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## Full Review

# Is genetic rescue of cystinosis an achievable treatment goal?

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## ABSTRACT

Cystinosis is an autosomal recessive metabolic disease that belongs to the family of lysosomal storage disorders. The defective gene is *CTNS*, which encodes the lysosomal cystine transporter, cystinosin. Cystine accumulates in all tissues and leads to organ damage including end-stage renal disease. In this review, we outline the studies that support that genetic rescue of cystinosis could be an achievable goal, even though cystinosis is a multi-compartmental disease and cystinosin an intracellular transmembrane protein. Using the mouse model of cystinosis, the *Ctns*<sup>-/-</sup> mice, we showed that transplanted hematopoietic stem cells (HSCs) were able to act as vehicles for the delivery of a functional *Ctns* gene to the different organs and led to the significant decrease of the tissue cystine content and tissue preservation. *Ex vivo* gene-modified *Ctns*<sup>-/-</sup> HSC transplantation using a lentiviral vector containing *CTNS* complementary DNA (cDNA) was also successful in the *Ctns*<sup>-/-</sup> mice and built the foundations for a clinical trial for autologous HSC transplantation for cystinosis. The capacity of HSCs for rescuing non-hematopoietic disease is controversial, and new insights into regenerative medicine could be gained from unraveling the underlying mechanism of action.

## CYSTINOSIS: A MULTI-SYSTEMIC DISEASE DUE TO A DEFECTIVE LYSOSOMAL TRANSMEMBRANE PROTEIN

The clinical presentation and the protein involved in cystinosis do not make this disease an easy target for gene rescue. Cystinosis is an autosomal metabolic disease that belongs to the family of the lysosomal storage disorders (LSDs), characterized by a lysosomal accumulation of cystine in all the cells of the

body [1]. Three allelic forms of the disease exist: infantile, juvenile and ocular. Clinically, they are distinguishable by the severity of symptoms and the age of onset. Patients with the infantile form typically present in their first year of life with symptoms of de Toni-debré-Fanconi syndrome characterized by severe fluid and electrolyte disturbances (vomiting, poor growth and rickets). Patients eventually progress to end-stage renal failure. Cystine accumulation also leads to multi-organ dysfunction and patients present with photophobia and blindness, hypothyroidism, hypogonadism, diabetes, myopathy and central nervous system defects. Patients with the juvenile form primarily develop progressive kidney dysfunction and photophobia around 12 years old, while the ocular form consists of photophobia and presents in adulthood. The gene underlying cystinosis, *CTNS*, is expressed in every cell and tissue and encodes a seven-transmembrane domain protein, cystinosin [2]. Cystinosin is a lysosomal H<sup>+</sup>-driven cystine transporter that allows cystine to exit the lysosomes [3, 4].

The only treatment for cystinosis is the drug cysteamine that reduces the intracellular concentration of cystine, and, if used early in the disease and in high doses, can delay the subsequent progression of renal glomerular damage and improve childhood growth [5]. However, the need for frequent dosing (every 6 h orally plus eye drops hourly) and severe side effects such as digestive intolerance and persistent odor render its chronic administration difficult, and compliance is a huge challenge in the pediatric population. Moreover, cysteamine does not prevent the proximal tubulopathy or the end-stage renal failure and only delays the onset of the complications [5, 6]. Patients with renal failure require dialysis or transplantation, both of which have significant negative health effects and due to the severe shortage of donor organs, patients may wait 3 to 6 years for transplantation. Thus, there is a pressing need for a new therapy with the potential to prevent the disease sequelae.

We tested gene rescue as a new therapeutic strategy in the mouse model for cystinosis, the *Ctns*<sup>-/-</sup> mice [7]. *Ctns*<sup>-/-</sup>

mice accumulate cystine and cystine crystals in all organs tested, and developed similar anomalies as those observed in affected patients, such as the ocular defects [7, 8]. *Ctns*<sup>-/-</sup> mice backcrossed on a C57BL/6 background develop renal dysfunction from 6 months of age and incomplete proximal tubulopathy [9]. By 15 months of age, the mice develop end-stage renal failure. Histologically, cystine crystals accumulate in the same tissue cell types observed in human, such as kupffer cells in liver. In the kidney, crystals accumulate progressively within proximal tubular cells and capsular interstitial cells, and proximal tubular cells appeared to be de-differentiated; the cells are flat without brush borders and the tubules have a thick basal membrane [7, 9]. The proximal tubules also exhibit the 'swan neck' deformity, found in mice and humans with cystinosis, resulting in atubular glomeruli. Finally, heavy infiltration of inflammatory cells can be observed in the kidney of the *Ctns*<sup>-/-</sup> mice. This model is a unique instrument for testing emerging therapies for cystinosis as therapeutic impact can be objectively tested through the measurement of tissue cystine levels and kidney function.

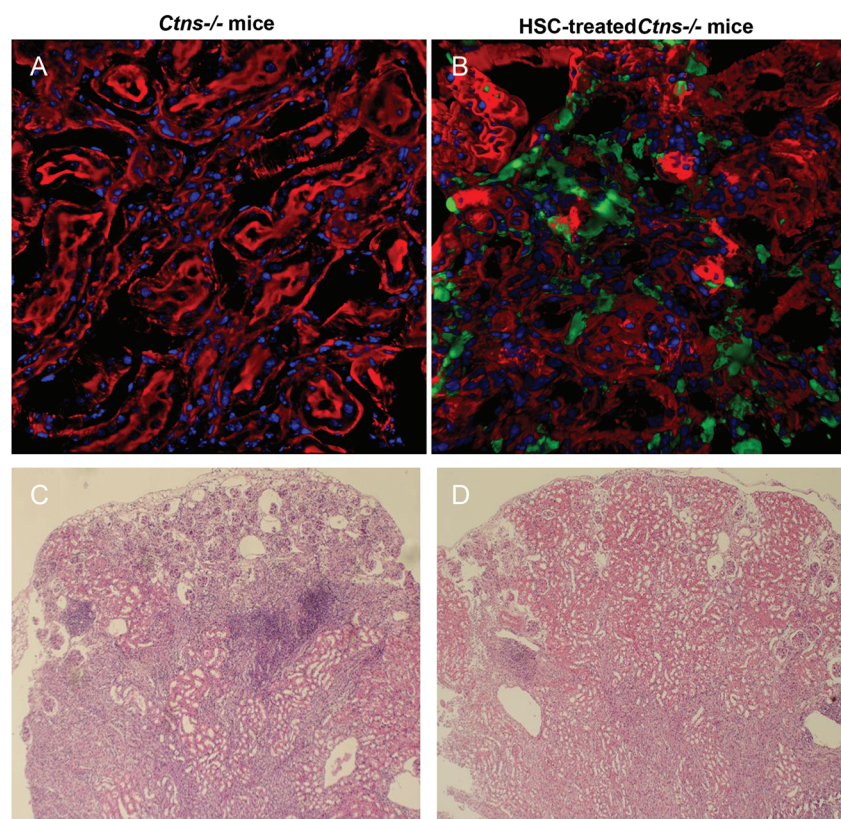
#### PROOF OF CONCEPT FOR USING HEMATOPOIETIC STEM CELLS AS A VEHICLE FOR GENE THERAPY IN CYSTINOSIS

Numbers of reports have established a proof of principle for allogeneic hematopoietic stem cell (HSC) transplantation in several LSDs [10]. However, a majority of them involve a secreted protein that can be recaptured by the adjacent cells and/or the main clinical phenotype is a neurodegenerative disease [11]. In contrast, the protein involved in cystinosis is a lysosomal transmembrane protein and the clinical entity is characterized by the progressive degeneration of multiple organs [2]. The genetic rescue of cystinosis would require the delivery of a functional *CTNS* gene to many cell types in most of the tissue compartments to address the serious global effects of this genetic disease. To date, no viral vector or gene-delivery system is capable of efficiently transducing cells in every tissue compartment. Thus, the use of a vehicle to bring the healthy gene to multiple organs is necessary. Adult bone marrow (BM) stem cells are an attractive vehicle as they are theoretically capable of integrating into every tissue compartment and are currently used in clinic [12]. Several BM cells can be used, the whole BM, the Hematopoietic Stem Cells (HSCs) and the Mesenchymal Stem Cells (MSCs). MSCs are multipotent stromal cells that can differentiate into a variety of cell types such as osteoblasts, adipocytes and chondrocytes [13, 14]. Clinical case reports for the use of MSCs in orthopedic applications have been published, though the effectiveness of these methods remains to be demonstrated [15]. HSCs are stem cells that give rise to blood cells. Their plasticity, i.e. their conversion to non-hematopoietic cell types, has been reported but is very controversial [16, 17]. The advantage of the HSCs is that they are routinely used in clinic, mainly to treat patients with cancers and other disorders of the blood and immune systems.

We tested these three types of adult BM stem cells in the mouse model of cystinosis [18]. We transplanted syngeneic BMCs, HSCs and MSCs isolated from GFP-transgenic mice into 2 months old *Ctns*<sup>-/-</sup> mice. As controls, *Ctns*<sup>-/-</sup> mice were transplanted with BMCs from *Ctns*<sup>-/-</sup> mice. All animals were lethally irradiated before transplantation. MSCs did not integrate efficiently and stably into any organ and only led to a short-term beneficial effect on the kidney function. This outcome is relevant with their paracrine mode of action rather than with cellular differentiation [19]. At 4 months post-transplantation in the mice treated with BMCs or HSCs, confocal microscopy and quantitative-PCR revealed a large quantity of transplanted BM-derived cells in all organs tested, from 5 to 19% of the total tissue cells (Figure 1; Table 1). Most of these cells were part of the intrinsic structure of the organ and were mainly resident-phagocytic cells such as kupffer cells in the liver and dendritic cells in the kidney. The organ-specific cystine content was reduced by 57–94% in all organs tested (Table 1). The natural progression of renal dysfunction was prevented, and deposition of corneal cystine crystals was significantly improved. We also transplanted BMCs and HSCs from luciferase-transgenic mice in *Ctns*<sup>-/-</sup> and wild-type mice to follow the fate of transplanted cells expressing a functional *Ctns* gene in live animals as a function of time. We observed abundant tissue colonization by BM-derived cells specifically in cystinosis mice, proving that tissue engraftment of BM-derived cells is a dynamic process and is linked to the underlying and progressive tissue injury.

We investigated the long-term effects of HSC transplantation in *Ctns*<sup>-/-</sup> mice 7–15 months post-transplantation [20]. The cystine content was significantly decreased in all tissues (from 54% in the kidney to 96.5% in the liver), proving that the treatment led to long-term and stably low levels of tissue cystine. We showed that syngeneic transplantation of HSC expressing a functional *Ctns* gene could provide long-term protection to the kidney and prevent the progression of the renal disease (Figure 1). However, we demonstrated that kidney preservation depends on achieving a relatively high level of donor-derived blood cell engraftment of *Ctns*-expressing cells, which is directly linked to the quantity of *Ctns*-expressing cells found within the kidney. These data suggest that the therapeutic benefits in the kidney after HSC transplantation are correlated with the quantity of transplanted stem cells expressing *Ctns* in the BM. These data also highlight the importance of testing the BM stem cell engraftment after transplantation to prevent the controversial data existing in the field of using adult stem cells for kidney repair [21]. In contrast, kidney preservation was not dependent on the age of the mice at the time of transplant, suggesting that if kidney injury is not too advanced the tissue could be rescued in older patients. Moreover, few to no cystine crystals were observed in all kidneys from treated mice, even the ones that were transplanted after 6 months of age. In contrast, abundant cystine crystals were consistently observed in the kidney from non-treated *Ctns*<sup>-/-</sup> mice. Thus, these results show that BM-derived cells specifically lead to the correction of cells containing cystine crystals.

This work represents the first proof of concept that transplantation of *Ctns*-expressing HSCs may lead to the successful



**FIGURE 1:** Confocal microscopy and histology analyses of kidney from *Ctns*<sup>-/-</sup> mouse control or transplanted with GFP-HSC. (A and B) Representative confocal microscopy pictures of kidney sections from *Ctns*<sup>-/-</sup> control (A) or transplanted with GFP-HSC (B) [18]. Nuclei stained by dapi are seen in blue, F-actin intermediate filament staining by Bodipy-Phalloidin is seen in red, and direct eGFP fluorescence is seen in green. Abundant GFP-positive BM-derived cells are observed in the *Ctns*<sup>-/-</sup> mice transplanted with GFP-HSC. (C and D) Representative kidney histology sections stained with hematoxylin/eosin from 15 to 17 months old *Ctns*<sup>-/-</sup> control (C) or transplanted with GFP-HSC (D) [20]. (C) *Ctns*<sup>-/-</sup> mice controls exhibit severe kidney anomalies characterized by atrophic proximal tubules, collapsed or sclerotic glomeruli, and large and multiple mononuclear infiltrates. (D) Treated *Ctns*<sup>-/-</sup> mice with high blood engraftment exhibited only focal renal anomalies and significantly less lymphoid infiltrates.

**Table 1. *Ctns* expression and cystine content decrease in tissues of *Ctns*<sup>-/-</sup> mice treated with wild-type BM-stem cells at 4 months post transplant**

	Brain	Eye	Heart	Kidney	Liver	Muscle	Spleen
<i>Ctns</i> expression, percentage of total cells	5.9	13.4	11.0	12.9	9.6	5.4	19.3
Percentage decreased of cystine content	57.3	70.4	81.9	70.0	94.4	65.6	86.8

treatment of cystinosis and could represent a new therapy for this disease. On 19 April 2012, Institutional Review Board approval was granted for an allogeneic transplantation protocol at the University of California, Los Angeles, to test the efficacy and toxicity of stem cell transplantation in patients affected with cystinosis. The clinical trial will include a maximum of six patients, aged  $\geq 18$  years, with significant signs of disease progression, or they can be children aged 13–17 years who do not tolerate cysteamine. Patients must also have a related BM donor who is HLA-matched on 10 of 10 alleles to minimize the risks of Graft-versus-host disease (GVHD). However, the strict HLA matching criterion considerably narrows down the

pool of potential trial participants. To date, none of the candidates for the cystinosis allogeneic transplantation trial has had a full-match sibling. Moreover, in the case of cystinosis, where the introduction and regular use of the drug cysteamine have permitted patients to live to adulthood, albeit with significant medical problems [22], the risk–benefit ratio may not justify allogeneic HSC transplantation in young patients. Indeed, the risk of morbidity and mortality associated with allogeneic transplantation is significant, the major complication being GVHD [23, 24]. In recent studies, acute GVHD grade II–IV occurred in 20–32% of patients and chronic GVHD in 16–59%, both significantly impacting survival of the recipients [25–27].

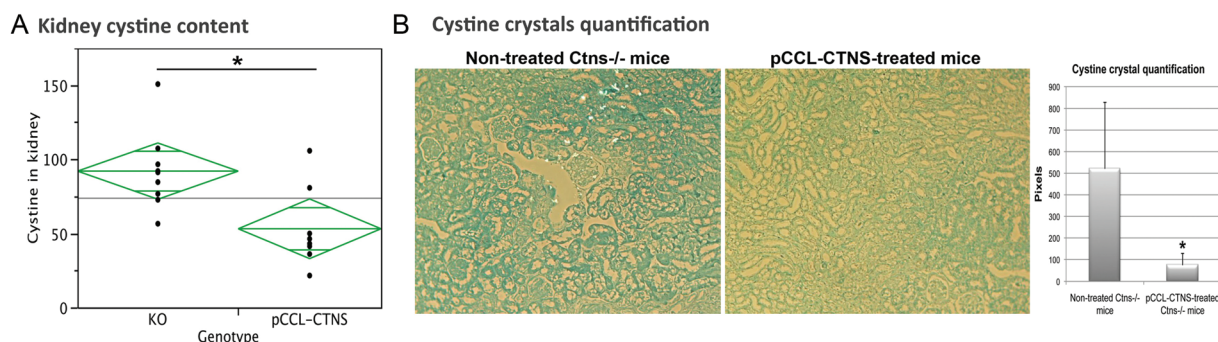
Moreover, high risks of infection related to the myeloablative regimen and immunosuppressive medications account for 16–19% of deaths [28].

### LENTIVIRAL-MODIFIED HEMATOPOIETIC STEM CELL TRANSPLANTATION FOR CYSTINOSIS: A SAFER STRATEGY

For the reasons described above, an autologous transplantation of HSCs *ex vivo* modified to express functional *CTNS* would be more appropriate for cystinosis. With regards to gene therapy, vectors derived from lentiviruses have replaced  $\gamma$ -retroviral vector due to their superior gene transfer efficiency and better biosafety profile [29–31]. Specifically, lentiviral vectors show greatly improved ability to transduce human HSCs compared to Murine Leukemia Virus (MLV)-based retroviral vectors, and deletions in their Long Terminal Repeats (LTRs) make the Self-Inactivated (SIN)-lentivirus vector a safer vector. Indeed, all cases of leukemogenic complications observed to date in clinical trials or animal models of gene therapy involved the use of retroviral vectors with LTR containing strong enhancer/promoters that can trigger distant enhancer activation [32–34]. In contrast, SIN-lentivirus vectors contain only one internal enhancer/promoter, which reduces the incidence of interactions with nearby cellular genes, and thus, decreases the risk of oncogenic integration [35, 36]. SIN-LTRs are also designed to prevent the possibility of developing replication competent lentivirus during production of the viral supernatants. Recently, a clinical trial using a SIN-LV to correct *ex vivo* HSCs in patients with X-adrenoleukodystrophy has achieved stable gene correction of ~20% of hematopoietic cells [37]. Cerebral demyelination was arrested in the two patients without further progression over 3 years of follow-up, which represents a clinical outcome that is comparable with that observed after allogeneic transplantation [38]. No adverse event and no evidence of clonal dominance have been reported so far.

Using the *Ctns*<sup>-/-</sup> murine model for cystinosis, we tested the use of HSCs genetically modified *ex vivo* to express a functional *CTNS* transgene using a SIN-lentiviral vector (pCCL-CTNS) [39]. We are working with human *CTNS* complementary DNA (cDNA) in this animal study and using a vector backbone currently in application in clinical trials [37] to establish the preclinical proof of concept for a human trial. We showed that transduced cells were capable of decreasing the cystine content in all tissues, kidney function improved and cystine crystals resolved within the kidney (Figure 2). However, in most tissues, the impact of wild-type HSC transplantation was superior to that of pCCL-CTNS-transduced HSCs, demonstrating that the quantity of progenitors expressing *CTNS* is more critical than the over-expression of *CTNS* per cell. These data also confirm our previous statement, suggesting a correlation between the therapeutic benefits and the quantity of stem cell expressing *Ctns* in the BM [20]. *Ex vivo* modified HSCs retained their differentiative capabilities, populating all tissue compartments examined, and allowing long-term expression of the transgene. Thus, *ex vivo* cytokine activation of HSCs did not alter the lineage commitment pathways of progenitor cells. As seen for the wild-type HSCs, we observed that the BM-derived cells were mainly tissue-resident phagocytic cells such as dendritic cells in the kidney, Kupffer cells in the liver and microglial cells in the brain. Direct correlation between the levels of lentiviral DNA present in the peripheral blood and the levels present in tissues was demonstrated, suggesting that this could be a useful clinical marker for following future transplant in patients.

It is our long-term goal to develop a new therapy for cystinosis using autologous transplantation of HSCs *ex vivo* modified using a lentiviral vector to introduce a functional version of *CTNS*. Successful engraftment of the gene-modified stem cells into the BM compartment of cystinosis patients could serve as a source of healthy stem cells for any injured tissue for the life of the patient. Thus, autologous transplantation of gene-modified HSCs could potentially represent a life-long therapy that may prevent kidney transplantation and long-term complications associated with cystinosis. We recently



**FIGURE 2:** Kidney cystine and cystine crystal levels in *Ctns*<sup>-/-</sup> mouse controls or transplanted with *Ctns*<sup>-/-</sup> HSCs *ex vivo* modified with a pCCL-CTNS lentivirus vector [39]. (A) Cystine content levels (nmol half cystine/mg protein) in male *Ctns*<sup>-/-</sup> mice transplanted with pCCL-CTNS-transduced *Ctns*<sup>-/-</sup> HSCs (pCCL-CTNS) are significantly decreased compared with control non-treated *Ctns*<sup>-/-</sup> mice (KO) at 8 months post-transplant. (B) Kidney sections stained with methylene blue in ethanol were used to observe and quantify cystine crystals. Abundant cystine crystals were observed in kidney sections from non-treated *Ctns*<sup>-/-</sup> mice in contrast to pCCL-CTNS-treated mice. Error bars are defined as mean  $\pm$  SD, \*P < 0.05.

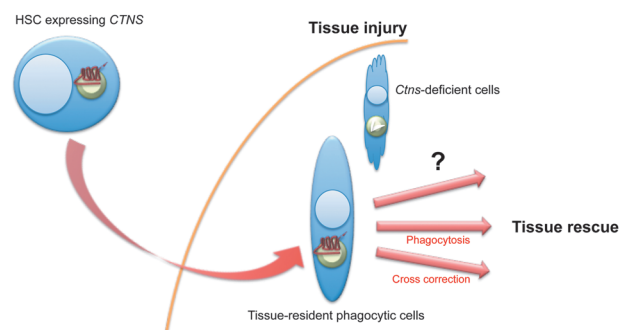
concluded a pre-Investigational New Drug (IND) meeting for this product with the Food and Drug Administration (FDA) and in order to obtain an IND for a phase I clinical trial for cystinosis, we are now starting the pharmacology/toxicology studies on test product safety.

## INSIGHT INTO THE MECHANISM FOR HSC-MEDIATED THERAPY FOR CYSTINOSIS

The observation that HSCs can rescue progressive tissue injury in multiple compartments in the context of non-hematopoietic genetic diseases is still controversial and the mechanism is unknown. Do the cells transdifferentiate within the tissues? Do the cells fuse with the host cells? Do they differentiate in hematopoietic lineage cells and have a paracrine effect? Could a combination of all these processes be involved?

In the context of cystinosis, we observed differentiation of the transplanted HSCs into tissue-resident phagocytic cells within tissues [18, 20, 39]. Tissue-resident phagocytic cells, macrophages and dendritic cells, are known to contribute to tissue homeostasis by, for instance, clearing apoptotic material [40]. We suspect that these cells have a major role in the pathogenesis of cystinosis. It is plausible that the demonstrated high level of cystine clearance after HSC transplantation is attributable to the phagocytosis of cystine-overloaded cells by the BM-derived cells expressing *CTNS* (Figure 3). Indeed, it has been shown that Kupffer cells contain 75% of the total cystine content in the liver, whereas they only represent  $\leq 10\%$  of the total cells in the liver [41].

Alternatively, these cells might transfer the functional cystinosis from the *CTNS*-expressing cells to the adjacent deficient cells. Cross correction has been well demonstrated in the case of secreted enzymes in several lysosomal storage disorders such as Hurler syndrome [42]. In this case and in the case of other lysosomal enzyme-deficient LSDs, the uptake of the enzymes from the extracellular milieu by neighboring cells occurs via the mannose-6-phosphate receptor that subsequently mediates endocytosis and lysosomal targeting [43, 44].



**FIGURE 3:** Fate of the HSCs after transplantation in the *Ctns*<sup>-/-</sup> mice. HSCs migrate to the injured tissues and differentiate into tissue-resident phagocytic cells. The question that remains unsolved is how these cells lead to tissue rescue? Is it by phagocytizing the cystine-overloaded cells, by transferring the functional cystinosis to adjacent cells or by another pathway?

However, the protein involved in cystinosis is a seven-transmembrane lysosomal protein. To test the existence of cross corrections in this context, we generated a new cystinosis mouse model that ubiquitously expressed the DsRed reporter gene. DsRed *Ctns*<sup>-/-</sup> HSCs were transduced with a lentiviral vector containing the *CTNS*-eGFP fusion cDNA and transplanted in our regular *Ctns*<sup>-/-</sup> mice [39]. Cystinosis-eGFP was observed in the lysosomes of DsRed-expressing BM-derived cells within the tissues. Remarkably, we also observed host cells containing eGFP-positive intracellular vesicles. *In vitro*, we also showed that cystinosis could be transferred to adjacent *Ctns*-deficient cells leading to their significant cystine content decrease. These data showed that, although cystinosis is a lysosomal transmembrane protein, a transfer to adjacent cells is possible and functionally active (Figure 3).

However, direct secretion of cystinosis is not possible. Microvesicles or exosomes containing *CTNS* genetic material, both released from the endosomal compartment, could be involved [45, 46]. Recently, Dr Iglesias *et al.* showed that human MSCs could release microvesicles containing cystinosis protein and mRNA that led to cystine decrease in human *CTNS*-deficient cells [47]. Additional studies are still necessary to demonstrate the route for cystinosis transfer *in vitro* and *in vivo* and the mechanisms by which HSCs can lead to tissue preservation in cystinosis.

## CONCLUSION: WHAT DOES THIS WORK BRING TO THE FIELD?

This work is important given the conflicting data and non-reproducible results that have led to negative pronouncements such as ‘unmanipulated HSCs are unlikely to provide organ repair beyond hematopoiesis’ [48]. Indeed, HSC plasticity is still not commonly accepted and whether HSCs can rescue progressive tissue injury in multiple compartments, including the kidney, is under debate [49]. Thus, we started with some reasonable skepticism that simply transplanting *Ctns*-expressing HSCs in the *Ctns*<sup>-/-</sup> mice would not be sufficient to address the serious global effects of this genetic disease. This was particularly a concern, given that cystinosis is an ubiquitous, intracellular, transmembrane lysosomal protein [2–4]. However, we were able to demonstrate that transplantation of HSCs expressing a functional *Ctns* gene could be therapeutic. The challenge is now to test this treatment in patients with cystinosis. HSC gene therapy will be the safest approach, compared with allogeneic HSC transplantation; however, it will require several years to conduct the safety studies required by the FDA.

If this therapy is successful in humans, this work might change the course of treatment for other genetic diseases for which curative therapy requires gene addition to many cells in multiple tissue compartments, where the protein involved is an intracellular transmembrane protein, and for which HSC transplantation has never been considered as an option. This work might also bring new insights into the field of tissue repair by HSC transplantation as applied to non-hematopoietic disorders. Our data suggest that no transdifferentiation

occurs with HSCs, but rather differentiation in tissue-resident phagocytic cells. Unraveling the mechanisms by which HSCs could lead to tissue repair in the context of cystinosis could lead to new concepts such as the existence of cross correction in the case of a lysosomal transmembrane protein. Therefore, not only do we believe that genetic rescue is indeed an achievable goal for cystinosis, this work might take regenerative medicine in a different direction that has not yet been explored.

## ACKNOWLEDGEMENTS

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## CONFLICT OF INTEREST STATEMENT

None declared.

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