Activation of the transcription factor EB rescues lysosomal abnormalities in cystinotic kidney cells

Laura R. Rega[1], Elena Polishchuk[2], Sandro Montefusco[2], Gennaro Napolitano[2], Giulia Tozzi[3], Jinzhong Zhang[4], Francesco Bellomo[1], Anna Taranta[1], Anna Pastore[5], Roman Polishchuk[2], Fiorella Piemonte[3], Diego L. Medina[2], Sergio D. Catz[4], Andrea Ballabio[2] and Francesco Emma[1]

[1]Division of Nephrology and Dialysis, Bambino Gesù Children's Hospital and Research Institute, Rome, Italy; [2]Telethon Institute of Genetics and Medicine, Pozzuoli (Naples), Italy; [3]Unit for Muscular and Neurodegenerative Diseases, Bambino Gesù Children's Hospital and Research Institute, Rome, Italy; [4]Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California, USA; and [5]Laboratory of Proteomics and Metabolomics, Bambino Gesù Children's Hospital and Research Institute, Rome, Italy

Nephropathic cystinosis is a rare autosomal recessive lysosomal storage disease characterized by accumulation of cystine into lysosomes secondary to mutations in the cystine lysosomal transporter, cystinosin. The defect initially causes proximal tubular dysfunction (Fanconi syndrome) which in time progresses to end-stage renal disease. Cystinotic patients treated with the cystine-depleting agent, cysteamine, have improved life expectancy, delayed progression to chronic renal failure, but persistence of Fanconi syndrome. Here, we have investigated the role of the transcription factor EB (TFEB), a master regulator of the autophagy-lysosomal pathway, in conditionally immortalized proximal tubular epithelial cells derived from the urine of a healthy volunteer or a cystinotic patient. Lack of cystinosin reduced TFEB expression and induced TFEB nuclear translocation. Stimulation of endogenous TFEB activity by genistein, or overexpression of exogenous TFEB lowered cystine levels within 24 hours in cystinotic cells. Overexpression of TFEB also stimulated delayed endocytic cargo processing within 24 hours. Rescue of other abnormalities of the lysosomal compartment was observed but required prolonged expression of TFEB. These abnormalities could not be corrected with cysteamine. Thus, these data show that the consequences of cystinosin deficiency are not restricted to cystine accumulation and support the role of TFEB as a therapeutic target for the treatment of lysosomal storage diseases, in particular of cystinosis.

Kidney International (2016) **89,** 862–873; http://dx.doi.org/10.1016/j.kint.2015.12.045

KEYWORDS: cystinosis; pediatric nephrology; proximal tubule; renal pathology

Correspondence: *L.R. Rega, Division of Nephrology and Dialysis, Bambino Gesù Children's Hospital, Piazza Sant'Onofrio, 4, 00165 Rome, Italy. E-mail: laurarita.rega@opbg.net*

Received 11 May 2015; revised 22 December 2015; accepted 30 December 2015

Cystinosis is a rare autosomal recessive lysosomal storage disease (LSD) with an incidence of 0.5 to 1.0 per 100,000 live births. The most severe and frequent form, affecting ~95% of patients, is the infantile nephropathic cystinosis (OMIM 219800).[1] All clinical forms of cystinosis are caused by bi-allelic mutations in the *CTNS* gene, which is located on chromosome 17p13. More than 100 mutations have been reported;[2] however, the most frequent mutation, found in northern European patients, is a large deletion of 57,257 base pairs, involving the first 9 *CTNS* exons and part of exon 10.[3] The *CTNS* gene encodes the lysosomal cystine transporter, cystinosin, which is a 367 amino acid lysosomal protein that contains 7 transmembrane domains.[4] Cystinosin is a proton-cystine symporter devoted to the excretion of both cystine and protons out of the lysosomal lumen.[5] Because cystinosin is ubiquitously expressed, its absence or malfunctioning causes accumulation of cystine into the lysosomes throughout the body. Moreover, cystine is poorly soluble, thus its accumulation leads to the formation of crystals. Kidneys are one of the first organs to be affected in cystinotic patients, which typically present with clinical signs of Fanconi syndrome, by 4 to 6 months of age. Fanconi syndrome is characterized by polyuria and abnormal urinary loss of amino acids, glucose, low-molecular-weight and intermediate-weight proteins, and other solutes. If untreated, patients progress to end-stage renal disease by the age of 10 years.[6] The mechanisms linking lysosomal cystine accumulation to cell dysfunctions, in particular to the prominent proximal tubular defect, remain unclear. Recent studies have generated various hypotheses indicating that proximal tubule damage is associated with increased sensitivity to apoptosis,[7,8] abnormal authophagy,[9,10] mitochondrial dysfunction,[11] adenosine triphosphate depletion,[11] increased reactive oxygen species production,[11] endoplasmic reticulum stress,[12] endo-lysosomal dysfunctions,[13] and cell dedifferentiation.[14,15]

Cysteamine was introduced in the late 1970s for the treatment of cystinosis and has been the only therapy available since then.[16,17] However, foul odor and gastric side effects of oral cysteamine make adherence extremely difficult. Moreover, cysteamine does not correct all the symptoms of cystinosis; in particular, it does not have an impact on the renal Fanconi syndrome.[1] Even well-treated patients will

eventually progress to envisage renal disease and will require kidney transplantation. Thus, novel therapeutic approaches are needed for cystinosis.

The recent discovery of a lysosomal gene network, the coordinated lysosomal expression and regulation network and of its master regulator, transcription factor EB (TFEB), has provided new insights in the studies of LSD.[18] TFEB activates the transcription of genes that encode lysosomal proteins involved in several aspects of cellular clearance, such as lysosomal biogenesis, exocytosis, and autophagy, as well as nonlysosomal proteins involved in the degradation of autophagy substrates.[19] The role of TFEB in promoting cellular clearance has been proven in several diseases such as multiple sulfatase deficiency,[20] Pompe disease,[21] and alpha-1-anti-trypsin deficiency.[22] Together these findings provide proof of concepts that drugs that stimulate TFEB activity may represent useful therapeutic tools to enhance mobilization of aberrant storage materials in LSD.

Herein, we have investigated the impact of TFEB activation on 3 cystinotic phenotypes, namely lysosomal cystine accumulation, delayed processing of endocytic cargo and aberrant lysosomal compartment morphology. We have observed that cystinosin-depleted cells have reduced expression of TFEB. Moreover, abnormalities of the lysosomal compartment induced by cystinosin deficiency are associated with TFEB nuclear translocation. Both chemical and genetic stimulation of TFEB activity promotes clearance of cystine storages within 24 hours. Overexpression of TFEB stimulates also delayed cargo processing. Prolonged increased expression of TFEB could also rescue other defects of the lysosomal compartment in cystinotic cells that are not sensitive to cysteamine. These results support the role of TFEB as a therapeutic target for the treatment of LSDs and provide promising perspectives in the treatment of cystinosis.

RESULTS

CTNS-depleted cells have reduced TFEB expression

Endogenous expression of TFEB was analyzed in conditionally immortalized proximal tubular epithelial cells (ciPTEC) derived from the urine of a healthy volunteer or a cystinotic patient bearing the homozygous 57-kb deletion of the *CTNS* gene. Immunoblotting experiments performed on total cell extracts revealed reduced levels of endogenous TFEB in $CTNS^{-/-}$ ciPTEC, compared with $CTNS^{+/+}$ ciPTEC (~40%) (Figure 1a). Quantitative real-time polymerase chain reaction (PCR) also showed similar reduction of TFEB mRNA levels in $CTNS^{-/-}$ ciPTEC (~40%) (Figure 1b). Depleting intracellular cystine in $CTNS^{-/-}$ ciPTEC by cysteamine treatments did not rescue the defect of expression of TFEB (Figure 1b). Cysteamine treatment produced ~90% reduction of total cystine levels in $CTNS^{-/-}$ ciPTEC (Supplementary Figure S1A).

To prove that this effect was not specific of this cell line, we confirmed these results with a RNA interference approach. To this end, human kidney-2 cells were transfected with nontargeting (CTRL) or CTNS short interfering RNAs (CTNS knock down) and analyzed for TFEB expression. Immunoblotting experiments showed reduction of TFEB in CTNS knocked down cells (~50%) (Figure 1c). Quantitative real-time PCR also revealed ~40% reduction of TFEB mRNA levels in human kidney-2 cells silenced for CTNS expression (Figure 1d). The efficiency of CTNS silencing was approximately 60% in human kidney-2 cells (Figure 1e). Reduction of TFEB mRNA was also shown in HepG2 cells silenced for CTNS expression (Supplementary Figure S1B and C).

Together these results indicate that lack of cystinosin impairs TFEB expression.

TFEB is translocated into the nucleus of cystinotic cells

To address TFEB intracellular distribution, we performed nuclear-cytoplasmic fractionation experiments. Immunoblotting experiments revealed increased nuclear-cytoplasmic ratio of TFEB protein levels in cystinotic $CTNS^{-/-}$ ciPTEC, compared with control $CTNS^{+/+}$ ciPTEC (~3.5-fold increase), indicating increased nuclear translocation of TFEB in cystinotic cells (Figure 2a).

Control and cystinotic ciPTEC were also transiently transfected with a TFEB–green fluorescent protein (GFP) plasmid. After 24 hours, fluorescence analysis showed a 2-fold increase in TFEB-GFP nuclear translocation in $CTNS^{-/-}$ ciPTEC, as compared to control $CTNS^{+/+}$ cells (Figure 2b).

TFEB activation promotes reduction of cystine stores in cystinotic cells

To stimulate activation of endogenous TFEB, cystinotic $CTNS^{-/-}$ ciPTEC were treated with genistein (100 μM) for 24 hours.[23] As expected, upon genistein treatment, cystinotic ciPTEC showed increased mRNA levels of representative coordinated lysosomal expression and regulation network genes, indicating genistein-mediated TFEB activation (~5-fold increase of SQSTM1, ~3-fold increase of both ASAH1 and CTSD, ~1.6-fold increase in SMPD1) (Supplementary Figure S1D). Next, we checked the impact of genistein treatment on intracellular cystine levels. After genistein treatment, cystinotic ciPTEC showed ~40% reduction of cystine levels compared with vehicle (dimethyl sulfoxide [DMSO])-treated cells (Figure 3a). These data were also confirmed in fibroblasts obtained from a cystinotic patient bearing a heterozygous mutation in *CTNS* gene (c.18-21del.GATC c.255+3 A>T mutation) (~40% reduction of cystine levels in genistein-treated fibroblasts, as compared to DMSO-treated cells) (Supplementary Figure S1E).

In addition, we analyzed the effect of exogenous TFEB overexpression on cell cystine levels. $CTNS^{-/-}$ ciPTEC were transduced with control GFP or TFEB-GFP lentiviral vectors. The GFP and TFEB-GFP transduced cells were sorted to 99% purity (data not shown) and cultured for 4 weeks. Overexpression of TFEB-GFP in cystinotic ciPTEC cells resulted in significant reduction (~60%) of total cystine levels, compared with cystinotic ciPTEC cells transduced with control GFP (Figure 3b). These data were further confirmed by transient transfection. After 24 hours, TFEB-GFP transfected