Supplementary appendix

Impaired renal HCO_3^- excretion in Cystic Fibrosis

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Running title: CFTR in the kidney

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Supplementary results

The secretin-induced urine alkalization is absent in pendrin KO mice

Deduced from the above results we anticipated that pendrin KO mice may be unable to respond to secretin with an alkalization of the urine. We studied the effect of secretin on pH_u in pendrin WT and KO mice. Fig. 1C shows that secretin treated WT animals responded after a lag time of approximately 5 min. with a marked, transient urinary alkalization reaching a peak mean value after ~ 25 min. of 0.397 ± 0.1 pH units (p=0.0019, n=7) as compared to vehicle treated. Importantly, no significant pH_u alkalizations were observed in pendrin KO mice (Fig. 1F). In contrast, somewhat surprisingly, in pendrin KO mice secretin induced a urinary acidification starting after some 20 min. post-injection. A urinary acidification was absent in pendrin WT mice, while a concurrent masked acidification might cause an underestimation of the activated HCO_3^- excretion observed in WTs. Secretin-treated WT mice had a significantly higher urinary [HCO_3^-] compared to vehicle treated mice with a mean of 1.08±0.3 mM vs. 0.1697±0.1 mM in controls (Fig. 1D,G, p=0.0082, n=6-7) 60 min. following injection. The urine HCO_3^- excretion rate was significantly increased in secretin-treated WT mice 60 min. after the secretin injection as compared to vehicle treated with a mean of 5.61±2.06 vs. 1.18±0.57 nmol/h/g BW (p=0.0221, n=6-7). No differences in urinary HCO_3^- excretion were observed in pendrin KO mice (Suppl. Fig. 7). KO mice had a more acidic urine at baseline as compared to WT with a mean difference of 0.26±0.17 pH units (p=0.0219, n=14, Fig. 1E). KO mice also had a tentatively lower baseline urinary [HCO_3^-] and HCO_3^- excretion rate than WT, though not reaching a level of significance (Fig. 1H and Suppl. Fig. 7). These results illustrate the absolute pendrin dependence of secretin-induced renal HCO_3^- excretion.

The secretin-induced urine alkalization is largely absent in global CFTR KO mice

Subsequently, we studied the effect of secretin on pH_u in global CFTR ($CFTR_G$) WT and KO mice. Fig. 2A shows that secretin treated WT animals responded after a lag time of a few min. with a marked transient urinary alkalization lasting about 35 min. and reaching peak mean alkalizations of 0.799±0.21 pH units (p=0.0025, n=6-7) in comparison to the control injected WT group. In CFTR KO mice, the secretin effect on urinary pH was virtually absent (Fig. 2D). A small increase (0.176±0.1189, p=0.13, n=7) was observed in secretin-treated CFTR KO mice, though not statistically different to the control injected CFTR KO mice.

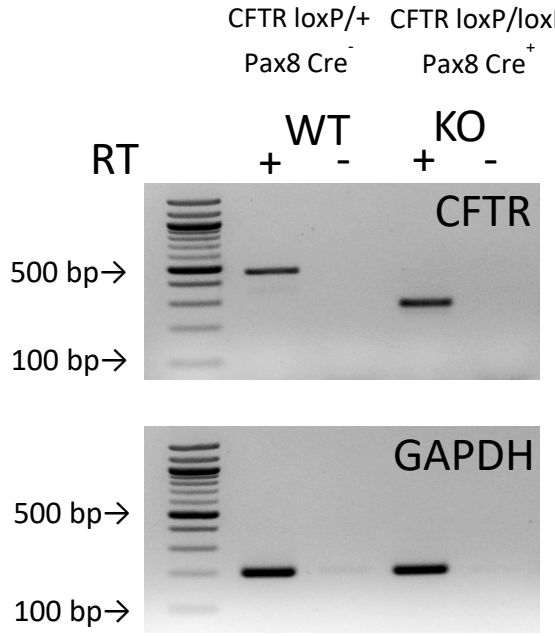
Upon secretin application urinary [HCO_3^-] increased markedly in CFTR WT mice and was significantly elevated as compared to the control group (Δ 90 min. 1.7±0.483 mM, p=0.0022, Fig. 2B). The urine HCO_3^- excretion rate was increased in WT animals as compared to controls after 60 min (ΔHCO_3^- excretion rate: 5.55±3.25 nmol/h/g BW (p=0.047, n=6, Suppl. Fig. 7). No significant differences in urine [HCO_3^-] or HCO_3^- excretion rates were observed at any point during the experiment in CFTR KO mice (Fig. 2E and Suppl. Fig. 7). Interestingly, resting urinary [HCO_3^-] and the urinary HCO_3^- excretion rate were significantly higher in WT as compared to CFTR KO-mice (Fig. 2F and Suppl. Fig. 7). Baseline urine pH values were tentatively lower in CFTR KO mice (Fig. 2C). These results demonstrate that functional CFTR is necessary to mediate secretin-dependent renal HCO_3^- excretion and that loss of CFTR causes lower baseline urine [HCO_3^-]. A small residual secretin-induced urine alkalization prevails in global CFTR KO mice though no effect on urine [HCO_3^-] was observed.

The secretin-induced urine alkalization is largely absent in tubule specific CFTR KO mice

The above data strongly support that the absence of renal epithelial CFTR is responsible for the inability to increase renal HCO_3^- excretion after secretin. To test this hypothesis further, the effect of secretin on pH_u in tubule specific CFTR ($CFTR_{TS}$) WT and KO mice was studied. Fig. 3A shows that secretin treated WT animals responded after a lag time of some min. with a marked transient urinary alkalization reaching peak mean alkalizations of 0.65±0.11 ($p<0.0001$, n=7) compared with control injected WT mice. In the $CFTR_{TS}$ KO mice a small increase was found, though not significantly different from control injected (0.2134±0.2125, p=0.053, n=7, Fig. 3D). A pronounced increase in urine [HCO_3^-] occurred in WT animals after secretin administration. The [HCO_3^-] elevation was highest in the first 30 min. following secretin administration, but stayed significantly higher as compared to controls for the remaining experiment (Fig. 3B). Likewise, a significant increase in the urine HCO_3^- excretion rate was observed 60 min. after secretin addition with a mean difference of 8.99±2.487 nmol/h/kg (p=0.0027, n=7) compared to control treated (Suppl. Fig. 7). No significant secretin-induced differences, in either urine [HCO_3^-] or urine HCO_3^- excretion rate, were found in $CFTR_{TS}$ KO-mice. Urine HCO_3^- measurements revealed a significantly lower baseline urinary [HCO_3^-] in $CFTR_{TS}$ KO mice compared to WT animals with a mean difference of 0.36±0.12 mM (p=0.0079, n=14, Fig. 3F). Baseline urine pH in $CFTR_{TS}$ KO mice were not different to those in WT mice. These data prove that renal tubular CFTR is necessary to permit secretin´s action to increase renal HCO_3^- excretion and that loss of renal CFTR causes lower baseline urine [HCO_3^-].

Supplementary figures

Suppl. Fig. 1:



Genotyping information of the 2 source mouse strains:
1. C57BL6 Pax8 Cre (Bouchard, M.; Souabni, A; Busslinger, M. (2004) Genesis 38, 105-109)

Genotyp for Cre recombinase positivity:
Primer forward: AATTTACTGACCGTACAC
Primer reverse: AATCGCCATCTTCCAGCAG
Expected PCR product size: 1024 bp

2. C57BL6/J CFTR fl10 (Hodges CA et al. Genesis 46: 546-552, 2008)

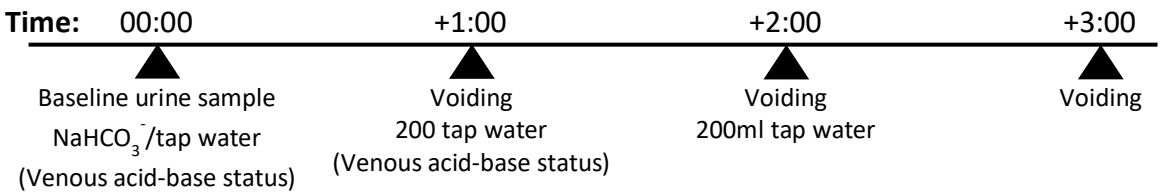
Genotyp for presence of floxed CFTR allele:
Primer forward: GTAGGGGCTCGCTCTTCTTT
Primer reverse 1: GTACCCGGCATAATCCAAGA
Primer reverse 2: AGCCCCTCGAGGGACCTAAT

Expected PCR product size:
408 bp product is for the floxed CFTR allele
353 bp product is for the wildtype CFTR allele

Renal tubule specific knockout of CFTR in mouse kidney (CFTRTS KO)

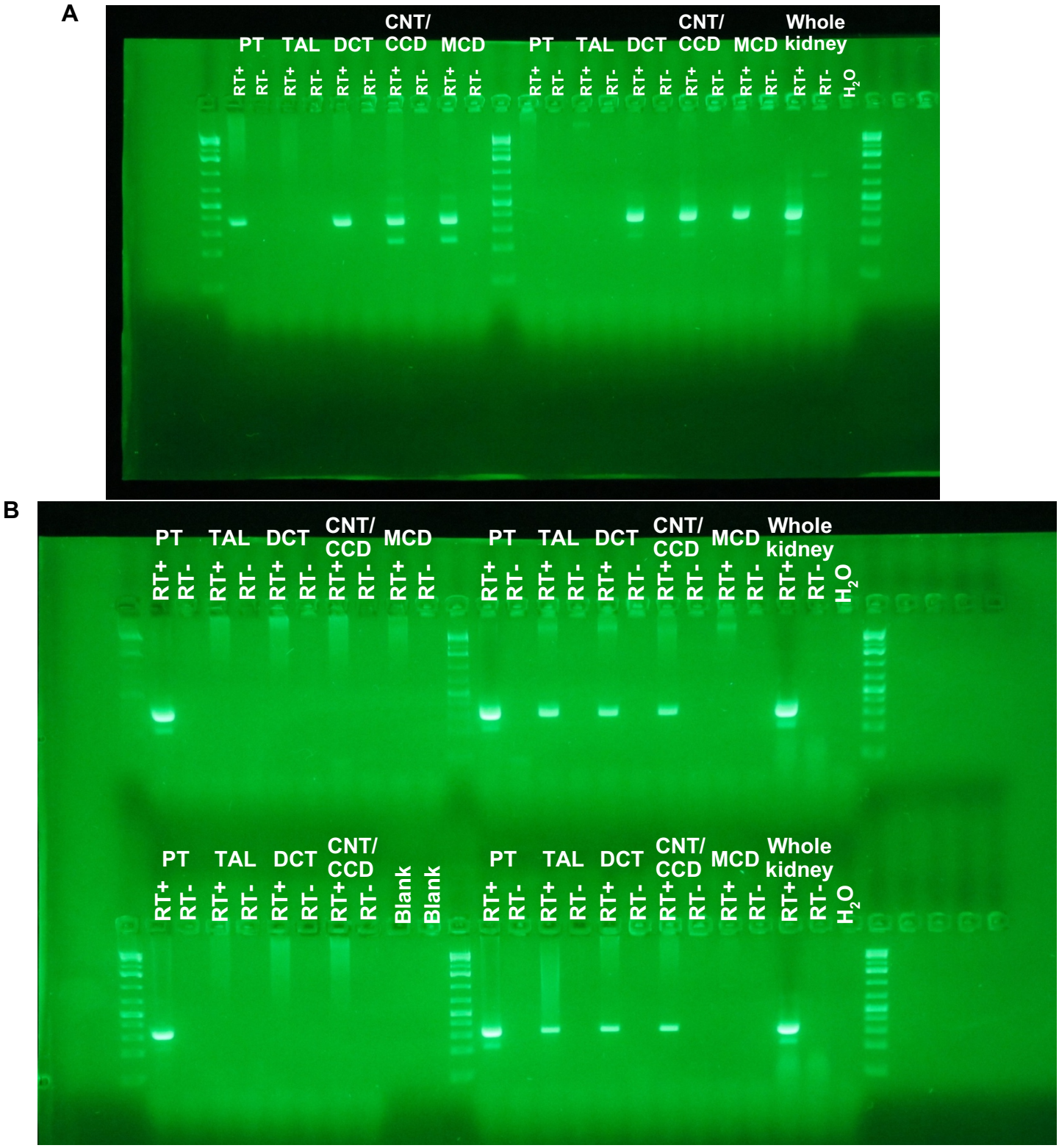
To verify renal specific knock out of CFTR by exon 10 deletion in Cftr loxP/loxP Pax8-Cre mice, RT-PCR with primer amplifying exon 10 region of CFTR mRNA was performed. Deletion of exon 10 is indicated by a PCR product of 293 bp compared to PCR product of WT-CFTR mRNA with 476 bp. GAPDH (200 bp), Reverse Transcriptase (RT). CFTR loxP/+ Pax8 Cre^- indicates a mouse example that was heterozygote for the lox P site and negative for the Cre recombinase = WT; CFTR loxP/loxP Pax8 Cre^+ indicates a mouse example that was homozygote for the lox P site and positive for the Cre recombinase = $CFTR_{TS}$ KO

Suppl. Fig. 2:



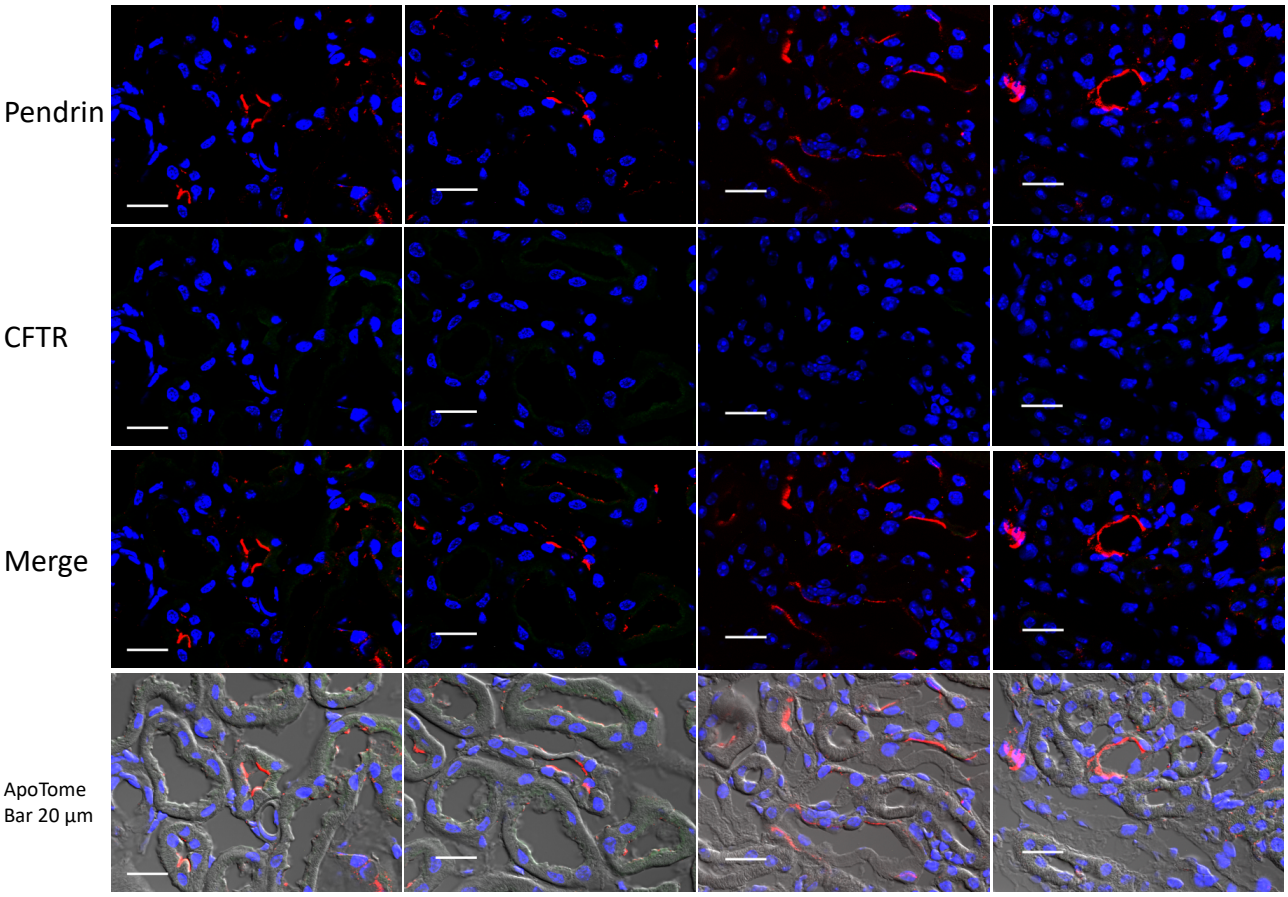
Schematic outline of the protocol for the CF urine test in humans

Suppl. Fig. 3:



Representative gel images from RT-PCR results along the mouse renal tubular system Probing for presence of **A** the SCTR secretin receptor mRNA and **B** CFTR

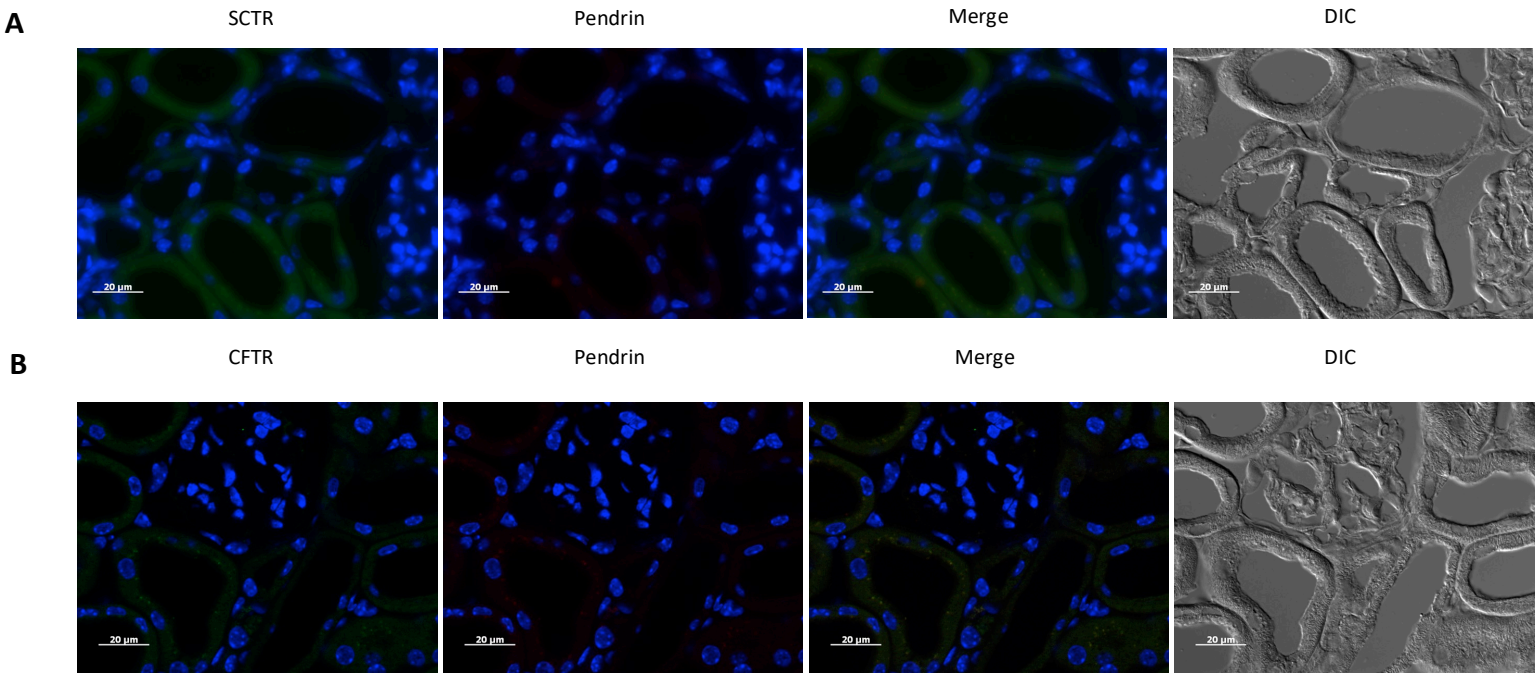
Suppl. Fig. 4:



Immunohistochemical localization of CFTR and pendrin in CFTR KO mice.

Tubular epithelial cells express pendrin (red) in the apical membrane, while no expression of CFTR (green) is found. Merged pictures show no co-expression of CFTR and pendrin. Bar 20 µm.

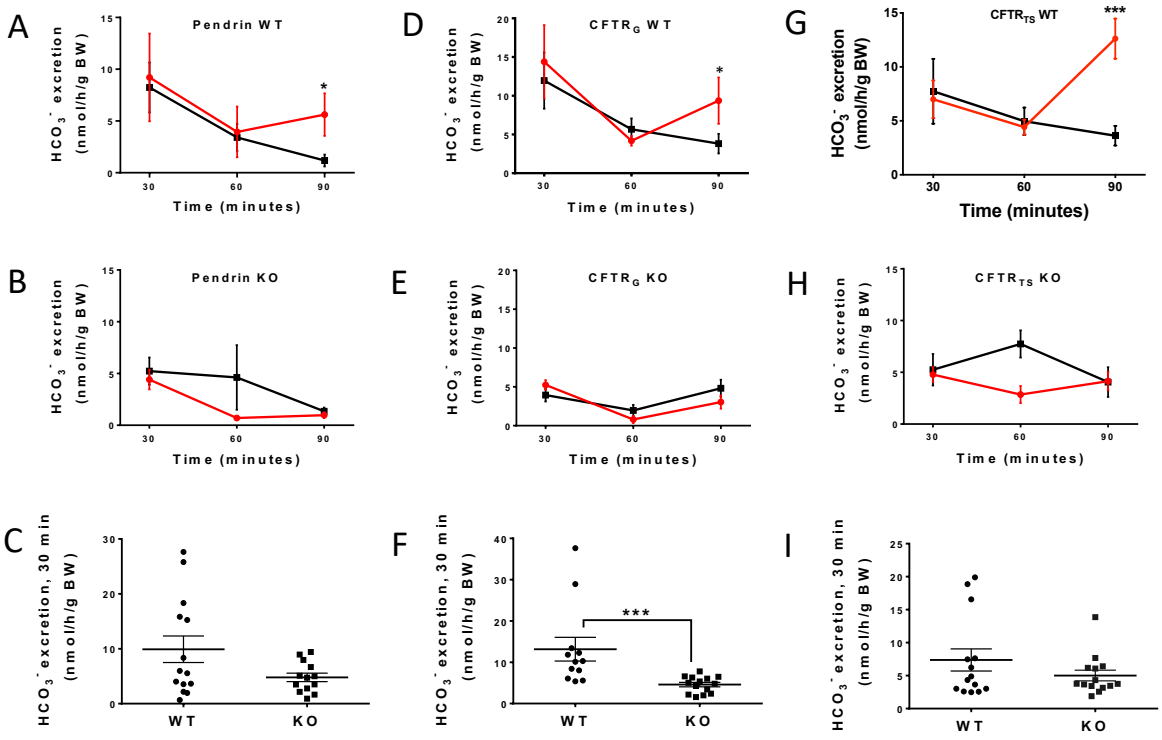
Suppl. Fig. 5:



Control staining without primary antibodies

A. SCTR/pendrin **B.** CFTR/pendrin. Bar 20 µm, differential interference contrast (DIC).

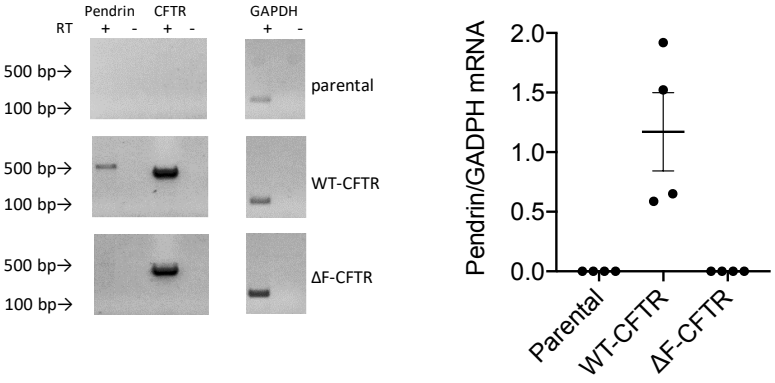
Suppl. Fig. 6:



Urinary HCO_3^- excretion rates from in vivo mouse experiments

Renal HCO_3^- excretion rates after secretin stimulation (red) or vehicle treatment (black) and under baseline conditions in Pendrin WT/KO- (**A, B, C**), global CFTR WT/KO- (**D, E, F**) and tubule specific CFTR WT/KO mice (**G, H, I**). *p<0.05, ***p<0.001. *t*-test.

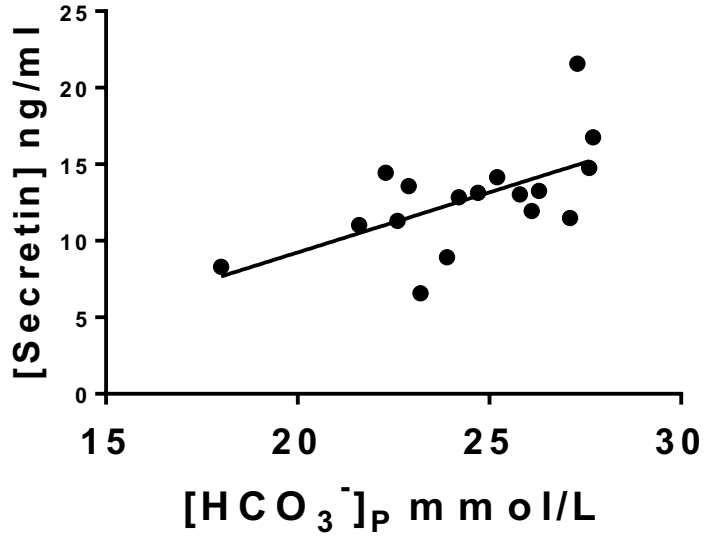
Suppl. Fig. 7:



mRNA expression of pendrin and CFTR in parental, WT CFTR- and deltaF508-transfected FRT cells

Pendrin mRNA is only present in WT CFTR transfected FRT cells.

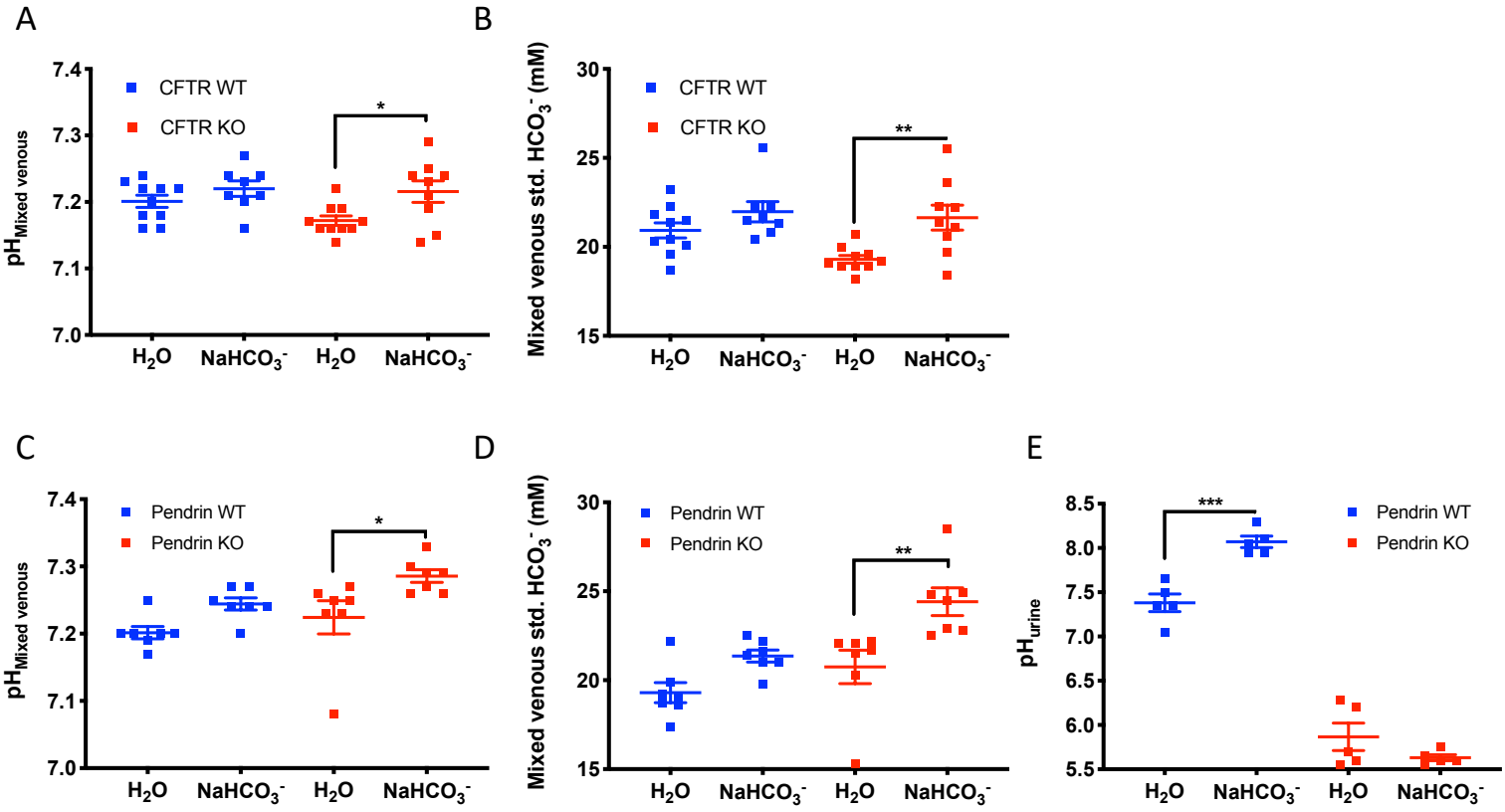
Suppl. Fig. 8:



Correlation of plasma secretion concentration plotted as a function of plasma HCO3- concentration

All dots are paired measurements from the data shown in Figure 7B, C. A significant linear regression is detected, p=0.012, R^2=0.36.

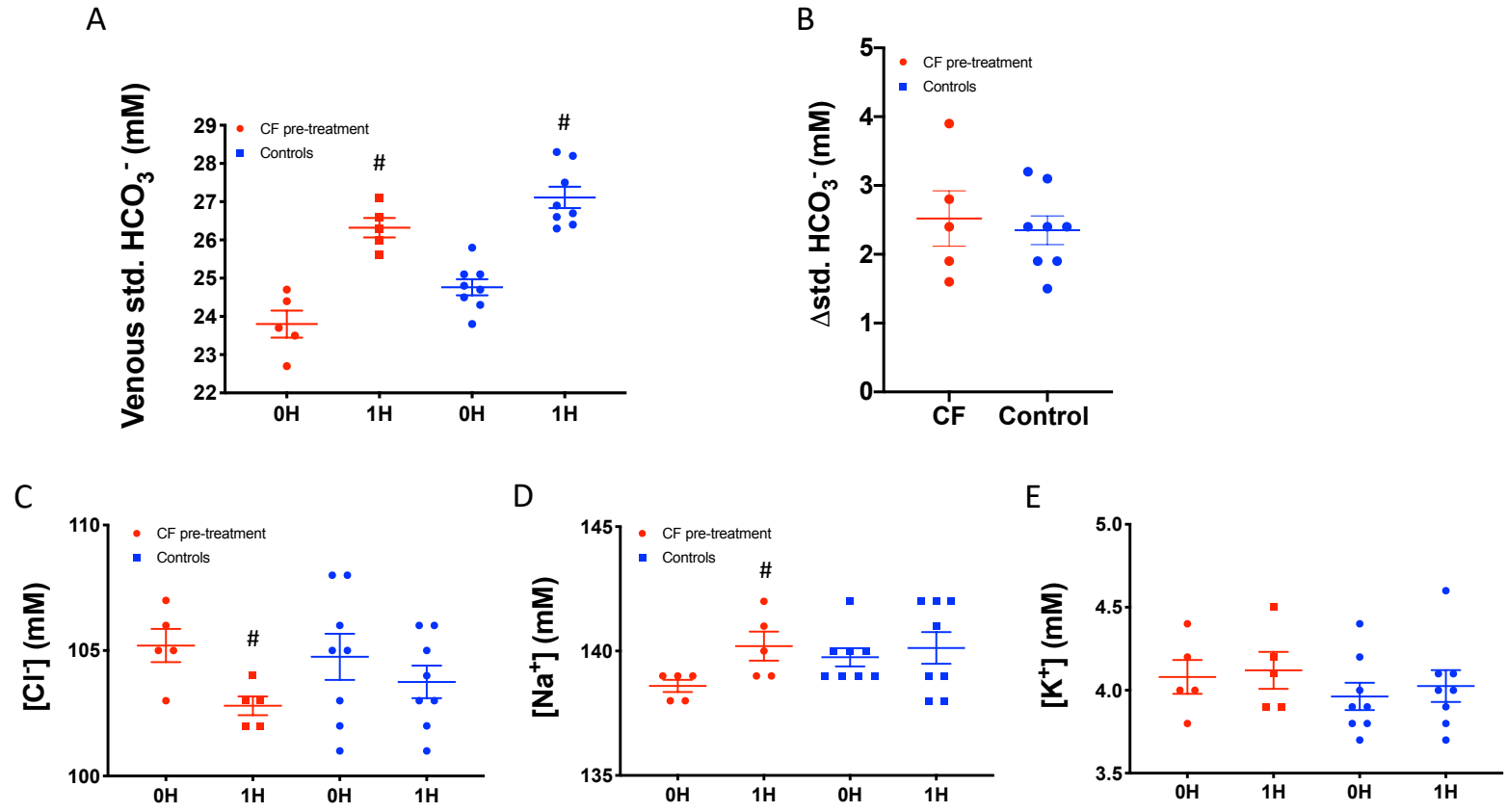
Suppl. Fig. 9:



Mixed venous blood gas parameters in CFTR- and pendrin KO/WT mice one hour after control or NaHCO3 gavage and urinary pH in pendrin WT/KO mice

A, B: Mixed venous standard HCO_3^- and pH in CFTR WT and KO mice one hour after subjection to either a control H_2O gavage or a 2.24mmol/kg BW $NaHCO_3$, one-way ANOVA. **C, D**: Mixed venous standard HCO_3^- and pH in pendrin WT and KO mice one hour after subjection to either a control H_2O or 2.24mmol/kg BW $NaHCO_3$, one-way ANOVA **E**: Urine pH in pendrin WT and KO mice one hour after either a control H_2O gavage or a 2.24mmol/kg BW $NaHCO_3$, one-way ANOVA.

Suppl. Fig. 10



Venous blood gas parameters before and one hour after a NaHCO3 challenge in healthy controls and CF patients prior CFTR modulator treatment

A, B: Venous standard HCO_3^- **C, D, E**: Venous Cl^-, Na^+ and K^+. # significant difference compared to before the challenge, paired t-test.

Supplementary tables

Suppl. Table 1

***In vivo* secretin experiments**		
Pendrin (129S1/SvImJ)	**Age (weeks)**	**Weight (g)**
WT Secretin (n=7)	11 CI: 9.2 to 12.9	25.4 CI: 23.2 to 27.6
WT control (n=7)	10.14 CI: 7.9 to 12.3	23.74 CI: 21.7 to 25.8
KO Secretin (n=7)	11.86 CI: 7.2 to 16.49	24.14 CI: 21 to 27.3
KO control (n=7)	12.14 CI: 8.8 to 15.5	23.01 CI: 20.1 to 26
CFTR global (C57Bl/6 and 129P2/OlaHsd)	**Age (weeks)**	**Weight (g)**
WT Secretin (n=6)	7.67 CI: 4.5 to 10.8	18.23 CI: 15.9 to 20.6
WT control (n=7)	7.33 CI: 3.9 to 10.8	18.58 CI: 15.7,21.5
KO Secretin (n=7)	6.14 CI: 5.5 to 7.8	15.86 CI: 13.5,18.2
KO control (n=7)	8.86 CI: 6.9 to 10.8	15.21 CI: 13,17.5
CFTR tubule-specific (C57Bl/6J)	**Age (weeks)**	**Weight (g)**
WT Secretin (n=7)	19.71 CI: 14.7 to 24.8	26.57 CI: 22.3 to 30.9
WT control (n=7)	23.29 CI: 21.6 to 25	28.5 CI: 24.73 to 32.3
KO Secretin (n=7)	18.71 CI: 11.6 to 25.8	25 CI: 22.6 to 27.4
KO control (n=7)	19.14 CI: 12.7 to 25.6	24 CI: 20.58 to 27.4

Mice characteristics for the mice used in in vivo secretin experiments

Values are presented as mean values followed by 95% CI.

Suppl. Table 2

Nephron segment	# CFTR bands/# samples	# SCTR bands/# samples
Proximal tubule	6 / 6	3 / 6
Thick ascending limb	2 / 6	1 / 6
Distal convoluted tubule	3 / 6	6 / 6
Connecting tubule / cortical collecting duct	2 / 6	6 / 6
Medullary collecting duct	0 / 5	5 / 5

RT-PCR results probing for presence of CFTR and secretin receptor mRNA along mouse renal tubular system