

# Minicircle DNA-based Gene Therapy Coupled With Immune Modulation Permits Long-term Expression of $\alpha$ -L-Iduronidase in Mice With Mucopolysaccharidosis Type I

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Mucopolysaccharidosis type I (MPS I) is a lysosomal storage disease characterized by mutations to the  $\alpha$ -L-iduronidase (*IDUA*) gene resulting in inactivation of the *IDUA* enzyme. The loss of *IDUA* protein results in the progressive accumulation of glycosaminoglycans within the lysosomes resulting in severe, multi-organ system pathology. Gene replacement strategies have relied on the use of viral or nonviral gene delivery systems. Drawbacks to these include laborious production procedures, poor efficacy due to plasmid-borne gene silencing, and the risk of insertional mutagenesis. This report demonstrates the efficacy of a nonintegrating, minicircle (MC) DNA vector that is resistant to epigenetic gene silencing *in vivo*. To achieve sustained expression of the immunogenic *IDUA* protein we investigated the use of a tissue-specific promoter in conjunction with microRNA target sequences. The inclusion of microRNA target sequences resulted in a slight improvement in long-term expression compared to their absence. However, immune modulation by costimulatory blockade was required and permitted for *IDUA* expression in MPS I mice that resulted in the biochemical correction of pathology in all of the organs analyzed. MC gene delivery combined with costimulatory pathway blockade maximizes safety, efficacy, and sustained gene expression and is a new approach in the treatment of lysosomal storage disease.

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## INTRODUCTION

The ultimate goal of gene therapy is to administer a safe delivery vehicle that mediates persistent, therapeutic levels of transgene expression. Considerations for this goal include the choice

of vector and any inherent drawbacks related to it as well as the potential ramifications of expressing a foreign transgene in an immune competent setting.

Options for delivering therapeutic genes include both viral and nonviral systems each of which has benefits and risks. Viral vectors can be delivered in a highly effective manner to multiple tissues; however, the packaging of a transgene into a virion can be laborious, and the cargo capacity of viral-based vectors is finite. In addition, they pose significant risks due to insertional mutagenesis.<sup>1</sup> In contrast, plasmid-based vectors are desirable due to their ease of production, less size restricted cargo capacity, and the fact that they can be maintained as an episome that reduces the likelihood of a deleterious insertional event. However, sustained *in vivo* transgene expression from plasmids can be difficult to achieve due to gene silencing. The mechanism by which this occurs appears to be due to the deposition of repressive heterochromatin on the noncoding bacterial backbone sequences required for plasmid bacterial preparation and propagation.<sup>2,3</sup>

A second hurdle in achieving sustained gene expression is the proinflammatory properties of either, or both, the vector and/or transgene itself. The broad tissue tropism of viral vectors is due to the interaction of the viral coat proteins with a cellular receptor; however, these proteins can serve as immunogens and result in immune system activation and targeting of vector-transduced cells.<sup>4</sup> In plasmid DNA the hypomethylated CpG motifs present in the bacterial backbone possess proinflammatory properties that can serve as a trigger for the initiation of an immune response.<sup>5</sup> Therefore, loss of gene expression can be due to silencing of the expression cassette by epigenetic changes or by loss of vector genomes as a result of immune-based clearance, which can adversely affect gene-based therapeutic interventions.

Vector modification strategies aimed at preventing the loss of gene expression have included the use of insulator elements, restricting gene expression to a particular target cell/tissue by

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utilizing tissue-specific promoters and/or including microRNA target sites (mirT) that prevent “off target” gene expression in antigen-presenting cells.<sup>6–8</sup> From a plasmid-based perspective a new technology has emerged that further prevents gene silencing known as the minicircle (MC). Devised by Chen *et al.* this system utilizes a phiC31 integrase recombination event to remove the bacterial backbone elements of the plasmid resulting in a DNA circle, *i.e.*, the MC, encoding the mammalian expression cassette of choice and a small *attR* footprint.<sup>9</sup> This species is resistant to gene silencing *in vivo*, is maintained as an extrachromosomal episome, and represents an ideal platform for gene replacement strategies.

A model candidate for gene replacement therapies is mucopolysaccharidosis type I (MPS I); an autosomal recessive disorder characterized by the loss of the lysosomal hydrolase  $\alpha$ -L-iduronidase (IDUA; EC 3.2.1.76) resulting in the gradual lysosomal accumulation of the glycosaminoglycans (GAGs) heparan and dermatan sulfate causing multi-organ system pathology.<sup>10</sup> Treatment for MPS I centers on the fact that extracellular IDUA can be endocytosed by the mannose 6-phosphate receptor on the cell surface and routed to the lysosome where it can degrade accumulated GAGs.<sup>11</sup> Multiple gene delivery platforms in numerous MPS animal models have been employed for the treatment of MPS I. A key consideration for IDUA-based therapies is the immune response to the IDUA protein that can include either or both B- and T-cell mediated responses. Strategies for circumventing immune activation has included gene delivery in immunologically immature neonatal animals as well as vector modifications

(*e.g.*, tissue-specific promoters) and direct immunomodulation in adult animals.<sup>7,12–19</sup>

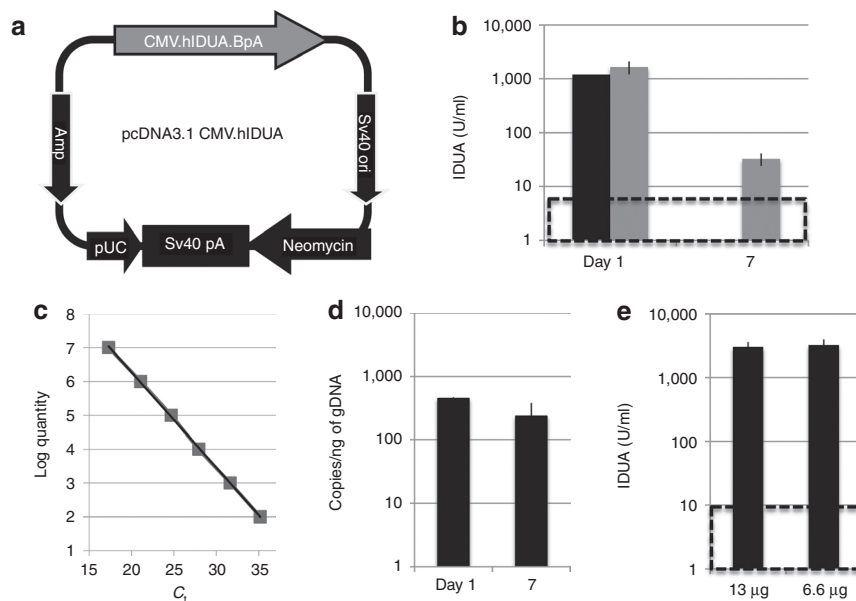
Due to the labor-intensive viral vector preparation procedures and the insertional mutagenesis risk associated with both viral and nonviral integrating vectors, we sought to implement the MC technology for IDUA gene expression. We further sought to define the optimal conditions for sustained transgene expression in the immune competent, IDUA deficient mouse strain that exhibits many of the characteristics of human MPS disease.<sup>20</sup>

We report the use of the MC technology for the gene therapy of MPS I. Delivery was mediated by hydrodynamic injection and expression was under the control of a liver specific promoter. However, neither this regulatory element nor the inclusion of mirT sequences resulted in sustained transgene expression. When an immunomodulatory strategy involving costimulatory blockade was used, we achieved prolonged therapeutic gene expression resulting in correction of many of the manifestations of the disease.

## RESULTS

### Loss of transgene expression from plasmids *in vivo* is neither due to the immune system nor loss of vector genomes

Our previous studies showed only short-term expression of IDUA was achievable in MPS I mice.<sup>21</sup> To determine whether the transient expression was the result of immune-mediated recognition of the “foreign” IDUA protein an IDUA plasmid regulated by the cytomegalovirus (CMV) promoter (Figure 1a) was injected



**Figure 1** Plasmid DNA expression *in vivo* is rapidly lost. Cytomegalovirus (CMV)-regulated transgene expression in immune-deficient mice. **(a)** CMV promoter driven expression cassette with the human  $\alpha$ -L-iduronidase gene and the BGH polyadenylation signal (BpA). Plasmid components include an ampicillin and neomycin resistance gene, the pUC origin of replication and SV40 regulatory elements. **(b)**  $\alpha$ -L-Iduronidase (IDUA) enzymatic activity was assessed in the plasma of immune-deficient NOD-SCID $\gamma$ c mice at which point the first group (black bar) was killed and liver genomic DNA harvested. A 24 hour and 7 day time point was collected for plasma IDUA on a second group (gray bars) followed by elective sacrifice and isolation of genomic DNA. Plasmid copy number. **(c)** A Taqman copy number assay was used by diluting known amounts of the CMV IDUA plasmid to generate a standard curve plotting cycle threshold ( $C_t$ ) (x axis) against log plasmid copy number (y axis) with  $y = -0.2816x + 11.905$ ;  $R^2 = 0.99968$ . **(d)** Plasmid copy numbers in animals 1 or 7 days postinjection. **(e)** Plasma IDUA levels of animals treated with the original 13.2  $\mu$ g dose compared to animals treated with one half of that amount (6.6  $\mu$ g) corresponding to the average loss of DNA copies in **d**. Black, hatched box indicates the normal level of IDUA present in a wild-type mouse.  $N = 3$ .

at a dose of 13.2  $\mu\text{g}$  *via* hydrodynamic tail vein injection into T-, B-, and natural killer (NK) cell deficient, macrophage defective NOD-SCID $\gamma\text{c}$  mice. The expression of IDUA reached supraphysiological levels 24 hours postinjection but was then largely lost by 7 days (Figure 1b). To determine whether loss of vector genomes by another mechanism (*e.g.*, degradation) was responsible for the decrease in IDUA expression, we analyzed the liver genomic DNA of the animals in Figure 1b and assessed the copy number of plasmids at the 24 and 7 day time points. A standard curve utilizing known amounts of the IDUA plasmid was generated by real-time PCR (Figure 1c) and animals at 7 days postinjection showed a modest (less than twofold) decrease in the number of vector genomes versus those analyzed 24 hours after gene delivery (Figure 1d). To determine whether this decrease in plasmid numbers would result in a concomitant decrease in IDUA enzyme we injected animals with the original 13.2  $\mu\text{g}$  dose and at a dose twofold less than that (6.6  $\mu\text{g}$ ) and assessed plasma IDUA at 24 hours and observed similar enzyme levels in both treatment groups (Figure 1e). These results show that neither immune-based clearance nor loss of vector genomes is responsible for the loss of IDUA gene expression and supports previous work showing that the presence of bacterial sequences in the plasmid become condensed with heterochromatin resulting in transgene silencing.<sup>2</sup>

### MC production and IDUA expression *in vivo*

To remedy this silencing defect inherent to plasmid-based vectors, Chen *et al.* developed the MC technology that allows for the specific removal of the bacterial backbone sequences by virtue of a recombination event between *attB* and *attP* sites that flank the mammalian expression cassette.<sup>2</sup> We inserted the liver-specific promoter-regulated human IDUA cDNA between these sites that are the recognition sites for the  $\phi\text{C31}$  recombinase that is present in two copies (Figure 2a). This construct is defined as the full length (FL) plasmid (Figure 2a(i)) that upon arabinose addition to the culture media results in a recombination event resulting in the generation of two products: the bacterial backbone that is subsequently linearized by I-Sce I making it subject to intracellular exonucleolytic degradation (Figure 2a(ii)) and the mammalian expression cassette (Figure 2a(iii)).

Resolution by agarose gel electrophoresis revealed a small amount of linearized vector (Figure 2b; top band in "Pre" lane) and supercoiled plasmid (Figure 2b; bottom band in "Pre" lane) in the FL prerecombination plasmid and following induction of the recombinogenic event small amounts of the unrecombined, FL product (Figure 2b(i)), circular backbone elements (Figure 2b(ii)) remain; however, the recombined MC species (Figure 2b(iii)) represents the major product.

To determine the efficiency of the FL versus the MC to mediate sustained gene expression we injected equimolar amounts of each into NOD-SCID $\gamma\text{c}$  mice. As was observed with the CMV promoter driven cassette the FL plasmid showed a rapid loss of gene expression whereas the MC injected animals exhibited long-term, stable levels of IDUA expression (Figure 2c). We next analyzed the status of the MC (*i.e.*, circular and episomal or linearized and integrated) in the liver genomic DNA of the MC treated animals in Figure 2c. Liver genomic DNA harvested 50 days postinjection was subjected to inverse PCR where the PCR primers are oriented

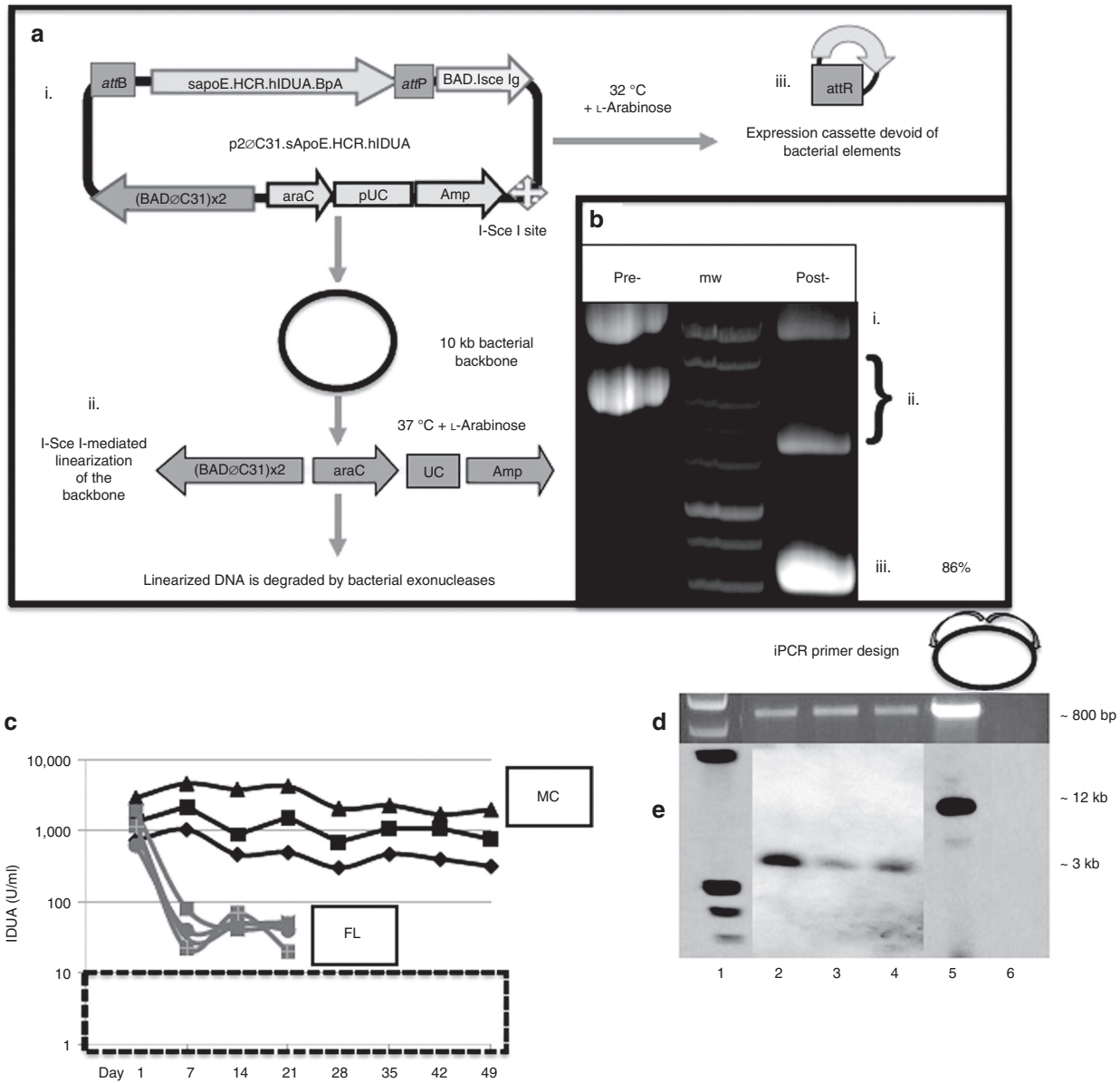
away from one another (diagram above Figure 2d) such that amplification would only occur if the template were circular. In all of the animals we detected the circular product showing that the MC can persist as a circular episomal DNA product (Figure 2d). To ascertain whether any linearization and subsequent genomic integration occurred, we performed Southern blotting on DNA isolated from the livers of MC-treated mice. Southern analysis revealed only the MC product a finding that is in agreement with previous studies<sup>2</sup> (Figure 2e).

### MC-mediated IDUA expression in immune competent and immune depleted animals

To determine the ability of the MC to mediate long-term expression in immune competent animals we injected an equal amount as delivered to NOD-SCID $\gamma\text{c}$  mice into C57Bl/6 mice. The plasma IDUA levels in these animals were lost by ~1 month (Figure 3a) indicating that an anti-IDUA immune response was operative.

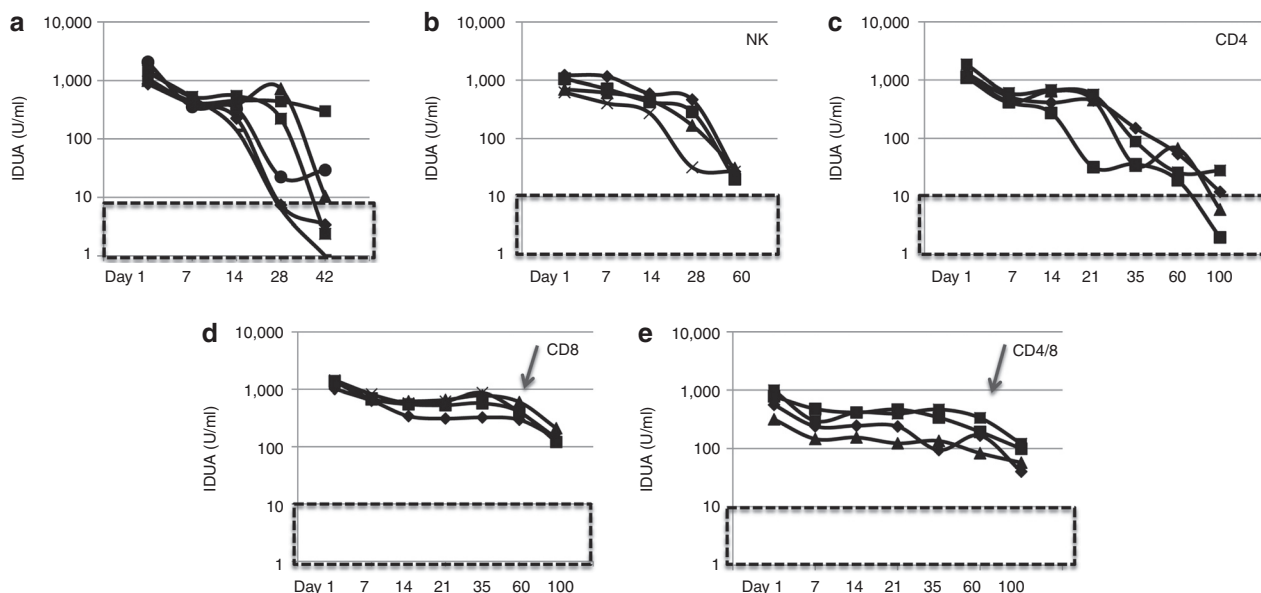
To assess the nature of this immune response we implemented an antibody-based immunodepletion strategy to remove different functional arms of the immune system. Depletion of NK cells (Figure 3b) or CD4 cells (Figure 3c) did not result in long-term IDUA expression. When either CD8 alone (Figure 3d) or CD4 and CD8 cells (Figure 3e) were depleted, gene expression was prolonged to at least 3 months. This expression was incumbent upon continuous, weekly injections of the depleting antibody as evidenced by diminished transgene expression following cessation of antibody depletion injections (indicated by gray arrows in Figure 3d,e) on day 60 with subsequent rebound of immune cell numbers.

The employment of a liver-specific promoter alone did not allow for long-term expression and sustained CD8<sup>+</sup> T-cell depletion is undesirable; therefore, we added a further layer of control to our vector by incorporating mirT into the 3' end of the cassette. The use of four copies of mir-142-3pT has been shown to result in therapeutic levels of factor IX without further immunomodulation due to prevention of gene expression in cells of the hematopoietic lineage.<sup>22,23</sup> It is thought that expression of the transgene in antigen-presenting cells can trigger immune cell activation that can negatively impact gene expression.<sup>24</sup> Therefore, we adopted the mir-142-3pT approach as well as expanded the mirT strategy to include mir155 that is upregulated in activated antigen-presenting cells.<sup>8</sup> The vector was modified to include, in the 3' UTR, four tandem copies of a 22 nucleotide sequence complementary to miR155 or four copies of the 23 nucleotide sequence perfectly complementary to the hematopoietic specific miR-142-3p miRNA or four copies of each arrayed so that the mir155T sequences were upstream of the mir-1423pT sequences. The different constructs were then hydrodynamically injected at a dose of 12.7  $\mu\text{g}$  into C57Bl/6 mice. At ~1 month after gene transfer three of four animals receiving IDUA-mir-142-3pT had lost gene expression (Figure 4a). Similarly, three of four animals treated with IDUA-mir155T showed declining levels of IDUA at 1 month (Figure 4b) as did three of four animals receiving the dual mir155/142 regulated plasmid in which IDUA levels declined more than tenfold in two animals (Figure 4c). Taken together the results from Figures 3 and 4 show the requirement of immunomodulation when using the IDUA transgene and that while regulation of the cassette by mirT

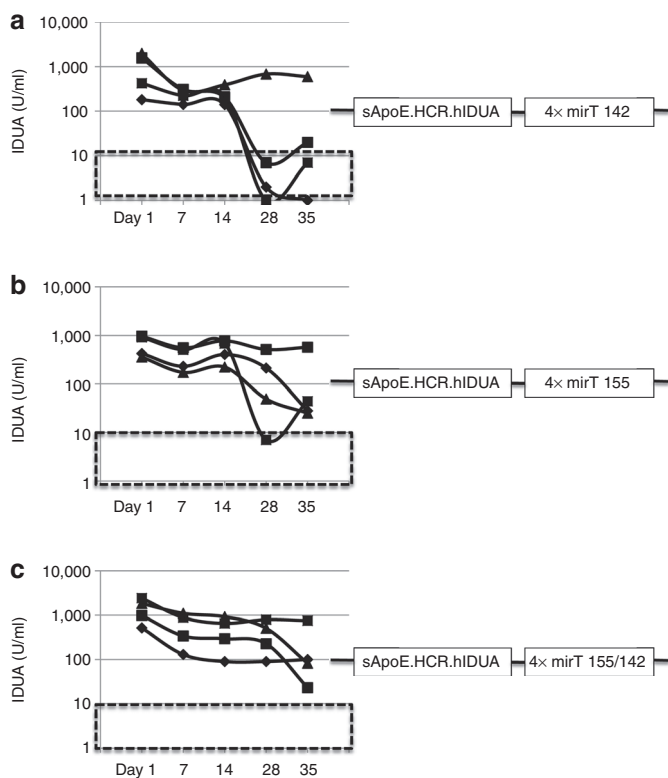


**Figure 2** IDUA-based minicircle cassette mechanism, purification, function, and maintenance as an episome *in vivo*. **(a)** Minicircle (MC) expression cassette and generation. The expression cassette, comprised of an enhancer promoter containing the human apolipoprotein E hepatic locus control region and the human  $\alpha$ -1-antitrypsin promoter (sApoE.HCR.hAATp) driving human  $\alpha$ -L-iduronidase (hIDUA) production with the bovine growth hormone polyadenylation signal (bpA), is flanked by the  $\phi$ -C31 attachment B ( $attB$ ) and attachment P ( $attP$ ) sites. The phiC31 recombinase gene is present in two copies (BAD C31x2) and it and the I-Sce I Ig gene are under the control of the arabinose inducible BAD promoter. The I-Sce I homing endonuclease site is denoted by the cross. Amp, ampicillin resistance gene; pUC, pUC plasmid origin of replication; *araC*, *araC* repressor. (i) The full-length prerecombination plasmid is approximately 12 kb. The addition of arabinose and incubation at 32°C for 2 hours results in the activation of the phiC31 genes and the generation of two products: (ii) the bacterial backbone (iii) the recombined MC. Supplemental arabinose is added followed by an increase of the culture temperature to 37°C for an additional 2 hours resulting in the activation of the I-Sce I gene causing linearization and degradation of the backbone elements. **(b)** Agarose gel electrophoresis of the plasmid in its prerecombination, FL state labeled "pre". The bands shown are the linearized and supercoiled species. The postrecombination MC species is shown at right. The differing plasmid species in the postrecombination MC are the: (i) ~12 kb linearized full length, (ii) bacterial backbone elements, and (iii) the ~3 kb MC, with the MC representing the major (~86%) product. **(c)** Treatment of mice with the unrecombined, full length (FL) plasmid or the MC. NOD-SCID $\gamma$ c mice ( $n = 4$  for FL and  $n = 3$  for MC) received equimolar amounts of either DNA species and plasma  $\alpha$ -L-iduronidase (IDUA) levels were measured at the specified time points. Maintenance of the MC as an episomal species. Liver genomic DNA from the MC treated animals in **2c** was isolated 50 days postinjection and analyzed by inverse PCR. The diagram above the agarose gel **(d)** show the primer design whereby human IDUA specific primers are pointing away from one another resulting in amplification of a DNA species that is circular. The PCR products were resolved on an agarose gel **(d)**. The same liver total genomic DNA was digested with *Bgl*II, an enzyme that cuts the MC or FL once, and hybridized with a human IDUA specific probe. Lane 1 of **d** and **e** are molecular weight (mw) standards, lanes 2–4 are the individual animals treated with MC from **2c**, lane 5 is a positive control containing plasmid DNA spiked into liver genomic DNA and lane 6 is a the negative control containing untreated mouse liver DNA. Black, hatched box indicates the normal level of IDUA present in a wild-type mouse.





**Figure 3** MC gene transfer *in vivo* into immune competent C57Bl/6 mice in the absence and presence of immunomodulation. C57Bl/6 mice were injected with minicircle (MC) in the absence of immune depletion or with targeted depletion of a specific arm of the immune system followed by MC injection and plasma  $\alpha$ -L-iduronidase (IDUA) activity. **(a)** No immune depletion. **(b)** NK-cell depletion with PK136 (anti-NK1.1) antibody. **(c)** CD4 depletion with GK1.5 antibody. **(d)** CD8 depletion with 2.43 antibody. **(e)** CD4 and CD8 cell depletion with GK1.5 and 2.43. Arrow indicates the point when immunomodulation was withdrawn. Black, hatched box is the endogenous level of IDUA in wild-type mice. *N* = 4 each group.



**Figure 4** micro RNA regulated IDUA MC gene transfer *in vivo*. Recombined minicircle (MC) with the IDUA 3' untranslated region (UTR) containing four copies of each of the miRNA target site (mirT) for **(a)** mir155 **(b)** mir-142-3p or **(c)** 155/142 miRNA was injected into C57Bl/6 mice (*n* = 4/group) and plasma  $\alpha$ -L-iduronidase (IDUA) levels were assessed weekly. Black, hatched box is the endogenous level of IDUA in wild-type mice.

**Table 1** Assessment of tissue specificity of apoE HCR MC-hIDUA DNA

	C <sub>t</sub> GAPDH	C <sub>t</sub> IDUA
MC-IDUA	Liver: 22.55 ± 0.28	29.225 ± 0.01
	Spleen: 22.32 ± 0.15	>35
MC-IDUA mir	Liver: 22.07 ± 0.04	28.275 ± 0.01
	Spleen: 23.58 ± 0.19	>35

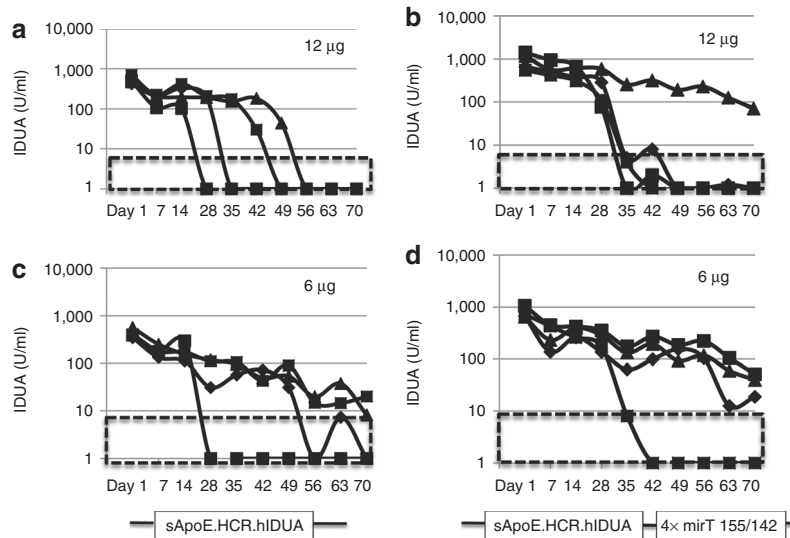
Mice were injected with MC-IDUA or MC-IDUA mirT142/155 expression cassette and real-time quantitative reverse transcription-PCR was performed on liver and spleen 1 week postdelivery. Taqman MGB Probes specific for murine GAPDH and human IDUA were used on liver and spleen. The average cycle threshold (C<sub>t</sub>) that each gene was detected at is shown ± SEM. *n* = 3/group. IDUA,  $\alpha$ -L-iduronidase; MC, minicircle.

showed some efficacy in achieving sustained expression it was incomplete.

**MC titration to determine the optimal delivery dose**

We considered that the partial ability of the liver-specific promoter driven MC-IDUA ± mirT regulation was potentially due to promiscuous gene expression outside of the liver and/or to saturation of the microRNA machinery as has been reported.<sup>25</sup> Therefore, we assessed the specificity of these regulatory elements by performing quantitative reverse transcription-PCR on the liver and spleen of treated animals. **Table 1** shows detectable levels of IDUA mRNA in the livers of animals treated with a liver-specific promoter-regulated cassette alone or in conjunction with mirT. However, in each instance no detectable IDUA mRNA was present in the spleen. These results show the specificity of the liver-specific promoter used in these studies.

We next performed a dose optimization study in C57Bl/6 mice to determine the appropriate amount of plasmid for gene



**Figure 5** Plasmid dosage effect on transgene expression. Recombined minicircle (MC) without or with the IDUA 3' untranslated region (UTR) containing mirT for 155/142 miRNA was injected into C57Bl/6 mice ( $n = 4/\text{group}$ ) at dosages of 12  $\mu\text{g}$  (**a** and **b**) or 6  $\mu\text{g}$  (**c** and **d**) with plasma  $\alpha$ -L-iduronidase (IDUA) levels assessed weekly. Black, hatched box is the endogenous level of IDUA in wild-type mice.

delivery *in vivo*. Animals were treated with a high (12  $\mu\text{g}$ ) or low (6  $\mu\text{g}$ ) dose of a non-miRNA regulated MC-IDUA or the MC-IDUA-mir155T/142-3pT cassette. In each instance a higher dose of MC showed gradual loss of gene expression (**Figure 5a,b**). The animals receiving the lower dose of plasmid in each case showed longer-term expression (**Figure 5c,d**) with the inclusion of the mirT sites resulting in a comparatively more stable expression than those animals not receiving mirT-regulated plasmids, although the enzyme levels declined by more than tenfold in all mice (**Figure 5c,d**). Taken together these results indicate that MC dose is a critical consideration for long-term expression and that the inclusion of multiple forms of gene regulation (*i.e.*, tissue-specific promoter and mirT sequences) in a MC DNA vector does not uniformly result in sustained IDUA expression.

### MC-IDUA delivery to MPS mice

Based on the preceding data, we devised a strategy for MC-based therapy in MPS mice. Because the mirT-regulated vectors trended toward providing the best levels of IDUA, we used these alone or in conjunction with an immunomodulatory strategy consisting of blockade of either the inducible costimulator (ICOS) or the costimulatory pathway. Costimulatory blockade of the B7/CD28 and CD40/CD40L pathways, which are critical in primary T-cell activation, has been used successfully in MPS.<sup>15</sup> ICOS, a CD28/cytotoxic T lymphocyte antigen 4 family member, is expressed on activated T cells and blocking this pathway has been shown to be effective in hemophilia gene therapy.<sup>26,27</sup>

Three groups of 6-month-old MPS animals were employed: a vector only group, a group receiving combined B7/CD28 and CD40/CD40L costimulatory blockade and a group receiving ICOS blockade. The vector only group showed a loss of transgene expression by ~1 month (**Figure 6a**). Similarly, ICOS depletion did not result in sustained transgene expression (**Figure 6b**). However, when costimulatory blockade with anti-CD40L (CD154), anti-B7.1 (CD80), and anti-B7.2 (CD86) antibodies was

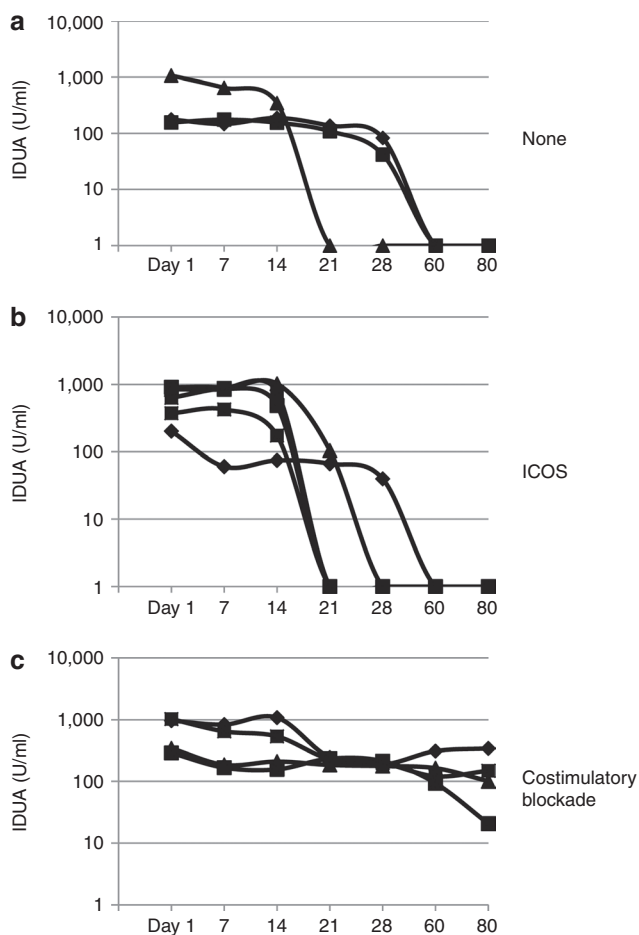
used, we observed a decline of more than tenfold in gene expression in only one of four animals followed until the animals were electively killed at 80 days after gene transfer (**Figure 6c**). In these three mice, IDUA levels of  $\geq 100$  U/ml were achieved throughout the course of the study, comparing favorably to that achieved with CD4<sup>+</sup> and CD8<sup>+</sup> T-cell depletion (**Figure 3e**).

### Assessment of humoral and cell-mediated immune response, tissue IDUA and GAG levels in MPS mice

To assess the nature of the immune response to MC delivery of the human IDUA transgene we tested for the presence of anti-IDUA IgG antibodies in the sera of animals 1 month after gene delivery. The animals that received plasmid without immunomodulation or in conjunction with ICOS depletion showed a robust antibody response whereas animals receiving costimulatory blockade did not develop antibodies (**Figure 7a**).

At 80 days after gene delivery, the animals were electively killed and to determine whether vector DNA remained in the liver of animals that lost expression and developed antibodies we performed PCR for the IDUA transgene on genomic DNA from the ICOS group. We were unable to demonstrate maintenance of the vector DNA in these animals (**Figure 7b**) indicating that the immune response to the human IDUA protein comprised both cell and humoral mediated responses (**Figure 7a,b**).

The animals that received MC DNA treatment along with anti-CD40L, anti-B7.1, and anti-B7.2 costimulatory blockade were further analyzed and compared to untreated, age-matched, sex-matched control MPS or wild-type C57Bl/6 mice for tissue IDUA enzyme levels as well as organ GAGs. These animals showed supranormal levels of IDUA in the liver with wild-type levels in the kidney and lung and small levels in the remainder of the organs analyzed (**Figure 7c**). In all tissues analyzed, the treated MPS mice showed a statistically significant (cerebellum:  $P \leq 0.01$ ; myocardium:  $P \leq 0.001$ ; lung:  $P \leq 0.001$ ; liver:  $P \leq 0.001$ ; spleen:  $P \leq 0.001$ ; kidney:  $P \leq 0.001$ ) decrease in total GAG content compared to



**Figure 6** MPS I MC gene transfer *in vivo* with immunomodulation. MPS I mice (~6 months of age) were injected with the minicircle (MC)-IDUA mirT142/155 expression cassette and plasma IDUA activity was assessed. **(a)** IDUA activity in MPS I mice ( $n=3$ ) in the absence of immune depletion. **(b)** Plasma IDUA in mucopolysaccharidosis type I (MPS I) mice ( $n=6$ ) that received inducible costimulator (ICOS) (7E.1769.67) antibody. **(c)** Plasma IDUA ( $n=4$ ) in mice that received costimulatory blockade with anti-B7.1 (16-10A1), anti-B7.2 (GL-1), and anti-CD40 ligand (MR-1).

untreated MPS mice and were comparable to those found in wild-type animals (Figure 7d). Because skeletal disease is a major component of MPS pathology, we also performed measurements to determine the width of the femur based upon similar analyses performed by Metcalf *et al.*<sup>16</sup> Our evaluation revealed a reduction in treated MPS mice compared to MPS controls ( $P=0.003$ , Figure 7e).

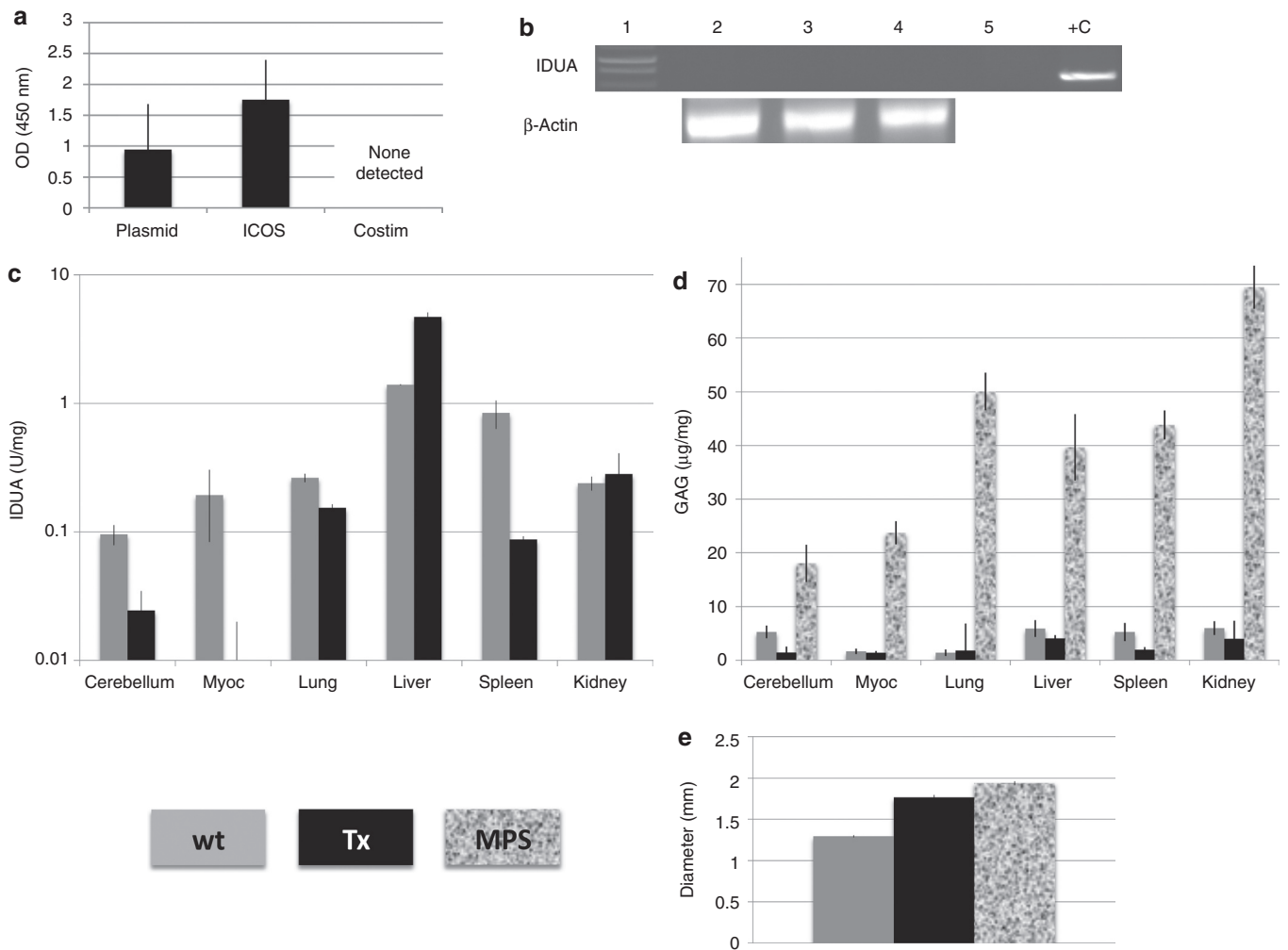
## DISCUSSION

In this study, we have demonstrated three key findings. First, the application of the MC technology is efficacious in the treatment of a lysosomal storage disease. Utilizing the murine liver as a depot organ for gene expression we show that delivery of a MC vector encoding IDUA is capable of ameliorating MPS I-related biochemical GAG accumulation. The liver was chosen as a target site for gene delivery due to its large size and ability to synthesize and secrete high levels of protein and is also easily accessible for gene transfer in the mouse by hydrodynamic injection of DNA, a procedure that is becoming applicable in larger animal models as well

as humans.<sup>28,29</sup> Moreover, because hepatocytes are terminally differentiated, these long-lived cells with a low turnover rate are well suited for nonintegrating vectors that are maintained as a stable episome.<sup>30</sup> A major limitation to this approach has been achieving sustained transgene expression due to transcriptional silencing of the plasmid expression cassette. This barrier was encountered in our studies in immunodeficient mice whereby we showed retention of plasmid DNA with a decrease in the amount of enzyme produced (Figure 1). With the observed loss of enzyme production and the maintenance of DNA in the immune defective environment of the NOD-SCID $\gamma$  mouse we were compelled to define the optimal delivery vehicle that maximized safety and efficacy. Groundbreaking work to show that the noncoding sequences inherent to the bacterial backbone of plasmids and the generation of the MC system that is resistant to silencing<sup>9</sup> provided us a means to deliver a therapeutic gene to the liver.

Therefore, to generate a mammalian expression cassette that is resistant to chromatin silencing whose expression is restricted to hepatic parenchymal cells we generated an MC vector with the sApoE.HCR.hAAT promoter driving expression of human IDUA (Figure 2a). Induction of the recombination event leads to a bacterial backbone circular DNA that is subsequently linearized and degraded leaving a small expression cassette that is purified by standard DNA procedures (Figure 2b).<sup>9,31</sup> Of the transcriptionally competent (*i.e.*, representing either the FL or MC versions capable of expressing the transgene) DNA species purified after the recombination induction ~86% was the MC version (Figure 2b). Injection of the prerecombination FL (Figure 2a(i)) versus the postrecombination MC (Figure 2a(iii)) showed that only the MC was capable of mediating sustained IDUA expression (Figure 2c). Therefore, even though there is “contaminating” FL/backbone sequences in the MC purification procedure they do not impede gene expression derived from the MC species. These findings are in agreement with other studies showing that the bacterial sequences must be present *in cis* for gene silencing to occur, and that when present *in trans*, even at an eightfold molar excess, do not negatively impact MC-mediated gene expression.<sup>2,32,33</sup> Therefore, any contaminating bacterial sequences will not result in silencing of the MC itself; however, the presence of these alternate species of DNA that copurify with the MC will preclude use in future clinical trials. To address this, a new version of the MC plasmid and a simplified and more efficient recombination and purification procedure are under development that will allow for a GMP suitable product.<sup>34</sup>

Molecular analysis of the MC in the livers of immune-deficient animals showed that it is maintained as a circular episome (Figure 2d,e). This is a critical finding and highlights a major advantage of the MC in that the risk of insertional mutagenesis is minimized. Many reports for MPS gene therapy have used integrating viral and nonviral vectors.<sup>15,16,18,35</sup> Most integrating viruses show a bias toward inserting into or near areas of transcriptional activity, thus posing a threat to dysregulation of endogenous gene function.<sup>1,36</sup> Even integration into areas not associated with active gene transcription can potentially be hazardous due to the fact that the majority of the genome is transcribed and noncoding regions possess critical regulatory functions.<sup>37</sup> Although we cannot completely rule out vector integration; however, at the level of



**Figure 7** Humoral and innate immune response in MPS mice, organ IDUA enzyme and glycosaminoglycan levels and assessment of skeletal pathology. **(a)** Humoral immune response. Sera from the treatment groups was assessed for the presence of anti-IDUA antibodies by incubating a 1:50 dilution with recombinant human IDUA that was adsorbed to a microtiter plate. IgG antibody was then detected by adding a goat anti-mouse horse radish peroxidase conjugated antibody and monitoring the color development at Abs 450. **(b)** Innate immune response. Liver genomic DNA from animals that received minicircle (MC)  $\alpha$ -L-iduronidase (IDUA) with inducible costimulator (ICOS) treatment or costimulatory blockade were assessed for the presence of IDUA vector genomes by PCR. Lane 1 is the molecular weight standard, lanes 2–4 are liver genomic DNA from ICOS treated mice, lane 5 is the no template control and lane 6 is liver genomic DNA from a mouse that showed detectable levels of plasma IDUA. Lower bands are PCR products for  $\beta$ -actin that served as a quality control. **(c)** Tissue IDUA and GAG Levels. Homogenates from wild-type ( $n = 3$ ), untreated MPS I  $^{-/-}$  mice ( $n = 4$ ) and mucopolysaccharidosis type I (MPS I) mice receiving IDUA MC in conjunction with costimulatory blockade ( $n = 4$ ) were analyzed for IDUA enzyme activity **(c)** and **(d)** biochemically for glycosaminoglycan (GAG) content. The means and SEM are shown. Levels are normalized to protein and for all tissues in untreated MPS mice versus treated MPS mice the  $P$  value was  $P < 0.01$  (Student's  $t$ -test). **(e)** Skeletal pathology. The femora of age matched, sex matched wild-type, MPS, and treated animals ( $\sim 9$  months of age) were measured at the midshaft point. For MPS control mice versus treated MPS mice the  $P$  value was  $P = 0.0003$  (Student's  $t$ -test). The small amount of substrate converted in the IDUA enzyme assay by untreated MPS mice was subtracted from the remaining groups. The OD value from untreated MPS mice for the antibody assay was subtracted from all groups. 'Wt', 'Tx', and 'MPS' denotes wild type, treated, or untreated MPS control mice, respectively.

sensitivity of Southern blotting we show no integration events and is consistent with previous reports (Figure 2d,e).<sup>9</sup>

Our vector contained a liver-specific promoter as well as mirT sequences aimed at preventing "off target" gene expression in nonparenchymal (*i.e.*, antigen-presenting cells). Our second key finding is that this dual regulatory strategy failed to prevent an immune response to the IDUA transgene. This is in contrast to a previous report in MPS I where a nearly identical promoter was capable of mediating long-term expression without immunomodulation.<sup>16</sup> Moreover, a vector containing a liver-specific promoter driving expression of factor IX with the 142-3pT sequences

in the 3' portion of the cassette resulted in long-term factor IX expression without immunomodulation resulting in phenotypic correction.<sup>22</sup> We confirmed by quantitative reverse transcription-PCR that expression of the IDUA transgene appears to be limited to the liver whether mirT are incorporated or not (Table 1). However, even at the exquisite level of sensitivity of the assay we cannot completely rule out "off target" gene expression in our constructs  $\pm$  mirT.

We propose three possible explanations for our alternative results. The first may be the differing expression profile between our studies using a plasmid-based system and the previous studies



using a retroviral delivery vehicle.<sup>16,22</sup> It has been suggested that a delay in gene expression may prevent the onset of an immune response and lentiviral vectors can exhibit a delay in expression postdelivery.<sup>38</sup> The significance of this is that following localized tissue damage in response to delivery of the viral vector the foreign transgene is not expressed and therefore is not available for presentation as an antigen.<sup>38</sup> Because the local inflammatory response is relatively brief, the “danger signal” from lentiviral vector delivery can resolve prior to transgene expression allowing for long-term expression of the foreign gene.<sup>39</sup> Following hydrodynamic injection there is significant, short-term inflammation as evidenced by increases in liver transaminases.<sup>40</sup> Moreover, the rapid onset of gene expression typical of plasmid vectors in this proinflammatory environment may result in enhanced immune system activation due to the presence of the transgene during the inflammation that occurs postinjection.<sup>41</sup>

Second, the ability of the cellular miRNA machinery to regulate the transgene may have been saturated, a phenomena that has also been documented with the use of viral vectors.<sup>25</sup> Moreover, a recent study using a plasmid DNA vector with a tissue-specific promoter as well as mirT showed an incomplete ability to prevent a transgene-specific immune response.<sup>42</sup> Indeed, our studies support these findings as evidenced by the initial testing of mirT in the IDUA cassette (**Figure 4**) as well as in the dosage studies performed (**Figure 5**). These data suggest that mirT may have benefit if a low enough dose can be used. However, a further decrement in plasmid dosage to the data presented in **Figure 5** resulted in an inability to delineate IDUA expression from the transgene from endogenous levels in immune competent, wild-type mice (data not shown). As such, any beneficial strategy involving mirT in plasmids must take into account the dosage given weighed against a concomitant decrease in gene expression potentially resulting in subtherapeutic levels of transgene expression. Lastly, the immunogenic profile of different transgenes can vary greatly. The strong immunogenicity of IDUA is well documented and therefore the inclusion of mirT may not be broadly applicable to highly immunogenic peptides.<sup>17,19</sup>

By performing antibody-based depletion of various immune system cells we identified the necessity to prevent CD8<sup>+</sup> T-cell activation in order to achieve long-term transgene expression (**Figure 3**). The studies that followed suggested a trend toward better expression with a lower dose of plasmid that contained mirT; however, this expression pattern was incomplete (**Figures 4,5**). We therefore used this construct and adopted a strategy for the MPS mouse experiments involving costimulatory blockade in a manner similar to Ma *et al.* who achieved long-term expression of non-mirT regulated IDUA<sup>15</sup> as well as a strategy involving ICOS blockade used by Peng *et al.* in factor IX gene replacement studies.<sup>27</sup> The animals receiving the plasmid only showed a rapid loss of gene expression that occurred sooner than in wild-type animals (**Figures 5,6**). The potential reasoning for this may be the presence of the immunogenic human IDUA protein in an IDUA null setting compared to the expression of human IDUA in the context of murine IDUA in wild-type animals. As such, IDUA null animals may mount a rapid immune response to the protein while the species similarity between human and mouse IDUA in wild-type animals may delay the onset of the immune response.

Similarly, the ICOS treated animals showed a loss of transgene expression at ~1 month (**Figure 6b**) whereas B7/CD28 and CD40/CD40L combined costimulatory blockade allowed for transgene expression over the course of the study (80 days) (**Figure 6c**). To assess the mechanism resulting in IDUA loss in MPS animals we determined whether an antibody response was generated against the human IDUA protein. In the plasmid only and ICOS groups, we demonstrated the presence of IgG antibodies for IDUA while the costimulatory blockade strategy prevented anti-IDUA antibodies (**Figure 7a**). The generation of anti-IDUA antibodies is well documented; however, the nature of this response appears not to impede enzymatic function.<sup>43</sup> To determine whether vector genomes are lost, which would indicate a cytotoxic T-lymphocyte response against vector-transduced cells, we harvested genomic DNA and performed an IDUA specific PCR that failed to detect any vector genomes (**Figure 7b**). Therefore, IDUA delivered in this fashion elicits both a humoral and innate immune response and strategies for modulating these arms of the immune system must be considered in order to achieve long-term gene expression.

Transgene expression was restricted to the liver but due to the ability of IDUA to cross-correct cells at a distance to the site of production we were able to detect IDUA enzyme in other tissues (**Figure 7c**). A critical component to MPS therapies is the ability of the vector system of choice to achieve sufficiently high levels of enzyme to result in biochemical reduction of GAGs. The liver showed supraphysiological levels of IDUA, the lung and kidney were comparable to wild-type mice and the cerebellum, myocardium, and spleen showed lower levels than in normal mice (**Figure 7c**). Despite sub-wild-type amounts of IDUA enzyme in these organs the levels achieved were sufficient to reduce GAGs to wild-type levels in every organ system analyzed (**Figure 7c,d**).

One surprising finding during the postmortem analyses was the reduction of GAGs in the cerebellum (**Figure 7d**). We have previously shown that the cerebellum is a major site of MPS pathology<sup>21</sup> and here show a significant reduction in cerebellar GAGs in treated versus untreated MPS mice (**Figure 7d**). This result highlights the third key finding of these studies and adds to a growing body of work suggesting that IDUA can gain access to the central nervous system by an unknown mechanism.<sup>15,35</sup> These postmortem findings precluded any live animal functional neurological readouts that will be the subject of future studies.

Over the course of this work, female C57Bl/6 mice were employed to define the optimal plasmid vector and immunomodulatory strategy. To ensure continuity and consistency, we used female MPS animals as well. Therefore, we restricted our analysis of the heart pathology to the biochemical quantification of GAGs in the myocardium. The rationale for this decision is based upon our recent findings that the severe aortic disease that is a major cause of morbidity in MPS I shows an incomplete penetrance in female mice.<sup>44</sup> Future studies will address the ability of the MC to ameliorate aortic disease in male mice. Lastly, we analyzed the femoral width in the treated and untreated animals because dysostosis multiplex leads to severe morbidity in MPS I.<sup>16,45</sup> We noted a significant reduction in the midshaft width in the treated versus untreated animals; however, the bones remained thickened compared to wild-type mice (**Figure 7e**). Thus, these findings document the ability of our strategy to ameliorate pathology not

only in the viscera but as well as two of the canonically treatment refractory tissues of the brain and skeleton.

In conclusion, we have shown the applicability of the MC system for the treatment of MPS I. Critical to this is the safety this vector system affords due to its maintenance as an extrachromosomal episome. When IDUA was under the control of the dual regulatory mechanisms of mirT and a tissue-restricted promoter we were unable to achieve sustained transgene expression in the absence of immunomodulation. We identified the clinically applicable approach of combined B7/CD28 and CD40/CD40L costimulatory blockade as the optimal immunomodulatory strategy that resulted in prolonged MC-mediated IDUA expression with concomitant reduction of MPS GAG pathology in all of the tissues analyzed.

## MATERIALS AND METHODS

**Plasmid construction.** The human IDUA cDNA (kindly provided by Dr Donald Kohn, Los Angeles, CA) was PCR amplified using forward: 5'-TGTGCTAGCATGCGTCCCCTGCGCCCCCGCGCCGCTGC-3' and reverse: 5'-TGTAGATCTTCATGGATTGCCCGGGATGG-3' that allowed for subsequent digestion with *NheI* and *BglII* for insertion into the same sites in the p20C31.hFIX MC plasmid or was amplified with forward: 5'-CACCATGCGTCCCCTGCGCCCCCGCGCCGCTGC-3' and the above reverse primer for insertion into the TOPO pcDNA3.1 D expression plasmid (Invitrogen, Carlsbad, CA). The mir-142-3pT sequences were obtained from the LV.ET.FIX.142-3pT plasmid<sup>22</sup> and the mir155 sequences were assembled using the oligonucleotides described by Brown *et al.*<sup>8</sup> Downstream of IDUA were restriction endonuclease sites that allowed for the direct insertion of one or both of the mirT sequences using PCR primers that are vector specific for the pBluescriptII.KS plasmid (containing mir-142-3pT) or the TOPO pcDNA3.1 D plasmid (containing mir155) that flank the mirT sites.

**MC generation.** The MC version of IDUA was prepared by an overnight culture in 1l of Terrific Broth (Invitrogen). The cells were spun at 3,000 rpm for 15 minutes and then resuspended in LB-broth containing 1% arabinose and then incubated at 32°C for 2 hours. Low salt LB supplemented with 1% L-(+)-arabinose (Sigma Aldrich, St Louis, MO) at a volume of 125 ml was then added following with an increase of the culture temperature to 37°C for an additional 2 hours. The cells were centrifuged and a maxi-prep using the PureLink HiPure Maxi-prep (Invitrogen) kit was performed using volumes of five times the amount described by the manufacturer for the re-suspension, lysis, and neutralization buffers. The lysates were then separated over four individual columns, pooled, and concentrated for use by ethanol precipitation.

**Gene delivery and animals.** Plasmid DNA at the indicated doses was injected into all animals using the hydrodynamic tail vein injection procedure.<sup>46</sup> The animal strains used were NOD-SCID<sub>γ</sub>, C57Bl/6, or, the IDUA<sup>-/-</sup> mouse (C67Bl/6 background),<sup>20</sup> respectively.

**Enzyme analysis.** At the indicated time points heparanized blood was obtained by retroorbital bleed and 5 μl of plasma was incubated with 4-methylumbelliferyl α-L-iduronide as the substrate (NBS Biological, Corston, Bath, UK) for 6 hours at 37°C as described.<sup>15</sup> For tissue IDUA organs were homogenized in a homogenization buffer consisting of 150 mmol/l NaCl, 0.2% Triton-X 100, 5 mmol/l EDTA, and 20 mmol/l Tris. The protein concentration of each lysate was determined using the Wide Range Protein Quantification kit (Dojindo, Gaithersburg, MD). Fluorescence was measured at an excitation spectra of 360 nm and an emission of 465 nm on a Synergy 2 Multi-Mode Microplate Reader plate reader (BioTek, Winooski, VT). One unit of enzyme is defined as the amount of enzyme that releases one nmol of 4-methylumbelliferone in 1 hour.

**Immune modulation.** The immune depletion strategy for the C57Bl/6 animals was a schedule consisting of injections at days 5, 2, and then weekly following delivery of the plasmid. Four hundred micrograms of anti-CD4 generously provided by Dr Frank Fitchm University of Chicago, Chicago, IL (GK1.5 hybridoma, rat IgG2b), anti-CD8 (2.43 hybridoma, rat IgG2b), and/or anti-NK kindly provided by Dr Gloria Koo, Merck-Sharp-Dohme, Whitehouse Station, NJ (NK1.1/PK136 hybridoma, mouse IgG2a) were diluted in phosphate-buffered saline and injected intraperitoneally. The antibody depletion schedule for the IDUA<sup>-/-</sup> mice was: day 8, day 4, day 2, day 0 (plasmid delivery), daily for the first week and then twice weekly for the remainder of the studies. The costimulatory blockade group received 200 μg each of anti-B7.1 kindly provided by Dr Hans Reiser, Dana-Farber Cancer Center, Boston, MA (hybridoma 16-10A1, human IgG), anti-B7.2 (hybridoma GL-1, rat IgG2a, American Type Culture Collection, Rockville, MD), and anti-CD40L (hybridoma MR-1BM, human IgG) in phosphate-buffered saline by intraperitoneal injection. The ICOS blockade group received 220 μg of anti-ICOS antibody (hybridoma 7E.17G9, rat IgG2b)<sup>47</sup> intraperitoneally. The identification of the hybridoma clones that produce the antibodies and the species of origin is provided in parentheses.

**Molecular analysis.** Animals were killed and liver samples were collected for genomic DNA isolation using the PureLink Genomic DNA MiniKit (Invitrogen). Inverse PCR was performed using PCR primers forward: 5'-TTCAGCCCAGACACAGGTGCTGTCTGG-3' and 5'-GGTGGG TGAAGTTGTAGCTCAGG-3' and the product was resolved on a 1.5% agarose gel. Southern blot was performed on the same genomic DNA samples using 10 μg of genomic DNA that was digested with *BglII* followed by transfer to a nylon membrane (Hoffmann-La Roche, Indianapolis, IN). A full length IDUA cDNA probe was generated by PCR using the DIG DNA labeling mix that was hybridized to the membrane in DIG Easy Hyb Buffer (both from Hoffmann-La Roche). Detection of the DIG labeled product was performed by using an anti-digoxigenin-AP conjugated antibody at a concentration of 1:20,000. Chemiluminescent detection of the product was achieved by using the CDP-STAR Reagent (Hoffmann-La Roche) followed by exposure to Xomat Blue Film (Eastman Kodak Company, Rochester, NY).

**Real-time PCR.** For the copy number assay a standard curve was generated using serially diluted IDUA plasmid. Genomic DNA from the liver was isolated using the PureLink Genomic DNA Mini kit (Invitrogen) and equal amounts were used in a TaqMan Assay using the Hs00164940\_m1 IDUA (Applied Biosystems, Foster City, CA). The assay was performed on the ABI 7300 instrument and results analyzed with ABI Relative Quantification Study software (Applied Biosystems). For quantitative reverse transcription-PCR, the organs were homogenized in Trizol reagent (Invitrogen) and total RNA was isolated using the PureLink Micro-to-Midi Total RNA Purification System (Invitrogen) followed by removal of genomic DNA using TURBO DNase (Applied Biosystems). Equal amounts of RNA were reverse transcribed using the SuperScript III First-Strand Synthesis System for reverse transcription-PCR (Invitrogen). A TaqMan Gene Expression Assay using the human IDUA probe from above and the murine Mm99999915\_g1 GAPDH probe were used as above.

**GAG pathology.** Tissues used for IDUA enzyme analysis were also used for GAGs. To one milligram of tissue homogenate 1 ml of a 1,9 dimethylmethylene blue association reagent (0.2 mol/l GuHCL, 0.2% formic acid, and 30 mmol/l sodium formate) was added and incubated at room temperature on a vortex. The samples were centrifuged, decanted, and 1 ml of disassociation reagent (50 mmol/l sodium acetate, 4 mol/l GuHCL, and 10% 1-propanol) was added. The samples were vortexed until the precipitate disappeared and then the optical density at 656 nm was determined using the Synergy 2 Multi-Mode Microplate Reader. A standard curve utilizing chondroitin sulfate was also generated.

**IDUA antibody.** IDUA antibody testing at the specified time points was done as described by Di Domenico.<sup>13</sup> Twenty nanograms of recombinant IDUA/Laronidase (Genzyme, Cambridge, MA) diluted in phosphate-buffered saline was coated on a microtiter plate overnight at 4°C. The plate was blocked with TBST-BSA (0.05% Tween, 1% BSA) for 1 hour and washed with phosphate buffer saline Tween 20. A 1:50 dilution (in phosphate-buffered saline) of serum was added for 1 hour and then washed with phosphate buffer saline Tween 20. Goat anti-mouse IgG (Abcam, Cambridge, MA) at a 1:1,500 dilution was added followed by substrate development using R&D Systems (Minneapolis, MN) Color Reagent A (stabilized hydrogen peroxide) and Color Reagent B (tetramethylbenzidine) mixed together in equal volumes. A stop buffer containing 2 N sulfuric acid was added and the plate was read using a Synergy 2 Multi-Mode Microplate Reader plate reader (BioTek).

**Statistical analysis.** Statistical significance was analyzed using the Student's *t*-test. *P* values <0.05 were considered significant.

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