Successful treatment of the murine model of cystinosis using bone marrow cell transplantation

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Cystinosis is an autosomal recessive metabolic disease that belongs to the family of lysosomal storage disorders. The defective gene is CTNS encoding the lysosomal cystine transporter, cystinosin. Cystine accumulates in every organ in the body and leads to organ damage and dysfunction, including renal defects. Using the murine model for cystinosis, Ctns−/− mice, we performed syngeneic bone marrow cell (BMC), hematopoietic stem cell (HSC), and mesenchymal stem cell transplantation. Organ-specific cystine content was reduced by 57% to 94% in all organs tested in the BMC-treated mice. Confocal microscopy and quantitative polymerase chain reaction revealed a large quantity of transplanted BMC in all organs tested, from 5% to 19% of the total cells. Most of these cells were not from the lymphoid lineage but part of the intrinsic structure of the organ. The natural progression of renal dysfunction was prevented, and deposition of corneal cystine crystals was significantly improved in the BMC-treated mice. HSC had the same therapeutic effect as whole BMC. In contrast, mesenchymal stem cell did not integrate efficiently in any organ. This work is a proof of concept for using HSC transplantation as a therapy for cystinosis and highlights the efficiency of this strategy for a chronic, progressive degenerative disease. (Blood. 2009; 114:2542-2552)

Introduction

Cystinosis is an autosomal metabolic hereditary disease characterized clinically by generalized proximal renal tubular dysfunction (Fanconi syndrome) and biochemically by lysosomal accumulation of cystine, which leads to the formation of cystine crystals. Cystinosis belongs to the family of lysosomal storage disorders (LSDs) characterized by the tissue accumulation of incompletely degraded substrates leading to multiple organ dysfunction. The gene underlying cystinosis, CTNS, encodes a 7-transmembrane domain protein, cystinosin,1 a lysosomal cystine transporter.2,3 Affected persons typically present before 2 years of age with symptoms of severe fluid and electrolyte disturbances (ie, dehydration, vomiting, poor growth, rickets). Without specific treatment, they progress to end-stage renal failure by the end of the first decade; in the United States, cystinosis accounts for approximately 1.4% of children on dialysis and 2% of pediatric kidney transplantsations (North American Pediatric Renal Trials and Collaborative Studies, 2008 Annual Report).5 Cystine accumulation eventually leads to multiorgan dysfunction and patients present with photophobia and blindness, hypothyroidism, hypogonadism, diabetes, myopathy, and central nervous system defects.6

Different treatments have been tested for LSD, including enzyme replacement, substrate depletion, and bone marrow transplantation.7 For cystinosis, substrate depletion with the drug cysteamine reduces the intracellular concentration of cystine. If used early in the disease and in high doses, it can reduce the subsequent progression of renal glomerular damage and other defects.8 However, the need for frequent dosing and multiple undesirable side effects, such as digestive intolerance and persistent odor, render its chronic administration difficult. Moreover, the proximal renal tubulopathy is not sensitive to cysteamine.

Over the past 2 decades, several reports have established a proof of principle for allogeneic bone marrow stem cell transplantation in several LSDs, including Hurler disease (MPS-I), globoid-cell leukodystrophy (Krabbe disease), and adrenoleukodystrophy.9,10 Treatment of Hurler patients performed before the age of 2 years has been the most gratifying, and the reconstitution of enzymatic activity is correlated with prolonged survival and, in some cases, normal or near-normal cognitive development and myocardial function.11-13

Cystinosin is a lysosomal transmembrane protein that cannot be secreted. Therefore, in contrast to Hurler syndrome, the normal enzyme produced by tissue-engrafted cells cannot spread to be recaptured by other cells lacking a functional Ctns gene. Therefore, a proof of concept for bone marrow cell transplantation as a therapy for cystinosis is necessary. We used the mouse model, Ctns−/− mice, which accumulate cystine and cystine crystals in all organs tested.14 Ctns−/− mice develop ocular changes similar to those observed in affected patients, bone and muscular defects, and behavioral anomalies. Ctns−/− mice backcrossed on a C57BL/6 background develop an incomplete proximal tubulopathy and renal failure by 15 months of age.15 Here we report that syngeneic bone marrow transplantation from wild-type (WT) donors into Ctns−/− mice successfully protected these animals from the progression of the kidney tissue injury and corneal cystine deposition that represent 2 of the major clinical problems faced by children and young adults with this genetic disorder. There was significant engraftment of donor bone


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marrow–derived cells in every tissue compartment tested as determined by confocal microscopy and quantitative polymerase chain reaction (PCR). Mechanistically, only engraftment of bone marrow cells producing a functional Ctns was able to reverse the disease process. Second, quantitative expression of functional Ctns and tissue colonization by bone marrow cell (BMC)–derived cells measured by luciferase imaging in these animals correlated in each tissue compartment with reduction of cystine levels of 57% to 94% as measured by tandem mass spectrometry.

**Methods**

**Mice**

C57BL/6 Ctns−/− mice were provided by Dr C. Antignac (Inserm U574) and bred continuously at the Scripps Research Institute. Transgenic mice constitutively expressing green fluorescent protein (GFP; C57BL/6-Tg(Actb-EGFP)10bsj) were obtained from The Jackson Laboratory. Transgenic mice constitutively expressing firefly luciferase were provided by Dr M. Geusz (Bowling Green State University). All protocols were approved by the Association for Assessment and Accreditation of Laboratory Animal Care–Accredited Institutional Animal Care and Use Committee of the Scripps Research Institute.

**BMC and HSC isolation, MSC generation, and cell transplantation**

BMCs were flushed from the long bones of 6- to 8-week-old mice and BMC and HSC isolation, MSC generation, and cell transplantation of the Scripps Research Institute. Methods were performed using the PAXgene Blood RNA Kit (PreAnalytix QIAGEN). Ctns-specific quantitative PCR was performed using 2 μL cDNA and 2× Universal TaqMan Master Mix (Roche Diagnostics). Ctns primer mix (in Ctns exon 8: Ctns oligo 1: TTGGTGGCTGCACTGGATATC, Ctns oligo 2: AGCTGTGATGAGAAGAGAAGA, and Ctns probe: CACATGT-GCTCCAGTTC (Applied Biosystems) and 18s primer mix (Applied Biosystems) on an Applied Biosystems 7900 HT.

**Immunofluorescence analysis**

Tissues were fixed in formaldehyde 5%, equilibrated in sucrose 20% overnight, and frozen in Tissue-Tek Optimal Cutting Temperature buffer at −80°C (Sakura Finetek). Twelve- to 20-μm-thick sections were cut and blocked with 1% bovine serum albumin, 10% donkey serum in phosphate-buffered saline. The blocking buffer was then diluted 1:5 in phosphate-buffered saline and antibody added in the dilutions denoted below and incubated with sections at room temperature for 1 hour; the lectins were incubated for 2 hours. Each tissue was stained with 4,6-diamidino-2-phenylindole (DAPI) and Bodipy-phalloidin (1:500 dilutions for 30 minutes; Invitrogen) or Cy5-conjugated anti-F4/80 antibody (1:50; Serotec) and biotinylated anti–mouse CD45 antibody (1:500 dilution; BD Biosciences Pharmingen) followed by Alexa 594–conjugated streptavidin (1:100; Invitrogen). Kidney sections were stained with rhodamine-conjugated Dolichos biflorus agglutinin (1:100 dilution), rhodamine-conjugated Ricinus communis agglutinin I (1:250 dilution), biotinylated Lotus tetragonolobus lectin (1:100 dilution; Vector Laboratories) followed by Alexa 594–conjugated streptavidin, and rabbit anti-human von Willebrand factor (VWF; 1:200 dilution; Dako) followed by donkey anti–rabbit IgG conjugated with Cy5 (1:100 dilution). Brain sections were stained with a mouse pan antiauxonal microfilament antibody (1:250 dilution; Covance) and a mouse antilgal fibrillary acidic protein (1:500 dilution; Millipore), followed by a donkey anti–mouse IgG conjugated with Alexa 594 (1:100 dilution; Jackson Immunoresearch Laboratories). Images were acquired using a Radiance 2100 Laser Scanning Confocal system attached to a Nikon TE2000-U inverted microscope (Bio-Rad–Carl Zeiss). All images were 8-bit optical image slices (0.5-μm interval step slices) acquired using LaserSharp 2000 software. Images were then analyzed with IMARIS 4.2 imaging software (Bitplane Scientific Solutions) to generate 3-dimensional reconstruction series of optical slices (Z-stacks).

**Tandem scanning reflectance confocal microscopy**

Confocal reflectance microscopy was performed on enucleated mouse eyes of various ages. Examinations were performed using a Tandem Scanning Confocal Microscope (Tandem Scanning Corporation) with a 24× surface-contact objective (numerical aperture, 0.6; working distance, 1.5 mm), Oriel 18 011 Encoder Mike Controller (Oriel Corp) for focal plane control, and Dage MTI VE-1000 camera (Dage MTI). One drop of artificial tear solution was placed on the tip of the objective as a coupling gel. All camera settings were kept constant throughout the experiment. For each examination, repeated through-focus datasets were obtained from the peripheral cornea and limbal region to identify cystine crystals and GFP-positive cells.

**Cystine content measurement**

Explanted tissues were grounded in 750 μL N-ethylmaleimide (Fluka Biochemika) at 650 μg/mL for cystine measurements. The proteins were precipitated using 15% 5-sulfosalicylic acid dihydrate (Fluka Biochemika), resuspended in NaOH 0.1 N, and measured using the Pierce BCA protein assay kit. The cystine-containing supernatants were sent to the University of
Urine to 90% in mice transplanted with WT BMCs and 3% to 6% in mice transplanted with BMCs. The survival rate for BMC-transplanted mice after lethal irradiation was 93%, and all the surviving mice exhibited benefits. The contribution of donor-derived cells within each lineage was determined for 5 mice transplanted with GFP-transgenic BMCs and for which the peripheral blood engraftment was 72% plus or minus 17%; 86% plus or minus 7% were GFP-positive T lymphocytes, 69% plus or minus 11% were B lymphocytes, and 45% plus or minus 21% were macrophages.

Kidney

In all the experimental groups described in the first paragraph of “Results,” renal function was assessed by measuring creatinine, urea, alkaline phosphatase, and phosphate levels in the serum and creatinine clearance, protein, and phosphate in 24-hour urine collections. Serum creatinine and urea of Ctns−/− mice transplanted with WT BMCs were significantly better than control Ctns−/− mice and Ctns−/− mice transplanted with Ctns−/− BMCs (Table 1). Moreover, kidney function of mice transplanted with WT BMCs was not significantly different from WT controls. Serum urea was increased in Ctns−/− mice treated with WT MSC compared with Ctns−/− mice treated with WT BMCs, but the creatinine levels were normal.

Kidney sections for each group of mice defined in the first paragraph were analyzed by confocal microscopy for the presence of GFP-positive cells. We did not observe any GFP-positive cells in Ctns−/− mice treated with Ctns−/− BMCs (Figure 1A-B) as a negative control for imaging of GFP in the experimental groups. Only few GFP-positive cells were observed in mice transplanted with GFP BMCs (< 5 cells per section; data not shown). WT mice treated with WT BMCs isolated from GFP-transgenic mice (GFP BMCs) also revealed very few GFP-positive cells in the kidney (Figure 1C-D). In contrast, Ctns−/− mice transplanted with GFP BMC showed abundant BMC-derived cells (Figure 1E-F).

Most of the GFP-positive cells in Ctns−/− mice transplanted with GFP BMCs were interstitial, but only a few were of lymphoid or macrophage lineage as defined by CD45 and F4/80 staining, respectively (Figure 2A). GFP-positive cells colocalized with proximal and distal tubular cells and some were in glomeruli (Figure 2B-D). Finally, GFP-positive cells were colocalized to tubular and glomerular basement membranes or with endothelial cells (Figure 2E-F).

Eye and brain

One eye per mouse was analyzed by confocal microscopy for the presence or absence of corneal cystine crystals and GFP-positive

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**Table 1. Serum and urine analyses for renal function (n = 12 per group)**

<table>
<thead>
<tr>
<th>Serum</th>
<th>Wild-type controls</th>
<th>Ctns−/− controls</th>
<th>WT BMCs</th>
<th>Ctns−/− BMCs</th>
<th>WT MSCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine, mg/dL</td>
<td>0.34 ± 0.06</td>
<td>0.49 ± 0.30†</td>
<td>0.35 ± 0.07</td>
<td>0.43 ± 0.10†</td>
<td>0.38 ± 0.11</td>
</tr>
<tr>
<td>Creatinine clearance, μL/min</td>
<td>79.40 ± 74.24</td>
<td>32.28 ± 42.58</td>
<td>65.65 ± 72.34</td>
<td>43.04 ± 34.46</td>
<td>85.35 ± 64.60</td>
</tr>
<tr>
<td>Urea, mg/dL</td>
<td>36.54 ± 1.55</td>
<td>56.92 ± 18.41†</td>
<td>23.83 ± 19.62</td>
<td>74.95 ± 17.32†</td>
<td>41.98 ± 4.85†</td>
</tr>
<tr>
<td>Phosphate, mg/dL</td>
<td>18.31 ± 3.06</td>
<td>23.09 ± 4.70†</td>
<td>10.95 ± 6.82</td>
<td>10.57 ± 7.53</td>
<td>11.86 ± 6.35</td>
</tr>
<tr>
<td>Alkaline phosphatase, IU/L</td>
<td>60.54 ± 23.57</td>
<td>67.35 ± 26.75</td>
<td>75.77 ± 21.10</td>
<td>67.76 ± 18.10</td>
<td>55.73 ± 16.20</td>
</tr>
</tbody>
</table>

- Wild-type controls indicate nontreated WT C57BL/6 mice; Ctns−/− controls, nontreated C57BL/6 Ctns−/− mice; WT BMCs, Ctns−/− mice treated with WT BMCs; Ctns−/− BMCs, Ctns−/− mice treated with Ctns−/− BMCs; and WT MSCs, Ctns−/− mice treated with WT MSCs.
- *P < .05 vs wild-type mice.
- †P < .05 vs Ctns−/− WT BMCs.

California, San Diego Biochemical Genetics laboratory for measurements by mass spectrometry.

**Live animal luciferase imaging**

Mice transplanted with BMCs isolated from luciferase transgenic mice were measured at 2, 3, and 4 months after transplantation using the IVIS Imaging System 200 Series (Caliper Life Sciences). First, the mice were anesthetized, and 75 μL luciferin (30 mg/mL; Caliper Life Sciences) was injected intraperitoneally 10 minutes before the observation. Quantitative signal analysis was done using the Living Image 2.5 software (Caliper Life Sciences).

**Statistics**

We expressed data as arithmetic mean plus or minus SD and performed statistical analysis using one-tailed t tests as the samples were large enough and normally distributed. A P value less than .05 was considered statistically significant. Corneal cystine crystals were recorded as a simple present or absent result, and statistics were done by χ² analysis.

**Results**

Freshly harvested BMCs were used for transplantation. MSCs were prepared by culture following Dr Mereilles’ protocol and used at passage 20. The phenotype of the MSCs was tested and confirmed by flow cytometry at the time of transplantation: positive for CD44, CD90.2, and CD105 and negative for CD45, CD31, and CD34 (data not shown). We transplanted 2 × 10⁷ WT C57BL/6 BMCs or 10⁵ MSC in lethally or sublethally irradiated Ctns−/− mice, respectively. As controls, we transplanted 2 × 10⁷ Ctns−/− BMCs into lethally irradiated Ctns−/− mice. The groups were WT mice, untreated Ctns−/− mice, WT BMC-treated Ctns−/− mice, and Ctns−/− BMC-treated Ctns−/− mice. Within each group, the animals were age-matched littermates and a mix of males and females. Mice were between 2 and 4 months of age when transplanted and killed 4 months later for analysis. These experiments were repeated 3 times (n = 5 mice) for a total of 15 mice per group. Three mice per group were killed 2 months after injection to determine whether tissue integration was increasing with time. To allow tracking of cells by confocal microscopy, we transplanted 4 Ctns−/− mice and 4 WT controls with BMCs from GFP-transgenic mice. All transplanted MSCs were from GFP-transgenic mice.

Cell engraftment was determined in peripheral blood by quantifying either GFP-positive cells by flow cytometry or by quantitative RT-PCR for the WT Ctns gene. Engraftment ranged from 5% to 90% in mice transplanted with WT BMCs and 3% to 6% in mice transplanted with MSCs. The survival rate for BMC-transplanted mice after lethal irradiation was 93%, and all the surviving mice exhibited benefits. The contribution of donor-derived cells within each lineage was determined for 5 mice transplanted with GFP-transgenic BMCs and for which the peripheral blood engraftment was 72% plus or minus 17%; 86% plus or minus 7% were GFP-positive T lymphocytes, 69% plus or minus 11% were B lymphocytes, and 45% plus or minus 21% were macrophages.
cells. Cystine crystals were detected only in mice more than 7 months of age. Of 7 Ctns/H11002/BMC-treated Ctns/H11002/BMC mice, 6 presented cystine crystals (Figure 3D). However, in 7 age-matched WT BMC-treated mice, only 3 had cystine crystals in the cornea (treated vs nontreated, \( P = .05 \)). GFP-positive cells were observed in the peripheral cornea, localized to the corneal stroma (Figure 3A) and adjacent to the basal cells of the corneal epithelium (Figure 3B). GFP-positive cells generally had a dendritic (Langerhans) cell–like morphology. At the vascularized limbus, a heavy perivascular infiltrate was also observed (Figure 3C).

Muscle, spleen, liver, and heart

Mice transplanted with MSCs present with very few GFP-positive cells in muscle, spleen, liver, and heart. In contrast, mice that received BMCs had a large quantity of transplanted BMC-derived cells in all these tissues. In the muscle, most of the GFP-positive cells were nonlymphoid lineage interstitial cells. However, the observation of some GFP-positive muscle cells indicated a subset of transplanted BMC-derived cells probably fused with muscle fibers (Figure 4A-C). The few GFP-positive cells were macrophages (white, arrows). In the liver, the GFP-positive cells were interstitial (data not shown). In the heart, most of the GFP-positive cells were Kupffer cells, colocalizing with macrophages (F4/80) and leukocyte lineage cells (CD45\(^+\); data not shown). Spleen demonstrated the most GFP-positive cells of any tissue. Surprisingly, the majority were not of the lymphoid lineage (CD45\(^+\) and
tissues in Ctns−/− BMC-treated mice as a function of time. In MSC-treated mice, a decrease in cystine level was significant only in heart at 2 months, but levels increased at 4 months. In contrast, as early as 2 months, Ctns−/− mice transplanted with WT BMCs demonstrated a decrease in cystine content compared with Ctns−/− BMC-treated mice. This cystine content decrease was observed in all the tissues, except the kidney, but was only statistically significant in the spleen and liver. By 4 months, cystine levels were significantly less in WT BMC-treated mice in all the tissues tested, ranging from a 57% decrease in the brain to 94% in the liver.

Quantitative RT-PCR with Ctns-specific primers was performed in the same tissues at 2 and 4 months after transplantation (Table 3). No Ctns expression was detected in Ctns−/− BMC-treated mice. The results confirmed the high level of engraftment observed for WT BMCs and the low engraftment of MSC-derived cells. Thus, Ctns expression decreased in MSC-treated mice in all tissues, except spleen between 2 and 4 months. Ctns expression was highest in mice treated with WT BMCs in the spleen, confirming the confocal microscopy data (Figure 4). To show the changes in time, we normalized the quantitative RT-PCR values for Ctns expression at 2 and 4 months by taking the ratio of the WT values to the Ctns−/− mice transplanted with WT BMCs. These results demonstrate that Ctns expression increased between 2 and 4 months after injection in every tissue (Figure 6B). By 4 months, Ctns expression ranged between 5.4% of the total Ctns expressed in WT mice in muscle to 19.3% in spleen.

Finally, to follow the fate of transplanted BMCs expressing a functional Ctns gene in live animals as a function of time, we transplanted 10 Ctns−/− mice and 5 WT controls with BMCs isolated from luciferase-transgenic mice. The mice were imaged at 2, 3, and 4 months after transplantation, and luciferase expression was quantitatively measured. At each time point, 4 pictures for each mouse were taken, representing the ventral, dorsal, left, and right side views. Figure 6A is a representative ventral view picture of the same Ctns−/− and WT mice at the 3 different time points. The significant increase of luciferase intensity and tissue area with time is clearly evident in the Ctns−/− mouse and correlates with our evidence for the engraftment of BMC-derived cells in multiple tissues. In contrast, in WT mice, the luciferase expression remains minimal over time.

HSCs

We showed that whole BMCs efficiently integrated in multiple organs, resulting in significant decreases of cystine content in these tissues. BMC is a heterogeneous mixture of cells that contains a small number of HSCs. For the purpose of a clinical application, we need to know if whole bone marrow is necessary to achieve our objectives or if similar results can be obtained with purified HSCs only. We lethally irradiated and transplanted 10 2-month-old Ctns−/− mice with Scal + HSCs isolated from GFP-transgenic mice. These were all killed at 4 months after transplantation for analysis. All these mice had high levels of peripheral blood engraftment of transplanted cells, ranging from 73% to 94% (hematopoietic lineage chimerism: 95% ± 3% for T lymphocytes, 65% ± 14% for B lymphocytes, and 50% ± 14% for macrophages). Controls included nontreated Ctns−/− mice and WT animals at the same ages. Mice transplanted with Scal + GFP HSCs showed abundant GFP-positive cells within all the tissues tested by confocal microscopy (data not shown). Quantitative RT-PCR with Ctns-specific primers showed a high level of engraftment of Scal + HSC-derived cells in all the tissues tested of Ctns−/−-treated mice (Table 4). The relative expression of Ctns gene in HSC-treated
**Table 2. Cystine content in different tissues of treated and control mice at 2 and 4 months after transplantation**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Wild-type controls</th>
<th>Ctns&lt;sup&gt;−/−&lt;/sup&gt; BMCS</th>
<th>WT BMCS</th>
<th>WT MSCs</th>
<th>Wild-type controls</th>
<th>Ctns&lt;sup&gt;−/−&lt;/sup&gt; BMCS</th>
<th>WT BMCS</th>
<th>WT MSCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>0.02 ± 0.00</td>
<td>0.70 ± 0.15</td>
<td>0.61 ± 0.29</td>
<td>0.75 ± 0.28</td>
<td>0.02 ± 0.00</td>
<td>1.38 ± 0.45</td>
<td>0.59 ± 0.32*</td>
<td>0.62 ± 0.39*</td>
</tr>
<tr>
<td>Eye</td>
<td>0.07 ± 0.20</td>
<td>20 ± 11</td>
<td>18 ± 11</td>
<td>27 ± 4</td>
<td>0.1 ± 0.02</td>
<td>45 ± 12</td>
<td>13 ± 8</td>
<td>36 ± 9</td>
</tr>
<tr>
<td>Heart</td>
<td>0.17 ± 0.10</td>
<td>25 ± 7</td>
<td>23 ± 16</td>
<td>10 ± 1*</td>
<td>0.08 ± 0.10</td>
<td>61 ± 15</td>
<td>11 ± 8</td>
<td>25 ± 9</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.18 ± 0.05</td>
<td>76 ± 16</td>
<td>135 ± 12</td>
<td>45 ± 24</td>
<td>0.25 ± 0.06</td>
<td>116 ± 63</td>
<td>35 ± 34*</td>
<td>59 ± 34*</td>
</tr>
<tr>
<td>Liver</td>
<td>0.02 ± 0.01</td>
<td>57 ± 3</td>
<td>6 ± 0.2*</td>
<td>62 ± 23</td>
<td>0.02 ± 0.02</td>
<td>62 ± 19</td>
<td>3 ± 1</td>
<td>51 ± 15</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.23 ± 0.13</td>
<td>17 ± 7</td>
<td>15 ± 2</td>
<td>10 ± 0.7</td>
<td>0.07 ± 0.20</td>
<td>30 ± 11</td>
<td>10 ± 6</td>
<td>22 ± 4</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.06 ± 0.04</td>
<td>91 ± 39</td>
<td>38 ± 5*</td>
<td>124 ± 35</td>
<td>0.12 ± 0.03</td>
<td>160 ± 50</td>
<td>21 ± 19*</td>
<td>95 ± 17*</td>
</tr>
</tbody>
</table>

Cystine content is stated in nmol half-cystine/mg protein.

*P* < .05 versus mice treated with Ctns<sup>−/−</sup> BMCS.
infused WT BMCs expressing functional Ctns. These BMC-derived cells were predominately nonlymphoid lineage interstitial cells but also colocalized with distal and proximal tubular, glomerular, and endothelial cells. Moreover, the treated mice exhibited normal serum urea and creatinine levels in contrast to control mice treated with Ctns−/− BMCs. The renal disease in children with cystinosis starts as a proximal tubulopathy before 1 year of age and then evolves to a progressive loss of glomerular function.6 Cystine crystals are not abundant in the kidney and are mostly observed in interstitial cells, occasionally in glomerular cells, and rarely in

**Table 3. Ctns expression in different tissues of treated and control mice at 2 and 4 months after transplantation**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Wild-type controls</th>
<th>Ctns−/− BMC</th>
<th>WT BMCS</th>
<th>2 months (n = 3 animals per group)</th>
<th>4 months (n = 12 animals per group)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>40 352 ± 41 063</td>
<td>ND</td>
<td>694 ± 842</td>
<td>2 ± 2</td>
<td>8362 ± 3814</td>
</tr>
<tr>
<td></td>
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<td></td>
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<td></td>
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</tr>
<tr>
<td>Eye</td>
<td>124 553 ± 17 116</td>
<td>ND</td>
<td>14 722 ± 20 816</td>
<td>12 ± 13</td>
<td>52 942 ± 35 772</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>44 510 ± 15 675</td>
<td>ND</td>
<td>3865 ± 5415</td>
<td>2 ± 1</td>
<td>27 089 ± 13 649</td>
</tr>
<tr>
<td></td>
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<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>304 504 ± 95 560</td>
<td>ND</td>
<td>6569 ± 1749</td>
<td>31 ± 54</td>
<td>47 709 ± 26 694</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td>Liver</td>
<td>38 616 ± 32 898</td>
<td>ND</td>
<td>674 ± 319</td>
<td>15 ± 23</td>
<td>180 281 ± 15 749</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscle</td>
<td>36 233 ± 15 457</td>
<td>ND</td>
<td>342 ± 119</td>
<td>ND</td>
<td>28 148 ± 12 428</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>185 567 ± 24 855</td>
<td>ND</td>
<td>1375 ± 968</td>
<td>3 ± 3</td>
<td>136 147 ± 11 703</td>
</tr>
</tbody>
</table>

Relative expression of Ctns as 18S-normalized fold changes is shown. ND indicates not detected.
tubular cells. Ctns mice develop an incomplete renal disease, but like the human patients, cystine crystals accumulate progressively within interstitial cells. Thus, BMC-derived interstitial cells in our transplanted mice may have a significant, positive effect on the progression of the underlying kidney disease.

On the other hand, MSCs did not integrate efficiently within the kidney in our model. Nonetheless, MSC transplantation led to some improvement in renal function compared with control Ctns mice transplanted with BMCs. However, after an initial decrease in cystine content at 2 months, cystine levels increased thereafter. In the literature, the potential for MSC transplantation to improve kidney failure remains controversial. Several studies showed that, in the setting of renal injury, transplanted MSCs can generate mesangial and tubular epithelial cells and restore renal structure and function. Others showed that MSC injections do not prevent Alport syndrome in COL4A3-deficient mice and MSCs do not differentiate into renal cells. The protective effects of MSCs seem to be the result of a paracrine effect of these cells by secretion of growth factors, such as vascular endothelial growth factor and transforming growth factor-β1, rather than cellular differentiation. This mechanism could explain the initial improvement we observed in the Ctns mice.

We evaluated the engraftment of BMC-derived cells in multiple tissues based on 3 metrics: GFP-positive cells identified by confocal microscopy, quantitative PCR for Ctns expression, and cystine content determined by mass spectrometry. The results by all these metrics showed high levels of engraftment in eye, brain, muscle, liver, spleen, and heart. The nature of cystinosis as a clinical entity is a progressive dysfunction of multiple organs caused by the accumulation of cystine in the tissues; thus, the findings here underline the potential utility of BMC transplantation for this genetic disease. Of particular importance is that, although cystine levels continued to increase in the Ctns mice as a function of time, our data demonstrated that successful transplantation of WT BMCs reversed this accumulation.

Table 4. Ctns expression in the different tissues of mice treated with Sca1 HSC compared with wild-type and Ctns controls

<table>
<thead>
<tr>
<th>Relative expression of Ctns as fold changes*</th>
<th>Wild-type mice</th>
<th>Ctns−/− mice</th>
<th>Ctns−/− mice Sca1 HSC</th>
<th>Percentage Ctns in Sca1-transplanted vs wild-type mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>93 381 ± 93 056</td>
<td>ND</td>
<td>1940 ± 2201</td>
<td>2.1</td>
</tr>
<tr>
<td>Eye</td>
<td>780 359 ± 699 835</td>
<td>ND</td>
<td>34 532 ± 26 910</td>
<td>4.4</td>
</tr>
<tr>
<td>Heart</td>
<td>204 857 ± 143 183</td>
<td>ND</td>
<td>8725 ± 3488</td>
<td>4.3</td>
</tr>
<tr>
<td>Kidney</td>
<td>72 120 ± 36 321</td>
<td>ND</td>
<td>6291 ± 7818</td>
<td>8.7</td>
</tr>
<tr>
<td>Liver</td>
<td>26 656 ± 21 901</td>
<td>ND</td>
<td>3588 ± 2558</td>
<td>13.5</td>
</tr>
<tr>
<td>Muscle</td>
<td>46 125 ± 11 840</td>
<td>ND</td>
<td>3288 ± 2923</td>
<td>7.1</td>
</tr>
<tr>
<td>Spleen</td>
<td>19 634 ± 3603</td>
<td>ND</td>
<td>15 218 ± 3590</td>
<td>77.5</td>
</tr>
</tbody>
</table>

ND indicates not detected.

*18S-normalized fold changes.
In patients, corneal cystine crystals appear from the first decade of life, resulting in photophobia and visual impairment. Cystine crystals are found first in the periphery of the cornea and then in the center. Ctns mice demonstrate similar ocular anomalies to patients, and cystine crystals are also first found at the periphery of the cornea. In addition to the decreased cystine content, BMC transplantation led to a significant decrease in the number of mice with corneal cystine crystals (43% WT BMCS vs 86% of Ctns BMCs; P < .05). In the third decade of life, patients with cystinosin exhibit a deterioration of the central nervous system characterized by mental deterioration, impaired cerebellar function, pyramidal signs, ischemic lesions, and severe impairment in visual short-term memory. Middle-aged Ctns mice also present with behavioral anomalies and spatial and working memory deficits that correlate with cystine accumulation in the brain. In the present study, we demonstrated Ctns expressing BMC-derived cells in the brain, differentiated into or fused with endothelial or smooth muscle cells, as well as some glial-like cells. For another LSD, metachromatic leukodystrophy, partial or total correction was observed after BMC transplantation in mice. BMC-derived cells also represent less than 10% of the total cells in the liver. It is also interesting to note that the addition of the CTNS gene to hematopoietic cells results in a significant decrease in cystine levels in our model as Ctns mice transplanted with Ctns BMCs still accumulated cystine and developed kidney dysfunction. The fact that lethally irradiated WT mice exhibited few transplanted BMC-derived cells in the tissues, as shown by confocal microscopy for GFP-positive cells and IVIS imaging for luciferase-expressing cells, proves that the extensive colonization of Ctns mice by WT BMC-derived cells is specifically the result of the impact of cystinosin. The few BMC-derived cells observed in WT mice might be myeloid and lymphoid lineage fusion hybrids that can occur after lethal irradiation of mice. In contrast, Ctns mice exhibited abundant BMC-derived cells (eg, GFP-positive) that colocalized with tissue-specific cell phenotypes, and the studies of luciferase BMC-derived cells in live animals confirmed the increase in engraftment over time. Moreover, most of these cells were not from the lymphoid lineage (specifically, not macrophages), even in the spleen, but were part of the intrinsic structure of the organ. Tissue engraftment of bone marrow–derived cells can occur through cell differentiation or cell fusion. For instance, bone marrow cells can give rise to endothelial, smooth muscle reticuloendothelial, and Kupffer cells through differentiation and skeletal muscle through fusion. However, this field is still controversial; and, in our case, the mechanism remains to be established.

Taken together, the literature on cystinosin and our data indicate that Ctns expressing BMC replace or fuse with cells that accumulate the most cystine crystals in each tissue. Thus, BMC transplantation creates a reservoir of healthy cells that migrate to the targeted organ as a function of progressive cellular injury and may replace or fuse with the cells accumulating the highest levels of cystine, many of which are interstitial and reticuloendothelial cells. Consistent with this conclusion, Hippert et al depleted 75% of the liver cystine content in Ctns mice by chemically depleting Kupffer cells representing less than 10% of the total cells in the liver. It is also interesting to note that the addition of the CTNS gene to Ctns hepatocytes in culture resulted in a significant decrease in cystine levels. We have confirmed these experiments using Ctns fibroblasts (data not shown). In sum, these data add additional support to the conclusion that there is a direct relationship between expression of a functional protein and the reduction in cystine levels.

Cystinosin is a transmembrane protein localized to the intracellular lysosomal membrane. Thus, cells lacking Ctns cannot recapitulate the normal protein from transplanted WT cells, even in close proximity. In contrast, in Hurler syndrome, normal enzyme can be secreted by BMC tissue-engrafted cells and taken up by adjacent host cells. Indeed, in a murine model of Hurler syndrome treated by transplantation of retroviral-transduced bone marrow cells, tissue engraftment of transgene-positive cells was low, primarily limited to spleen and liver. Nonetheless, glycosaminoglycan levels decreased in all the tissues tested, and that effect was explained by...
uptake of the secreted enzyme by adjacent cells.\textsuperscript{50,51} In contrast, in our studies, the mechanism underlying the decrease of cystine in all tissues involves the localization of cells expressing the functional gene and, thus, is correlated with the abundant engraftment of BMC-derived cells. Therefore, BMC transplantation for cystinosis is an example where therapeutic efficacy requires the local integration of cells with the functional protein rather than local delivery of a missing enzyme like many LSDs.

HSC transplantation, modeled in mice by purification of Sca1\(^+\) HSCs, has the same therapeutic effects as whole bone marrow cell transplantation in the mouse model of cystinosis, including high levels of tissue engraftment and significant decreases in cystine content. This is important because human HSCs (CD34\(^+\)) are readily isolated from peripheral blood after growth factor-mediated mobilization (ie, granulocyte-macrophage colony-stimulating factor) and this population is significantly enriched for the true HSCs. Moreover, future ex vivo gene delivery to introduce a functional Ctns gene will be more efficient with purified HSCs than with whole BMCs.

This work represents the first step toward a clinical trial of using bone marrow cell transplantation as a therapy for cystinosis. This disease, like many lysosomal storage disorders, is a slowly progressive process that represents an ongoing cystine accumulation, leading downstream to cell stress, cell death, tissue injury, and eventually organ dysfunction, including kidney failure, blindness, and progressive myopathy. The permanent engraftment of bone marrow cells expressing a fully functional Ctns gene would provide a continuous source of healthy cells that could traffic to different tissue compartments where cells are stressed or dying from cystine accumulation. We have demonstrated the high efficiency of this strategy in the cystinosis model as determined objectively by significant levels of bone marrow–derived cell engraftment in multiple tissue compartments correlated with improvements in measured cystine content and renal function. These results suggest that bone marrow cell or hematopoietic stem cell transplantation is particularly suitable for the chronic and progressive injury characteristic of cystinosis and potentially other lysosomal storage disorders in which mutations of intracellular and transmembrane proteins are involved.

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Authorship

Contribution: K.S., F.H., M.T., and S.C. performed research and analyzed data; S.C. and D.R.S. designed the project and wrote the manuscript; J.V.J. and J.S. performed the eye analysis; and S.R. contributed to the brain analysis.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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