Mechanisms Underlying the Fanconi Syndrome in Cystinosis 2009-2010

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The research is concerned with the mechanisms responsible for the emergence of the Fanconi Syndrome in the renal proximal tubule (RPT) in cystinosis. Possibly, the reduced transport result from an altered redox state. Alternatively, the decreased activity of apical Na^+/solute cotransport transport systems are due to alterations affecting trafficking of apical transporters in cystinotic RPT cells. In order to evaluate these hypotheses primary rabbit kidney proximal tubule (RPT) cells have been treated with cystinosin siRNA to knockdown cystinosin. By this means an 83 +/- 4% reduction in the level of cystinosin and an 81 +/- 2% reduction in cystinosin mRNA was obtained, as shown in Figure 1.

Figure 1

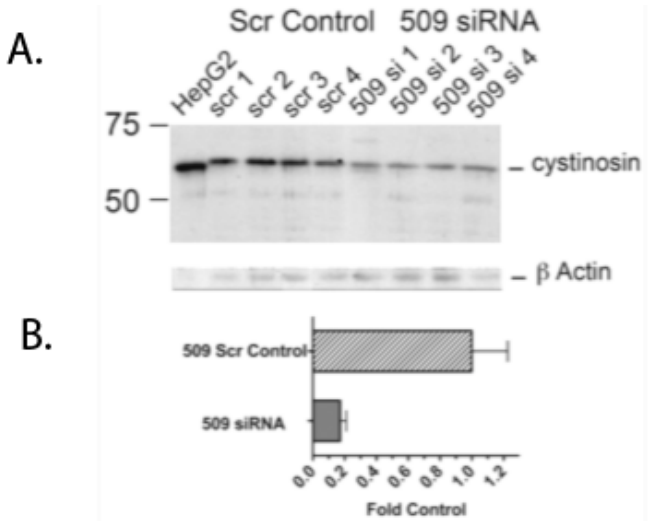


Figure 1. Effect of Cystinosin siRNA on the Expression of Cystinosin. Primary RPTs were transfected with 200 nM 509 cystinosin or scrambled siRNA with lipofectamine 3 times over 4 days. The sequence of the sense strand of 509 cystinosin siRNA was GAC AAU ACG UCU UGC UGC CCA GUU A, and the sequence of the equivalent scrambled siRNA was GAC GCA UUU CUG UCG ACC CGA AUU A. A. The level of cystinosin was examined by Western analysis , in parallel β actin. B. The level of Cystinosin mRNA was determined by RT-PCR as described in Materials and Methods. Relative cystinosin mRNA values were calculated from Ct values (1) as averages (+/-) SEM of triplicates.

In order to determine whether the reduction in the level of cystinosin over this time period, resulted in the emergence of the Fanconi Syndrome, transport by 2 apical

transport systems, the Na^+/Pi cotransport system and the Na^+/glucose cotransport system, was examined. Fig. 2 shows that the Pi uptake rate was reduced by 52 +/- 5% in the presence of 140 mM NaCl in cultures treated with cystinosin siRNA, which could be explained by a 70 +/- 6% reduction in the Na^+ dependent component of Pi uptake. The uptake of ^{14}C-αMethyl-D-Glucoside, a substrate of the Na^+/glucose cotransport system, was also reduced, albeit to a lower extent (28 +/- 4%).

Figure 2

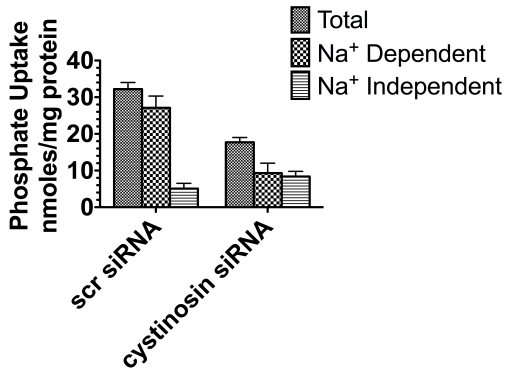
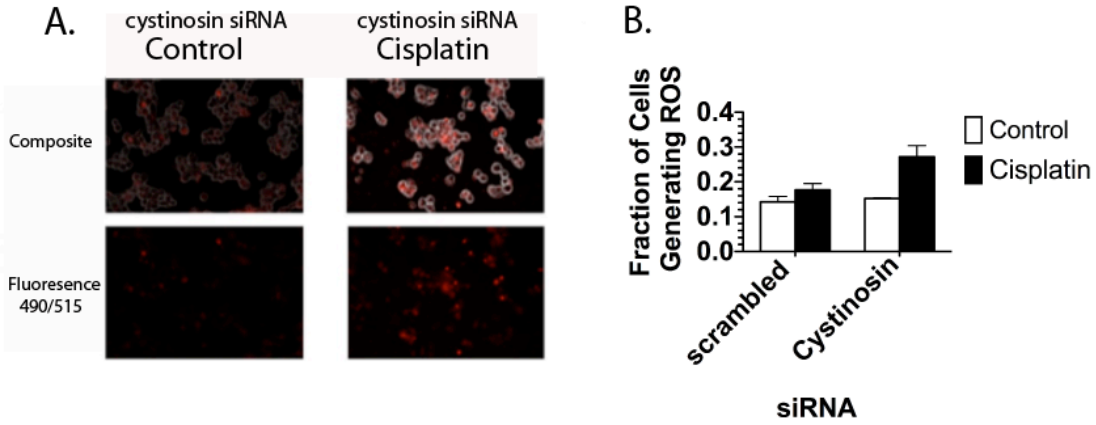


Figure 2. Effect of Cystinosin siRNA on Transport in Primary RPT cells transfected with cystinosin and scr siRNA. ^{32}Pi Uptake (1 mM) was determined after 30 min in the presence of 140 mM NaCl or 140 mM KCl. The Na^+ dependent component was calculated.

According to an alternative hypothesis, the Fanconi Syndrome emerges in cystinosis as a result of a reduction in the number of proximal tubules. Indeed, we observed that growth of RPT cells was reduced by 45 +/- 10% following treatment with cystinosin 509 siRNA (vs. scr controls). Reduced growth may due to oxidative stress.

In order to examine this hypothesis, primary RPT cells were treated with 20 μM cisplatin, which caused an increase in Reactive Oxygen Species (ROS) (which was visualized using Aminophenyl Fluorescein (APF), as shown in Fig. 3A). Fig. 3B shows that cisplatin treatment increased ROS 1.8 +/- 0.2 fold in cystinosin siRNA transfected cultures, unlike scr controls (1.1 +/- 0.1 fold increase.

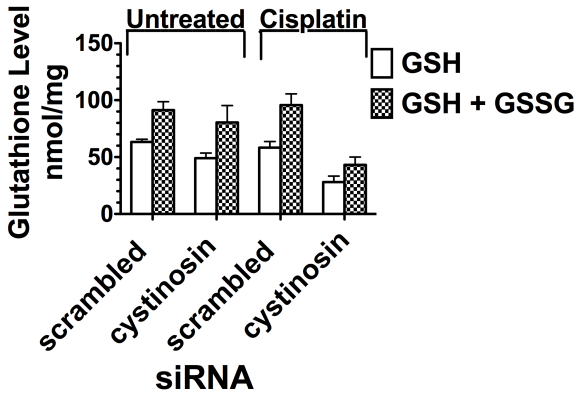
Fig.3



Primary RPTs were transfected with siRNA and then treated 4 hr. with 5 μM cisplatin. APF was added, and fluorescence observed (490/515) (3). The proportion of stained cells was determined in 20 microscope fields using NIH ImageJ. Values are averages +/- SEM of triplicate determinations.

Cisplatin also had an affect on the level of GSH in the primary RPT cells. Fig. 4 shows that in the absence of cisplatin, the level of reduced glutathione (GSH) was lower in cystinosin siRNA treated primary RPTs than in scr controls (73 +/- 2 % of the scr controls). Following a 4 hr incubation with cisplatin, the GSH level was further reduced in cystinosin siRNA transfected cultures (44 +/- 8% reduction), unlike scr controls.

Fig. 4



Primary RPTs transfected with siRNA (Fig. 3) were treated 4 hrs with 5 μM cisplatin (or untreated). GSH (and GSH + GSSG) was determined in triplicate (Promega GSH-Glo). Values (averages +/- SEM) are representative of 3 studies

In order to determine whether the increased oxidative stress is associated with an increased frequency of apoptosis, the effects of TNF α (which activates apoptosis through an extrinsic pathway) and cisplatin (which activates apoptosis through an intrinsic pathway) were examined. 30 ng/ml TNFα + 2.5 µg/ml Actinomycin D caused increased cell death and apoptosis. The frequency of apoptosis and cell death increased in cystinosin siRNA treated cultures (as indicated by a 2.4 fold higher level of caspase 3 activity compared with control cultures). Similarly, primary RPTs treated with 20 µM cisplatin became apoptotic and died. In primary RPTs treated with cystinosin siRNA, 31 +/- 3% of the cells died following a 12 hr incubation with 20 µM cisplatin, a value 2.2 fold higher than in RPTs treated with scrambled siRNA. The increased cell death was associated with a decreased level of reduced glutathione in primary RPT cells treated with cystinosin siRNA as compared with scrambled controls.

To summarize, an 80% knockdown in cystinosin siRNA results in decreased transport by apical transport systems including the Na^+/phosphate cotransport system (NaPi2a) and the Na^+/glucose cotransport system (SGLT2). The primary RPT cells also exhibited an increased sensitivity to killing by TNFα and cisplatin. The increased sensitivity to killing by cisplatin was associated with a decrease in reduced glutathione, presumably a consequence of reduced cytoplasmic cysteine levels. Further studies are in progress to evaluate these hypotheses.