Efficient downregulation of the lung liquid clearing γ–ENaC subunit by RNAi

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Abstract:
Cystic fibrosis (CF) is caused by mutations in the gene encoding for cystic fibrosis transmembrane conductance regulator (**CFTR**). CFTR functions as chloride channel on the apical membrane of epithelia thereby regulating the transport of chloride and also sodium ions indirectly, through its inhibitory effect on epithelium sodium channel (**ENaC**) (1,2,3). The detail mechanism is not completely understood. It seems that the regulation of ENaC fails due to the mutated CFTR protein. And it is assumed that ENaC plays a major role in the pathogenesis of chronic lung disease in CF-patients. Several observations suggest that a downregulation of ENaC restores the perciliary liquid layer, thereby rehydrating the mucus and improving ciliary clearance in the lung – as the major aims in CF therapy. The lung liquid clearing γ-ENaC subunit is critical for Na^+- and K^+-transport (4). The increasing expression of the α- and γ-ENaC subunits in fetal animal lungs is leading to increased capacity for Na^+-absorption (4). γ-ENaC subunit also shows higher contribution in the regulation of channel gating (5). Therefore, we specifically downregulated the γ-ENaC subunit expression *in vitro* by RNAi and could show the knockdown on mRNA level (qRT-PCR) and also on protein level, using siRNA and shRNA, respectively.

Introduction:

CF is one of the most common life-shortening, childhood-onset autosomal recessive diseases in Caucasian populations. It affects many organs and 90 % of the patients die in their 30s from lung failure. The most consistent aspect of therapy is minimizing and treating the lung destruction caused by thick mucus and infections. The current low volume hypothesis suggest that hyperactivation of ENaC leads to an increased absorption of Na^+ by epithelial cells and to water hyperabsorption, causing a thick mucus leading to chronic lung disease.

Materials and Methods:

M-1, a murine cell line derived from cortical collecting duct was cultivated in PC-1 medium (Lonza, Wuppertal) without antibiotics (6). M1 were incubated with dexamethasone (DEX), a synthetic glucocorticoid (500 nM or 0 nM as negative control). Cells were harvested after indicated time points and western blot analysis were performed (Fig. 1).
In the next approach the cells were transfected (HiPerFect Transfection Reagent, Qiagen, Hilden/Germany) with custom designed siRNAs or shRNAs for γ-subunit of ENaC. The siRNAs were synthesized by Qiagen and were used in a concentration of 50 nM for each transfection. The selected siRNA sequences were m-γ-ENaC_I: GGATTTCAGTTGTGCTCAA (Pos. 694), m-γ-ENaC_II: GCTCTTGATATTCTACAAA (Pos. 1596) and m-γ-ENaC_III: AAATCATCGAAGTCTT CTTCA (Pos. 1730). The shRNA (subcloned by Geneart, Regensburg/Germany) sequences are located at nt 694, nt 1596 and nt 773. After indicated time points, the cells were harvested and qRT-PCR (Fig. 2a, 3a) or western blot analysis (Fig. 2b, 3b) were performed (primary antibody: Gamma-ENaC, ABR, Golden,CO).

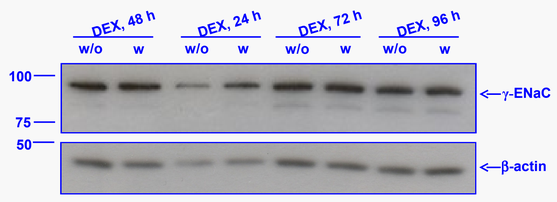

Figure 1: **γ-ENaC expression influenced by cell density in M1 cells**.

3x10^5 M1 cells were seeded in 6 well plates, treated with 500 nM dexamethasone (DEX) and harvested after 24 h, 48 h, 72 h and 96 h. The western blot analysis didn't show significant difference compared to the not treated cells. We suggest that the increasing expression level of γ–ENaC protein depends on cell density and increasing over time, not due to the used concentration of DEX.

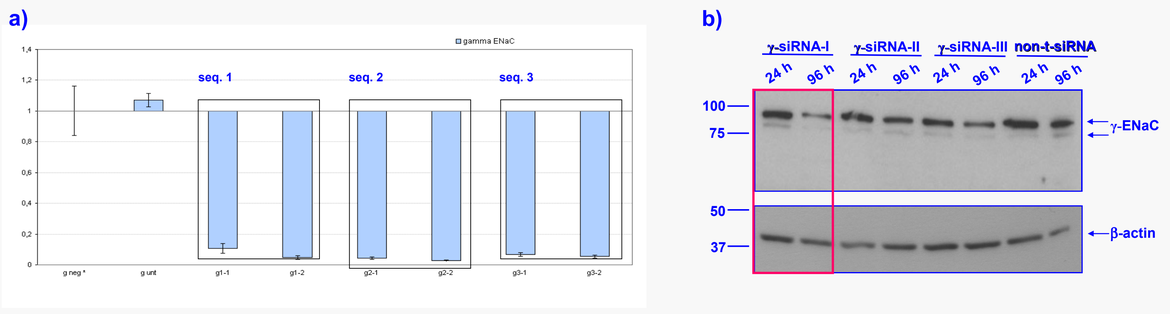

Figure 2: Inhibition of murine γ-ENaC with siRNA.

M1 cells were transfected with three different siRNAs (50 nM) against γ–ENaC subunit. **a)** Total RNA isolation was done after 72 h. γENaC mRNA levels were determined by qRT-PCR showing efficient knockdown of approximately 90 %. Samples having been normalized for levels of GAPDH and CFTR and expressed relative to mock treated controls with setting their knockdown activity to 1. The results are means ± SD from at least three independent experiments.
b) A second set of M1 were transfected with same siRNAs and harvested after two time points. 15 µg of total protein were loaded and subjected to a 7.5 %-PAGE. The WB analysis shows a reduction after 96 h of siRNA incubation compare to 24 h incubation. **loading control:** β-actin, non-t = non-targeting siRNA.

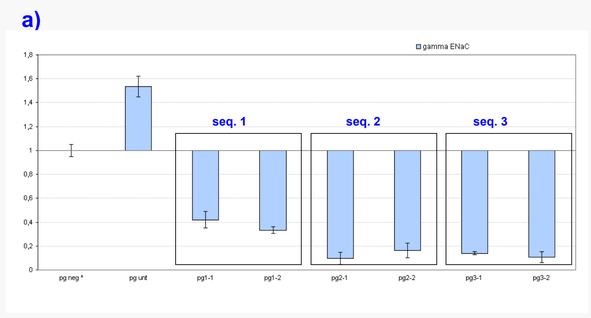

Figure 3: Inhibition of murine γ-ENaC via shRNA.

a) M1 cells were transfected with three different shRNAs (4 µg/sample) against the γ–ENaC subunit and total RNA isolation was done after 72 h. γENaC mRNA levels were determined by qRT-PCR showing efficient knockdown of around 90 %. Samples having been normalized for levels of GAPDH and CFTR and expressed relative to mock treated controls with setting their knockdown activity to 1. The results are means ± SD from at least three independent experiments.
b) Furthermore the γ–ENaC protein levels were monitored at 72 h post transfection by western blot analysis. 20 µg of total proteins were loaded and transferred onto PVDF membrane. The shRNA sh_γ-II treated cells show the most reduction of γ–ENaC protein after 72 h. The amount of β-actin was used as a loading control.

Results and Discussion

The silencing effects of siRNAs for γ-ENaC in M1 epithelial cells are detectable after 72 h as the expression level of γ-ENaC is fully reached after a certain percentage of cell density.
The results show a knockdown of the γ-ENaC subunit after short dsRNA transfection. m-γ-ENaC_I-siRNA and m-γ-ENaC_II-shRNA seem to be the most effective in downregulation of γ-ENaC subunit in M1 cells on protein level.
As one of the short dsRNA sequences posses similarity to inmunostimulating motifs (5'-UGUGU-3'), siRNA modification such as methylation of the ribosyl ring at the strand termini (but also other modifications) could help minimizing the off-target occurrence with no impact on siRNA potency. These results establish the basis for *in vivo* studies. As CF targets the lung in particular, it is interesting to apply the siRNA locally and not only systemically.

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