Hematopoietic Stem Cell Transplantation and Lentiviral Vector-Based Gene Therapy for Krabbe’s Disease: Present Convictions and Future Prospects

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Currently, presymptomatic hematopoietic stem and progenitor cell transplantation (HSPCT) is the only therapeutic modality that alleviates Krabbe’s disease (KD)-induced central nervous system damage. However, all HSPCT-treated patients exhibit severe deterioration in peripheral nervous system function characterized by major motor and expressive language pathologies. We hypothesize that a combination of several mechanisms contribute to this phenomenon, including 1) nonoptimal conditioning protocols with consequent inefficient engraftment and biodistribution of donor-derived cells and 2) insufficient uptake of donor cell-secreted galactocerebrosidease (GALC) secondary to a naturally low expression level of the cation-independent mannose 6-phosphate-receptor (CI-MPR). We have characterized the effects of a busulfan (Bu) based conditioning regimen on the efficacy of HSPCT in prolonging twi mouse average life span. There was no correlation between the efficiency of bone marrow engraftment of donor cells and twi mouse average life span. HSPCT prolonged the average life span of twi mice, which directly correlated with the aggressiveness of the Bu-mediated conditioning protocols. HSPCT transduced with lentiviral vectors carrying the GALC cDNA under control of cell-specific promoters were efficiently engrafted in twi mouse bone marrow. To facilitate HSPCT-mediated correction of GALC deficiency in target cells expressing low levels of CI-MPR, a novel GALC-AErdb fusion whose uptake by host cells is mediated by an IGF2R-independent pathway may resolve this limitation. It appears that an aggressive busulfan conditioning protocol significantly extends the life span of twi mice.

SIGNIFICANCE

Although presymptomatic hematopoietic stem and progenitor cell transplantation (HSPCT) is the standard therapeutic modality for Krabbe’s disease, it fails to provide a curative solution. Newly developed lentiviral vectors carrying myeloid- and erythroid-specific promoters improve the therapeutic potential of HSPCT by facilitating GALC delivery to host HSPC without compromising their ability to engraft the host bone marrow. Low levels of CI-MPR (also known as IGF2R) may limit the efficacy of HSPCT for curing KD. A novel GALC-AErdb fusion whose uptake by host cells is mediated by an IGF2R-independent pathway may resolve this limitation. It appears that an aggressive busulfan conditioning protocol significantly extends the life span of twi mice.

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Neonatal bone marrow transplantation (BMT) provides significant (although incomplete) protection for early infantile Krabbe’s disease (KD) patients’ central nervous system (CNS), preserves their cognitive function, and appears to lengthen their survival (mean of 23–25 months without treatment) up to the second decade of life (Escolar et al., 2005; Duffner et al., 2009, 2011; Wasserstein et al., 2016). However, BMT cannot effectively alleviate the motor and sensory deficiencies caused by peripheral demyelination. This therapeutic approach is premised on the ability of the donor’s hematopoietic stem and progenitor cells (HSPC) and their progenies to colonize the host CNS and to secrete functional GALC, which upon mannose-6-phosphate (M6P)-mediated uptake compensates for the lack of host GALC function. Failure to efficiently complete any of these processes potentially contributes to the mechanism that renders KD incurable by HPCT. Host conditioning is aimed at generating space for donor HSPC in host target niches and is considered critical for efficient engraftment. Although not applicable for KD patients, total body irradiation (TBI) is the conditioning method employed in most HSPCT-based preclinical studies of KD (Lin et al., 2007; Gentner et al., 2010). Notwithstanding the major impact of the conditioning protocol on the clinical outcome of HSPCT in lysosomal storage disorder (LSD) patients, the effects of the aggressiveness of the conditioning protocol on the pathologic course of KD have not been evaluated in a preclinical model. The ability of GALC-deficient host cells to take up extracellular GALC efficiently is the premise of the HSPCT-based therapy for KD. Both the cation-dependent and the cation-independent M6P receptors (CD-MPR and CI-MPR) mediate intracellular trafficking of lysosomal enzymes. However, only the CI-MPR (also known as IGF2R) facilitates uptake of extracellular proteins via the M6P receptor (M6PR) pathway (Munier-Lehmann et al., 1996; Dahms et al., 2008; Stein et al., 2010). Several research groups have demonstrated that expression of IGF2R in rodent and human CNS is cell type specific (Nisletsky et al., 1993; Gonzalez-Parra et al., 2001; Hawkes and Kar, 2003, 2004; Jofre et al., 2009). Thus, naturally low levels of IGF2R expression in various cell populations could potentially reduce the therapeutic efficacy of HSPCT for KD patients. The ability to mediate cellular uptake of GALC via an M6P-independent pathway should address this potential weakness. Recent publications described the development of a fusion protein comprising the ApoE1-receptor binding domain (AErbd) and the lysosomal protein α-L-iduronidase (IDUA; Wang et al., 2013; Dai et al., 2014). This facilitated cellular uptake and transcytosis of the novel fusion protein to the CNS via the low-density lipoprotein (LDL) receptor-related protein 1 (LRP-1) pathway, independently of the M6P pathway. Premised on this technology, a novel GALC-AErbd fusion protein can potentially be used to treat cell populations that naturally do not express sufficient IGF2R.

To date, preclinical trials employing viral vectors for GALC delivery failed to significantly alter the pathologic course of KD in the twi mouse model (Lin et al., 2005, 2007; Gentner et al., 2010; Ungari et al., 2015). Notwithstanding the ongoing growth in the pool of potential bone marrow (BM) donors, it is still possible that the lack of a matched allogeneic donor will render KD patients unsuitable for HSPCT. The ability of lentiviral vectors to efficiently transduce and maintain long-term transgene expression in patients’ HSPC and their differentiated progenies has been employed in human clinical trials to establish therapeutic autologous HSPC-based gene replacement protocols for adrenoleukodystrophy (Cartier et al., 2009), metachromatic leukodystrophy (Biffi et al., 2013) and Wiscott-Aldrich syndrome (Aiuti et al., 2013) patients for whom a matched donor could not be identified. However, cytotoxicity associated with lentiviral vector-mediated GALC expression in HSPC poses a potential hurdle in employing the aforementioned therapeutic approach for KD patients (Gentner et al., 2010; Visigalli et al., 2010). By incorporating a tandem of target sequences of the HSPC-specific micro-RNA mir126 investigators (Gentner et al., 2010; Ungari et al., 2015) minimized GALC expression in HSPC and efficiently engulfed human and mouse HSPC that had been transduced with lentiviral vectors from which the human PGK promoter regulated expression of functional GALC mRNA. We assert that overexpression of target sequence of host miRNAs may alter the natural miRNA system (Ebert and Sharp, 2010). On the other hand, lentiviral vectors that express functional GALC under the control of cell-specific promoters would further enhance expression of GALC in the relevant HSPC progenies (e.g., microglia), with minimal GALC expression in HSPC. This strategy should facilitate therapeutic autologous HSPCT in KD patients without posing risk/benefit concerns associated with the overexpression of decoy micro-RNA targets.

MATERIALS AND METHODS

Cells

THP-1 cells were cultured in RPMI1640 (Hyclone, Logan, UT) with 10% FBS (Atlantic Biologicals, Miami, FL), 2 mM glutamine (Corning Cellgro, Manassas, VA), 100 U/ml
penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B (Corning Cellgro). Murine erythro leukemia (MEL) and 293T cells were maintained in DMEM High-Glucose (Hyclone) supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B. During the differentiation, MEL cells were induced by 5 mM hexamethylene bisacetamide (HMBA) and supplemented with 20% FBS. Lineage-negative cells purified using a mouse lineage cell depletion kit (Miltenyi Biotechnology, San Diego, CA) were cultured in Stem Span SFEM medium (Stemcell, Vancouver, British Columbia, Canada) with 100 ng/ml mouse stem cell factor (SCF), 100 ng/ml Flt3 ligand, 100 ng/ml IL3, and 100 ng/ml TPO (Sigma, St. Louis, MO).

**Plasmids**

The lentiviral vector packaging cassette ΔNRF and the VSV-G envelope plasmid were described previously (Kafri et al., 1999). Promoter sequences and codon-optimized GALC cDNA’s were synthesized by GeneArt (Thermo Fisher). The relevant DNA fragments were cloned into lentiviral vectors (pTK134 or pTK208). Vector structures were verified by restriction enzyme analysis and/or sequencing.

**Luciferase Assay**

Luciferase expression was measured by the Luciferase Assay system from Promega (Madison, WI). Briefly, virus-transduced cells were lysed with lysis buffer and analyzed on 1420 Multilabel Counter Victor 3 (PerkinElmer, Waltham, MA). Activities were further normalized with protein concentration and viral copy number (VCN) per cell. Protein concentration was determined by the Bradford method.

**VCN Quantification**

VCN was quantified by multiplex PCR (Suwanmanee et al., 2013) on an ABI7300 real-time PCR system. NotI794 primer/prober set (left primer 5'-taagaccaccgacagca-3', right primer 5'–cactctcagttgcra3'; No. 25; Roche Universal Probe Library [UPL]) was used for vectors detection, and paired with two different reference genes, mouse GAPDH primer/probe set or human GUSB primer/probe set (Roche, Indianopolis, IN). DNA samples were treated with dNTP 1 to minimize plasmid contamination before PCR analysis.

**293T Uptake and GALC Activity Assay**

Cells were incubated with medium containing different GALC variants at 37°C for 3 hr. After three PBS washes, cells were lysed with RIPA buffer on ice for 30 min. Cell lysates were cleared by centrifugation at 12,000 rpm for 5 min at 4°C and assayed for GALC activity. For M6P inhibition, 293T cells were pretreated with or without 1 mM M6P for 30 min, followed by incubation of conditioned media with different GALC proteins.

GALC activity assay was performed as described previously (Martino et al., 2009). Briefly, cells were lysed in RIPA buffer supplemented with protease inhibitors (Sigma). Proteins (10 μl, ~5–10 μg) were incubated with the artificial fluorogenic substrate 4-methylumbelliferone-galactopyranoside (1.5 mmol/liter) resuspended in 100 μl 0.1/0.2 mol/liter citrate/phosphate buffer, pH 4.0, in the presence of 11 μmol/liter AgNO3 at 37°C for 30 min, followed by treatment with 0.2 M sodium carbonate buffer. Fluorescence of liberated 4-MU was measured on the 1420 Multilabel Counter Victor 3. Free 4-methylumbelliferone (4-MU; Sigma) was used as a standard to calibrate β-galactosidase activity. Results were normalized with protein concentration.

**Primary Fibroblast Culture and GALC Activity Assay**

Human fibroblasts derived from two patients and two unaffected healthy donors (GM06806, GM04913, GM00041, GM08333; Coriell Institute) were seeded at a density of 10,000 cells/cm² in growth medium (DMEM, 15% FBS, 2 mM L-glutamine, nonessential amino acids, penicillin/streptomycin 100 U/ml; Thermo Scientific, Pleasanton, CA). After 2 days, the medium was replaced and changed daily with growth medium supplemented with supernatant derived from cells overexpressing GALC or GALC-AErdb and from cells transfected with the sole vector as a control. Sister cultures were also treated with 2.5 mM M6P. This treatment was carried out in duplicate for 3 days, after which the cells were washed twice with PBS, collected, pelleted, and resuspended in distilled H2O for GALC activity analysis. Cell suspensions were sonicated (three pulses, 3 sec each, 30% intensity) and used to perform the GALC activity assay, as described by Wiederschain et al. (1992). Briefly, 10 μl lysate was added to 20 μl of a substrate solution containing 6-hexadecanoylamino-4-methylumbelliferonyl-β-D-galactoside (HMU-β-GAL), mixed, and incubated for 17 hr at 37°C. After incubation, the reaction was terminated with a solution containing 0.2% SDS and Triton X-100, pH 10.7, and the fluorescence measured (ex 370 nm, em. 535 nm) by fluorometry. Results were normalized for protein content.

**Animals**

Female BoyJ mice (B6.SJL-Ptplca Pepcb/BoyJ; RRID:IMSR_JAX:002014) at age ~6–8 weeks were purchased from the Jackson Laboratory. Heterozygous twitcher (GALC+/−) mice on a congenic C57BL/6 background (RRID:IMSR_JAX:000845) were kindly provided by Dr. Steven J. Gray in Gene Therapy Center, University of North Carolina at Chapel Hill (UNC). The mouse colony was maintained under the supervision of T.K., and all procedures were approved by the Institutional animal care and use committee of UNC (IACUC 13-195.0). Genotyping was carried out by PCR with clipped toe DNA’s before postnatal day 8 (date of birth counted as day 0). Briefly, the toes were lysed in 25 mM NaOH/0.2 mM EDTA at 98°C for 90 min, followed by neutralization with same volume of 40 mM Tris (pH 5.5). PCR (98°C 3 min, followed by 40 repeated cycles of 98°C 10 sec, 62°C 15 sec, 72°C 20 sec) was performed with toe DNA and primer pair (left primer 5’-CACACACCCCCAGTTACTCAACC-3’, right primer 5’-GATGGCCCATGTCTTCCAG-3’; Precision Melt Supermix; Bio–Rad, Hercules, CA). Melting curve of knockouts, wild type, and heterozygotes was determined by using a Roche light Cycle 480. (The method was developed by Steven J. Gray in the Gene Therapy Center at UNC.) Endpoint reaching animals were euthanized by CO2 asphyxiation.
in accordance with UNC IACUC protocol (13-195.0). Endpoint criteria included: weight loss of more than 25% of body weight, difficulties in drinking, respiratory distress and severe hind leg paralysis.

**Brain Immunohistochemistry of L-Cycloserine-Treated twi Mice**

Animals received 25 mg/kg L-cycloserine (Sigma Aldrich) subcutaneously three times per week starting at postnatal day 8. On the next day, fresh donor bone marrow (BM) cells from BoyJ mouse tibia, femur, and pelvis were isolated, counted and resuspended in PBS. Each conditioned animal received approximately 5 x 10^7 total BM cells via i.p. injection.

For transduced lineage-negative BM cells, total BoyJ bone marrow were isolated on the same day of mice conditioning. Isolated Lin^- cells were transduced with lentiviral vectors (vTK1667 or vTK1784) at m.o.i. 50 (based on 293T cells) in culture medium with an additional 10 µg/ml rapamycin (Sigma) for ~14–16 hr. Transduced Lin^- cells were washed with PBS and mixed with fresh isolated total BM cells for transplantation. Each Bu-conditioned mouse received 1 x 10^7 Lin^- cells transduced with vTK1667, 1 x 10^6 Lin^- cells transduced with vTK1784, and 1 x 10^7 fresh isolated total BM cells from BoyJ mice.

**Viral Vector Production, Concentration, and Titration**

Lentiviral vector production transient three-plasmid transfection into 293T cells, and viral concentration were described by Kafri et al. (Kafri et al., 1999). The following plasmid amounts were used: 15 µg transfer cassette, 10 µg ΔNRF packaging construct, and 5 µg of the VSV-G envelope plasmid pMD.G. Viral vectors were concentrated by sucrose gradient ultracentrifugation. The emergence of replication competent retroviruses (RCRs) was ruled out by three independent safety assays (GFP rescue assay, Tat transfer assay, and Gag transfer assay) as described earlier. Titers of physical vector particles were determined by p24^agg ELISA using the National Institutes of Health p24 Antigen Capture Assay kit, as previously documented (Kantor et al., 2009). Infectious unit titers were determined by measuring VCN following infection on 293T cell.
therapeutic regimens for LSD. This reality renders KD patients for whom an HSPC donor could not be identified practically untreatable. The ability of lentiviral vectors to efficiently deliver functional cDNAs to patients' HSPC and to maintain therapeutic levels of transgene products in their progenitors opens a promising therapeutic avenue that can circumvent the need for a matching donor. However, recent studies focusing on lentiviral vector-mediated GALC delivery to HSPC indicated that GALC overexpression is toxic to HSPC and inhibits the engraftment in hematopoietic tissues (Gentner et al., 2010; Visigalli et al., 2010). We hypothesized that novel lentiviral vectors from which functional GALC cDNA is expressed under control of either myeloid or erythroid/megakaryocytic promoters would minimize toxic GALC expression in HSPC and would facilitate efficient engraftment of vector-transduced HSPC in hematopoietic tissues. To test this hypothesis, we developed a series of novel vectors from which the GFP and the firefly luciferase reporter genes, as well as a docod-optimized (CO) human GALC cDNA, were expressed under control of either a myeloid- or erythroid/megakaryocytic-specific promoters. The myeloid promoter was synthesized based on the nucleotide sequence of the 146gp91 promoter described earlier (He et al., 2006; Barde et al., 2011). The erythroid/megakaryocytic promoter IHK was developed and characterized earlier (Moreau–Gaudry et al., 2001). Lentiviral vectors carrying these cDNAs under control of a CMV promoter served as controls (Fig. 1A). Cell-specific gene expression from the aforementioned vectors was characterized in relevant cell lines. Specifically, THP-1 and hexamethylene bisacetamide (HMBA)-induced mouse erythroleukemia cells (MELs) served as myeloid and erythroid reporter cell lines, respectively. Vector-transduced 293T cells served as controls. As shown in Figure 1B–D and Table II, highly cell-specific gene expression was exhibited for the 146gp91 and the IHK promoters in the relevant THP-1 and HMBA-induced MEL cells, respectively. Furthermore, GFP expression under control of the 146gp91 promoter in BM cells expressing the myeloid marker Mac1 was significantly higher than the expression levels detected in vector-transduced Lin−/Kit−/CD11c− (LSK) HSPC (Fig. 1B). On the other hand, efficient GFP expression from lentiviral vectors carrying the CMV promoter was detected in total cultured lineage-negative (Lin−) cells or LSK cells following transduction with lentiviral vectors carrying the IHK promoter. Furthermore, a remarkable increase in GFP and GALC expression from the IHK promoter was observed following HMBA-mediated differentiation of MEL cells (Fig. 1B). In contrast, negligible GFP expression was detected in either total cultured lineage-negative (Lin−) cells or LSK cells following transduction with lentiviral vectors carrying the IHK promoter. In addition, CMV containing vectors exhibited significantly higher levels of transgene expression in 293T and LSK cells compared with the expression levels detected in vector-transduced Lin−/Kit−/CD11c− (LSK) HSPC (Fig. 1C). No GFP expression was detected in either total cultured lineage-negative (Lin−) cells or LSK cells following transduction with lentiviral vectors carrying the IHK promoter. The findings indicate that efficient lentiviral vector-mediated delivery of GALC expression cassettes to donor HSPCs. As described below and in Figure 3B and Table III, vector-transduced HSPC efficiently engrafted the host BM following conditioning. Thus, BM chimerism of donor cells containing one or more vector genomes expressing the GALC under these cell-specific promoters was not lower than the chimerism of nontransduced HSPC. These findings indicate that efficient lentiviral vector-mediated delivery of GALC expression cassettes comprising cell specific promoters can be achieved without posing biosafety concerns associated with altering the host miRNA system.

### Table I. Primary Antibodies Used

| Antigen | Description of Immunogen | Source, host species, catalog No., clone or lot No., RRID | Concentration used |
|---------|--------------------------|----------------------------------------------------------|-------------------|
| Insulin-like growth factor 2 Receptor (IGF2R/CI-M6PR/CDD223) | Synthetic peptide corresponding to residues surrounding Phe1379 of human IGF-II receptor | Cell Signaling Technology, rabbit monoclonal, 15128 | 1:100 (immunostaining) |
| Leukocyte common antigen Ly5.1 (CD45.1) | SJL mouse thymocytes and splenocytes | Bio Legend, mouse monoclonal, 110708 RRID:AB_313497 | 2 μg/ml/10^6 cell (flow cytometry) |
| Leukocyte common antigen Ly5.2 (CD45.2) | B10.S mouse thymocytes and splenocytes | BD Biosciences, mouse monoclonal, 561874, RRID:AB_10894189 | 2.5 μg/ml/10^6 cell (flow cytometry) |
| CD11b/Mac-1a | Mouse splenic cells CD11b | BD Biosciences, rat monoclonal, 553112, clone M1/70, RRID:AB_398535 | 2 μg/ml/10^6 cell (flow cytometry) |
| Lymphocyte antigen Ly-6A/E (Sca-1) | IL-2-dependent mouse T cell line CTL-L | BD Biosciences, rat monoclonal, 561021, RRID:AB_20340121 | 2 μg/ml/10^6 cell (flow cytometry) |
| SCF receptor (SCFR); stem cell factor receptor CD117 (cKit) | Mouse bone marrow mast cells | BD Biosciences, rat monoclonal, 561074, RRID:AB_10563023 | 2 μg/ml/10^6 cell (flow cytometry) |
| Lineage antibody cocktail, with isotype control | mouse T-cell receptor (CD3ε), mouse splenic cells (target CD11b); mouse abelson leukemia virus-induced pre-B tumor cells (target B222); mouse fetal liver (target Ter119, erythroids marker); Ly6G/6C | BD Biosciences, hamster antibody cocktail, 561301, RRID:AB_10611731 | 20 μl/100 μl/10^6 cell (flow cytometry) |

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Fig. 1. Novel lentiviral vectors support erythroid- and myeloid-specific transgene expression. A: Novel self-inactivating (SIN) vectors carrying erythroid (IHK)- and myeloid (146gp91)-specific promoters. ssp, Synthetic secretory signal peptide; psp, parental secretory signal peptide; co-GALC, codon optimized mouse GALC cDNA; myc, myc tag; 146gp91, myeloid-specific promoter; IHK, erythroid/megakaryocyte-specific promoter; IRES, internal ribosome entry site; WP, woodchuck hepatitis virus posttranscriptional regulatory element; ΔU3 LTR, Self-inactivating (SIN) LTR, deleted of the parental enhancer promoter. B: Lentiviral vectors carrying the IHK promoter support erythroid-specific transgene expression in vitro. FACScan analysis of GFP expression in mouse erythroleukemia (MEL) cells, human 293T cells, mouse Lin− cells, and mouse Lin− Sca11 Kit1 (LSK) cells following transduction with lentiviral vectors carrying either the IHK promoter (pTK1580 and pTK1582) or the CMV promoter (pTK945). MEL and 293T cells were analyzed either before or after HMBA-induced erythroid differentiation. Untransduced MEL and 293T cells served as controls. Percentage of GFP-positive cells is shown. Mean fluorescence intensity (MFI) presents levels of GFP expression. Note that GFP expression from IHK-containing lentiviral vectors was detected only in HMBA-induced MEL cells. C,D: Lentiviral vectors carrying the 146gp91 promoter support myeloid-specific transgene expression in vitro. C: Lentiviral vectors carrying the GFP reporter gene under control of either the myeloid 146gp91 promoter (pTK1607) or the CMV promoter (pTK945) were employed to transduce human 293T cells, cells of the human THP-1 monocyte cell line, the above-mentioned mouse LSK cells, and mouse BM cells expressing the macrophage surface marker Mac11. FACScan analysis of GFP expression was employed as described for B. Note that high levels GFP expression from the CMV promoter were detected in all target cells excluding the Thp1 cells. CMV-regulated expression was higher in LSK than in Mac11 cells. On the other hand, GFP expression driven by 146gp91 was high in THP1 and very low in 293T cells. Furthermore, GFP expression level in Mac11 cells was significantly higher than the level of expression detected in LSK cells. D: Lentiviral vectors carrying the firefly luciferase under control of either a myeloid promoter (pTK1607) or a CMV promoter (pTK993) transduced 293T and THP-1 cells. Luciferase activity in relative light unit (RLU) was normalized per milligram protein and VCN per cell. Luciferase activity generated by each vector in 293T served as a reference baseline. The ratio or fold expression of luciferase activity from these vectors in THP1 cells relative to luciferase expression in 293T cells was calculated. Note that CMV expression in THP-1 cell was dramatically lower than its expression in 293T cells. On the other hand, luciferase expression per vector genome from the myeloid promoter increased 33-fold relative to its expression in 293T cells.
TABLE II. Novel Lentiviral Vectors Carrying the IHK Promoter Demonstrate Erythroid Specific Expression of GALC in Differentiated MEL Cells*

| Galc activity (nmol/hr/mg protein) | Cont pTK1580 | pTK1582 |
|-----------------------------------|-------------|---------|
| Naïve MEL                         | ND          | 6.46    | 8.47    |
| Differentiated MEL (HMBA)         | ND          | 30.70   | 34.03   |

*MEL cells were transduced with lentiviral vectors carrying codon-optimized murine GALC cDNA under control of the erythroid/megakaryocyte-specific promoter IHK (pTK1580, pTK1582). Vector-transduced MEL were induced to undergo erythroid differentiated by 5 mM HMBA. GALC activity in vector-transduced MEL cells prior to and after differentiation was determined and served as a surrogate marker for IHK promoter activity. Note the robust increase in IHK activity following HMBA-induced differentiation.

L-Cycloserine Enhances IGF2R Expression in Twi Mouse CNS

The cornerstone of cell- and gene therapy-based therapeutic protocols for LSDs is the phenomenon of “cross-correction,” in which donor cell-secreted, M6P-comprising enzymes are taken up by host enzyme-deficient cells (either in the periphery or in the CNS). Sufficient expression of IGF2R, the CI-M6PR in treated cells, is essential to facilitate this therapeutic pathway. However, supraphysiological levels of functional GALC following viral vector-mediated gene delivery (either alone or in combination with BMT) to twi mouse CNS and PNS resulted in only moderate prolongation of average mouse life span (Lin et al., 2007). Similarly, a substrate reduction approach using L-cycloserine, an inhibitor of sphingolipids synthesis (which should also decrease accumulation of toxic undegraded metabolites such as psychosine), failed to prolong twi mouse life span (Hawkins-Salsbury et al., 2015). However, by combining L-cycloserine treatment with BMT and AAV vector-based GALC delivery, Hawkins-Salsbury et al. dramatically prolonged the life span of treated twi mice. Premised on these studies, we theorized that an L-cycloserine-mediated increase in IGF2R expression facilitated uptake of functional GALC secreted from donor HSPC and from AAV vector-transduced cells by twi cell populations that naturally express insufficient levels of the M6P receptor. To test this hypothesis, we employed a semiquantitative immunohistochemistry analysis to characterize IGF2R levels in either L-cycloserine treated or naïve twi mice. As shown in Figure 2A,B, L-cycloserine-treated mice exhibited significantly higher levels of cellular IGF2R (Student’s t test, n = 3, \( P = 0.006 \)). These data further support the notion that insufficient IGF2R expression limits the efficacy of BMT and gene therapy-based therapeutic protocols to cure KD.

Efficient M6P-Independent Uptake of a Novel GALC-ApoE1 Receptor Binding Domain (GALC-AErbd) Fusion Protein

Intrigued by the notion that low levels of IGF2R in various CNS and PNS cell populations potentially limit the efficacy of HSPC transplantation and gene therapy protocols for treating KD, we sought to facilitate cellular uptake of secreted GALC independently of the M6P pathway. To this end, we developed a novel fusion protein comprising the GALC and the AErbd.

This approach was premised on earlier studies in which fusion of the AErbd to the lysosomal protein α-L-iduronidase (IDUA) facilitated uptake of the novel protein by blood–brain barrier (BBB) endothelial cells, transcytosis to the CNS, and delivery to astrocytes and neurons throughout the cerebral cortex via the LRP-1 pathway (independently of the M6P pathway) (Wang et al., 2013; Dai et al., 2014; El-Amouri et al., 2014). The novel GALC-AErbd cDNA was cloned into lentiviral vectors under control of a CMV, an IHK, or a 146gp91 promoter (Fig. 2C). To characterize the efficiency of LRP-1-mediated GALC uptake, 293T cells were transduced with lentiviral vectors expressing either GALC or GALC-AErbd cDNAs. Conditioned media were collected from vector-transduced cells and applied to 293T cells and human fibroblasts derived from KD patients, in either the absence or the presence of M6P. Cellular activity of GALC (Wiederschain et al., 1992) in conditioned medium-treated cells served as a surrogate marker for cellular uptake of GALC and GALC-AErbd. Activity of GALC in untreated healthy and KD human fibroblasts served as reference controls. As shown in Figure 2D,E the presence of M6P, a competitive inhibitor of the IGF2R–mediated protein uptake pathway, efficiently inhibited uptake of conditioned media GALC by both 293T cells and human KD fibroblasts. On the other hand, M6P treatment had a minimal yet measurable effect on cellular uptake of GALC-AErbd. These data indicate that cellular uptake of the novel GALC-AErbd fusion protein can be mediated independently by the IGF2R– and the LRP-1–specific pathways. This attribute of GALC-AErbd broadens the spectrum of target cells that can benefit from the “cross-correction” phenomenon in current and future therapeutic regimens for KD (Polavarapu et al., 2007; Lillis et al., 2008).

Twi mouse life span following HSPCT correlates with the aggressiveness of the preconditioning protocol.

Earlier studies suggested that both host conditioning with either irradiation or chemotherapy and damage to the CNS are required for efficient donor HSPC engraftment in rodent brains (Priller et al., 2001; Mildner et al., 2007; Davoust et al., 2008).

Note that Bu was the major chemotherapy agent employed in earlier lentiviral/HPCT–based human clinical trials of neurodegenerative diseases (Cartier et al., 2009; Aiuti et al., 2013) as well as in allogeneic HPCT for KD (Escolar et al., 2005), yet irradiation-conditioning protocols have been employed in most HSPCT-based preclinical trials investigating KD (Lin et al., 2007; Gentner et al., 2010; Hawkins-Salsbury et al., 2015). This limits our ability to accurately interpret the therapeutic outcomes described in the above-mentioned preclinical studies. Furthermore, the correlation between the levels
| Survival study included | Age (days) | CD45.1 (%) Ave ± SD | CD45.2 (%) Ave ± SD | CD45.1 (%) Ave ± SD | CD45.2 (%) Ave ± SD | CD45.1 (%) Ave ± SD | CD45.2 (%) Ave ± SD | VCN |
|-------------------------|------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|-----|
| A K67L2 12.5 KO, ♀ | 89 | 93.5 ± 4.7  0.7 | 28.5 ± 3.4  35.4 | 64.0    | 25.7 |
| K68L1 12.5 KO, ♀ | 80 | 94.2 ± 2.1  1.0 | 38.3 ± 2.6  26.0 | 58.0    | 26.2 |
| K68L4 12.5 KO, ♀ | 77 | 93.6 ± 1.2  1.2 | 36.5 ± 3.2  38.6 | 58.6    | 28.2 |
| K69L3 12.5 KO, ♀ | 86 | 94.6 ± 1.1  0.9 | 25.8 ± 3.1  34.6 | 34.3 ± 3.7  57.8 | 59.6 ± 2.9  29.2 | 27.3 ± 1.7 |
| B K51R2 12.5 KO, ♀ | 74 | 90.0 ± 2.1  2.1 | 89.3 ± 3.9  3.9 | 60.7    | 28.2 |
| K51R3 12.5 KO, ♀ | 65 | 91.1 ± 4.1  4.1 | 58.6 ± 2.1  2.1 | 58.6    | 28.2 |
| K51R4 12.5 KO, ♀ | 108 | 98.9 ± 2.7  4.4 | 2.7 ± 1.6  2.7 | 96.8    | 96.8 |
| C K65L1 12.5 He, ♀ | 202 | 97.5 ± 0.5  0.2 | 7.6 ± 2.5  2.5 | 89.8    | 89.8 |
| K67L1 12.5 WT, ♀ | 103 | 41.3 ± 54.8  54.8 | 4.8 ± 85.6  85.6 | 2.0    | 96.6 |
| K69L2 12.5 WT, ♀ | 87 | 93.7 ± 28.5  28.5 | 13.7 ± 27.2  27.2 | 16.6 ± 3.2  32.9 | 74.0 ± 9.5  9.5 | 7.9 ± 2.6  2.6 | 84.0 | 89.8 ± 5.1 |
| D K67R4L4 25 KO, ♀ | 90 | 96.2 ± 0.5  0.5 | 40.8 ± 24.9  24.9 | 53.5    | 28.8 |
| K68L4 25 KO, ♀ | 78 | 95.1 ± 0.6  0.6 | 40.5 ± 25.3  25.3 | 46.7    | 40.1 |
| K68L3 25 KO, ♀ | 80 | 96.8 ± 1.1  1.1 | 37.9 ± 21.9  21.9 | 57.3    | 23.9 |
| K51R4 25 KO, ♀ | 80 | 92.7 ± 0.2  0.2 | 50.9 ± 19.5  19.5 | 62.2    | 26.2 |
| K69L1 25 KO, ♀ | 87 | 96.5 ± 1.7  1.7 | 0.5 ± 0.4  0.4 | 27.2 ± 3.5  3.5 | 41.9 ± 26.7  26.7 | 54.8 ± 5.4  5.4 | 24.1 | 28.6 ± 6.7 |
| E K48L2 25 KO, ♀ | 170* | 96.8 ± 0.1  0.1 | 23.9 ± 56.8  56.8 | 7.8 ± 83.2 |
| K48L3 25 KO, ♀ | 176 (dead) | 97.7 ± 0.8  0.8 | 17.6 ± 62.9  62.9 | 7.1 ± 85.6 |
| K50R2 25 KO, ♀ | 140 | 95.0 ± 1.5  1.5 | 10.4 ± 73.9  73.9 | 4.4 ± 92.3 |
| K51R3 25 KO, ♀ | 72 | 91.3 ± 2.8  2.8 | 0.9 ± 0.6  0.6 | 92.3    | 92.3 |
| F K58R2 25 WT, ♀ | 203 | 97.1 ± 0.4  0.4 | 23.9 ± 56.8  56.8 | 7.8 ± 83.2 |
| K59R1 25 He, ♀ | 264 | 98.9 ± 0.5  0.5 | 17.6 ± 62.9  62.9 | 7.1 ± 85.6 |
| K59R1 25 WT, ♀ | 221 | 98.0 ± 0.4  0.4 | 10.4 ± 73.9  73.9 | 4.4 ± 92.3 |
| K67R1L4 25 WT, ♀ | 103 | 94.3 ± 2.0  2.0 | 0.3 ± 0.1  0.1 | 11.3 ± 15.8  15.8 | 78.2 ± 67.9  67.9 | 5.6 ± 6.2  6.2 | 90.7 | 87.9 ± 4.2 |
| G K53R3 25 KO, ♀ | 160* (vet) | 96.5 ± 0.0  0.0 | 37.4 ± 17.0  17.0 | 64.4 ± 19.6  19.6 | 0.46 |
| K59R4L2 25 KO, ♀ | 203* | 96.5 ± 0.0  0.0 | 37.4 ± 17.0  17.0 | 64.4 ± 19.6  19.6 | 0.46 |
| K53L1 25 KO, ♀ | 98 (dead) | 98.6 ± 0.2  0.2 | 11.1 ± 0.1  0.1 | 1.24 |
| K59L1 25 KO, ♀ | 128 | 96.6 ± 0.2  0.2 | 11.1 ± 0.1  0.1 | 1.24 |
| K59L2 25 KO, ♀ | 203 (dead) | 94.6 ± 1.1  1.1 | 0.1 ± 0.1  0.1 | 1.24 |
| K59R4L1 25 WT, ♀ | 201 | 95.8 ± 0.4  0.4 | 19.9 ± 64.7  64.7 | 10.4 ± 81.9  81.9 | 0.63 |
| K64L3 25 WT, ♀ | 182 | 96.8 ± 0.2  0.2 | 30.4 ± 33.5  33.5 | 18.6 ± 64.2  64.2 | 0.35 |
| K53L3 25 WT, ♀ | 241 | 97.1 ± 0.4  0.4 | 11.0 ± 19.9  19.9 | 60.2 ± 53.0  53.0 | 14.1 ± 5.0  5.0 | 10.0 ± 6.2  6.2 | 87.7 | 79.5 ± 10.5 0.82 |
| Boyj — WT, ♀ | ~150 | 96.8 ± 0.1  0.1 | 80.7 ± 0.6  0.6 | 93.3 ± 0.2  0.2 | 96.2 |
| K53R1L1 — WT, ♀ | 256 | 0.2 ± 94.8  94.8 | 0.1 ± 84.0  84.0 | 0.1 ± 96.2  96.2 |

*The dose of Bu employed in the conditioning protocol is indicated. Mice used for the survival study are marked. Mice in groups A–F received 5–6 × 10⁸ total donor BM cells. Mice in groups G and H received 1 × 10⁶ pTK1784-transduced Lin⁻ cells, 1 × 10⁶ pTK1667-transduced Lin⁻ cells, and 1 × 10⁶ donor total BM cells. “Age” denotes the age of the mouse at the time of death or when sacrificed experimentally. Only mice involved in the “survival study” died from natural causes or were euthanized in accordance with UNC IACUC protocol. “Dead” indicates that the specific mouse was found dead in its cage; “vet”: denotes that tissues of euthanized mouse were not available; asterisk indicates the mice sacrificed for data collection. LSK, bone marrow Lin⁻Sca1⁻Kit⁺ cells; CNS enriched microglial and leukocytes were isolated by percoll gradient from perfused brain.
of donor/host chimerism in the bone marrow and the therapeutic benefits of HPCT in KD has not been established. Thus, it is imperative that we further characterize the effects of the conditioning protocol and host health status on the therapeutic outcome of HSPCT in the twi mouse model of KD. To this end, as shown in Figure 4A and Table III, healthy donor total BM cells (BoyJ expressing CD45.1) were transplanted into host mice (C57B6 expressing CD45.2) including either twi (GALC−/−; groups A, B, D, and E) or healthy hosts (groups C and F). Note that the healthy host population comprised heterozygous twi (GALC1/−) and wild type mice (GALC+/+).

Specifically, at postnatal day 8, host mice were conditioned by i.p. injection of either 12.5 (groups A–C) or 25 mg/kg Bu (groups D–H) and 1 day later were given naïve donor total bone marrow cells (5–6 × 10⁷ cells/mouse, groups A–F) i.p. Mouse body weight was measured every 2–3 days. To facilitate characterization of
donor cells engraftment in twi host CNS, mice were sacrificed at postnatal days 77–90 (groups A and D). All other twi mice died naturally or were euthanized in accordance with UNC Institutional Animal Care and Use Committee (IACUC) protocol. As shown in Figure 3A,B, untreated twi mice maintained close to normal body weight until week 5, after which dramatic weight loss followed by death occurred in all mice before postnatal day 46. Healthy BMT-treated mice, which were conditioned with Bu 12.5 mg/kg demonstrated a body weight increase curve that was not significantly different from the curve of untreated healthy mice. However, conditioning of healthy mice with 25 mg/kg Bu inhibited gain in mouse body weight for approximately 2 weeks, after which a continuous increase in body weight was observed. However, at postnatal day 180, the average body weight of this group of mice was still significantly lower than the body weight of untreated or 12.5 mg/kg Bu-treated mice. For the first 7 weeks of life, the body weight curve of twi mice treated with 25 mg/kg Bu was not significantly different from the body weight curve of their healthy counterparts, which received the same treatment. Interestingly, this group of mice failed to gain weight after postnatal week 7. The maximal average body weight of these mice was 12.5 g; however, in contrast to untreated twi mice, death in this group was not preceded by body weight loss. Because of the milder conditioning protocol, twi mice treated with 12.5 mg/kg of Bu demonstrated early gains in body weight, which started to decline at postnatal week 5 and stabilized within 1 week. The average body weight at death of this group of mice was not significantly different from the average body weight of their twi counterparts that were conditioned by body weight loss. Because of the milder conditioning protocol, twi mice treated with 12.5 mg/kg Bu demonstrated early gains in body weight, which started to decline at postnatal week 5 and stabilized within 1 week. The average body weight at death of this group of mice was not significantly different from the average body weight of their twi counterparts that were conditioned with 25 mg/kg of Bu. On the other hand, a highly significant difference was observed in the effects of the two conditioning protocols on the life span of HSPCT-treated twi mice. As shown in Figure 3C, the average life span of twi mice conditioned with 25 mg/kg Bu was significantly longer than the average life span of those conditioned with 12.5 mg/kg Bu (Student’s t-test, n = 4 and 9, P = 0.014).

The average life span of twi mice following HSPCT did not correlate with the level of donor/host chimerism in the BM.

Next we sought to investigate whether the longer life span of twi mice following high-dosage of Bu (25 mg/kg) conditioning protocol was secondary to a more efficient engraftment of donor HSPC in the host BM compartment. To this end mouse pluripotent hematopoietic Lin·Sca1·Kit+ (LSK) cells isolated from bone marrow of all mouse groups A–H were FACSanayzed for CD45.1 (donor)– and CD45.2 (host)–expressing cells. As shown in Figure 4A,C and Table III, with the exception of a single mouse (group C mouse K67L1) all mice in all treatment groups exhibited high levels of chimerism (>90%). These data strongly suggest that the levels of BM chimerism cannot explain the difference in average twi mouse life span following conditioning with high and low Bu doses. No difference in chimerism was observed between wild-type and twi mouse hosts.

The levels of donor/host cell chimerism in twi mouse hosts CNS were significantly higher than those found in wild-type hosts’ CNS.

To understand better the mechanism by which the conditioning protocol affects the life span of BMT–treated twi mice, we characterized donor/host chimerism in the

Fig. 2. Induction of IGF2R expression in mouse CNS by L-cycloserine and efficient uptake of a novel GALC-AErdbd fusion protein via an M6P-independent pathway. A,B: Analysis of IGF2R expression in L-cycloserine (L-cys)-treated and untreated mouse CNS. A: Representative sections from untreated (I–III) and L-cys-treated (IV–VI) twi mouse diencephalon. Hematoxylin and eosin (HE) staining (I, IV) shows no significant morphological differences between L-cys-treated and untreated mice. Immunohistochemical (IHC) staining (II, V) shows enhanced expression of IGF2R in L-cys-treated mouse diencephalon.

To semiquantify expression of IGF2R in treated and untreated twi brain, the IHC-stained sections were masked by red (3 positive), orange (2 positive), yellow (1 positive), and blue (no signal; III, VI). All panels are X20. Scale bar = 100 μm. B: Semiquantification of IGF2R expression using IHC-stained sagittal brain sections of L-cys treated and untreated twi mice. Red and orange signals were quantified as positive cells. The percentage of positive cells between two groups were compared by two-tailed Student’s t-test, N = 3 in each group, P = 0.006. C: Depiction of the lentiviral vectors used to characterize uptake of the novel GALC-AErdbd and its usage: ssp, Synthetic secretory signal peptide; psp, parental secretory signal peptide; co-GALC, codon optimized mouse GALC cDNA; myc, myc tag; AErbdb, ApoE receptor binding domain; 146gp91, myeloid-specific promoter; IKK, erythroid/megakaryocyte-specific promoter; WP, woodchuck hepatitis virus post-transcriptional regulatory element; AU3 LTR, SIN LTR, deleted of the parental enhancer promoter. D: Uptake of GALC and GALC-AErdb by 293T cells via the IGF2R and the LRP-1 pathways. 293T cells were cultured either in the presence or in the absence of 1 mM M6P (a competitive inhibitor of protein uptake via the IGF2R pathway) in conditioned media containing either GALC (generated by pTK1557-transduced cells) or GALC-AErdbd (generated by pTK1664-transduced cells). The GALC activity in these 293T cells served as a surrogate marker for GALC uptake. Cellular GALC activity in the absence of M6P served as a reference value, considered to be 100%. The ratio of cellular GALC activity in the presence of M6P relative to GALC activity of the same protein without M6P was calculated. Uptake of GALC (pTK1557) was efficiently inhibited by the presence of 1 mM M6P, whereas uptake of the GALC-AErdbd fusion protein (pTK1664) was only slightly affected. E: Uptake of GALC and GALC-AErdbd by healthy and KD human fibroblasts cells via the IGF2R and the LRP-1 pathways. Human fibroblasts isolated from healthy donors (Cont) and KD patients (Krabbe) were cultured either in the presence or absence of M6P (a competitive inhibitor of protein uptake via the IGF2R pathway) in conditioned media containing either GALC (generated by pTK1557-transduced cells) or the GALC-AErdbd (generated by pTK1665-transduced cells). The levels of GALC activity in naïve KD and healthy human fibroblasts are shown. Note that the presence of 2.5 mM M6P efficiently inhibited GALC uptake, whereas GALC-AErdbd uptake was not significantly affected in the presence of M6P.

Journal of Neuroscience Research
To this end, either CNS enriched microglia and leukocytes or CD11b-gated cells were analyzed by FACscan for CD45.1(donor) or CD45.2 (host) expression. As shown in Figure 4B,C and Table III, no significant differences in the levels of donor/host chimerism in the CNS were found between low (12.5 mg/kg, group A)- and high (25 mg/kg, group D)-dose Bu-treated twi mice. On the other hand, the level of chimerism in twi mouse CNS was always significantly higher than the chimerism level in the CNS of their healthy counterparts (Student’s t-test, n = 4, P = 0.002 for CNS enriched microglia and leukocytes and P = 1.6 × 10^{-7} for CD11b-gated cells in low-dose groups; P = 0.002 for CNS enriched microglia and leukocytes and P = 7.3 × 10^{-7} for CD11b-gated cells in high-dose groups). These data suggest that KD-induced pathology may enhance the efficiency of engraftment and colonization of hematopoietic-derived donor cells in the host CNS.

Transduction of HSPC with lentiviral vectors carrying the GALC-AErbd cDNA, under the control of either a myeloid- or an erythroid/megakaryocyte-specific promoter, does not reduce engraftment efficiency in host BM.

To increase the efficacy of BMT for KD, we sought to overexpress the novel GALC-AErbd protein in HSPC-derived cells. We expected that high levels of donor cell-secreted GALC-AErbd, whose uptake by
GALC-deficient host cells is mediated by two independent pathways (M6P and LRP-1 pathways), would further extend the life span of BMT-treated twi mice. To this end, Lin<sup>−</sup> cells isolated from donor BoyJ (CD45.1) mice were transduced at an m.o.i. of 50 with lentiviral vectors from which the GALC-AErbd cDNA is expressed...
under control of either the myeloid-specific promoter 146gp91 (pTK1667) or the erythroid/megakaryocyte-specific promoter IHK (pTK1784). At postnatal day 8, twi and wild-type control mice were conditioned by i.p. injection of Bu (25 mg/kg). At postnatal day 9 mice were injected i.p. with the respective vector-transduced cells (1 × 10^6 cells/mouse of pTK1667-transduced Lin^−_ cells and 1 × 10^6 cells/mouse of pTK1784 vector-transduced Lin^−_ cells) along with 10^7 naive total BM cells (Table III, groups G and H). Wild-type and twi mice receiving naive total BM cells (5–6 × 10^7 cells/mouse) served as controls. Mouse weight was determined every 2–3 days. Mice were euthanized according to UNC IACUC protocol. 

Vehicle total BM cells was determined by qPCR. In addition donor/host chimerism in isolated LSK cells and in treated mouse CNS were determined by FACScan analysis as described above. As shown in Figure 3C, HSPC transduced with lentiviral vectors carrying the GALC-Arbd did not extend the life span of BMT-treated twi mice beyond the average life span of these mice following BMT with nontransduced donor HSPC. Similarly, the weight gain curve of either twi or wild-type mice treated with vector-transduced HSPC was not significantly different from the weight gain curve of their counterparts, which received donor nontransduced total BM cells (Fig. 3B). These data indicated that, under the conditions described above, lentiviral vector delivery of GALC-AErbd to donor HSPC did not increase the therapeutic benefits of BMT to twi mice. To evaluate the efficiency of lentiviral vector transduction and engraftment of vector-transduced HSPC, we characterized donor/host chimerism and VCN in the BM of wild-type and twi mice. As shown in Table III, the efficiency of engraftment and colonization of HSPC transduced with lentiviral vectors from which the GALC-AErbd is expressed under the control of erythroid- and myeloid-specific promoters is similar to that of BM engraftment of nontransduced HSPC. VCN in BM of twi mice (ranging between 0.46 and 1.24) was higher than the VCN in BM of wild-type mice (ranging between 0.02 and 0.82). Altogether these data indicated that HSPC transduced with lentiviral vectors carrying GALC under control of cell-specific promoters could colonize the BM compartment in wild-type and twi mice. Note that in earlier studies significantly higher VCN (five to eight copies) in host mouse BM was required to moderately prolong the life span of GALC deficient mice (Gentner et al., 2010; Ungari et al., 2015). Clearly, additional studies using higher m.o.i. to achieve higher VCN in host BM are required to accurately evaluate the therapeutic potential of the GALC-AErbd fusion protein.

**DISCUSSION**

Allogeneic HSPCT with HLA-matched healthy donor HSPC has been the standard therapeutic approach for a plethora of rare metabolic genetic disorders (such as LSDs, including KD). Since the early 1980s more than 2,000 HSPCTs were performed to treat genetic disorders (Boelens et al., 2014). The degree of success of this approach is mainly disease specific. With regard to LSDs, the spectrum of endpoint therapeutic benefits ranges between largely inefficient even in animal models (Helderson et al., 2013) to highly beneficial in human patients (Boelens et al., 2013). However, even in the...
most successful scenario—HSPCT applied to MPS-IH (Hurler disease) patients—a complete cure is far from being achieved (Aldenhoven et al., 2015). Treating pre-symptomatic KD patients with HSPCT preserves most of their cognitive function. However, progressive deterioration of the patients’ PNS severely affects gross motor function and expressive language (Escolar et al., 2005; Duffner et al., 2009). We assert that the limited efficacy of the current HSPCT protocols for curing KD is linked to the basic biological processes on which the HSPCT therapeutic approach for KD is premised. These include the ability of donor HSPC to efficiently colonize the host in proximity to cell populations that are vulnerable to GALC deficiency. Furthermore, the therapeutic effects of HSPCT are heavily dependent on the “cross-correction” phenomenon, so it is imperative that these host target populations express sufficient levels of IGF2R to facilitate efficient uptake of donor-derived GALC. Overall, this study was premised on the notion that two independent mechanisms potentially limit the efficacy of current HSPCT protocols at curing KD. Specifically, we hypothesized that both inefficient conditioning protocols (which limit the number and biodistribution of donor-derived microglia in host CNS) and inefficient host cell uptake of donor cell-secreted GALC (resulting from lack of sufficient IGF2R) should be addressed to maximize the therapeutic benefits of HSPCT protocols for KD patients.

In addition, we have characterized some of the obstacles and potential solutions associated with the strategy of combining lentiviral vector delivery of GALC with HSPCT-based therapy for KD. The ability of lentiviral vectors to deliver and maintain long-term transgene expression in HSPC and their progenies opens new directions in HSPCT-based therapy for genetic disorders in general and for KD in particular (Miyoshi et al., 1999; Cartier et al., 2009; Aiuti et al., 2013; Biffi et al., 2013). Specifically, lentiviral vectors carrying functional GALC to autologous HSPC circumvent the need to identify an HLA-matched donor. In addition, overexpression of GALC in vector-transduced cells should in theory broaden the spectrum of target cells that can benefit from the phenomenon of cross-correction. However, in recent preclinical studies using mouse models of KD, lentiviral vector-mediated GALC delivery to HSPC revealed potential limitations in this approach. Overall only a very mild effect on GALC-deficient mice life span could be achieved, and only in mice showing very high VCN in engrafted donor cells (Gentner et al., 2010; Ungari et al., 2015). Furthermore, GALC cytotoxicity, which inhibited engraftment of vector-transduced HSPC, necessitated incorporation of an HSPC-specific miRNA target sequence to minimize GALC expression in and to facilitate efficient engraftment of vector-transduced HSPC. Notably, a heterogeneous cell population with an average VCN of 8 usually contains cells with VCN higher than 25. The combination of very high VCN and vector design comprising the target sequence to host miRNA raises biosafety concerns. These include the possibility of inadvertently altering the host miRNA system via the “sponge effect” described earlier in which high levels of exogenous miRNA target sequence served as a competitive inhibitor decay that suppressed specific miRNA function (Ebert and Sharp, 2010). We assert that weaknesses in the lentiviral vector system should be addressed by further vector development rather than by manipulating host biological pathways. To this end, we developed novel lentiviral vectors from which the reporter genes GFP and firefly luciferase (as well as the GALC and the GALC-AErbd fusion protein) were expressed under control of either an erythroid/megakaryocyte (IHK)- or a myeloid (146gp91)-specific promoter (Moreau-Gaudry et al., 2001; He et al., 2006; Barde et al., 2011). The novel vectors demonstrated cell-specific expression in vitro and facilitated efficient GALC-AErbd delivery to and engraftment of vector-transduced HSPC. Donor/host chimerism in host BM following transplantation with vector-transduced cells was equivalent to the chimerism achieved following engraftment of untransduced HSPC. VCN of the novel vectors in twi mouse BM ranged from 0.46 to 1.24. Because, in addition to the vector-transduced Lin- cells, the transplanted donor cell population included 10^7 total BM cells, we speculate that VCN in engrafted transduced cells was higher than that described above.

The mild effect of high lentiviral VCN on the average life span of HSPCT-treated GALC-deficient mice (Gentner et al., 2010; Ungari et al., 2015) suggested that high levels of vector/donor cell-derived functional GALC would not cure KD patients. This phenomenon was in line with similar studies in which supraphysiologic levels of AAV vector-delivered GALC failed to significantly alter the life span of twi mice (Lin et al., 2007). We theorized that lack or insufficient levels of IGF2R in GALC-deficient host cells rendered the cross-correction phenomenon inefficient. This notion is supported by several studies showing that expression of IGF2R is developmentally and gender dependent (Nissley et al., 1993; Gonzalez-Parras et al., 2001; Hawkes and Kar, 2003, 2004; Romano et al., 2005; Jofre et al., 2009). Furthermore, analysis of IGF2R expression in healthy and Alzheimer’s disease human brains demonstrated regional and cell type specificity (Kar et al., 2006). GALC was shown to express primarily in neurons and not on other cell types in the CNS (astrocytes, oligodendrocytes) that can benefit from the cross-correction phenomenon. The fact that IGF2R is biallelically expressed in human tissues (Kalischuever et al., 1993) and is genomically imprinted in mice (Barlow et al., 1991) limits the ability to interpret some of the results obtained in mouse models. Here we demonstrated for the first time an L-cycloserine-mediated increase of IGF2R. These findings support our notion that the dramatic prolongation in twi mouse life span upon combining L-cycloserine treatment with HSPCT and AAV vector-mediated GALC delivery is secondary to an L-cycloserine-induced increase in IGF2R (Hawkins-Salsbury et al., 2015). Additional characterization of this phenomenon with identification of novel small molecules that enhance IGF2R expression may improve current...
The GALC-AErbd approach was developed to circumvent the need to upregulate IGF2R expression by directing GALC uptake via an IGF2R-independent pathway. Unlike the IGF2R-independent pathway, which is dependent on the IGF2R and LRP-1 pathways, our GALC-AErbd approach utilizes the IGF2R and LRP-1 pathways. The goal of this approach is to maximize the clinical outcome of HSPCT in the twi mouse model. We achieved this by employing this approach to treat other LSDs, which have a broad spectrum of target cells in the CNS. However, further optimization of the conditioning/HSPCT protocols is needed to maximize the clinical outcome of HSPCT in the twi model of KD. We anticipate that the results of this study will pave the way for the development of new GALC fusion proteins with a broadened spectrum of target cells in the CNS.
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