

# Lentiviral-Transduced Human Mesenchymal Stem Cells Persistently Express Therapeutic Levels of Enzyme in a Xenotransplantation Model of Human Disease

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**Key Words.** Mesenchymal stem cells • Xenotransplantation • Ex vivo gene therapy • Adult stem cells • Lysosomal storage disease • Lentiviral vector

## ABSTRACT

Bone marrow-derived mesenchymal stem cells (MSCs) are a promising platform for cell- and gene-based treatment of inherited and acquired disorders. We recently showed that human MSCs distribute widely in a murine xenotransplantation model. In the current study, we have determined the distribution, persistence, and ability of lentivirally transduced human MSCs to express therapeutic levels of enzyme in a xenotransplantation model of human disease (nonobese diabetic severe combined immunodeficient mucopolysaccharidosis type VII [NOD-SCID MPSVII]). Primary human bone marrow-derived MSCs were transduced ex vivo with a lentiviral vector expressing either enhanced green fluorescent protein or the lysosomal enzyme  $\beta$ -glucuronidase (MSCs-GUSB). Lentiviral transduction did not affect any in vitro parameters of MSC function or potency. One million cells from each population were transplanted intraperitoneally into separate groups of neonatal NOD-

SCID MPSVII mice. Transduced MSCs persisted in the animals that underwent transplantation, and comparable numbers of donor MSCs were detected at 2 and 4 months after transplantation in multiple organs. MSCs-GUSB expressed therapeutic levels of protein in the recipients, raising circulating serum levels of GUSB to nearly 40% of normal. This level of circulating enzyme was sufficient to normalize the secondary elevation of other lysosomal enzymes and reduce lysosomal distention in several tissues. In addition, at least one physiologic marker of disease, retinal function, was normalized following transplantation of MSCs-GUSB. These data provide evidence that transduced human MSCs retain their normal trafficking ability in vivo and persist for at least 4 months, delivering therapeutic levels of protein in an authentic xenotransplantation model of human disease. *STEM CELLS* 2008;26:1713–1722

Disclosure of potential conflicts of interest is found at the end of this article.

## INTRODUCTION

Since the original description of mesenchymal stem cells' (MSCs) role in the maintenance of the hematopoietic system [1, 2], subsequent research has described a surprising amount of lineage differentiation capability, with evidence of their role as progenitors of bone, fat, and cartilage [3, 4]. Recent renewed investigation into the biology of these cells has shown isolation of MSCs is possible from tissue origins outside the marrow niche, including lipoaspirate material [5], umbilical cord [6], and placenta [7]. This ability of MSCs to interface with diverse tissue environments, coupled with the well-documented immune tolerance elicited by these cells [8–10], has presented MSCs as an attractive platform for cellular and gene therapy.

Convergent engineering of differentiation protocols with biocompatible matrices has produced promising results in ap-

plications of limited organ systems, including articular [11–15] and intervertebral [16] cartilage repair, tendon damage [17, 18], segmental bone repair [19, 20], and adipose tissue generation for space filling and soft tissue defects [21, 22]. MSCs have also been genetically engineered for improvement of hematopoietic engraftment following myeloablative transplantation regimens [23–25], targeted delivery of antitumor factors [26, 27], and secretion of growth factors and cytokines [28–31]. Success in these applications has led to several clinical trials using MSCs for treatment of inherited disorders, producing promising results in osteogenesis imperfecta [32], metachromatic leukodystrophy, and Hurler's syndrome [33]. However, although the data are promising for the development of MSC-based therapeutics, elucidation of the basic biology, trafficking after transplantation, and characterization using authentic in vivo models of systemic disease are still lacking.

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We previously reported the widespread distribution of human MSCs following transplantation into immune-deficient mice [34]. Here we have exploited this ability, coupled with gene transfer technology, to examine the utility of human MSCs for treatment of an inherited disorder of enzyme deficiency, mucopolysaccharidosis type VII (MPSVII). MPSVII results from the genetic deficiency of the enzyme  $\beta$ -glucuronidase (GUSB), a ubiquitously expressed lysosomal acid hydrolase necessary for the degradation of glycosaminoglycans (GAGs) [35]. Absence of this enzyme leads to the accumulation of incompletely processed GAGs within cells, causing a multitude of clinical manifestations in nearly every organ system, including skeletal abnormalities, cognitive deficits, impaired vision and hearing, as well as shortened lifespan [36]. A spontaneously arising mouse model of GUSB deficiency has been described that closely mirrors the human course of disease [37–40]. This strain was recently backcrossed onto the nonobese diabetic severe combined immunodeficient (NOD-SCID) background, thus producing a mouse model of MPSVII disease that is capable of accepting transplanted human cells [41]. These mouse models of MPSVII disease, and several large animal models of MPSVII, have provided a considerable body of data on possible therapeutic approaches. However, no current regimen of therapeutic intervention has proven able to address the multiplicity of complications from this disease.

Recent work in this NOD-SCID MPSVII mouse model has shown improvement in many facets of disease following bone marrow transplantation of normal GUSB+ human CD34+ cells [41]. Continuing investigations reported similar success using lentiviral-mediated gene replacement therapy to correct GUSB-null CD34+ cells from a human patient, which were then transplanted into the NOD-SCID MPSVII mouse [42, 43]. Although the results obtained in this cohort of experiments were encouraging, the low efficiency of lentiviral transduction into the CD34+ cells and inefficient correction of disease in several organ systems have left room for improvement.

In the current study, we transduced normal human MSCs with lentiviral vectors expressing either enhanced green fluorescent protein (MSCs-eGFP) or GUSB (MSCs-GUSB) and transplanted the cells separately into cohorts of NOD-SCID MPSVII mice to examine the utility of these cells to correct the disease. There was no decrease in the number of transduced MSCs in vivo at either 2 or 4 months following intraperitoneal transplantation to neonatal MPSVII animals. Although the endogenous level of enzyme produced by MSCs-eGFP was insufficient to decrease the accumulation of GAG, MSCs-GUSB expressed supraphysiologic levels of GUSB that increased serum levels of enzyme to nearly 40% of normal. This level of enzyme resulted in normalization of the secondary elevations of another lysosomal enzyme and decreased lysosomal storage in multiple organ systems. Significant correction of retinal rod and cone function, as measured by electroretinography, was also observed. Overall, these data suggest that genetically engineered human MSCs are highly efficient at systemic protein delivery and may be an effective cellular therapy for inherited metabolic disorders.

## MATERIALS AND METHODS

### Human MSCs

Primary human MSC cultures were established following bone marrow aspiration from healthy volunteers according to the human subject guidelines of the Washington University School of Medicine (St. Louis) and approved informed patient consent. Whole marrow aspirations were collected into heparinized vials and passed

through 70- $\mu$ m nylon mesh screens. Cells in the flow-through were washed in red blood cell lysis buffer (BD Biosciences, San Jose, CA, <http://wwwbdbiosciences.com>), pelleted by centrifugation, and plated on tissue culture coated plates at an approximate density of  $5 \times 10^3$  mononuclear cells/cm<sup>2</sup>. Original plating and all subsequent maintenance cultures used a variation on Dexter's original medium (DOM), consisting of Iscove's Modified Dulbecco's Medium with 15% fetal bovine serum (Atlanta Biomedical, Lawrenceville, GA, <http://www.atlantabio.com>), 15% donor horse serum (Atlanta Biomedical),  $10^{-4}$  M 2-mercaptoethanol (Sigma-Aldrich, St. Louis, <http://www.sigmaaldrich.com>),  $10^{-6}$  M hydrocortisone (Sigma-Aldrich), 50 U/ml penicillin, 50  $\mu$ g/ml streptomycin sulfate, and 2 mM L-glutamine (Invitrogen, Carlsbad, CA, <http://www.invitrogen.com>). Both bovine and equine sera were from lot-selected batches tested in our laboratory for potency in maintenance of MSC cultures. Cultures were rinsed gently with PBS (Tissue Culture Support Center, Washington University School of Medicine, St. Louis) after 12 hours, and maintained at subconfluence throughout the experiment.

### Lentiviral Vector Generation

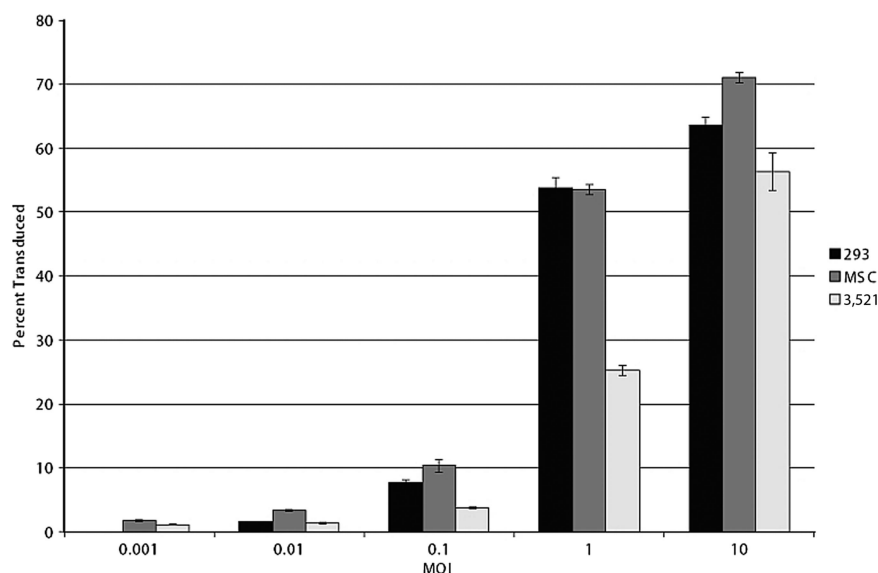
Human immunodeficiency virus-based lentiviral vectors expressing either eGFP or human GUSB in this experiment were based on the same backbone construct to ensure comparable expression profiles. Both lentiviral vectors used a modified Moloney murine leukemia virus-based promoter sequence (MND), as previously described (a kind gift from D. Kohn) [44–46]. Recombinant vector stocks were produced using calcium-phosphate to transiently transfect 293T cells with the transfer plasmid and three helper plasmids (pMDG, pMDLg, and RSVREV; a kind gift from Cell Genesys, San Francisco, <http://www.cellgenesys.com/>) [47]. The transfectate was removed after 24 hours, and unconcentrated viral supernatants were collected 24 hours after that. Virus was concentrated by ultracentrifugation at 25,000 rpm for 90 minutes at 4°C. Viral concentrates were titered against both 3521 cells (a GUSB-null murine fibroblast cell line) and 293T cells to establish relative baselines for each vector and allow expression of multiplicity of infection (MOI) of both vectors as 293T transducing units per target cell [42, 43].

### Lentiviral Transduction

For all transductions, cells were transduced within the first three passages following initial plating, and at less than 30% confluence, exposed to virus for 16 hours, then thoroughly rinsed with PBS and returned to basal media. At 48 hours following transduction, all cohorts of cells were analyzed by flow cytometry for transduction efficiency. Transduction efficiency titering assays were performed using triplicate cultures of MSCs, 293T cells, and 3521 cells exposed to eGFP virus at log-fold increasing MOIs from 0.001–10 (Fig. 1). These triplicate titering cultures were used for calculation of average transduction efficiency  $\pm$  SEM (Fig. 1) and determination of the optimal experimental MOI of 10 for all experiments. Transduced MSCs were further evaluated using a flow cytometry-based analysis of cell surface protein expression to ensure that the cells maintained their native phenotype.

### Flow Cytometry Analysis

MSC cultures were harvested using Cell Dissociation Buffer (Invitrogen) according to the manufacturer's instructions, and single cell suspensions were created containing at least  $1 \times 10^6$  cells for each staining cohort. MSCs were kept at 4°C throughout the staining procedure and were tested for expression of the following: CD11b, CD14, CD18, CD19, CD31, CD34, CD38, CD44, CD45, CD54, CD62L, CD73, CD79a, CD90, CD105, CD106, CD117, CD133, CD144, CD166, and CD271 (BD Biosciences). Staining was performed on ice for 30 minutes in the presence of 2.4G2 hybridoma (HB-197; American Type Culture Collection [ATCC], Manassas, VA, <http://www.atcc.org>) cell-free supernatant to block nonspecific Fc receptor binding. Samples were rinsed thoroughly with ice-cold PBS and analyzed using a Cytomics FC-500 Series Flow Cytometer and CXP software (Beckman Coulter, Fullerton, CA, <http://www.beckmancoulter.com>).



**Figure 1.** Transduction efficiency of various fibroblast cell lines. Log-fold increasing multiplicity of infection (MOI) using an enhanced green fluorescent protein (eGFP)-expressing lentiviral construct. The transduction efficiency (percentage transduced) was determined for 293 cells (filled bars), primary human mesenchymal stem cells (MSCs) (shaded bars), and 3521 cells (open bars) by flow cytometry 48 hours after transduction. Transductions were performed in triplicate; error bars represent SEM. A significantly ( $p < .05$ ) higher percentage of MSCs, compared with 3521 cells, were transduced at every MOI except 0.001. A significantly ( $p < .05$ ) higher percentage of MSCs, compared with 293T cells, were transduced at MOIs of 0.001, 0.01, and 0.1 but not at either MOI of 1 or 10.

Determination of GUSB lentiviral transduction efficiency into the 3521 cell line was accomplished using the ImaGene Green C<sub>12</sub>FDGlcU GUS Gene Expression Kit (Invitrogen) according to the manufacturer's instructions. Briefly, this kit contains a lipophilic substrate that allows for detection of intracellular GUSB activity following cleavage of a fluorescent tag. The fluorescent molecule is retained within the cell during the analysis and thus GUSB+ cells can be enumerated by flow cytometry.

### Transwell Culture System

Cultures were established using primary human MSCs, 293T cells (ATCC), and 3521 cells. These cultures were split into two cohorts, receiving either the GUSB-expressing lentivirus under identical conditions described above, or no lentiviral treatment. For each cell type assayed,  $5 \times 10^5$  unmanipulated or GUSB-transduced cells were plated in the lower chamber of a 6-well transwell system. Forty eight hours following transduction,  $5 \times 10^5$  untransduced 3521 cells were cultured in the upper chamber, separated by a 0.4- $\mu$ m membrane, as recipients of soluble GUSB secreted by cells in the lower chamber. Cultures were maintained for 24 hours, and then the media, transduced cells in the lower chamber, and untransduced cells in the upper well were all harvested separately and quantified for GUSB activity as described. Transwell assays were performed in serum-free media to eliminate background GUSB activity present in bovine serum, and cell populations and media were all harvested separately. For in vitro GUSB expression assays, results are expressed as the average of four independent experiments, with error shown as standard deviation, and significance determined as  $p < .05$ .

### NOD-SCID MPSVII Mice

The NOD-SCID MPSVII strain is the result of extensive backcrossing of the mutant GUSB allele from the B6.C-H-2<sup>bmi1</sup>/ByBirgus<sup>m<sup>psl</sup>+</sup> mouse onto the NOD/LtSz-scid background and has been previously characterized in detail [41–43]. Animals were bred and maintained at the Washington University School of Medicine under approved animal care protocols. Affected animals were generated by breeding mice heterozygous for the MPSVII mutation. Homozygous GUSB-deficient pups were identified at birth by biochemical analysis of toe tissue [48, 49]. Within 3 days of birth, mutant pups received intraperitoneal transplant of either  $1 \times 10^6$  human MSCs expressing eGFP (MSCs-eGFP) or an equal number of MSCs overexpressing GUSB (MSCs-GUSB). Injections were delivered in Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free, Hepes-buffered PBS in a total volume of approximately 250  $\mu$ l.

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### Flash Electroretinography

Flash electroretinography (ERG) was performed as previously described [50]. At approximately 2 months and 4 months of age, age-matched cohorts of wild-type, untreated MPSVII, MPSVII receiving MSCs-eGFP, and MPSVII mice receiving MSCs-GUSB were tested. To assess dark-adapted (principally rod function) responses, animals were anesthetized using a ketamine (80 mg/kg)/xylazine (15 mg/kg) mixture following overnight dark adaptation. The animals were exposed to five flashes from a Grass Instruments PS33-Plus xenon white light flash (Grass Technologies, West Warwick, RI, <http://www.grasstechnologies.com/index.html>) at 0.1/second. Responses were recorded using a Grass platinum wire electrode (Grass Technologies) fashioned into a 2-mm loop and placed lightly on the eye, band-pass filtered at 0.1–1000 Hz, and averaged. Reproducibility of the recorded responses was assessed by repeating trials using slightly different electrode positions. To assess light-adapted (principally cone function) responses, animals were exposed to room light for at least 20 minutes prior to testing. Fifty identical flashes were presented at 1/second against a brightly lighted background. ERG b-wave amplitudes, which reflect both photoreceptor and inner retinal integrity, were measured conventionally (negative-to-positive peak amplitude in microvolts). Results are expressed as the average measurements attained from replicates as detailed above,  $\pm$  SEM for MPSVII mice receiving MSCs-GUSB ( $n = 9$ ), untreated mutants ( $n = 8$  in dark adapted,  $n = 5$  in light adapted), and untreated wild types ( $n = 12$ ).

### Histochemical and Biochemical Analyses of Enzyme Activity

Following sacrifice, small portions of organs were harvested and frozen in Optimal Cutting Temperature embedding media (Sakura, Torrance, CA, <http://www.sakura.com>) and sectioned in 12  $\mu$ m-thick slices. GUSB-specific histochemical analysis was performed as described [41], using naphthol-AS-BI- $\beta$ -D-glucuronide (Sigma-Aldrich) as a substrate, followed by counterstaining with methyl green.

Alternative methods were used for quantitative measurement of GUSB levels in organs, serum level concentrations, and transwell experiments [41]. Homogenates were prepared from either tissue samples or cell pellets harvested following the transwell experiments. Samples were homogenized in buffer containing 10 mM Tris (pH 7.5), 150 mM NaCl, 0.2% Triton X-100, and 1 mM dithiothreitol (DTT). For serum samples, peripheral blood samples were centrifuged and cell-free supernatant was analyzed. A portion of each sample was then incubated at 37°C for 1–6 hours in 100  $\mu$ l of 5 mM 4-MU- $\beta$ -D-glucuronide substrate (Sigma-Aldrich) dissolved in 0.1 M sodium acetate (pH 4.6). Reactions were stopped by the addition of 1 ml of 0.1 M sodium carbonate. The samples were

measured fluorimetrically and then compared with standard solutions of known concentrations of 4-MU for quantitation of GUSB activity. For measurement of the secondary lysosomal enzyme  $\alpha$ -galactosidase ( $\alpha$ -GAL), the exact same method is used with substitution of 4 mM 4-MU- $\alpha$ -D-galactoside as the substrate. Bradford-based (Bio-Rad, Hercules, CA, <http://www.bio-rad.com>) protein determinations were used to normalize all measurements and calculate specific activity of each enzyme assayed.

Results of serum sample measurements are expressed as the average percentage of GUSB, compared with wild-type animals, in the serum of three individual animals of each cohort per time point. Measurements are expressed as the average  $\pm$  SD at biweekly intervals. Tissue sample measurements indicate the average activity obtained from duplicate measurements made on homogenates from three individual animals per cohort. Results are expressed as averages  $\pm$  SD, with significance set as  $p < .05$ .

### Quantitative Real-Time Duplex Polymerase Chain Reaction Analysis

Quantitation of human cell engraftment was performed using a modified version from Bensidhoum et al. [51] as previously published by our laboratory [34]. Tissue samples were mechanically dissociated and homogenized via standard protocols, followed by extraction and purification of DNA (<http://www.cshprotocols.org>). Samples were assayed in a duplex real-time reaction using TaqMan detection chemistry (Applied Biosystems, Foster City, CA, <http://www.appliedbiosystems.com>) for simultaneous examination of the human  $\beta$ -globin (F-GTGCACCTGACTCCTGAGGAGA, R-CCT-TGATACCAACCTGCCAG) gene and the mouse *rapsyn* gene (F-ACCCACCCATCTGCAAAT, R-ACCTGTCCGTGCTGCAGAA). Primer pairs and corresponding internal dual-label 6FAM-TAMRA probe sets were selected using Primer Express software (Applied Biosystems). As previously described in detail [34], triplicates of log-fold dilutions of human DNA in mouse, and mouse DNA into human were created, to generate standard curves and establish the lower limits of both detection and linearity. Accepted results generated slope curves from  $-3.1$  to  $-3.6$ , indicating efficiency between 90%–110%, with linear range detection to 0.005 ng of DNA in 100 ng of opposite species DNA (data not shown). Assuming an average of 5 pg DNA/mammalian cell, this indicates the detection of one cell per 100,000 (0.001% engraftment). For determination of total human cell contribution, the calculated number of human cells/sample was multiplied by each sample's fraction of the total organ wet weight. Experimental samples were prepared from three animals per cohort, and run in triplicate. Results are expressed as average human cells/10,000 mouse cells  $\pm$  SEM.

### Histopathologic Analysis

At the time of sacrifice, portions of organs were fixed using 4% paraformaldehyde/2% glutaraldehyde in PBS. These samples were then embedded in Spurr's resin and sectioned into 0.5- $\mu$ m slices onto slides. Sections were stained with toluidine blue and evaluated for lysosomal distention in a double-blinded manner.

## RESULTS

### MSC Transduction, GUSB Expression, and Secretion

Primary human MSCs used in this study uniformly expressed high levels of CD105, CD73, and CD90, did not express antigens indicative of other cell lineages, and maintained this expression profile throughout viral manipulation and in vitro propagation (data not shown). This molecular profile is in accord with our prior studies [34] as well as recent minimal criteria established by the International Society for Cellular Therapy [52]. MSCs transduced at an MOI of 10 with lentiviral vectors expressing either eGFP or GUSB attained a mean transduction efficiency of  $71.1\% \pm 1.33\%$  (SEM) during log phase growth and within passage three of initial plating. In comparison, 293T and a murine GUSB-deficient fibroblast cell line (3521) in

similar log phase growth were transduced at  $63.6\% \pm 2.21\%$  and  $56.3\% \pm 5.05\%$  (SEM), respectively (Fig. 1), resulting in a significantly improved transduction efficiency of MSCs versus 3521 ( $p = .041$ ) but not 293 ( $p = .063$ ). These efficiencies are in line with previously reported transductions using eGFP marking of primary MSC cultures [34]. Increasing the MOI beyond 10 did not significantly increase transduction efficiency (data not shown).

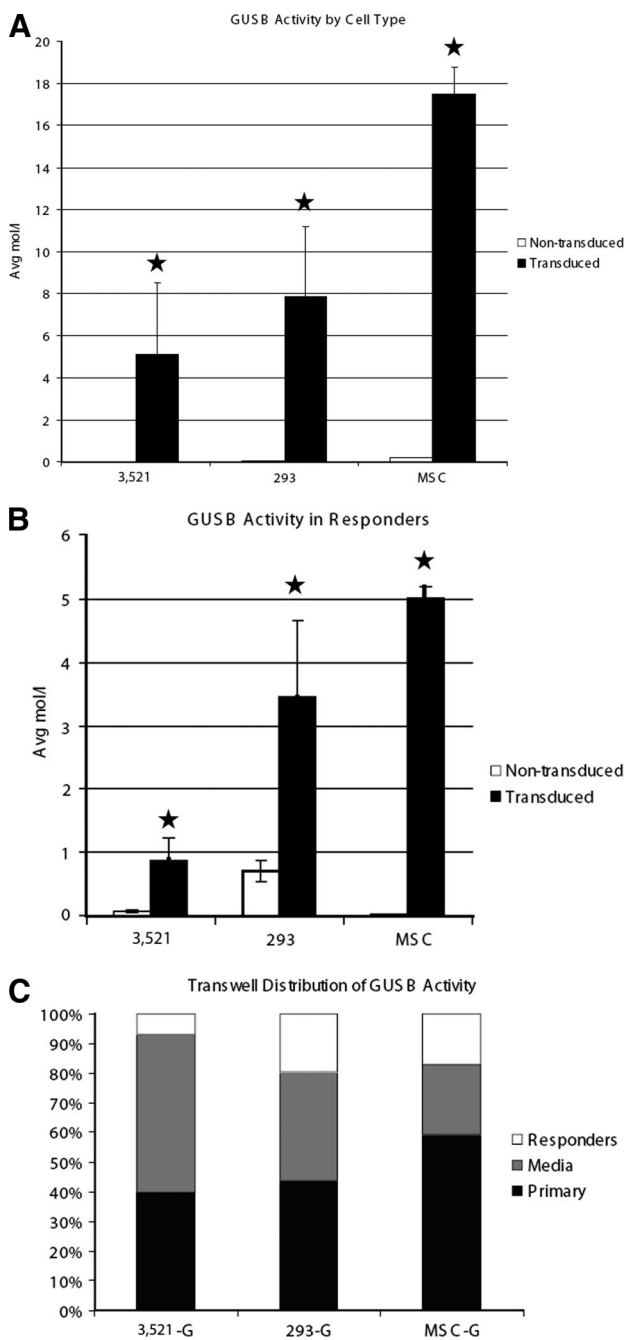
A transwell system was used to determine GUSB expression, secretion, and subsequent endocytosis by untransduced GUSB-deficient cells. This analysis was performed on untransduced and transduced MSCs, 293T cells, and 3521 cells. Unmanipulated MSCs produced very low amounts of GUSB (Fig. 2A). This finding is in accord with previous observations on the endogenous production of GUSB by fibroblastic cells [41, 42]. Following lentiviral transduction, all cell types demonstrated a marked increase in total production of enzyme. Transduced human MSCs and 293T cells demonstrated a 91- and 256-fold increase in GUSB activity, respectively, compared with untransduced cells ( $n = 4$ ,  $p = .00012$  and  $.019$ ). However, transduced MSCs expressed at least twofold greater total amounts of GUSB compared with transduced 293T cells ( $n = 4$ ,  $17.48 \pm 1.3$  vs.  $7.83 \pm 3.3$  M,  $p = .021$ ).

Endocytosis of exogenous lysosomal enzymes via mannose-6-phosphate receptors is highly efficient and can be quantified in the transwell system. The total amount of GUSB activity in the untransduced cells in the upper chamber exposed to transduced MSCs was approximately 1.5-fold greater than the activity in the upper chamber cells exposed to transduced 293T cells (Fig. 2B). However, transduced 293T cells secreted (media) a higher proportion of their GUSB activity compared with MSCs (Fig. 2C). The increase in GUSB activity in the untransduced cells in the upper chamber is likely due to the ability of MSCs to overexpress GUSB from the lentiviral vector rather than an increased secretory capacity. The utility of MSCs as a platform for cell-based therapies is also highlighted by the fact that MSCs express higher levels and secrete a greater amount of GUSB compared with a skin-derived fibroblast cell line (3521).

### MSC-Mediated In Vivo Expression

From 4–16 weeks after transplantation, recipient mice were bled from a peripheral vein and the serum was analyzed for GUSB activity. Animals that received a transplant of MSCs-GUSB demonstrated persistent expression of the transgene in vivo. This resulted in consistent serum GUSB levels of nearly 40% of normal (Fig. 3A). This relatively high level of circulating activity was a surprising finding, given the relatively small initial transplant dose ( $10^6$  total cells). In contrast, endogenous expression and secretion of GUSB from untransduced MSCs is quite low, as indicated from the transwell assay system. Consequently, transplantation with MSCs-eGFP was insufficient to elevate circulating GUSB levels above background.

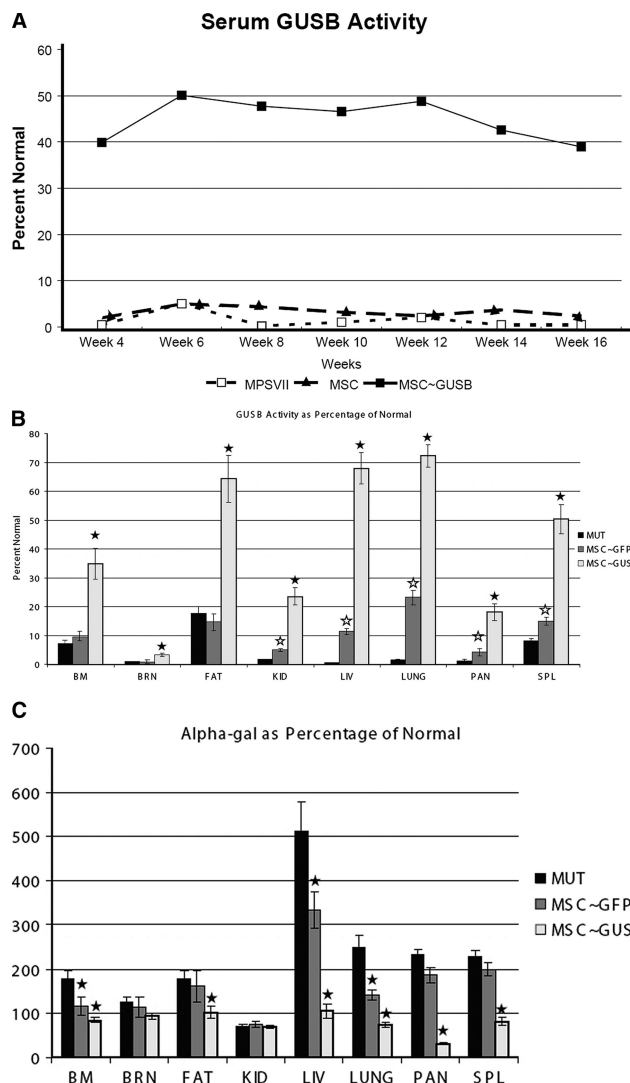
Animals receiving MSCs-GUSB not only demonstrated increased serum GUSB activity but also had significantly increased levels of enzyme in all organs surveyed (Fig. 3B,  $p < .05$  vs. untreated mutants). Animals that received a transplant of MSCs-GUSB had enzyme levels that were more than 30% above normal levels in the bone marrow, omental fat pad, liver, lung, and spleen (Fig. 3B). Although animals that received a transplant of MSCs-eGFP had significantly increased GUSB activity in the kidney, liver, lung, and spleen (Fig. 3B,  $p < .05$  vs. untreated mutants), the absolute activity detected in this cohort was less than half that observed in mice that had received a transplant of MSCs-GUSB, and most tissues did not have significantly increased activity over baseline.



**Figure 2.** In vitro characterization of  $\beta$ -glucuronidase (GUSB) expression, secretion, and uptake. (A): Total GUSB activity (average moles 4 MU liberated/L) in 3521 cells, 293 cells, and primary mesenchymal stem cells (MSCs). (B): Total GUSB activity in untransduced cells (Responders) in upper chamber of the transwell system. Increased activity is due to secreted GUSB from the transduced cells (3521 cells, 293 cells, or MSCs) in the lower chamber. (C): Distribution of GUSB contained in the transduced cells (filled bars), media (shaded bars), and upper responding cells (open bars) expressed as a percentage of the total activity in the transwell system. Results are expressed as the average of four independent measurements,  $\pm$  SD; significance as  $p < .05$  by Student's  $t$  test.  $\star$  indicates a significant difference between non-transduced and transduced groups.

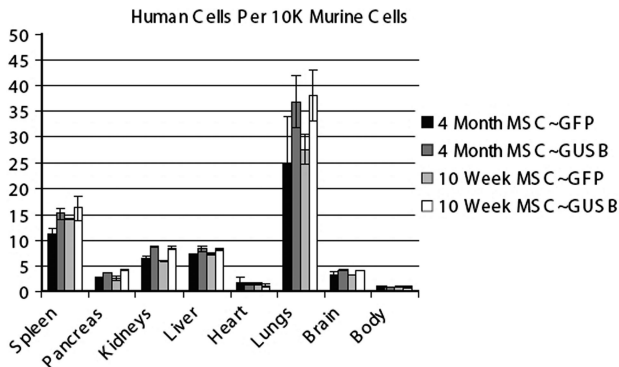
Overall, these results are superior to previous reports of systemic ex vivo gene therapy for MPSVII disease. Transplantation of genetically corrected human hematopoietic progenitor cells into the NOD-SCID MPSVII mouse was shown to result in

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**Figure 3.** In vivo  $\beta$ -glucuronidase activity in serum and tissues. (A): Average serum  $\beta$ -glucuronidase (GUSB) levels in untreated mucopolysaccharidosis type VII (MPSVII) animals ( $n = 3$ , narrow dashed line), animals that had received mesenchymal stem cells (MSCs) secreting endogenous levels of -GUSB ( $n = 3$ , wide dashed line), and animals that had received MSCs-GUSB ( $n = 3$ , solid line). Results are expressed as the average percentage of wild-type serum GUSB levels over time following transplantation  $\pm$  SD;  $p < .05$  for all MSCs-GUSB time points versus both MSCs and untreated mutants. (B): Average GUSB-specific activity per organ in MSCs-GUSB recipients (open bars), MSCs expressing enhanced green fluorescent protein (MSCs-eGFP) recipients (shaded bars), and untreated mutant MPSVII mice (filled bars) at 4 months of age. GUSB activity is expressed as the average percentage of normal levels ( $\pm$  SD) obtained from duplicate measurements of three animals per cohort. Statistical significance ( $p < .05$  by two-tailed Student's  $t$  test) was achieved in indicated organs using GUSB versus enhanced green fluorescent protein (eGFP) ( $\star$ ) and eGFP versus mutant (MUT) ( $\star$ ) comparisons. (C): Average  $\alpha$ -galactosidase-specific activity in the same organs and groups described in (B), expressed as the average percentage of normal ( $\pm$  SD). A decrease in  $\alpha$ -galactosidase activity toward wild-type values is a surrogate indication of disease correction. The stars indicate statistical significance compared with untreated mutants ( $p < .05$  by two-tailed Student's  $t$  test).

marrow correction with approximately 80–100 nmol/mg per hour activity detected [42]. MSCs-eGFP in this study were almost able to attain this level of expression in the marrow ( $\sim$ 60 nmol/mg per hour; Fig. 3B) despite undetectable engraftment in



**Figure 4.** Quantitation of human cells per 10,000 murine cells as detected by duplex quantitative polymerase chain reaction. Tissue samples are from 10-week-old mucopolysaccharidosis type VII (MPSVII) mice injected with mesenchymal stem cells expressing enhanced green fluorescent protein (MSCs-eGFP) (lightly shaded bars) or MSCs- $\beta$ -glucuronidase (GUSB) (open bars) and 4-month-old MPSVII mice injected with MSCs-eGFP (heavily shaded bars) or MSCs-GUSB (filled bars). Each cohort consists of three animals, with samples run in triplicate. Results are expressed as  $s \pm$  SEM. This assay is linear to 0.001% engraftment (one cell per 100,000).

the marrow itself. Further, animals that had received transduced MSCs more than doubled this marrow activity, producing just more than 200 nmol/mg per hour of activity, or nearly 35% of normal levels (Fig. 3B). We have attributed this high level of marrow correction without engraftment to the high level of donor MSC residence within highly vascular organs, such as liver, spleen, lung, and kidney, and the subsequent high serum content of GUSB.

A common feature of lysosomal storage disease is a dramatic elevation in other lysosomal enzymes. Measurement of  $\alpha$ -GAL activity represents a sensitive biochemical surrogate of disease progression and response to therapy. Bone marrow, omental fat, liver, lung, pancreas, and spleen from animals that received a transplant of MSCs-GUSB had nearly normal levels of  $\alpha$ -GAL (Fig. 3C). This is consistent with the robust expression of GUSB in these tissues. The only significant decreases in  $\alpha$ -GAL activity in animals transplanted with MSCs-eGFP were observed in the liver and lung (Fig. 3C,  $p = .027$  and  $.023$ , respectively, vs. untreated mutants). However, those levels were still significantly elevated, compared with normal.

### Distribution of Transduced MSCs

Given the surprisingly high level of GUSB activity in vivo and the relatively small number of cells initially transplanted ( $10^6$  transduced MSCs), we set out to determine the number and distribution of human MSCs. As previously described [34], we developed a duplex quantitative polymerase chain reaction (PCR) method for the detection and quantification of human cells within mouse tissue. This method uses the simultaneous PCR detection of a single-copy human and mouse gene, compared against 10-fold limiting dilution curves of human DNA in mouse DNA. This method has been shown to be linear down to 0.001% engraftment [34, 53]. In mice that were 4 months of age, donor MSCs were detected in every tissue analyzed (Fig. 4). The relative distribution of MSCs is virtually identical to that observed following transplantation of nontransduced MSCs into the same xenotransplantation model [34]. Assuming an average of 5 pg of DNA per mammalian cell, we are able to estimate the number of donor human cells per sample, based on these PCR data. By multiplying this number by each sample's fractional weight of the total organ, we were able to estimate the total

number of human cells in each organ. When the total number of MSCs in the animals was calculated, there were approximately  $2-4 \times 10^6$  MSCs remaining at 4 months of age. These data suggest that transduced MSCs persist in vivo for prolonged periods of time without dramatically expanding.

### Disease Correction

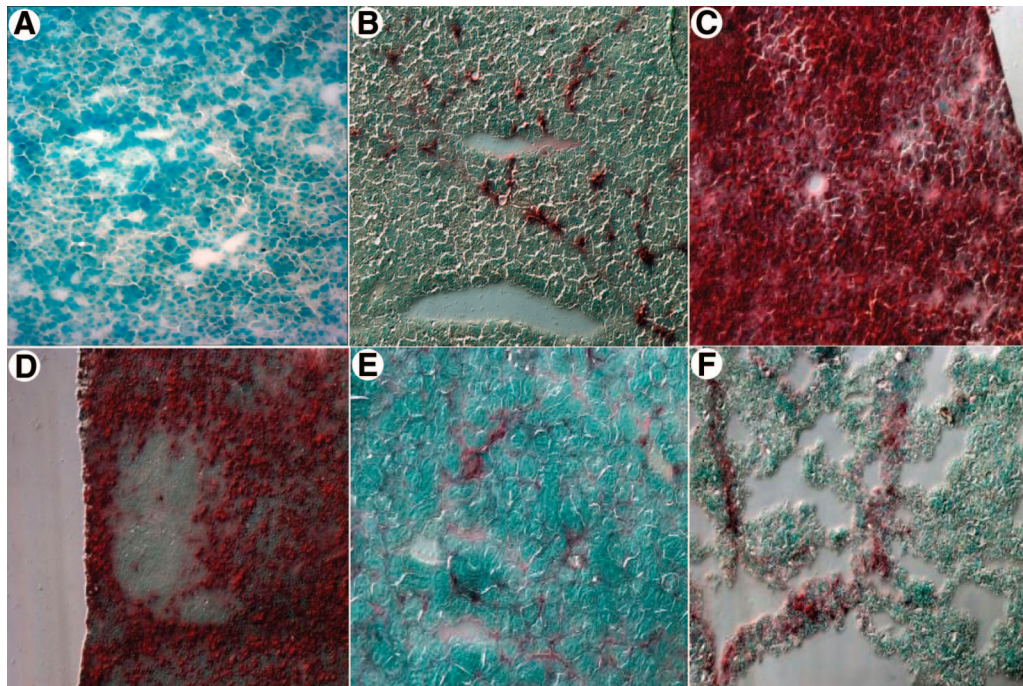
Histochemical localization showed widespread high levels of GUSB activity in the spleen, liver, lung, and kidneys of animals that received a transplant of MSCs-GUSB (Fig. 5). Animals that received a transplant of MSCs-eGFP did not show this widespread robust staining in any organ. However, infrequent, intense punctate staining was observed in several tissues—likely due to the rare engrafting MSCs present in the organ (data not shown).

Tissue samples from each cohort were fixed and sectioned for double-blind evaluation of lysosomal distension. Organs of untreated animals were found to have markedly distended lysosomes not present in wild-type animals (Fig. 6). In contrast, mutant animals that received MSCs-GUSB showed a nearly complete lack of this distension in several tissues. MSCs-GUSB cohorts demonstrated a moderate to robust resolution of lysosomal distension in spleen and liver, with a mild correction of pathology in the heart and brain. Animals that received a transplant of MSCs-eGFP had no appreciable decrease in lysosomal distension in any tissue.

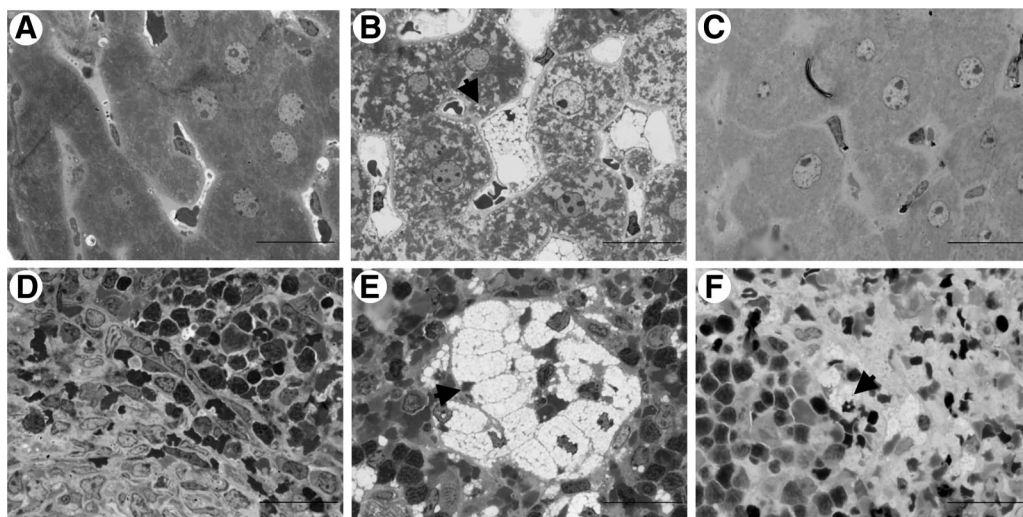
MPSVII mice have progressive retinal degeneration that leads to decreased amplitudes of both dark-adapted (rod) and light-adapted (cone) electroretinographic signals. Retinal function has been used as one physiological measure of therapeutic efficacy [50]. Unexpectedly, whereas no animals in any treatment group showed consistent decreases in lysosomal distension in the cornea or retinal pigmented epithelial layer, 4-month-old animals that received a transplant of MSCs-GUSB showed significantly improved retinal function. Improved retinal function was observed using both dark-adapted (Fig. 7A) and light-adapted (Fig. 7B) paradigms. Animals that received a transplant of MSCs-eGFP showed no improvements in retinal function. Unfortunately, only two animals that received a transplant of MSCs-eGFP could be evaluated by ERG at 4 months of age since the remaining animals that underwent transplantation died during the procedure, as did three untreated mutant animals. Neither of the two MSCs-eGFP animals had improved retinal function compared with untreated MPSVII animals (data not shown). This is further indirect evidence that animals that received a transplant of MSCs-GUSB had less severe disease than animals that received a transplant of MSCs-eGFP, since all eight of the animals that received a transplant of MSCs-GUSB survived the procedure whereas only two of the eight animals that received a transplant of MSCs-eGFP survived. In both light-adapted and dark-adapted responses, age-matched MSCs-GUSB cohorts were significantly improved over their untreated mutant littermates ( $p = .0338$  and  $.0049$  for light and dark adapted, respectively) and were not significantly different from untreated wild-type animals.

## DISCUSSION

We tested the utility of human bone marrow-derived MSCs as a platform for ex vivo gene therapy in a xenotransplantation model of inherited metabolic disease. Consistent with previous reports, our data show that MSCs are highly susceptible to lentiviral transduction and the majority of primary human MSCs can be infected with a relatively low multiplicity of infection (MOI = 10). It has been shown previously that measurable



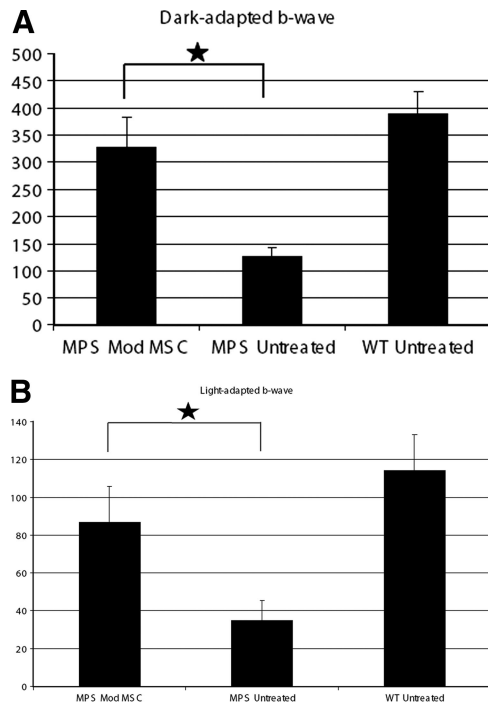
**Figure 5.**  $\beta$ -glucuronidase (GUSB) distribution in various tissues following transplantation. (A): There is no demonstrable GUSB activity in the liver of a 4-month-old mucopolysaccharidosis type VII (MPSVII) mouse that did not undergo transplantation. (B): Low numbers of GUSB-positive cells (red stain) are observed in the liver of a 4-month-old MPSVII mouse that received a transplant of mesenchymal stem cells expressing enhanced green fluorescent protein (MSCs-eGFP). The number of GUSB-positive cells is in the same range as the number of human cells detected by quantitative polymerase chain reaction (Fig. 4). (C): Nearly every cell in the liver of a 4-month-old MPSVII mouse that received a transplant of MSCs-GUSB is GUSB positive. (D): Most of the cells in the white pulp of the spleen from an MPSVII mouse that received a transplant of MSCs-GUSB are GUSB positive. Isolated GUSB-positive cells are also observed in the (E) kidney and (F) lung of 4-month-old MPSVII mice that received a transplant of MSCs-GUSB.



**Figure 6.** Lysosomal storage was nearly eliminated in the liver and spleen of mucopolysaccharidosis type VII (MPSVII) animals transplanted with mesenchymal stem cells expressing  $\beta$ -glucuronidase (MSCs-GUSB). There is no lysosomal distention observed in normal liver (A), but extensive storage (filled arrow) is observed in the liver of an untreated MPSVII animal (B). In contrast, there is little or no demonstrable lysosomal distention in the liver of an MSCs-GUSB-treated MPSVII mouse (C). Similar levels of storage as those observed in the livers were observed in the corresponding spleens of normal (D), untreated MPSVII (E), and MSCs-GUSB-treated MPSVII (F) mice. The bar indicates 50  $\mu$ m.

levels of circulating proteins can be obtained following transplantation of genetically modified MSCs into experimental animal models [30, 54]. This high level of circulating proteins was attributed to a greater secretory capacity of MSCs compared with other cell types [24]. However, the secretory capacity of MSCs was not directly measured. In the current study, we also show that transduced MSCs can mediate relatively high and

consistent levels of a circulating lysosomal enzyme (GUSB) for at least 4 months. On careful analysis, our *in vitro* data suggest that the high levels of circulating enzyme may be due in large part to the ability of MSCs to overexpress the protein rather than to secrete higher levels. The proportion of total GUSB secreted by transduced MSCs was intermediate between a murine skin-derived fibroblast (3521) and 293T cells. However, transduced



**Figure 7.** Retinal function is improved following transplantation of mesenchymal stem cells expressing  $\beta$ -glucuronidase (MSCs-GUSB). (A): Flash electroretinography (microvolts [ $\mu$ v]) showing increased amplitudes of the dark-adapted b-wave (rod function) in MSCs-GUSB-treated mucopolysaccharidosis type VII (MPSVII mice) (MPS Mod MSC) compared with age-matched untreated MPSVII mice (MPS Untreated). (B): Flash electroretinography in the same animals show an improved light-adapted b-wave response (cone function) in MSCs-GUSB-treated MPSVII animals compared with untreated MPSVII animals. Stars represent a significant difference ( $p = .0338$  and  $.0049$  for light and dark adapted, respectively). The amplitudes of the dark-adapted and light-adapted b-waves in MSCs-GUSB-treated MPSVII animals were not significantly different from untreated normal animals (WT Untreated).

MSCs expressed approximately 5- and 1.5-fold higher levels of enzyme compared with 3521 and 293T cells, respectively. The higher level of expression in MSCs compared with 293T cells cannot be attributed to differences in transduction efficiency since both cell types transduce with similar efficiency at equal MOI.

The high (40% normal) levels of circulating enzyme were surprising given the relatively small number ( $10^6$  transduced cells) of cells transplanted into the newborn animals. As mentioned above, we believe that this could be due to the dramatic overexpression of GUSB from the lentiviral vector. However, an alternate hypothesis is that the transduced MSCs had greatly expanded in vivo, thus producing more total enzyme. We believe that this is unlikely since (a) there were no gross lesions or masses observed in any of the animals that underwent transplantation, suggesting that the MSCs had not formed tumors, (b) quantitative PCR indicated that the MSCs had not expanded greatly after transduction and transplantation, and (c) if the transduced MSCs had expanded over time then circulating enzyme levels would likely increase, however, the enzyme levels remained stable for the duration of the study.

The quantitative PCR results also provide several other important insights. These data suggest that neither the transduction protocol nor overexpression of GUSB negatively effects the in vivo distribution of MSCs. In fact, the distribution of transduced MSCs in this study is nearly identical to

the distribution of unmanipulated human MSCs in a previous study performed in the same xenotransplantation model [34]. These data also suggest that the number of MSCs did not decrease during the course of this study. This seems rather remarkable since the animals were not conditioned in any way prior to transplantation. Finally, consistent with previous bone marrow transplantation studies, there is no apparent selective advantage for GUSB-positive MSCs in the MPSVII mouse.

The xenotransplantation model of MPSVII is ideally suited both for studying the distribution of human cells in vivo and for determining the ability of human cells to treat an inherited metabolic disease [41, 42]. As demonstrated above, transduced human MSCs persist in the NOD-SCID MPSVII model and produce relatively high levels of circulating GUSB. The transduced MSCs also result in relatively high levels of GUSB activity in a number of tissues. The majority of activity in tissues appears to be from secreted enzyme rather than from the infiltration of GUSB-positive cells into the various tissues. This is dramatically illustrated in the liver and spleen where the majority of cells are GUSB positive by histochemical staining but the number of infiltrating MSCs is between only 5 and 15 cells/10,000 murine cells. In contrast, there is good concordance between the number of GUSB-positive MSCs-eGFP present in the liver and spleen detected histochemically and the quantitative PCR data.

It has been shown previously that relatively low levels (1%–5% normal) of GUSB can reduce the secondary elevations of other lysosomal enzymes and reduce lysosomal distension in some tissues [55–57]. Our data are consistent with these findings. The relatively high levels of circulating GUSB normalize the secondary elevations of  $\alpha$ -GAL in many tissues and reduce lysosomal distension in several tissues. Interestingly, although we were able to systematically analyze the brain of only a single animal that received a transplant of MSCs-GUSB, there appeared to be a substantial decrease in lysosomal distension in both neurons and non-neuronal cells. This is consistent with several previous studies showing that sustained low levels of circulating GUSB could decrease lysosomal storage in the brains of MPSVII mice [58, 59].

Although low levels of GUSB can improve certain biochemical and histological features of disease, a previous study showed that circulating levels of GUSB between 2.5% and 5% normal are insufficient to provide substantial clinical benefit [59]. One measure of clinical benefit is retinal function, which is only minimally improved with 2.5%–5% normal levels of circulating enzyme [59]. Consistent with these data is the fact that control animals that received a transplant of MSCs-eGFP showed no improvement in retinal function. However, retinal function in animals that received a transplant of MSCs-GUSB was significantly improved compared with untreated MPSVII animals and was not significantly different from untreated normal control animals. The degree of functional improvement in the retina with 40% normal levels of circulating GUSB in the current study is greater than that observed in a previous study with 2.5%–5% normal circulating levels of GUSB. The results obtained in the current study are similar to those obtained from transplantation of lentiviral-transduced human HPCs [41, 42], however the level of expression and consequent correction observed with transduced MSCs was notably more beneficial.

## SUMMARY

In summary, this study demonstrates the ability of human MSCs to be transduced efficiently with a lentiviral vector and to

express high levels of protein in vitro and in vivo. Neither transduction with a lentiviral vector nor overexpression of a therapeutic protein alters the ability of human MSCs to traffic throughout the vasculature and to home into multiple tissues within a xenotransplantation model. Importantly, the transduced MSCs persist in vivo for the duration of the study and express consistent levels of enzyme. Although the endogenous level of GUSB expression from primary control MSCs was insufficient to attenuate the disease in NOD-SCID MPSVII mice, genetically modified MSCs produced levels of GUSB that resulted in biochemical, histological, and clinical improvements. These data support the use of MSCs as an independent or adjunct therapy for the treatment of inherited metabolic disorders such as lysosomal storage diseases.

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## DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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**Lentiviral-Transduced Human Mesenchymal Stem Cells Persistently Express  
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