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Research paper

Repeated siRNA application is a precondition for successful mRNA γENaC knockdown in the murine airways

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ABSTRACT

The volume of the airway surface liquid is regulated by Na^+ absorption and Cl^- secretion by the respiratory epithelium. In cystic fibrosis, Na^+ hyperabsorption caused by the absence of functional CFTR protein leads to an altered airway surface liquid composition and finally to a deteriorated mucociliary clearance. It has been suggested that down regulation or inhibition of the amiloride-sensitive epithelial Na^+ channel (ENaC) could restore the disrupted airway hydration. Therefore, targeting ENaC by RNA interference could be of therapeutic relevance. In this context, we investigated whether RNAi could lead to a reduction in γENaC expression in epithelia *in vitro* and *in vivo* in mice. Transfection of cells with specific siRNA sequences for γENaC subunit reduced expression to ~10% relative to control. For *in vivo* experiments, siRNA sequences specific for the γENaC subunit were administered to the murine nasal cavity and, 72 h later the animals were killed. In the first approach, only a single application of naked siRNA was given. In the second approach, repeated siRNA applications were performed. The single application of siRNA sequences had no effect on mRNA content of the targeted γENaC subunit, whereas repeated siRNA application resulted in a significant reduction in γENaC mRNA in the respiratory tissue. We conclude that repeated siRNA application is necessary for γENaC knockdown in the murine airways.

1. Introduction

CF is an autosomal recessive inherited disease caused by mutations in the CFTR gene. This leads to a lack of functional or to a dysfunctional CFTR protein on the apical membrane of ciliated epithelial cells and serous cells of the submucosal glands in the airways. CFTR is a chloride channel exchanging chloride ions between the cytoplasm and the airway surface liquid (ASL). In CF, it has been hypothesized that the missing function of CFTR causes an increased ENaC-mediated sodium uptake from the luminal secretions of the airways, depleting the airway surface liquid and leading to a defective mucociliary clearance [1–3]. This promotes bacterial infections, resulting in a slow progressive destruction of the airway tissue. Due to the central role of ENaC-mediated sodium uptake by the respiratory epithelium for the pathogenesis of CF lung disease, it has been postulated that a reduction in ENaC activity could attenuate CF lung disease. Pharmacological approaches to inhibit the function of ENaC have been studied. Most studies used aerosolized amiloride which is an orally active potassium-sparing diuretic. However, the low potency and rapid absorption of aerosolized amiloride by the respiratory epithelia translated into only short duration of efficacy in CF patients [4–7]. Long-acting ENaC blockers are problematic due to potential renal side effects.

Downregulation of ENaC expression by RNAi could be an alternative therapeutic approach to improve mucociliary clearance in CF. With regards to this strategy, specific siRNA sequences for the mRNA ENaC subunits have to be developed. ENaC is a multimeric complex composed of three subunits, α-, β- and γENaC [8,9]. Each ENaC subunit consists of two transmembrane segments, a large extracellular domain and cytoplasmic N and C termini [10–12]. ENaC is expressed in ciliated airway epithelia cells of small and large airways, as well as in nasal mucosa. It is strongly suggested that ENaC in ciliated airway epithelia is controlled by local rather than by systemic signaling [13–15]. It has been demonstrated that the extent of epithelial Na^+ transport through ENaC channels is regulated through trafficking mechanisms that control ENaC expression at the cell surface. All three ENaC subunits have been identified in airway epithelial cells [8,9,16,17]. Cell surface delivery of ENaC out of the endoplasmic reticulum (ER) requires assembly of the α-, β- and γ-subunits into a multiunit complex. Thus, ENaC trafficking out of the ER is limited by the number of available individual subunits. Therefore, transcriptional regulation of a single ENaC subunit could influence the number of ENaC

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channels at the cell surface. Recent work using amongst others a renal collecting duct cell line (M1) suggested that expression of γENaC might limit formation of functional channels [16,19]. To address this hypothesis and take advantage of the suggested local regulation of ENaC, we investigated the impact of RNAi *in vitro* and *in vivo*. ENaC-specific small interfering (si)RNAs were designed, synthesized, and first used in cell culture and in a second approach tested in a mouse model. We decided to target the γ-subunit as it evidently has a strong contribution to the ENaC function [19–22].

Our results showed that *in vitro* a single application was sufficient to decrease ENaC mRNA levels, whereas *in vivo* repeated administrations were necessary to obtain a significant reduction for γENaC mRNA.

2. Experimental procedures

2.1. Tissue culture and transfection of cells

M-1 is a murine epithelial cell line derived from the cortical collecting duct. Cells were purchased from American Type Culture Collection (CRL-2038) and routinely cultivated under standard conditions (37 °C, 5% CO_2) in PC-1™ serum-free complete medium with supplementary growth factors provided by Lonza Walkersville, Inc. (Wuppertal, Germany). The medium was also supplemented with 5 mM GlutaMAX™ I (Invitrogen, Karlsruhe, Germany). Cells were split when reached 85% of confluency. The experiments were performed until passage 16 maximally.

To determine the effect of siRNAs on γENaC subunit, several non-commercial algorithms were used to design siRNAs oligonucleotides (provided by Dharmacon si*DESIGN*® Center, Invitrogen BLOCK-iT™ RNAi Designer and GenScript GenScript's design algorithm). The theoretical best three siRNA sequences were ordered and synthesized by Qiagen (Hilden, Germany). Negative control siRNAs: AllStars Neg. Control siRNA and Negative Control siRNA Alexa Fluor 488 (Qiagen, Hilden Germany).

For cell culture-based screening of siRNAs approximately $3–5 \times 10^5$ cells/well in 6-well plates were transfected with 50 nM of siRNA complexed with HiPerFect Transfection Reagent (Qiagen, Hilden, Germany) 24 h after seeding. SiRNAs were prepared according to the manufacturer's protocol. qRT-PCR and Western blot analysis were performed after 72 h, unless indicated otherwise. SiRNA sequences for murine γENaC (Accession number: NM_011326): γENaC_I: GGATTTCAGTTGTGCTCAA; γENaC_II: GCTCTTGATATTCT ACAAA; γENaC_III: AAATCATCGAAGTCTTCTTCA.

2.2. RNA isolation from cells and from tissue

Total RNA was isolated from cells using RNeasy® Mini Kit (Qiagen, Hilden/Germany) according to the manufacturer's instructions. The RNA concentration was determined photometrically (Nanodrop ND-1000, Peqlab, Erlangen/Germany), and the RNA was stored at −80 °C until qRT-PCR experiments were performed.

For RNA extraction, tissue samples (lungs and nasal epithelia) were immediately submerged in RNAlater RNA Stabilization Reagent (Qiagen, Hilden/Germany) after harvesting. RNA was extracted using the RNeasy® Micro Kit (Qiagen, Hilden/Germany) for nasal epithelia and RNeasy® Protect Mini Kit (Qiagen, Hilden/Germany) for lung in accordance with the manufacturer's instructions.

2.3. Quantitative real-time PCR

For quantification, isolated RNA was reverse transcribed into cDNA using the iScript select kit (Bio-Rad, Hercules, CA, USA). The cDNA was amplified by iCycler using iQ™ SYBR Green SuperMix (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. Reactions were carried out in duplicates or triplicates in a final volume of 25 μl. The real-time PCRs for the different conditions (negative, non-specific, target) and the actin controls were done on the same 96-well microplate to compare the results. The sequences of the gene-specific primers (Metabion, Martinsried/Gemany) were as follows: *γENaC*: 5′-aaacatctac aatgctgcctattcc-3′ (forward) and 5′-cagccacatttttccaccatct-3′ (reverse); *β-actin*: 5′-GCTCTGGCTCCTAGCACCAT-3′ (forward) and 5′-CACCGATCCACACAGAGTACTTG-3′ (reverse); *mGAPDH*: 5′-ggcctacatggcctccaa-3′ (forward) and 5′-ggg atagggcctctcttgct-3′ (reverse); *mCFTR*: 5′-tcgtgatcacatcagaaattattg ataat-3′ (forward) and 5′-ccacctctctcaagttttcaatcat-3′ (reverse) and *TNF-α*: ATCAGTTCTATGGCCCAGACCCT (forward) and CTACTTTGGAGTCATTGCTCTGTG (reverse). Reactions were incubated at 95 °C for 3 min followed by 40 cycles of 95 °C for 20 s, 62 °C for 20 s and 72 °C for 20 s (5-min final step) in skirted 96-well microplates sealed with StarSeal™ advanced polyolefin film (Starlab, Ahrensburg/Germany). Ct values were obtained using iCycler iQ software 3.1 (Bio-Rad, Hercules, CA, USA) that automatically calculated baseline cycles and threshold positions. The assays were normalized to β-actin RNA levels or mouse GAPDH or mouse CFTR.

2.3.1. Western blotting

SiRNA-transfected cells were washed in 1xPBS, harvested and lysed in lysis buffer: 50 mM Tris/HCl, pH 7.5/150 mM NaCl/5 mM EDTA/1% Triton X-100/1 mM DTT and protease inhibitor cocktail (Roche Applied Science, Mannheim/Germany) on ice. Protein concentrations were determined using Bio-Rad Protein Assay (Bio-Rad, Munich/Germany). Equal amounts of total protein were diluted in SDS sample-loading buffer (62.5 mM Tris/HCl pH 6.8/2% SDS/10% glycerol/2% DTT/0.001% bromophenol blue), boiled for 5 min, separated on a 7.5% Tris/HCl gel (Bio-Rad, Munich/Germany) and transferred to PVDF membrane (Millipore, Schwalbach/Germany). Membranes were blocked with 1 × TBS-T (20 mM Tris/HCl, pH 7.6/137 mM NaCl/0.1%, Tween-20) containing 5% skim milk powder (Sigma Aldrich, Deisenhofen/Germany) for 1 h at RT. The following primary antibodies were used as indicated: γENaC (polyclonal, 1:2000, ABR Hamburg/Germany) and mouse anti-actin (monoclonal, 1:100,000 Millipore, Schwalbach/Germany). Membranes were washed with 1 × TBS-T and incubated with HRP-conjugated secondary antibodies for 1.5 h at RT. Proteins were visualized with enhanced chemiluminescence reagents (ECL, Amersham Biosciences-GE, Freiburg/Germany) following the manufacturer's instructions. The exposed films were scanned and analyzed with ImageJ 1.37v software (Wayne Resband, NIH, USA).

2.3.2. Densitometric analysis of Western blot bands

Gel images were analyzed with ImageJ 1.37v software (Wayne Resband, NIH, USA). A plot profile was obtained for each band. The area under the function curve was taken as the band density. The density for each γENaC band was normalized by the density of the corresponding actin band.

2.4. Intranasal administration of synthetic siRNA in mice

All animal procedures were approved and controlled by the local ethics committee and carried out according to the guidelines of the German law of protection of animal life. Mice were maintained under specific pathogen-free conditions and were acclimatized for at least 7 days before performing the intranasal administration.

White female BALB/c mice (Elevage Janvier, Le Genest-Saint-Isle/France) were 8 weeks old (n = 5/group; n = 4/negative group), weighing 18–21 g were anesthetized intraperitoneally with midazolam (5 mg/kg Pharmaplus GmbH, Hameln/Germany), fentanyl

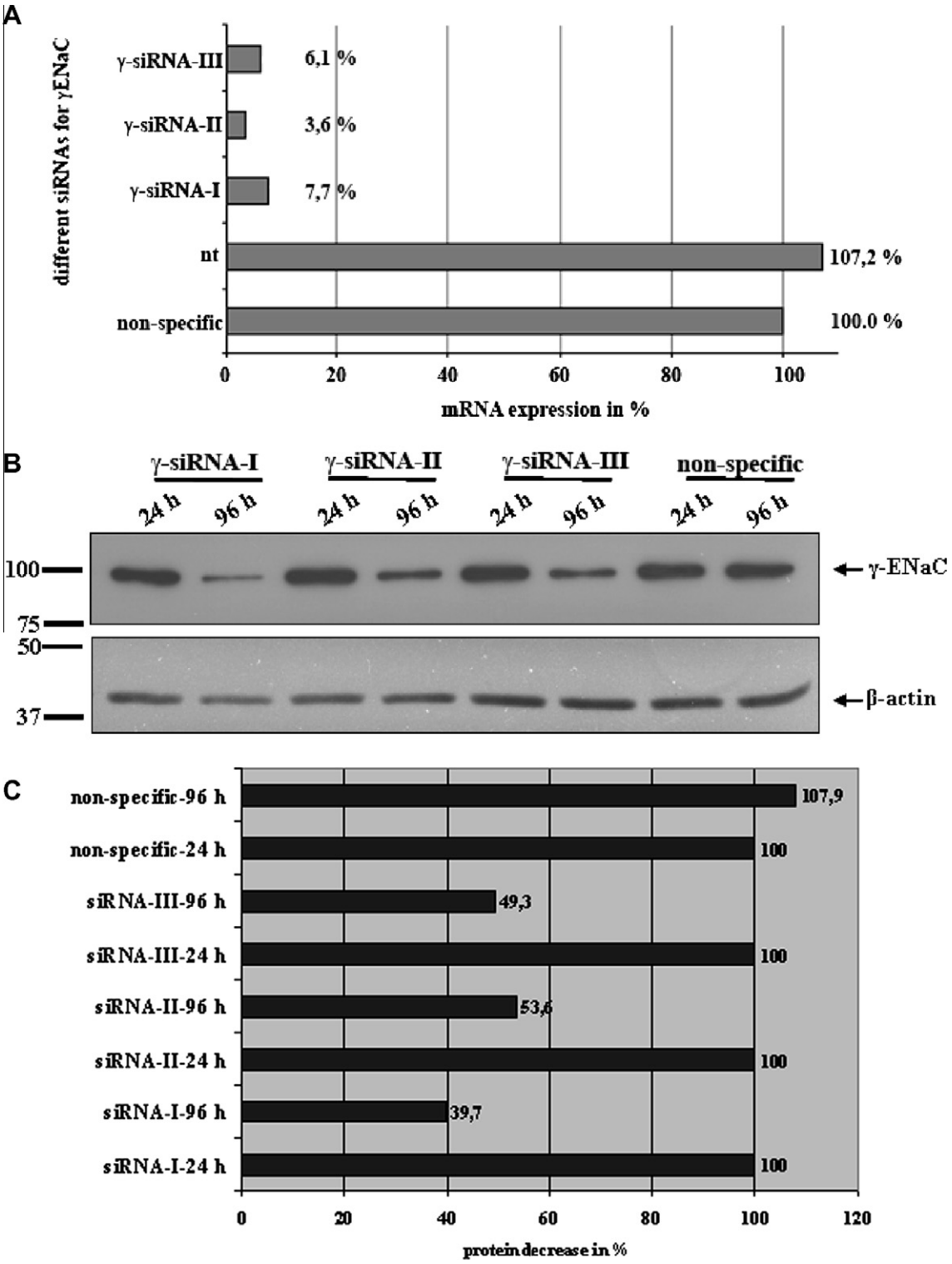

Fig. 1. Inhibition of murine γENaC subunit *in vitro* with different siRNAs. (A) M1 cells were transfected with three different siRNA sequences for γ-subunit and total RNA isolation was done after 72 h. The mRNA levels for the three different siRNAs were determined by qRT-PCR showing efficient knockdown of more than 90% for the γ-subunit. Data are shown in relation to the non-specific control, which is assumed to have an expression value of "100%". The results were confirmed in at least three independent experiments. (B) γENaC protein levels were monitored at 24 h and 96 h post transfection by Western blot analysis. The amount of β-actin was used as a loading control. The results show reduction in γENaC protein after 96 h. As control the cells were transfected with non-specific siRNA which was labeled with Alexa (non-specific). Numbers to the left of the gel represent the mobility of Bio-Rad Precision Plus Protein Dual Color standards in kDa. The results were confirmed in at least two independent experiments. (C) The result in 1B was quantified (ImageJ 1.37v), and the inhibition of protein expression is given in %.

(0.05 mg/kg, Janssen-Cilag GmbH, Neuss/Germany) and medetomidin (0.5 mg/kg, Pfizer Pharma GmbH, Karlsruhe/Germany). Once anaesthetized, the mice were placed onto heated boards in the supine position. A fine-tip catheter (VWR, Darmstadt/Germany) was placed 2.5 mm within the right nostril [23], and 80 μg siRNA [24] per mouse in 80 μl RNAse-free water (Qiagen, Hilden/Germany) were perfused at a rate of 6.7 μl/min using a peristaltic pump (Aladin-1000, WPI, Sarasota/USA). During the perfusion,

the mice were kept in an inverted position. Oxygen was substituted during the whole procedure. After the nasal perfusion, mice were allowed to wake up with a cocktail mix of flumazenil (0.4 mg/kg, Hikma-Pharma GmbH, Nieder-Olm/Germany), naloxone (0.5 mg/kg, Pharmaplus GmbH, Hameln/Germany) and atipamezole (0.375 mg/kg, Pfizer Pharma GmbH, Karlsruhe/Germany) injected subcutaneously. The mice were perfused either once or thrice (every second day), and 72 h after the last administration of siRNA (γ-ENaC_I: 5′-GGATTTCAGTTGTGCTCAA-3′; non-specific control siRNA VIII: 5′-ACUCUAUCUGCACGCUGAC-3′; Dharmacon, Bonn/Germany), the animals were culled by cervical dislocation and nasal epithelia and lungs were harvested. Untreated animals were not perfused at all.

2.5. Statistical analysis

Differences in mRNA expression between groups were analyzed by pair wise fixed reallocation randomisation tests with REST 2005 software [25]. Data are presented as the normalized mean ± SEM. (standard error of mean).

3. Results

3.1. Efficient knockdown of ENaC mRNA and protein after siRNA application in M1 cells

To determine the effect of siRNA application on ENaC subunit mRNA and protein content, several non-commercial algorithms were used to design specific siRNA sequences for γENaC subunit. The theoretical best three siRNA sequences were tested. After performing transfection experiments, cells were harvested after 72 h and total RNA was isolated as described in experimental procedures. For γENaC (Fig. 1A), all three tested siRNAs resulted in approximately 90% of mRNA knockdown. In a second set of transfection, the cells were harvested after 24 h and after 96 h. After cell lysis, total proteins were subjected to Western blot analysis. We could also show a significant reduction of γENaC on the protein level in the Western blot analysis (Fig. 1B). After quantifying those Western blots with ImageJ, we found a reduction in the protein content to ∼40% of controls 96 h after transfection with siRNA_I (Fig. 1C).

3.2. No γENaC reduction in murine nasal epithelia and in murine lung tissue after single application of γENaC-siRNA_I

For *in vivo* application, we decided to use the most efficient siRNA sequence of our *in vitro* tested sequences against γENaC, which was γENaC-siRNA_I. The Dharmacon synthesized siRNAs were resuspended in RNase-free water. Female Balb/c mice were administrated once with 80 μg of "naked" γENaC-siRNA_I or with non-specific control siRNA (Dharmacon). The animals were examined every day for any external signs of side effects like weight loss, piloerection, lethargy or any respiratory problems. There were no signs of behavioral alteration. After 72 h, the nasal epithelia and the lungs were harvested and qRT-PCR was performed. The qRT-PCR for γENaC mRNA did not show any significant difference between untreated (neg.) control (non-specific) and γENaC-siRNA_I (target)-treated animals (Fig. 2) in both nasal and lung tissue.

3.3. γENaC reduction in murine nasal epithelia and in murine lung tissue after triple siRNA application

In a second set of experiments, mice were treated under the same conditions as previously, but the siRNA against γENaC was administered repeatedly three times (every second day). After the daily examination of the treated animals, we could observe a slight weakness due to anesthesia, but no other behavioral changes were noted. Seventy-two hours after the last administration, the organs were harvested.

In contrast to the single application, the triple application of γENaC-siRNA led to a significant reduction of γENaC mRNA in the nasal epithelia (relative expression 0.660 [95% CI: 0.590–0.779], $P = 0.004$) and in the lungs (relative expression 0.384 [95% CI: 0.363–0.403], $P < 0.001$) (Fig. 3). The following Western blot

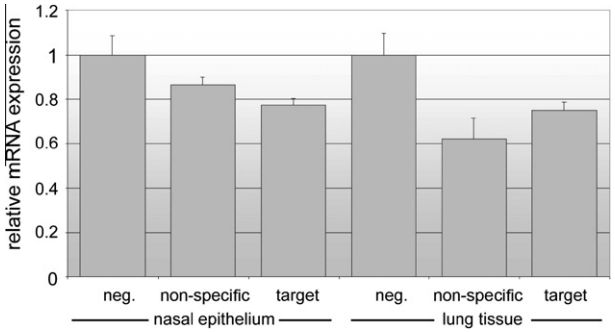

Fig. 2. Intranasal single application. Female Balb/c mice were treated (intranasal administration) once with 80 μg γENaC-siRNA (target) or non-specific siRNA (non-specific) diluted in distilled water. After 72 h, the nasal epithelia and the lungs were harvested and qRT-PCR was performed in duplicates. Mean normalized expression values ± SEM are shown for each mouse. Neg.: mice were not treated with siRNA.

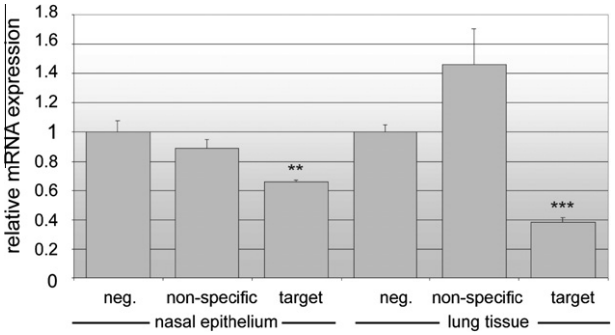

Fig. 3. Intranasal triple applications. (A) Female Balb/c mice were treated (intranasal administration) three times (every second day) with 80 μg γENaC-siRNA (target) or non-specific siRNA (non-specific) diluted in distilled water. After 72 h, the nasal epithelia and the lungs were harvested and qRT-PCR was performed in duplicates. Mean normalized expression values ± SEM are shown. Neg.: mice were not treated with siRNA.

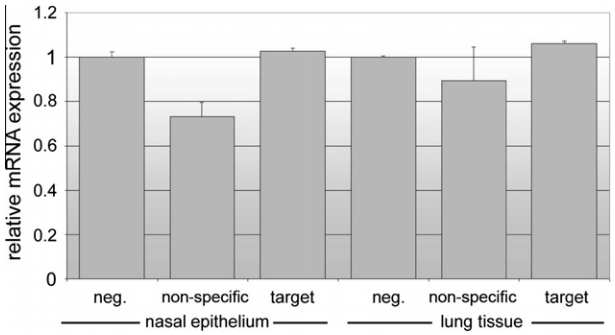

Fig. 4. No significant signal shift of TNF-α in tissue samples after the triple siRNA dose. qRT-PCRs were performed for TNF-α in duplicates after the triple administrations of siRNA as described above. Mean normalized expression values ± SEM are shown. Neg.: mice were not treated with siRNA. target: γENaC-siRNA; non-specific: non-specific-siRNA.

analysis of the lung tissue revealed a γENaC protein reduction in two of five γENaC-siRNA_I treated animals (data not shown). Expression of αENaC mRNA or βENaC mRNA was not changed by transfections with γENaC-siRNA_I (data not shown).

3.4. No impairment of the cytokine TNFα after triple application of γENaC-siRNA in tissue samples

To analyze a possible inflammatory response after siRNA application, the cytokine TNFα was measured in nasal and lung tissue after triple administration of γENaC-siRNA. There was no difference in the concentration of TNFα in the analyzed tissue samples between untreated, non-specific- and target-siRNA-treated animals (Fig. 4).

4. Discussion

The results of our study showed that only repeated application of "naked" siRNA led to a significant reduction of targeted mRNA in the murine airways. Whereas in *in vitro* experiments, a single administration was efficient enough to downregulate the targeted mRNA.

Administration of naked siRNA into the airways has the advantage that toxic side effects caused by the nucleic acid carriers like cationic liposomes or polymers could be avoided. There are a number of publications on the use of siRNA application in combination with lipid-based or polymer-based siRNA carrier. Griesenbach et al. perfused antisense oligonucleotides (asODN) and siRNAs complexed to a cationic liposome (GL67) into the mouse nose. The amount of the administered nucleic acids (80 μg/mouse) and the perfusion procedure were quite identical to our *in vivo* experiments [24]. In their study, an uptake of FITC-labeled asODN or siRNAs complexed to GL67 into nasal epithelial cells was only seen for asODN. Griesenbach et al. could not detect changes in a functional assay for ENaC, nor any reduction in αENaC mRNA after application of anti-ENaC asODN to the murine nose. In contrast to Griesenbach et al., we used more potent siRNA sequences than the asODN used in their studies. In our *in vitro* studies, the administered specific siRNAs reduced γENaC mRNA by approximately 90%. On protein level, we reached 60% of reduction. The discrepancy of the level of mRNA and protein after siRNA administration could be explained by a pool of unprocessed ENaC subunits in the cytoplasm of the target cells which cannot be reduced so efficiently by inhibiting the translation of newly formed ENaC subunits. Hughey et al. already could show two independent pools of ENaC at the cell surface, where one of the pools is unprocessed [26].

In this study, we have not performed functional analysis on the relevance of this reduction for the ENaC-mediated sodium uptake. Recently, we could show [17] that in human bronchial epithelial cells. a reduction of γENaC mRNA to 50% of control resulted in a significant reduction in the amiloride-sensitive current. Very similar results were found on epithelia obtained from CF patients. In addition, the physiologic relevance of siRNA-mediated ENaC downregulation on the fluid transport of bronchial epithelia was measured, and it could be shown that the fluid absorption from the apical side of the epithelium was reduced to 45% of the absorption measured in control epithelia.

In accordance with the study by Griesenbach et al., we did not detect a change in mRNA expression of γENaC subunit after a single application. A reduction in the targeted mRNA expression in our study was only seen after triple application of siRNAs. Repeated applications will most likely increase the number of siRNA molecules in the cytoplasm leading to a more pronounced reduction in mRNA expression. Zegarra-Moran et al. has recently shown that the expression of mRNA of ENaC subunits was reduced for up to seven days after a single application of siRNA sequences in respiratory epithelial cells (Zegarra-Moran, personal communication). Therefore, it is most likely that an additive effect of the single application resulted in a significant and measurable reduction in gene expression. In addition, the osmolarity of the instilled solution seems to be an important efficacy parameter for the uptake of nucleic acids into the epithelial cells of the airways. In contrast to the study by Griesenbach et al., we suspended the siRNA in RNase-free water. This could have caused an increased uptake of the siRNA molecules due to a "hypoosmotic shock" induced in the respiratory epithelia [27].

The Western blot analysis showed only in two of the five γENaC-siRNA_I-treated animals a detectable γENaC protein reduction in the lung tissue. Due to the variability of *in vivo* experiments, larger number of animals has to be analyzed for a detectable protein reduction and a functional effect. Still, these data are encouraging that improved application procedures might result in a more pronounced effect also on the protein level of ENaC inhibition.

For future clinical use, repeated applications would be necessary therefore inflammatory responses caused by the applied siRNAs are of major concern. In this context, it has to be mentioned that our *in vivo* administrated siRNA posses similarity to immunostimulating motifs (5′-UGUGU-3′) in its sense/passenger strand. Although no obvious visible sign of pain or immune response e.g. piloerection could be determined, it remains to investigate the blood sera and plasma for increased factors (transaminases, NF-κB, INF-γ or other cytokines) due to a possible immune response. Additionally performed qRT-PCR approaches did not show any α-, β-ENaC (data not shown) nor TNFα shifts after target siRNA treatment, so that our results indicate a real siRNA down regulation without obvious sign of inflammation. Therefore, an unspecific effect of ENaC knockdown, which has been reported by others is unlikely [28,29].

ENaC function is tightly regulated due to its central role for Na + homeostasis. In contrast to a number of voltage- and ligand-gated ion channels, which are regulated through rapid changes in channel opening and closing, ENaC is regulated mainly through its expression on the cell surface. This opens the possibility to interfere with its function by means of inhibition of expression. In this context, the focus of this study was to analyze whether RNAi has the potential to knockdown the expression pattern of a single subunit of this heteromultimeric complex.

The use of RNAi as a strategy for gene silencing may provide an opportunity for the treatment of cystic fibrosis lung disease and CF-like lung diseases. It has been postulated that an increased ENaC-mediated sodium uptake is responsible for the changed composition of airway surface liquid and as consequence for the inefficient mucociliary clearance in CF lung disease [1].

In the past, pharmacological strategies have been addressed to downregulate ENaC function. This has been problematic due to the short half-life of amiloride, and the potential renal side effects of long-acting inhibitors. The first time Boucher and his group postulated in 1995 that this chloride channel is regulating and inhibiting the apical epithelial sodium channel (ENaC) and that a failure of CFTR leads to accelerated ENaC activity and the known symptoms [1]. As murine ENaC is composed of three subunits α, β and γ, we had to clarify whether there is any redundancy and the importance of the subunits for the channel function. We decided to choose the γ-subunit as it evidently has a strong contribution to the ENaC function [20–22]. It is known that the COOH part of β- and γENaC is essential for channel regulation [30,31]. Furthermore, it has been shown that a region following close to the second transmembrane domain is crucial for the correct channel function [21].

Even though the importance of γENaC is shown for the normal, ENaC function and the loss of γENaC function [21] already leads to

decreased short-circuit currents [20], it has to be ruled out, whether the downregulation of γENaC under our chosen conditions is sufficient enough to reduce ENaC functionality and to moderate CF symptoms *in vivo*. Taken together, these data show for the first time that intranasal perfusion of naked siRNA under repeated applications could downregulate an endogenously expressed mRNA in murine respiratory epithelial tissue.

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