. Subsequently, tissue cylinders were made with a 5 mm diameter motor-driven coring tool. Precision-cut kidney slices (250 µm thick) were prepared within 1 h after excision of the tissue in ice-cold oxygenated Krebs–bicarbonate–Ringer solution, pH 7.4 (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) with a Krumdieck tissue slicer [31]. Slices were incubated individually in 12-well culture plates (Costar 3512;

Corning Glassworks, Corning, NY) in 1.3 ml of Williams Medium E with Glutamax-I, supplemented with 25 mM D-glucose, penicillin (100 U/ml) and streptomycin (100 µg/ml) and 50 ng/ml TNFα. SAINTarg (15 nmol/ml) was added to 3 slices per donor at $t=2$ h. All slices were placed at 37 °C and incubated for 24 h under humidified carbogen (5% CO_2/95% O_2) on an orbital shaker (90 rpm), harvested and frozen for immunohistochemical analysis. ATP level measurements, performed at different time points as described previously [32], demonstrated maintenance of viability throughout the experiment.

2.10. Immunohistochemical detection of E-selectin and SAINTarg in human kidney biopsies

Five-µm cryosections of human kidney slices were fixed in acetone for 10 min. Cryosection of slices incubated in the absence of SAINTarg were stained for expression of E-selectin by incubation for 45 min with H18/7 monoclonal mouse anti-human E-selectin antibody. Slices incubated in the presence of SAINTarg complexed with control siRNA were stained for localization of the SAINTarg delivery device by direct incubation for 45 min with preabsorbed unlabeled rabbit anti-mouse antibodies (Vector Laboratories Inc., Burlingame, CA, USA). Detection was performed using Envision+ system-HRP kit (DakoCytomation Denmark, A/S), according to the manufacturer's protocol, including a blocking step for endogenous peroxidase activity. Cryosections were counterstained with Mayer's hematoxylin (Klinipath, Duiven, The Netherlands). Between all incubation steps cryosections were washed extensively with PBS.

2.11. RNA isolation and quantitative RT-PCR analysis

RNA was isolated from cells with RNeasy mini plus kit (Qiagen Benelux, Venlo, The Netherlands). RNA integrity was studied by standard laboratory methods. RNA was reverse transcribed using SuperscriptIII reverse transcriptase (Invitrogen, Breda, The Netherlands) and random hexamer primers (Promega, Leiden, The Netherlands). Quantitative PCR amplifications were performed in triplicate for each sample according to the manufacturer's protocol on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Relative gene expression of VE-cadherin (assay number: Hs00174344_m1), CD31 (Hs00169777_m1) TNFR2, (Hs00153550_m1), and Tie-2 (Hs00176096_m1) was normalized for the expression of the house keeping gene GAPDH (Hs99999905_m1) according to the comparative Ct method (Ct = number of amplification cycles needed to achieve a fixed threshold value; $\Delta Ct = Ct_{\text{gene of interest}} - Ct_{\text{housekeeping gene}}$). Comparison of gene expressions in different samples was performed based on the differences in ΔCt of individual samples (ΔΔCt).

2.12. Western blot analysis

HUVEC transfected with VE-cadherin siRNA and non-silencing siRNA were harvested on day 4, lyzed in ice-cold RIPA buffer (Pierce), sonicated for 2×5 s and centrifuged to get rid of cell debris. Protein concentration was determined by BCA Protein Assay (Pierce). Proteins (4 µg per lane) were separated on a 10% polyacrylamide gel by SDS-PAGE with Mini-Protean II apparatus (BioRad Laboratorium, Veenendaal, The Netherlands) and transferred by Trans-Blot Electrophoretic Transfer system (BioRad) to polyvinylideenfluoride membrane (PVDF; GE Healthcare). The membrane was blocked at room temperature for 60 min in Tris buffered saline (TBS)/5% skimmed milk/0.1% Tween 20 (Sigma-Aldrich Chemie)/1% polyvinylpyrrolidone (PVP; Serva Electrophoresis, Heidelberg, Germany). The membrane was horizontally cut through the 70 kDa prestained marker and the upper part of the blot was incubated overnight at 4 °C with polyclonal rabbit anti-human VE-cadherin antibody (product number AHP628Z, AbD Serotec, Düsseldorf, Germany). For loading control the lower part of the blot was incubated for 1 h at room temperature with monoclonal mouse anti-GAPDH antibody (Fitzgerald Industries, Concord, MA). Membranes were washed in TBS/0.1% Tween/1% PVP and incubated at room temperature for 1 h with peroxidase-conjugated goat anti-rabbit immunoglobulin G (Southern Biotech Birmingham, AL)/goat anti-mouse $F(ab)_2$ fragment (Jackson ImmunoResearch Europe, Suffolk, UK). Blots were washed as described above and the signal was detected by enhanced chemiluminescence, using ECL Supersignal West Dura Detection Kit (Pierce). Signals were quantified by densitometric analysis using QuantityOne quantification software (BioRad).

2.13. Statistic analysis

Statistical significance of differences was studied by means of the two-sided Student's *t*-test, assuming equal variances. Differences were considered to be significant when $p<0.05$.

3. Results

3.1. SAINT effectively transfected endothelial derived cell lines but not primary endothelial cells

The efficacy of gene silencing by three sequence-specific siRNAs, TNFR2, CD31 and VE-cadherin, was analyzed in endothelial cells using the synthetic amphiphilic SAINT delivery system, previously shown to successfully deliver DNA, siRNA and proteins [12]. All siRNAs effectively silenced the corresponding genes by approximately 80–95% in two endothelial derived cell lines, HMEC-1 and ciGEnC (Fig. 1a). No relation was observed between the level of gene expression *per se* and the level of knock-down effect. In ciGEnC transfection with an siRNA specific for TNFR2, which has a relative low basal expression resulted in similar knock-down effect as an siRNA specific for the highly expressed VE-cadherin. The silencing of VE-cadherin was stronger in HMEC-1 than in ciGEnC, but CD31 and TNFR2 were silenced to a similar extent in both cell lines. In contrast, notable inefficient silencing on the expression of all three genes was observed in the primary endothelial cell HUVEC (Fig 1b). Only VE-cadherin expression was significantly reduced, whereas expression levels of CD31 and TNFR2 were unchanged after transfection. Transfection of HMEC-1, ciGEnC, and HUVEC with non-silencing control siRNA did not reduce the expression of any of the genes analyzed (Fig. 1c). In HMEC-1 transfection with non-silencing siRNA resulted in a slight increase of the expression of VE-cadherin, CD31, and TNFR2, but both in ciGEnC and HUVEC gene expression was unaltered by non-silencing siRNA.

We hypothesized that cell-type specific uptake capacities could explain the observed variations in gene silencing between cell lines on one hand, and HUVEC on the other hand. To address this, an experiment of HUVEC and HMEC-1 was performed with radioactively labeled scrambled control siRNA transfected with SAINT. This experiment demonstrated that approximately 4 times as much siRNA was taken up by HMEC-1 than HUVEC (Fig. 1d). This suggested that uptake capacity of primary endothelial cells and endothelial derived cell lines is different and that siRNA uptake likely presents a rate limiting step in transfection of HUVEC.

3.2. Synthesis and binding specificity of Ab_{Esel}SAINT conjugate to activated HUVEC

To improve the transfection procedure of activated primary endothelial cells we designed a novel delivery system with the aim to specifically deliver siRNA via E-selectin. For that reason, monoclonal anti-E-selectin antibody was covalently conjugated to SAINT-aminolinker molecules according to the scheme in Fig. 2a. Based on Ab_{Esel} input, overall protein yield coupled to SAINT-aminolinker ranged from 10 to 25%. MALDI-TOF mass spectrometry demonstrated

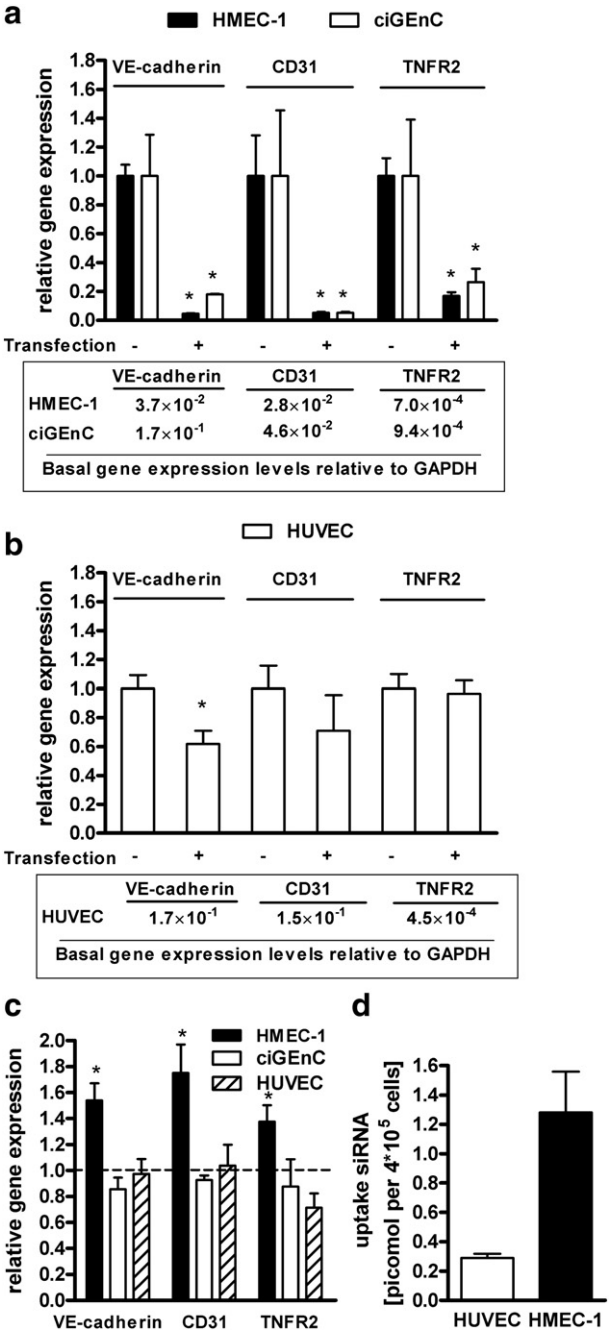


Fig. 1. Transfection of endothelial cell lines and primary endothelial cells is dependent on siRNA uptake. (a) Transfection of endothelial cell lines HMEC-1 and ciGEnC with SAINT and a gene-specific siRNA for VE-cadherin, CD31 and TNFR2. (b) Transfection of the primary endothelial cells HUVEC with SAINT and a gene-specific siRNA for VE-cadherin, CD31 and TNFR2. (c) Transfection of HMEC-1, ciGEnC and HUVEC with non-silencing control siRNA does not knock-down expression of VE-cadherin, CD31, or TNFR2. (a–c) Cells were harvested at $t = 48$ h. Bars depict relative gene expression with expression levels of untransfected cells arbitrarily set at one. (a, b) Levels of basal gene expression relative to GAPDH in HMEC-1, ciGEnC, and HUVEC are shown in insets. (d) SAINT mediated uptake of [^{32}P] radiolabeled siRNA into HMEC-1 and HUVEC. Cells were transfected for 4 h. (a–d) Data are presented as mean values ± SD, $n = 3$. *, $p < 0.05$ compared to untransfected cells.

that on average 1.34 ± 0.70 SAINT-aminolinkers were attached to one Ab_{Esel} monoclonal antibody (Fig. 2b).

We analyzed the ability of Ab_{Esel}SAINT conjugate to recognize and bind to E-selectin expressed by activated HUVEC. Flow cytometric analysis demonstrated specific and concentration dependent binding of Ab_{Esel}SAINT conjugate to the cells (Fig. 3a). The binding of Ab_{Esel}SAINT conjugate to HUVEC was identical to that of the parental Ab_{Esel}, while no binding was observed to resting endothelial cells. During this study Ab_{Esel}SAINT conjugate was synthesized 4 times with reproducible protein yield and chemical characteristics. We tested at different time points the E-selectin recognition and the chemical stability of different Ab_{Esel}SAINT conjugate batches, and concluded that the conjugates were stable for at least 1.5 years after synthesis, when stored at 4 °C (Table 1).

3.3. Synthesis, characterization, and specific binding of SAINTarg to activated endothelial cells in human kidney slices

Just prior to transfection experiments SAINTarg was formulated by complexing Ab_{Esel}SAINT conjugate with SAINT and thereafter siRNA was added to the mixture, resulting in a complex as schematically shown in Fig. 4. In general the siRNA–SAINTarg complex contained Ab_{Esel}SAINT conjugate:SAINT:siRNA in a molar ratio of 7.5:15,000:60 (protein:lipid:nucleotide) with a size of approximately 250 nm and a positive charged zeta-potential (Table 2). Table 2 also shows the size and zeta-potential of SAINT lipoplexes, and other SAINTarg compositions used throughout our study. Addition of siRNA in the highest concentration used (60 pmol/ml) lowered the mean diameter of SAINT- and SAINTarg lipoplexes slightly (from 250 to 200 nm), possibly indicating a more tight and energy favorable complexation in the presence of the negatively charged siRNA. SAINT- and SAINTarg lipoplexes exposed a positive zeta-potential, which was lowered, but not reversed, in the presence of 60 pmol/ml siRNA (Table 2).

To analyze the binding ability of SAINTarg to activated endothelial cells in a tissue context, 250 μm thick precision-cut slices of human kidney biopsies were incubated with SAINTarg. Kidney slices were activated by TNFα-containing medium for 2 h before adding SAINTarg to the solution. Slices harvested at 24 h were immunohistochemically analyzed for E-selectin expression (Fig. 5a) and the localization of SAINTarg (Fig. 5b). E-selectin was not expressed in the kidney microvasculatures at the start of the incubation, yet became abundantly expressed by peritubular capillaries during stimulation with TNFα. SAINTarg specifically associated to activated endothelial cells, following exactly the expression pattern of E-selectin.

3.4. Targeted transfection of siRNA to activated HUVEC

To analyze if SAINTarg improved transfection efficiency to activated primary endothelial cells, HUVEC were activated with TNFα and transfected with fluorescently labeled siRNA using SAINT (standard transfection protocol) and SAINTarg (targeted transfection protocol). Transfection of Alexa-tagged siRNA was significantly increased by the targeted transfection, as demonstrated both by fluorescence microscopy (Fig. 6a) and flow cytometric analysis (Fig. 6b). SAINTarg mediated transfection of activated HUVEC, was completely and specifically blocked by co-incubation with excess Ab_{Esel}, while irrelevant antibody did not affect the binding (Fig. 6c).

Quantitation of delivery of siRNA was performed by formulating radioactively labeled ^{32}P-siRNA with SAINT and SAINTarg, respectively. To analyze the effect of the ratio Ab_{Esel} antibody:SAINT lipid in SAINTarg formulations on siRNA delivery capacity we prepared SAINTarg using different concentrations of Ab_{Esel}SAINT conjugate complexed with a fixed SAINT lipid concentration (Fig. 7a). Transfection of activated HUVEC with SAINTarg delivered significantly more siRNA into the cells compared to SAINT based transfection when the concentration of Ab_{Esel} antibody was 7.5 pmol/ml or lower. When increasing the molar ratio of Ab_{Esel} antibodies, transfection with SAINTarg became less effective, when looking at the absolute amount of siRNA delivered. Subsequent SAINTarg transfection experiments were performed with 7.5 pmol/ml Ab_{Esel} antibody and 15 nmol/ml lipid.

Binding and uptake studies with radioactively labeled ^{32}P-siRNA furthermore demonstrated that targeted transfection with SAINTarg

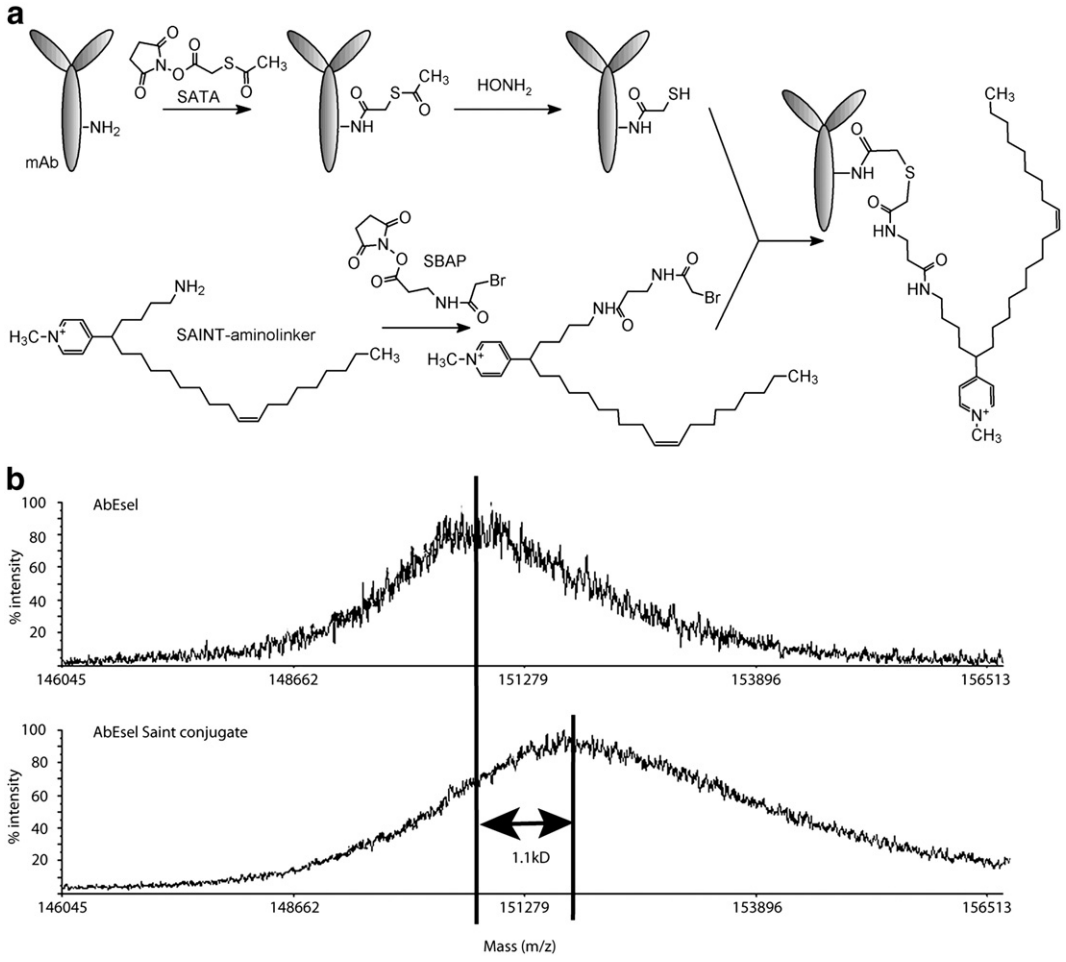


Fig. 2. Ab_{Esel}SAINT conjugate. (a) Schematic representation of the synthesis of the Ab_{Esel}SAINT conjugate. (b) A typical MALDI-TOF mass spectrometric analysis of Ab_{Esel}SAINT conjugate and parental Ab_{Esel} antibody demonstrating an increase in mass from 150,750 to 151,850 after conjugation with SAINT, corresponding to 2 SAINT molecules conjugated to one antibody molecule. On average four different batches of Ab_{Esel}SAINT conjugate resulted in conjugation of 1.34 ± 0.70 SAINT molecules per one antibody molecule.

was based on an active internalization into the endothelial cells, as more radioactivity associated with cells at 37 °C compared to 4 °C (Fig. 7b). This indicated that transfection with SAINTarg resulted not only in binding (measured at 4 °C) but also in uptake of siRNA into activated endothelial cells. Cellular uptake was further verified by confocal microscopic analysis of fluorescently labeled siRNA in living endothelial cells after SAINTarg mediated transfection. Images, taken at 1 μm depth intervals, showed that siRNA was present throughout the cytoplasm of the cell and was not restricted to the cell membrane (Fig. 7c).

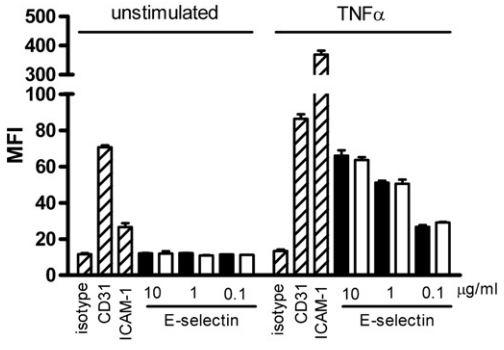


Fig. 3. Anti-E-selectin antibody preserves its antigen recognition after coupling to SAINT. Ab_{Esel}SAINT conjugate (filled bars) and parental anti-E-selectin antibody (open bars) bind specifically to TNFα-activated HUVEC. In resting cells MFI (mean fluorescent intensity) is identical to an isotype control. Control experiments are shown by hatched bars and include incubation with an isotype matched non-specific antibody, and detection of CD31 and ICAM-1. The endothelial cell marker CD31 is constitutively expressed in HUVEC, while ICAM-1 expression is induced by TNFα. Data are presented as mean values ± SD, $n = 3$.

3.5. Improved knock-down of VE-cadherin expression by targeted transfection of activated endothelial cells with VE-cadherin specific siRNA

HUVEC were transfected with siRNA specific for VE-cadherin using either SAINT or SAINTarg. Transfections were carried out with SAINTarg formulations of 7.5, 3.75, 1.88, and 0.94 pmol $Ab_{[Esel]}$ per 15 nmol SAINT. All SAINTarg formulations down regulated VE-cadherin to a similar extent as transfection with SAINT alone in resting HUVEC (Fig. 8a). However, when HUVEC were activated with TNFα, transfections with SAINTarg were more effective than with SAINT to reduce VE-cadherin gene expression. As expected, all SAINTarg formulations were more effective in activated HUVEC than in rested HUVEC. A control transfection experiment with either SAINT or SAINTarg, and a non-silencing siRNA did not alter VE-cadherin expression (Fig. 8a). Similarly, expression of an unrelated gene, Tie-2, was not modified by siVE-cadherin transfected by SAINT or SAINTarg (Fig. 8b), indicating the specificity of the method.

We next analyzed silencing of VE-cadherin on a protein level. Western blot analysis demonstrated that delivery of siRNA by SAINTarg resulted in a stronger down regulation of VE-cadherin in

Table 1
Stability of Ab_{Esel}SAINT conjugates.

Storage time	Antigen recognition			MALDI-TOF mass spectrometry			
	0 year	>1 year	>1.5 year	0 year	>0.5 year	>1 year	>1.5 year
Ab_{Esel}SAINT conjugate 1	+	+	+	+	n.d.	n.d.	+
Ab_{Esel}SAINT conjugate 2	+	+	n.d.	+	n.d.	+	n.d.
Ab_{Esel}SAINT conjugate 3	+	n.d.	n.d.	+	+	n.d.	n.d.
Ab_{Esel}SAINT conjugate 4	+	n.d.	n.d.	+	n.d.	n.d.	n.d.

+ Antigenic recognition = binding of conjugate to activated HUVEC identical to parental antibody.
+ MALDI-TOF mass spectrometry = increased mass of conjugate compared to parental antibody.
n.d. = not determined.

activated HUVEC than in resting cells, while silencing effects of VE-cadherin protein expression after transfection with SAINT was similar in resting and activated HUVEC (Fig. 8c).

The presented data suggest that SAINTarg formulation of siRNA lead to improved delivery of functional active siRNA into difficult-to-transfect primary endothelial cells. In general endothelial cell lines are more easily transfected than primary cells, as *e.g.* was observed with ciGEnC. Standard transfection protocol with SAINT and 60 pmol/ml VE-cadherin specific siRNA blocked VE-cadherin gene expression in TNFα-activated ciGEnC by more than 90%. This silencing effect was siRNA-concentration dependent. Lowering the VE-cadherin siRNA concentration, but keeping SAINT concentration the same, diminished the knock-down effect in a concentration dependent manner (Fig. 9).

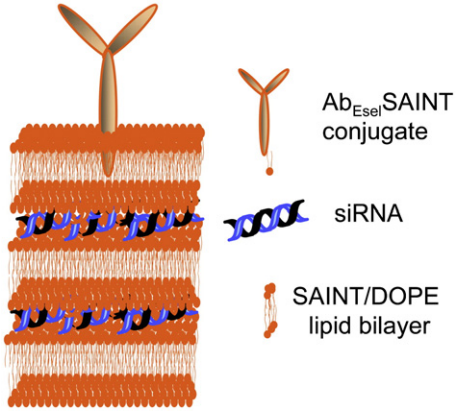


Fig. 4. SAINTarg. Schematic representation of SAINTarg complexed with siRNA, based on multilamellar structure of nucleotide–cationic lipid complexes as described by Rädler et al., [40].

Table 2
Particle size and zeta-potential.

Sample	[SAINT] (nmol/ml)	[siRNA] (pmol/ml)	[Ab_{Esel}] (pmol/ml)	Mean diameter (nm)
SAINT	15	–	–	252
SAINT + siRNA	15	0.6	–	259
	15	60	–	200
SAINTarg	15	–	30	223
	15	–	7.5	249
	15	–	1.88	251
SAINTarg + siRNA	15	0.6	7.5	232
	15	60	7.5	200
				Zeta-potential (mV)[a]
SAINT	15	–	–	46
SAINT + siRNA	15	60	–	8
SAINTarg	15	–	7.5	31
SAINTarg + siRNA	15	60	7.5	25

[a] Just prior to zeta-potential measurements, solutions were diluted 12 times with distilled water.

When formulating siRNA with SAINTarg, VE-cadherin silencing was also concentration dependent, yet the effect was not as rapidly lost by lower siRNA concentration. Transfection with SAINTarg and 12 pmol/ml siRNA was as efficient as transfection with SAINT and 60 pmol/ml siRNA. Lowering siRNA concentration even to 3 pmol/ml siRNA, SAINTarg mediated transfection was as efficient in terms of VE-cadherin silencing, as SAINT mediated transfection with 12 pmol/ml siRNA (Fig. 9). This indicated a general advantage of the targeted

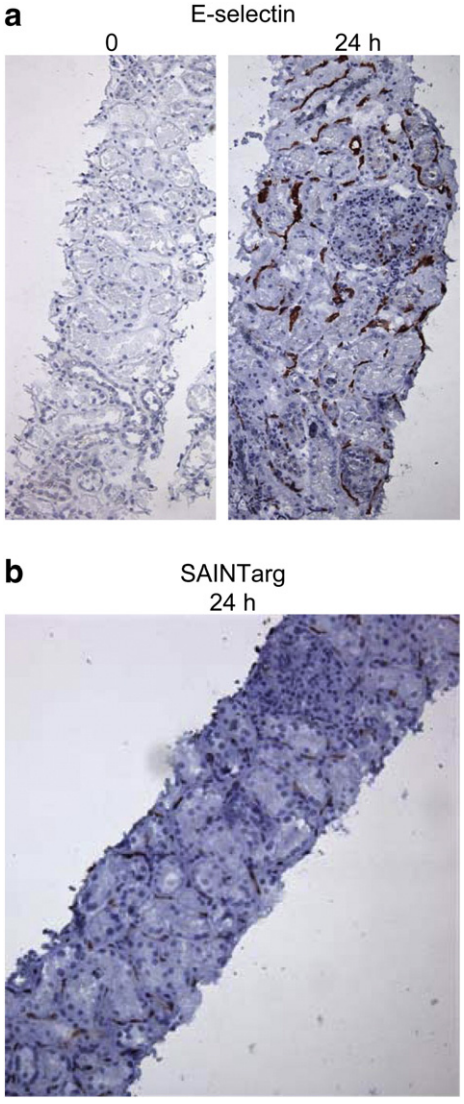
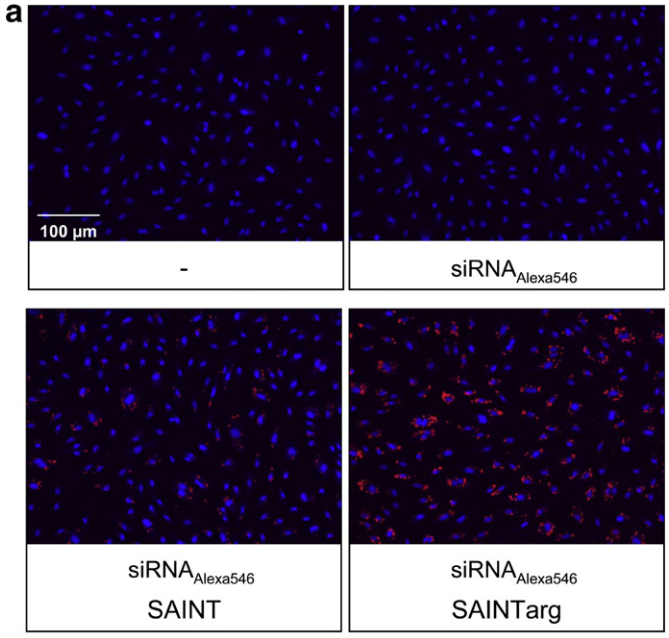


Fig. 5. SAINTarg binds specifically to activated endothelial cells in whole tissue environment. Immunohistochemical staining of (a) E-selectin and (b) SAINTarg. Precision-cut tissue slices of human kidney biopsies were incubated in TNFα-containing medium. The figures depict a typical result of 3 experiments with slices from independent donor kidney biopsies.



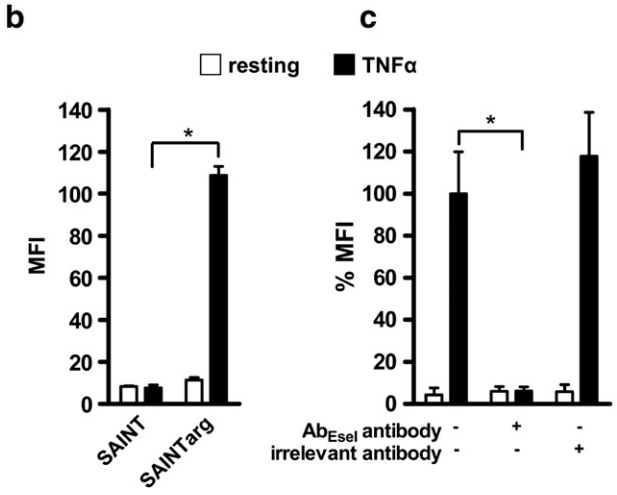


Fig. 6. SAINTarg increases transfection efficiency in activated primary endothelial cells. Transfection of activated endothelial cells with fluorescently labeled siRNA was enhanced with formulation of SAINTarg as demonstrated by (a) fluorescence microscopy, and (b, c) flow cytometric analysis of HUVEC. Nuclei are stained blue by DAPI. (a) HUVEC was activated with TNFα and either not transfected, or transfected for 4 h with naked Alexa$_{546}$–siRNA, SAINT + Alexa$_{546}$–siRNA, and SAINTarg + Alexa$_{546}$–siRNA, respectively. (b) Resting and TNFα-activated HUVEC were transfected for 4 h with SAINT + Alexa$_{488}$–siRNA, and SAINTarg + Alexa$_{488}$–siRNA, harvested and analyzed by flow cytometry. (c) Competition of transfection with free Ab$_{Esel}$. Transfection was performed as in (b), except for including co-incubation of 100 times excess amount of free Ab$_{Esel}$ or irrelevant antibody. SAINTarg mediated transfection in TNFα-activated HUVEC was completely blocked by Ab$_{Esel}$, but not by irrelevant antibodies. (b, c) MFI = mean fluorescent intensity. Data are presented as mean values ± SD, $n = 3$. *, $p < 0.05$.

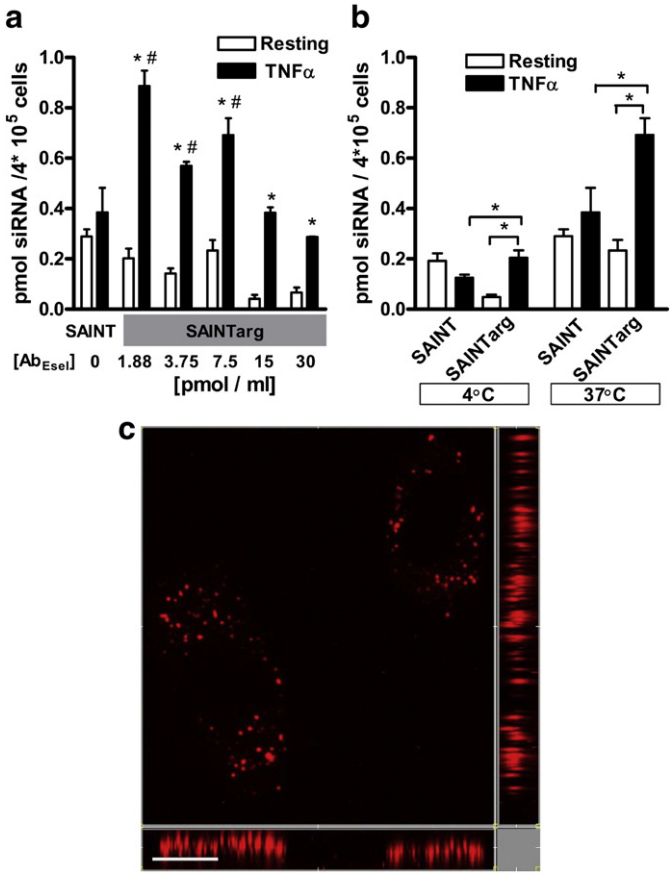


Fig. 7. Binding and uptake of siRNA in HUVEC. (a, b) Quantification of radioactive labeled siRNA associated to endothelial cells. (a) Transfection with different formulations of SAINTarg, consisting of fixed amounts of SAINT lipid (15 nmol/ml) and different amounts of Ab$_{Esel}$ antibodies. Data are presented as mean values ± SD, $n = 3$. *, $p < 0.05$ compared to transfection of resting HUVEC; #, $p < 0.05$ compared to transfection with SAINT. (b) Transfection with SAINTarg containing 15 nmol/ml lipid, 7.5 pmol/ml Ab$_{Esel}$ antibodies at 4 °C allowed binding but no uptake into the cells, while at 37 °C both binding and uptake found place. Data are presented as mean values ± SD, $n = 3$. *, $p < 0.05$. (c) Transfection with SAINTarg delivers siRNA into the cytoplasm of endothelial cells. Confocal image of activated HUVEC transfected with SAINTarg containing 15 nmol/ml lipid, 7.5 pmol/ml Ab$_{Esel}$ antibodies and Alexa$_{546}$-tagged siRNA showing a single *z*-axis plane. Insets below and to the right represent the confocal *z*-axis multiplane stack through the cells, demonstrating the presence of siRNA throughout the cytoplasm of the cells. Bar represents 15 µm in the *x*-axis. (a–c) Cells were transfected for 4 h.

transfection procedure, being that transfection of activated endothelial cells with SAINTarg encompasses a more efficient siRNA delivery process and less siRNA required for gene silencing than when using regular transfection technology.

4. Discussion

The current study describes a successful design of a cell selective targeted transfection vehicle for the delivery of siRNA into E-selectin expressing endothelial cells. Targeted transfection increased the uptake of functional active siRNA in both difficult-to-transfect primary endothelial cells and in endothelial cell lines. It was more effective in functional siRNA delivery than regular transfection technology as shown by significantly stronger silencing of a target gene, *i.e.*, VE-cadherin.

The cationic amphiphilic lipid SAINT is a highly effective transfection agent. In our laboratory transfection of primary endothelial cells with SAINT was similar, and in the presence of serum, superior to other commercially available non-viral cationic lipoplexes (data not shown), but transfection capability remained low compared to transfection of endothelial derived cell lines, which has also been reported previously [15]. To date the mechanism of action of lipoplex-mediated transfection is not fully understood. A correlation between clathrin-mediated endocytosis and lipoplex-mediated transfection has been established by various experimental evidence, although involvement of different pathways, such as macropinocytosis cannot be excluded [13,33]. Release of nucleotides to the cytosol requires endosomal escape, a process that is enhanced with the pH dependent fusiogenic helper-lipid DOPE [34].

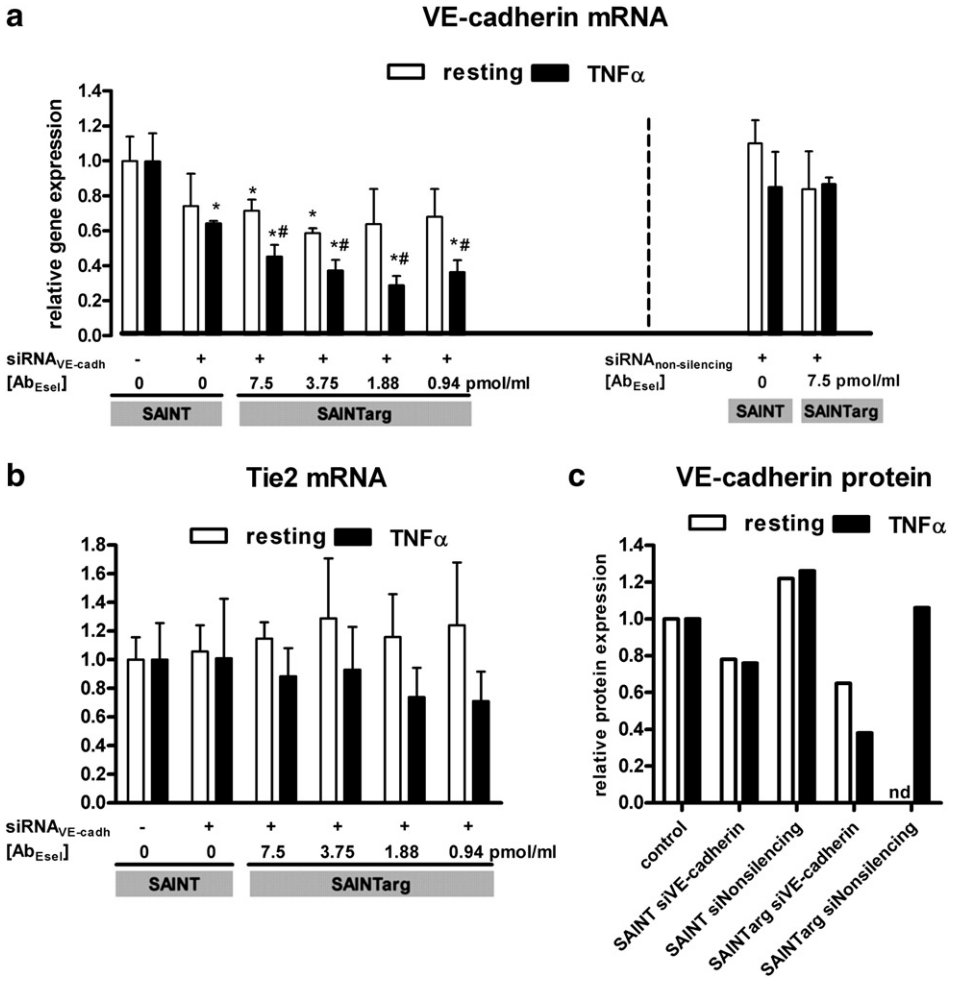


Fig. 8. SAINTarg mediated transfection increases silencing of VE-cadherin in activated primary endothelial cells. siRNA mediated silencing of VE-cadherin in resting and activated HUVEC. (a) Knock-down of VE-cadherin gene expression in activated HUVEC is enhanced by transfection with SAINTarg, while knock-down in resting HUVEC after transfection with SAINT or SAINTarg was identical. Control transfection experiments with SAINT and SAINTarg and non-silencing siRNA did not modify VE-cadherin expression. (b) Transfection of HUVEC with siRNA specific for VE-cadherin does not alter expression of Tie2. (c) Knock-down of VE-cadherin on protein level after transfection with SAINT or SAINTarg (7.5 pmol Ab_{Esel} per 15 nmol SAINT). Data in (a and b) are presented as mean values ± SD, $n = 3$. *, $p < 0.05$ compared to non-transfected control cells, arbitrarily set at one, #, $p < 0.05$ compared to transfection with SAINT. Data in (c) depict a representative result of 2 independent experiments, bars represent densitometric analysis of Western blots. Expression in non-transfected control cells is arbitrarily set at one; nd = not determined.

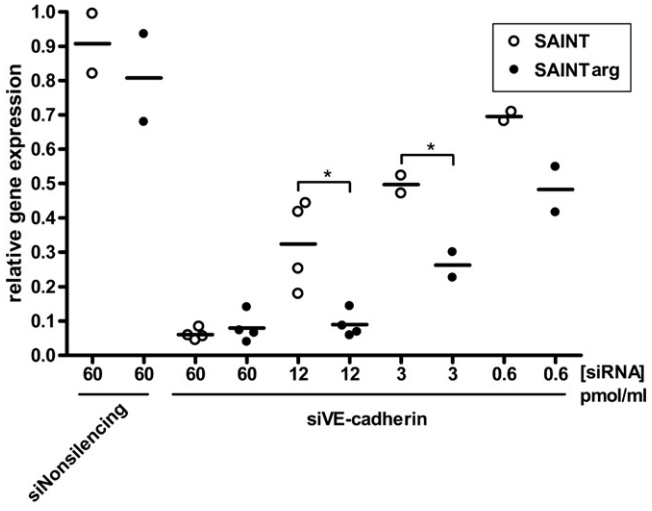


Fig. 9. SAINTarg mediated transfection increases silencing of VE-cadherin in glomerular endothelial cells ciGEnC. Increased knock-down of VE-cadherin by SAINTarg mediated transfection. Activated ciGEnC were transfected with SAINT (15 nmol/ml) and SAINTarg (7.5 pmol Ab_{Esel} per 15 nmol SAINT), respectively, and different concentrations of siRNA specific for VE-cadherin. *, $p < 0.05$.

In endothelial cells no data is available regarding the relationship between cellular uptake of siRNA and the efficiency in down regulation of gene expression. Our quantitative studies on the uptake of radioactively labeled siRNA in HUVEC and HMEC-1 cells showed that highly efficient gene silencing in HMEC-1 and lack thereof in HUVEC related to only a 4 times higher siRNA availability in HMEC-1, compared to HUVEC. Our results also indicated that mRNA levels do not determine the level of knock-down by siRNA, as in HUVEC mRNA levels of the genes analyzed were similar to those in HMEC-1 and ciGEnC. Instead, we conclude that differences in the capability of cellular uptake and/or intracellular trafficking of SAINT siRNA complexes are responsible for the observed differences in transfection efficiency.

The current study addressed the issue of specific delivery of siRNA into a target cell, which is important for therapeutic applicability. Activated endothelial cells play an important role in inflammatory processes and are therefore a relevant target for therapeutic interference. Successful knock-down of endothelial genes has been demonstrated by cationic lipid-mediated transfection *in vivo* [22]. One can envision that specific delivery of siRNA to endothelial cells at the place of inflammation can enhance therapeutic efficacy and limit potential side-effects of the treatment. To increase the specificity of transfection we have chemically conjugated anti-E-selectin antibodies to SAINT.

The coupling procedure described here consisted of a covalent conjugation of a monoclonal antibody to a SAINT-aminolinker molecule, and diverges from the protocol described by van Zanten et al., who coupled an antibody to the helper-lipid DOPE [35]. Our protocol ensured that the antibody was covalently bound to a cationic lipid entity. The antibody–lipid conjugate was characterized by MALDI-TOF mass spectroscopy to establish the average number of lipid molecules covalently bound to the antibody. The cationic lipid part of the conjugate enabled the conjugate to be inserted into SAINT lipoplex to obtain targeted cationic delivery complex (SAINTarg), in such a way that the amount of antibody–lipid conjugate in the lipoplex could be tightly controlled and optimized for most favorable delivery.

Evidently, coupling specific antibodies toward cell surface receptors to lipoplexes does not *per se* increase transfection efficacy. After binding, subsequent internalization is required and, as described above, the nature of the internalization pathway may control transfection efficacy [14]. The choice for E-selectin as a target molecule on the surface of activated endothelial cells was based on our previous observations that anti-E-selectin targeting constructs are rapidly internalized via receptor mediated endocytosis [36,37]. Targeting SAINT lipoplexes with anti-E-selectin antibodies therefore provided us with a tool not only to deliver siRNA to the surface of activated endothelial cells, but also to deliver the siRNA into these cells. Internalization of siRNA was substantiated by confocal microscopy, clearly visualizing a cytosolic localization.

SAINTarg synthesis was highly reproducible and transfection with different batches of SAINTarg resulted repeatedly in increased delivery of siRNA into activated HUVEC compared to transfection with SAINT. Moreover, knock-down of the expression of the endothelial gene VE-cadherin was significantly improved compared to transfection with SAINT, which indicates that the internalization processing of SAINTarg–siRNA complexes results in cytosolic delivery of functional active siRNA. That targeting to E-selectin delivers functional active siRNA into the cytosol is in agreement with our previous reports on successful intracellular delivery of pharmacologically active dexamethasone by E-selectin targeting both *in vitro* and *in vivo* [8,10].

Uptake of siRNA by SAINTarg in activated HUVEC was dependent on the antibody concentration. When the concentration of the antibody was increased above the critical value of 7.5 pmol per 15 nmol lipid the transfection efficiency by SAINTarg decreased dramatically (Fig. 7a). We hypothesize that steric hindrances of the antibody can prevent stable lipoplexes to be formed. Optimization of antibody:lipid ratio was previously studied for tumor cell specific antibodies coupled to SAINT [35]. Although no quantitative data on cellular uptake were reported, the authors demonstrated differences in selective binding of SAINT lipoplexes to target cells depending on antibody:lipid ratio. Interestingly, in our study the preferential delivery of siRNA into activated HUVEC above resting HUVEC remained, even when using relatively high Ab_{Esel} concentration, although the total uptake of siRNA was lower.

The advantage of targeted transfection of primary endothelial cells with SAINTarg to increase both specificity and efficacy of transfection was apparent, but not limited to primary endothelial cells. Although endothelial cell lines are generally easily transfected, our data showed that transfection with SAINTarg can significantly reduce the amount of siRNA necessary for the experiments.

A major pharmaceutical challenge in successful gene silencing *in vivo* concerns the development of efficient intracellular siRNA delivery strategies into the appropriate cell type in the body following systemic administration [38,39]. The results presented in the current study demonstrate a successful and specific siRNA delivery into primary endothelial cells *in vitro*, and justify further investigation into the *in vivo* behavior of SAINTarg mediated delivery of siRNA into activated endothelial cells. The *in vivo* analysis will consider both pharmacokinetics and therapeutic consequences of cell selective delivery of siRNA.

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