UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

Edição genômica por CRISPR/Cas9 para as mucopolissacaridoses – de modelos celulares ao desenvolvimento de novas terapias

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Orientador: Prof. Dr. Guilherme Baldo
Coorientadora: Profa. Dra. Ursula da Silveira Matte

Porto Alegre, fevereiro 2021.

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Tese submetida ao Programa de Pós-Graduação em Genética e Biologia Molecular da UFRGS como requisito parcial para a obtenção do grau de ***Doutor em Genética e Biologia Molecular****.*

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"*We may be nearing the beginning of the end of genetic diseases*"

Jennifer A. Doudna

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SUMÁRIO

LISTA DE ABREVIATURAS

AAV – vírus adenoassociados
Alo-TCTH – transplante alogênico de células-tronco hematopoiéticas
ANVISA – Agência Nacional de Vigilância Sanitária
BHE – barreira hematoencefálica
Bu – bussulfano
Cas9 – CRISPR *associated protein* 9
CCR5 – receptor de quimiocina C-C tipo 5
CRISPR/Cas9 – *Clustered Regularly Interspaced Short Palindromic Repeats/ CRISPR-associated protein 9*
CTH – células-tronco hematopoiéticas
DECH – doença do enxerto contra o hospedeiro
EMA – *European Medicines Agency*
FACS – *fluorescence activated cell sorting*
FDA – *Food and Drug Administration*
GAGs – glicosaminoglicanos
GBA – glicocerebrosidase
GFP – proteína verde fluorescente
gRNA – RNA guia
HDR – *homology-directed repair*
IDS – iduronato-2-sulfatase
IDUA – alfa-L-iduronidase
iPSC – *induced pluripotent stem cells*
M6P – manose-6-fosfato
M6PR – receptor de manose-6-fosfato
MPS – mucopolissacaridose
MPS I – mucopolissacaridose do tipo I
MPS II – mucopolissacaridose do tipo II
NHEJ – *non-homologous end joining*
NSG – NOD.*Cg-Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ
PAM – *protospacer adjacent motifs*
RNP – ribonucleoproteína / complexo ribonucleoproteico
SNC – sistema nervoso central
SUS – Sistema Único de Saúde
TALENs – *transcription activator-like effector nucleases*
TBI – *total body irradiation*, irradiação de corpo inteiro
TCTH – transplante de células-tronco hematopoiéticas
TLR – receptores tipo Toll
TRE – terapia de reposição enzimática
ZFN – *zinc finger nucleases*

LISTA DE FIGURAS

LISTA DE TABELAS

RESUMO

A edição genômica consiste na modificação precisa de sequências de DNA. Com o intuito de desenvolver modelos celulares para as mucopolissacaridoses do tipo I e II (MPS I, MPS II), doenças causadas por deficiências em enzimas, gerando acúmulo de glicosaminoglicanos (GAGs), utilizamos o sistema CRISPR/Cas9 para induzir mutações em células HEK293. Obtivemos células nocautes para ambas as doenças, com níveis indetectáveis de enzima e acúmulo significativo de GAGs. Com a mesma metodologia, criamos um modelo de MPS II utilizando células SH-SY5Y, que constituem um modelo relevante para estudos fisiopatológicos. Nestas células, observamos alterações morfológicas condizentes com o fenótipo de MPS II, como aumento de autolisossomos e de mitocôndrias. Ao serem diferenciadas em neurônios, as células apresentaram densidade de neuritos diminuída. Além da geração de modelos celulares, a edição genômica pode ser a peça principal de novos produtos de terapia. Utilizando CRISPR/Cas9 e um vetor viral, editamos eficientemente células-tronco hematopoiéticas humanas saudáveis para superexpressarem alfa-L-iduronidase, a enzima ausente na MPS I. Ao serem transplantadas em camundongos MPS I imunocomprometidos e pré-condicionados com bussulfano, as células editadas enxertaram eficientemente, chegando a mais de 90 % de quimerismo humano em alguns animais. Isso culminou na correção completa dos parâmetros bioquímicos avaliados nos tecidos viscerais. O uso de bussulfano, em comparação com a irradiação, aumentou significativamente a migração de células editadas ao sistema nervoso central, demonstrada pela atividade enzimática superior, com redução significativa de GAGs. Demonstramos, assim, que a edição genômica para terapia genica *ex vivo* é muito eficaz e promissora, com poucas lacunas a serem preenchidas em estudos pré-clínicos antes da tradução aos pacientes. Uma delas é a avaliação da eficácia e segurança em modelos animais imunocompetentes. Com vistas à continuidade do estudo, avaliamos e definimos o melhor protocolo de transplante em camundongos neonatos, cuja via de administração que apresenta maior alcance das células no cérebro foi definida como sendo a via retro-orbital. Conjuntamente, demonstramos que a edição genômica contribui não só para o estudo da fisiopatologia das doenças, como também pode atuar como agente terapêutico, sendo utilizada com eficiência em protocolos de terapia gênica.

PALAVRAS-CHAVE: edição genômica, CRISPR/Cas9, mucopolissacaridose, doenças lisossômicas, modelos celulares, células-tronco hematopoiéticas, bussulfano.

ABSTRACT

Genome editing is the precise modification of DNA sequences. Aiming at developing new cellular models for mucopolysaccharidosis type I and type II (MPS I, MPS II) – lysosomal diseases caused by enzyme deficiencies leading to glycosaminoglycan storage (GAGs) – we employed the CRISPR/Cas9 system to cause mutations in HEK293 cells. Knockout cells were obtained for both diseases, with undetectable enzyme levels and significant GAG storage. Using the same method, we developed a relevant MPS II cellular model using the SH-SY5Y line. In this model, morphologic alterations were pronounced and related to the MPS II phenotype, as increased autolysosomes and mitochondria. Moreover, upon differentiation into neurons, MPS II cells presented diminished neurite density. Besides developing cellular models, genome editing tools can be the main piece in new therapeutic products. Using CRISPR/Cas9 and a viral vector, we efficiently edited human hematopoietic stem cells to express alfa-L-iduronidase, the deficient enzyme in MPS I. Edited cells were transplanted in immunocompromised MPS I mice conditioned with busulfan, where they engrafted robustly, reaching over 90 % of human chimerism in few mice. Consequently, full biochemical correction was observed in visceral organs. Comparing to irradiation, conditioning with busulfan allowed significantly higher cell migration towards the central nervous system, as demonstrated by higher enzyme activity and significant reduction in GAG levels in the brain. Thus, we have demonstrated that genome editing for ex vivo gene therapy is efficient and a promising approach to be translated to clinic, with few gaps to be filled before. One of these gaps is the efficacy and safety in immunocompetent mice. To address this, we have evaluated the best protocol for cell transplantation into newborn mice. The administration route that presented the best outcome in delivering cell to the brain was the retroorbital; additionally, this was also easy to perform and had few failures. Together, we demonstrate that genome editing contributes not only to physiopathology studies but can also be used as a therapeutic agent in gene therapy protocols, with high efficiency in both tasks.

KEYWORDS: genome editing, CRISPR/Cas9, mucopolysaccharidoses, lysosomal diseases, cellular models, hematopoietic stem cells, busulfan.

A ERA DA EDIÇÃO GENÔMICA

Edição genômica, ou edição gênica, é a modificação precisa de DNA, seja por adição, remoção ou substituição de sequências em locais específicos do genoma. A ideia de edição genômica existe desde as décadas de 1980 e 1990, quando os mecanismos de recombinação e de reparo de DNA foram mais profundamente investigados, observando-se que a ocorrência de quebras na dupla-fita de DNA favorecia a recombinação e abrindo a possibilidade de modificações intencionais no genoma (JASIN, 1996). Em 1996, surge a primeira plataforma de edição genômica, as nucleases dedos de zinco (do inglês *zinc-finger nucleases*, ZFN) (KIM *et al.*, 1996), sendo seu primeiro uso em células humanas descrito em 2003 (PORTEUS *et al.*, 2003). Em 1998, é descrita a técnica utilizando meganucleases para substituição de genes (COHEN-TANNOUDJI *et al.*, 1998). Pouco depois, em 2010, surge outra plataforma um pouco mais simplificada, as TALENs (do inglês *trancription activator-like effector nucleases*), cujo uso em células humanas já se deu no ano seguinte, em 2011 (CHRISTIAN *et al.*, 2010). Foi em 2012, porém, que surgiu a técnica que revolucionou a edição genômica e a pesquisa biológica – o CRISPR/Cas9 (do inglês *Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated 9)* (JINEK *et al.*, 2012).

Como a clivagem da dupla-fita do DNA propicia a edição genômica

O objetivo da maioria das ferramentas de edição genômica é induzir quebras no DNA dupla-fita em locais de escolha, de forma precisa e específica. A explicação é que, assim que a clivagem do DNA dupla-fita é induzida, a célula eucariótica encarrega-se do reparo imediato, uma vez que a presença desse tipo de lesão é altamente danosa e leva à morte celular se não reparada. Dois são os mecanismos celulares de reparo utilizados: junção terminal não-homóloga (do inglês *non-homologous end joining,* NHEJ) e reparo por recombinação homóloga (*homology-directed repair,* HDR). Ambos são eficientes no reparo da sequência de forma correta, embora diz-se que o primeiro é sujeito a erro por, eventualmente, induzir pequenas mutações no local quando a demanda de reparo é alta e mecanismos alternativos ao canônico são utilizados pela célula (BETERMIER *et al.*, 2014; JACKSON *et al.*, 2009).

O reparo por NHEJ recruta enzimas de processamento terminal, polimerases e ligases que processam (o que pode ou não alterar a sequência) e ligam as fitas no local da quebra. Nas vias alternativas do NHEJ, alguns nucleotídeos são excisados no processo, resultando em pequenas indels (inserções e/ou deleções) no genoma. O HDR, por outro lado, utiliza sequências homólogas ao local da quebra para serem usadas como molde para o reparo. Primeiramente, diversas proteínas são recrutadas ao local, excisando uma das fitas do DNA e associando-se à fita remanescente. O complexo nucleoproteico, composto pela fita simples de DNA e proteínas, busca por sequências homólogas para prosseguir com o reparo. Estas sequências podem ser tanto cromátides irmãs, quanto sequências exógenas – e é fazendo uso desse sistema de reparo que é possível induzir modificações precisas no genoma (CARUSILLO *et al.*, 2020) (Figura 1).

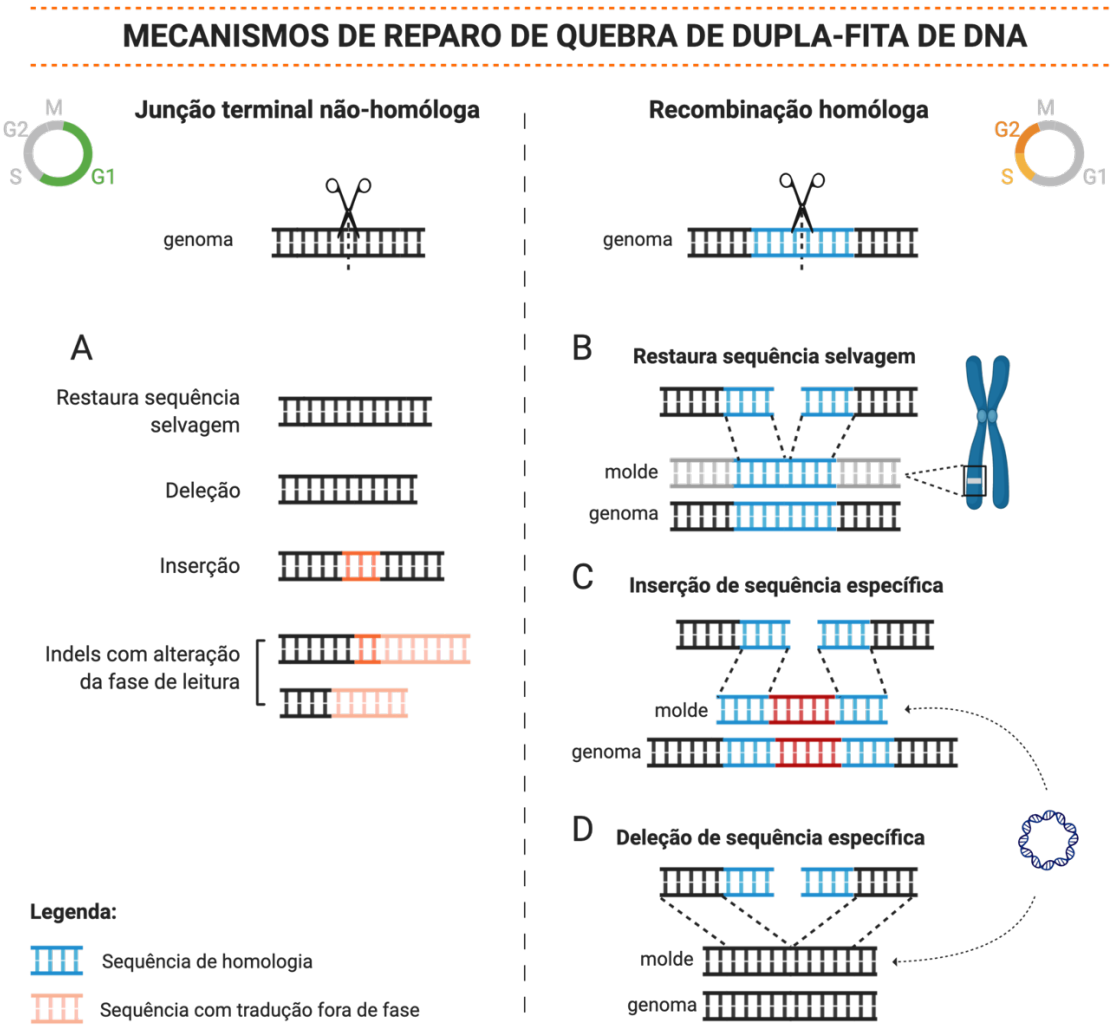

Figura 1. Mecanismos de reparo de quebra de fita-dupla de DNA. A) Reparo por junção terminal não-homóloga, que pode resultar em reparo completo do DNA sem alterações na sequência; inserções

ou deleções de múltiplos de três, alterando a sequência da proteína traduzida em poucos aminoácidos ou, ainda, *indels* que causem alteração da fase de leitura e alteração completa da sequência proteica a partir da mutação. Já o reparo por recombinação homóloga necessita de um DNA molde contendo sequências homólogas flanqueando o local da quebra para ocorrer. O DNA molde pode ser proveniente da cromátide irmã e resultar em B) restauração da sequência selvagem; ou pode ser proveniente de moléculas exógenas, como DNA plasmidial, e C) inserir uma sequência de interesse ou D) deletar uma sequência específica no genoma. Nos cantos superiores da figura são demonstradas as fases do ciclo celular em que os eventos ocorrem majoritariamente.

O mecanismo de NHEJ é o preferencial das células, utilizado especialmente por células quiescentes e células em G1; por outro lado, o HDR é estimulado em células em divisão, pois só nas fases S e G2 do estágio do ciclo celular é que há a presença de cromátides irmãs para a recombinação (CARUSILLO *et al.*, 2020). Importante ressaltar que, embora haja o mecanismo favorito dependendo do ciclo celular, ambos podem, inclusive, acontecer na mesma célula ao mesmo tempo, sendo cada alelo corrigido por um mecanismo distinto (DOUDNA, 2020). Sendo assim, baseado nas características de cada mecanismo de reparo, ao induzir quebras na dupla-fita de DNA utilizando as ferramentas de edição genômica podemos a) induzir a formação de indels por NHEJ e anular a função de um gene ou sequência ou b) introduzir sequências específicas por HDR, tanto para adição de genes inteiros quanto para modelar mutações específicas.

Plataformas baseadas em domínios proteicos: ZFN e TALENs

As primeiras técnicas customizáveis de edição genômica utilizam domínios proteicos de ligação ao DNA fusionados a uma endonuclease. Em eucariotos, o dedo de zinco é um dos domínios de ligação ao DNA mais comuns; cada dedo é capaz de se ligar a três nucleotídeos no DNA e, ao serem sintetizados em tandem (3-6 dedos), formam proteínas únicas de ligação para cada DNA alvo (9-18 pb) (URNOV *et al.*, 2010). Por outro lado, na plataforma TALEN, cada nucleotídeo do DNA é reconhecido por sequências repetidas de 33-35 aminoácidos que, quando ligadas, formam arranjos longos e específicos para o alvo. A especificidade de cada módulo para cada um dos quatro nucleotídeos se dá pelos aminoácidos nas posições 12 e 13 (KIM *et al.*, 2014). Para a formação da ferramenta de edição propriamente dita, estes domínios de ligação ao DNA são fusionados a uma endonuclease, *Fok*I, que possui seu sítio catalítico independente do seu sítio de ligação ao DNA – assim, seu sítio de ligação foi substituído pelos domínios customizáveis para que a ligação ao

DNA pudesse ser programável (MAEDER *et al.*, 2016). Entretanto, para a indução da quebra da dupla-fita pela *Fok*I, a enzima precisa ser dimerizada no alvo, o que requer o desenho de dois domínios de ligação: um específico para a fita senso e outro para a fita antisenso, de forma que fiquem adjacentes e com espaçadores de 5-7 pb ou 12-21 pb para ZFN e TALEN, respectivamente (KIM *et al.*, 2014). Com a ligação dos dois monômeros, a *Fok*I dimeriza e cliva a dupla-fita de DNA.

Ambas plataformas demonstraram ser eficientes no alvo e terem baixa atividade em locais indesejados (*off-target*), tanto que as ZFNs já estão sendo utilizadas em ensaios clínicos (POLETTO *et al.*, 2020). Suas desvantagens são inerentes à complexidade de desenho e de produção, uma vez que trata-se de domínios proteicos complexos que devem ser sintetizados para reconhecimento de ambas as fitas do DNA. Além disso, vetores de entrega com baixa capacidade de empacotamento ou carreamento não conseguem entregar os produtos em vetores únicos, necessitando de sistemas mais elaborados para a entrega eficiente de todos os componentes necessários (KIM *et al.*, 2014). A Figura 2 apresenta as principais características de cada plataforma.

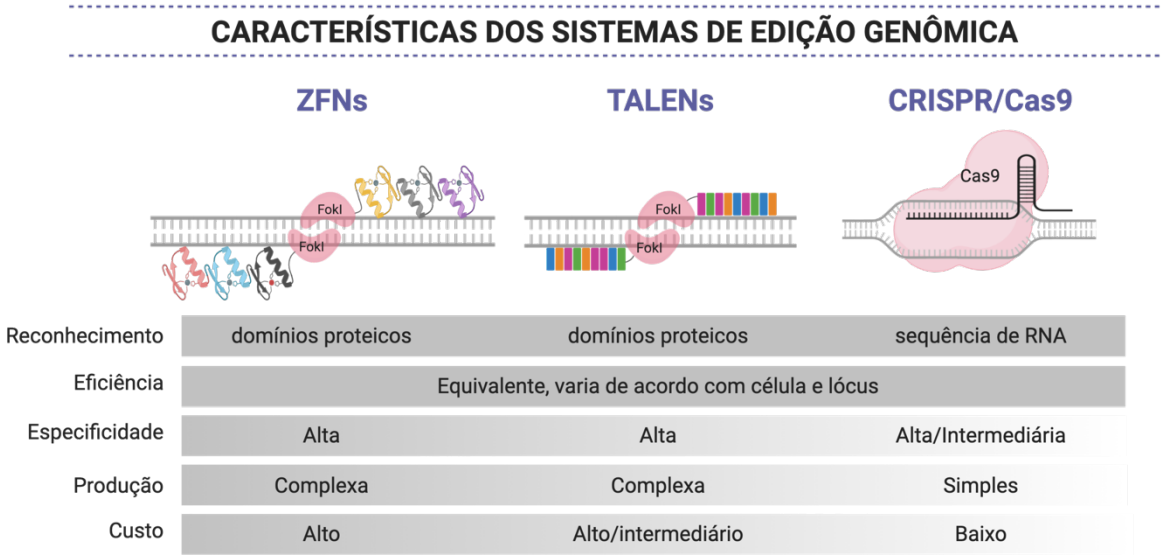

Figura 2. Características, vantagens e desvantagens das principais plataformas de edição genômica. As nucleases dedo de zinco (ZFNs) e as TALENs utilizam domínios proteicos de reconhecimento ao DNA e são muito específicas (por requererem a ligação de cada domínio de reconhecimento em cada uma das duas fitas de DNA), porém, são complexas de serem produzidas e possuem custo alto. Já o CRISPR/Cas9 é de baixo custo e de produção e desenho simples, uma vez que o reconhecimento do DNA é feito por complementariedade a uma molécula de RNA. Todas as plataformas são equivalentes quanto à eficiência de edição, sendo que o tipo celular e o lócus onde a edição deve ocorrer interferem na eficiência final.

Nucleases guiadas por RNA: o sistema CRISPR/Cas

A edição genômica por CRISPR/Cas9 revolucionou a área biotecnológica pela sua simplicidade e facilidade em comparação com as técnicas antecessoras supracitadas. Em 2020, o reconhecimento da importância da técnica para o mundo científico se deu pela atribuição do Prêmio Nobel em Química às pesquisadoras Emmanuelle Charpentier e Jennifer A. Doudna, oito anos após a publicação de seu trabalho descrevendo a nova técnica de edição genômica, que utiliza uma endonuclease guiada por RNA complementar à sequência de DNA (JINEK *et al.*, 2012).

O sistema CRISPR/Cas está presente em arqueas e em bactérias e corresponde a uma forma de imunidade adaptativa contra bacteriófagos e plasmídeos conjugativos. Parte das sequências encontradas no lócus CRISPR foi descrita ainda na década de 1980, muito antes do acrônimo CRISPR ser cunhado – o que se deu somente nos anos 2000 (JANSEN *et al.*, 2002). O lócus CRISPR foi descrito como sendo localizado em regiões intergênicas, composto de múltiplas sequências curtas e repetidas, interespaçadas por sequências não conservadas, chamadas de protoespaçadores. O lócus, porém, também possui genes próximos, cuja função era desconhecida à época e que foram chamados de CRISPR associados (Cas) (JANSEN *et al.*, 2002). Em 2005, foi observado que as sequências não conservadas possuíam alta homologia com sequências de bacteriófagos. Curiosamente, as sequências eram provenientes de fagos cujas bactérias eram resistentes e assim foi sugerido, pela primeira vez, que o sistema poderia estar envolvido com imunidade adquirida desses organismos (MOJICA *et al.*, 2005). Esta hipótese não foi bem recebida pela comunidade científica na época, mas foi confirmada experimentalmente poucos anos após (BARRANGOU *et al.*, 2007).

O mecanismo de ação do sistema CRISPR/Cas contra invasores é similar ao do RNA de interferência. Brevemente, as sequências chamadas de protoespaçadores são provenientes de infecções prévias por invasores, cuja parte do genoma foi incorporada entre sequências repetidas do lócus CRISPR bacteriano. Este arranjo, contendo repetições e protoespaçadores, é transcrito integralmente em um único pré-RNA CRISPR (pré-crRNA). Também há a transcrição paralela de outra sequência do lócus, cujo RNA resultante é chamado de RNA CRISPR transativador (tracrRNA). Por serem complementares, o tracrRNA hibridiza com as sequências repetidas do pré-crRNA que, por sua vez, é proces-

sado e clivado por RNases, resultando em fragmentos menores de RNA dupla-fita. O complexo crRNA-tracrRNA processado se liga a proteínas CRISPR associadas, como a Cas9, que é guiada pela porção variável de 20 nucleotídeos do crRNA – proveniente originalmente de sequências invasoras. Assim que o complexo Cas9 + RNA reconhece uma sequência com alta homologia à porção variável do crRNA, a nuclease cliva a dupla-fita de DNA para eliminar o possível invasor. O discernimento entre sequências próprias e sequências invasoras é feito pela obrigatoriedade de um motivo adjacente ao protoespaçador (PAM, do inglês *protospacer adjacent motifs*), presente somente nos invasores, que seja próximo ao local da clivagem. Uma vez que a Cas9 encontra o alvo com a sequência PAM apropriada, o DNA dupla-fita é dissociado e há invasão da fita de RNA, a fim de formar um híbrido DNA-RNA. O pareamento perfeito do híbrido gera uma conformação estrutural que é reconhecida pela Cas9 e que ativa os sítios ativos da enzima, induzindo a clivagem da dupla-fita do DNA, três pares de base à jusante da PAM (JIANG *et al.*, 2017).

CRISPR/Cas9 como ferramenta de edição genômica

O desenvolvimento da ferramenta de edição genômica CRISPR/Cas9 se deu em 2012 (JINEK *et al.*, 2012). Na ocasião, verificou-se que ambos crRNA e tracrRNA eram fundamentais para o funcionamento da técnica pois, ao hibridizarem, adotam uma conformação secundária necessária para a ligação à Cas9 e estabilização do complexo. Para simplificar ainda mais o sistema, foi desenvolvido o RNA guia (gRNA), um RNA quimérico de aproximadamente 100 nucleotídeos que associa ambos crRNA e tracrRNA em uma única molécula por um *loop* de ligação, mantendo a dupla-fita de RNA necessária e facilitando o desenho da técnica. Assim, para a indução da quebra da dupla-fita de DNA utilizando o sistema CRISPR/Cas9, são necessários apenas dois componentes: o gRNA, contendo os 20 nucleotídeos específicos desenhados de acordo com a sequência alvo no genoma e adjacentes a uma sequência PAM, e a Cas9. Para a modificação de sequências com precisão, adiciona-se à lista de itens um DNA molde ou vetor doador, necessário para o reparo por recombinação homóloga.

Logo que a quebra é induzida, o reparo celular é ativado. A via NHEJ é muito rápida e repara as quebras em menos de uma hora (CARUSILLO *et al.*, 2020). A ação do sistema CRISPR/Cas9 é ágil também, clivando o DNA segundos após chegar ao alvo. En-

tretanto, a permanência do sistema CRISPR/Cas9 na célula é de pelo menos algumas horas, o que acaba induzindo inúmeras e consecutivas quebras no mesmo alvo (LIU *et al.*, 2020). Após reparos precisos e consecutivos no mesmo local pela via NHEJ, eventualmente algum erro será gerado, o que altera a sequência de ligação do gRNA ou a variante recém induzida seja permanente. De modo similar, a HDR também poderá ocorrer inúmeras vezes na mesma célula caso o dano seja recorrente. Assim, no desenho do vetor doador, é importante que a sequência modificada possua mutações silenciosas em possíveis sequências PAM e/ou na sequência de anelamento do gRNA, a fim de que o DNA já editado não seja clivado novamente (JIANG *et al.*, 2017).

Os componentes da maquinaria CRISPR/Cas9 podem ser entregues de diversas maneiras às células. A maneira mais simples e acessível é o uso de plasmídeos que, em um único vetor, expressam o gRNA e a Cas9, inclusive códon-otimizada para maior expressão em células eucarióticas. Embora este sistema seja muito eficiente em linhagens celulares de fácil transfecção, cultivos primários geralmente não toleram a presença de grandes quantidades de DNA exógeno – como é o caso das células-tronco hematopoiéticas (CTH), cuja eficiência de edição com vetores plasmidiais é praticamente zero (HENDEL *et al.*, 2015). Além disso, outra desvantagem é o aparente aumento de atividade de clivagem fora do alvo, uma vez que o plasmídeo se mantém ativo na célula por dias, aumentando a chance de atividade inespecífica do sistema (LIANG *et al.*, 2015). Também podem ser utilizadas somente moléculas de RNA, tanto o gRNA quanto o mRNA para Cas9; essa forma, apesar de eficiente para edição, induz repressão transcricional global em células-tronco hematopoiéticas, por exemplo, efeito não observado com a utilização dos outros métodos (CROMER *et al.*, 2018). A terceira e mais eficiente forma é a pré-complexação da proteína Cas9 ao gRNA, formando um complexo ribonucleoproteico (RNP) que é entregue à célula, geralmente por métodos não-virais. Dessa maneira, o complexo possui ação imediata ao chegar no núcleo celular por não precisar ser traduzindo nem transcrito e, ao mesmo tempo, tem duração menor, o que minimiza a ação *off-target* (LIANG *et al.*, 2015). Essa forma de entrega tem se mostrado muito eficiente inclusive para células-tronco pluripotentes induzidas (do inglês *induced pluripotent stem cells*, iPSC) (MARTIN *et al.*, 2019).

Para que a maquinaria CRISPR/Cas9 de fato entre nas células alvo, é necessário que se faça uso de técnicas de transfecção ou de transdução. A transfecção do plasmídeo

CRISPR ou do complexo RNP é simples e costuma ter eficiência satisfatória, dependendo do tipo celular e, por isso, costuma ser o método de escolha na maioria dos casos. São utilizados, principalmente, eletroporação ou lipídeos e polímeros catiônicos (RUI *et al.*, 2019). Os vetores virais como lentivirus e vírus adenoassociados (AAV), por outro lado, são escolhidos para entrega do vetor doador ou mesmo para entrega de sequências codificadores do gRNA e Cas9, quando o alvo são células quiescentes e de difícil transfecção, ou em abordagens de terapia *in vivo* (DOUDNA, 2020).

Aplicações da edição genômica: modelos *in vitro*

A edição genômica de forma acessível permitiu o avanço de pesquisa básica de diversas maneiras. Uma delas é pela geração de modelos celulares, seja a partir de células-tronco ou a partir de linhagens celulares imortais, por exemplo. Utilizando uma ferramenta como CRISPR/Cas9, genes causadores de doenças monogênicas podem ser nocauteados facilmente em diversos tipos celulares para geração de modelos da doença *in vitro*, removendo a necessidade de coletas frequentes de células de pacientes. Assim, pode-se desenvolver modelos de doenças monogênicas em células sanguíneas, epiteliais ou, até mesmo, células de microglia e neuronais, sem dificuldade e com tempo de cultivo virtualmente ilimitado. Mesmo que células imortais apresentem aberrações cromossômicas e/ou fenótipo canceroso, esses modelos desenvolvidos podem ser muito úteis para análise de mecanismos fisiopatológicos simples, para análise de efeito de variantes novas e para varredura e teste iniciais de novos tratamentos, agilizando e tornando mais acessível a pesquisa básica (MIRABELLI *et al.*, 2019) (Figura 3).

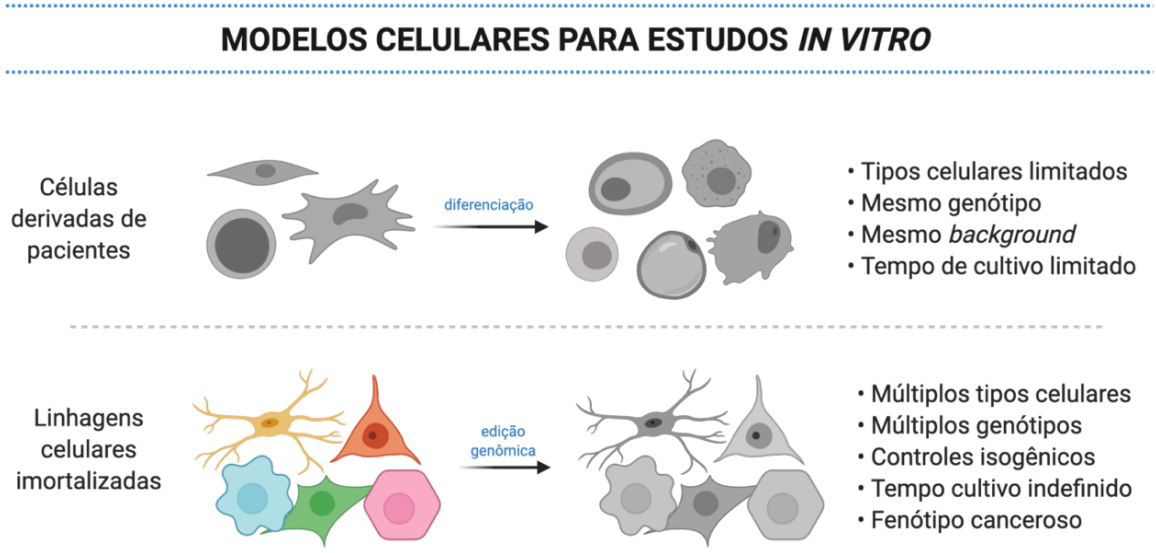

Figura 3. Características dos diferentes modelos celulares de doenças genéticas para estudos *in vitro*. Ainda que algumas células derivadas de pacientes possam ser diferenciadas em outros tipos celulares, a oferta ainda é pequena se comparada à linhagens celulares imortalizadas. Representadas em cinza são as células com genótipo causador de doença monogênica.

TERAPIA GÊNICA

Terapia gênica é a aplicação de um produto composto de ácidos nucleicos recombinantes utilizado para regular, reparar, substituir, adicionar ou deletar uma sequência genética, objetivando ação terapêutica, preventiva ou diagnóstica (WIRTH *et al.*, 2013). De forma simplificada, é a modificação genética das células de um indivíduo com o objetivo de tratar alguma doença ou condição (COTRIM *et al.*, 2008). Essa modificação pode ser por inserção de material genético no núcleo das células (que terá expressão episssomal) ou por alterações diretamente no genoma – seja por modificações controladas (edição genômica) ou por inserções randômicas ou semi-randômicas de transgenes (resultado de vetores retrovirais e transposons). A terapia gênica pode ser realizada *in vivo*, na qual a administração do vetor e a modificação do genoma ocorrem diretamente no indivíduo; ou *ex vivo*, procedimento no qual células específicas são coletadas do paciente, modificadas em laboratório e transplantadas de volta ao paciente (HIGH *et al.*, 2019).

Vetores de entrega

Os vetores de entrega são moléculas que auxiliam a inserção do DNA terapêutico na célula, podendo ser classificados em vetores virais e não-virais (Figura 4). Dentre os vetores virais, os lentivirus e os vírus adenoassociados (AAV) são os mais utilizados devido às suas características intrínsecas, inclusive com utilização na clínica e produtos de terapia aprovados. Eles são empregados há anos na terapia gênica "clássica" para entrega de genes inteiros, com cDNA e elementos regulatórios que aumentam a expressão do transgene (POWELL *et al.*, 2015). Ambos apresentam baixa imunogenicidade ao serem administrados e, após a transdução, possuem expressão estável em longo-prazo. Os lentivirus são vetores capazes de carrear em torno de 8 kb, enquanto os AAV são limitados a ~ 4,5 kb. Outras diferenças importantes são a capacidade de infecção – lentivirus transduzem somente células na fase G1 do ciclo celular e são vetores integrativos; já vetores AAV não possuem exigências quanto ao ciclo celular e raramente integram seu genoma ao genoma hospedeiro, mantendo-se episssomal (ANGUELA *et al.*, 2019). Diferentemente dos lentivirus, os AAV apresentam diversos sorotipos mapeados com afinidade diferencial para tipos celulares no hospedeiro – o AAV8, por exemplo, possui tropismo por células musculares, tanto músculo esquelético quanto cardíaco (LI *et al.*, 2020; NASO *et al.*, 2017).

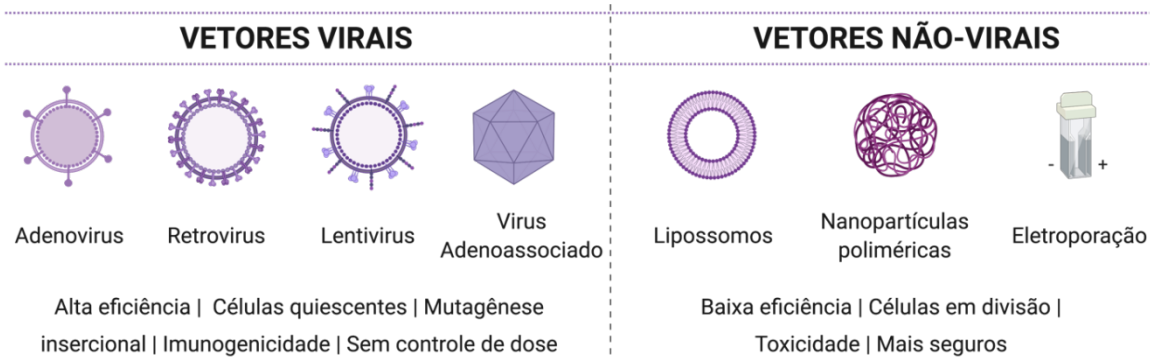

Figura 4. Características dos vetores de entrega mais utilizados para terapia gênica.

A principal desvantagem do vetor lentiviral é, justamente, sua capacidade integrativa, que ocorre de forma semi-randômica, pois possui preferência por áreas gênicas de alta atividade transcricional (SERRAO *et al.*, 2016). Por isso, há o risco de mutagênese insercional e consequente desenvolvimento de tumores. Mesmo sendo majoritariamente de ex-

pressão episssomal, os vetores AAV também podem ter eventos integrativos, mesmo que raros (WARD *et al.*, 2012). Entretanto, a maior desvantagem do uso de AAV é a existência de imunidade prévia – pois muitos indivíduos já tiveram contato com AAV e já desenvolveram imunidade, mesmo que os AAV não sejam patogênicos. Desse modo, a imunidade prévia pode diminuir a eficácia de uma possível terapia gênica *in vivo* utilizando AAV de mesmo sorotipo (LI *et al.*, 2020).

Recentemente, os vetores lentivirais e AAV também passaram a atuar na entrega de ferramentas de edição genômica. Os AAV, por serem episssomais e por possuírem pequena capacidade de empacotamento, são muito utilizados para a entrega dos vetores doadores para a recombinação homóloga. Já os vetores lentivirais, por serem naturalmente integrativos, foram modificados para serem integrase-deficientes, de modo que sejam expressos somente de forma episssomal pelo tempo necessário para que a edição genômica ocorra. Considerando a capacidade de empacotamento superior em relação ao AAV, os lentivirus integrase-deficientes são capazes de entregar tanto o vetor doador, quanto a sequência para expressão de nucleases em uma única partícula viral (CHEN *et al.*, 2016).

Produtos aprovados

Alguns produtos de terapia gênica já estão comercialmente disponíveis no Brasil e no mundo e muitos ensaios clínicos estão sendo conduzidos. Os primeiros produtos de terapia gênica no Brasil foram aprovados pela Agência Nacional de Vigilância Sanitária (ANVISA) em 2020, mesmo ano da publicação da Resolução da Diretoria Colegiada n° 338, que dispõe sobre o registo de produtos de terapia avançada no país. Também em 2020, a agência reguladora americana FDA (do inglês *Food and Drug Administration*) apresenta 18 produtos de terapia gênica ou celular licenciados, sendo seis de terapia gênica (EUA, 2020). Dentre os alvos dos produtos aprovados, estão câncer e doenças monogênicas raras, e as abordagens são tanto *in vivo* quanto *ex vivo*. A agência europeia EMA (do inglês *European Medicines Agency*), por sua vez, conta com dois produtos adicionais aprovados em seu catálogo (Figura 5).

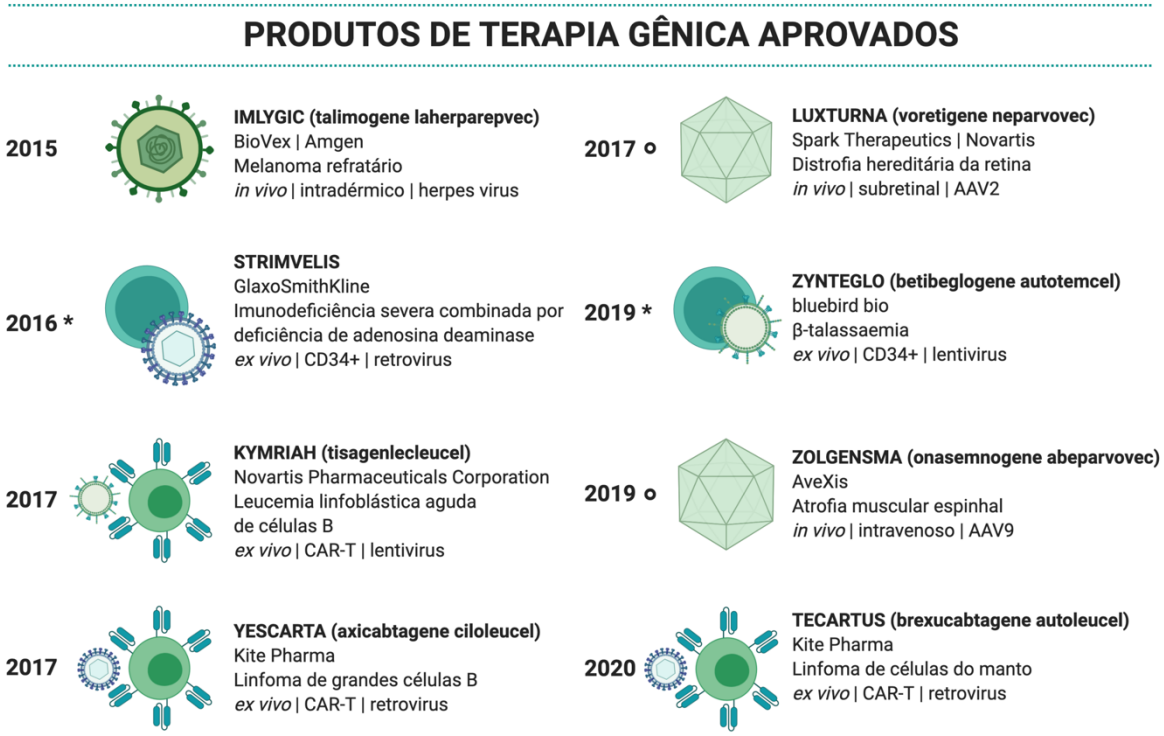

Figura 5. Produtos de terapia gênica aprovados pelas agências regulatórias ANVISA, FDA e EMA. Para cada produto, o ano corresponde à primeira aprovação em uma das três agências regulatórias. * aprovado apenas pela EMA; º aprovado inclusive pela ANVISA.

CÉLULAS-TRONCO HEMATOPOIÉTICAS E O TRANSPLANTE AUTÓLOGO

As células-tronco hematopoiéticas (CTH) são as únicas células multipotentes e com capacidade de auto renovação do sistema hematopoiético – multipotentes porque podem se diferenciar em todas as células do sistema sanguíneo (tanto em linhagens linfoides quanto mieloides), enquanto a auto renovação é a habilidade de se dividir em células-filhas idênticas (SEITA *et al.*, 2010). Sabe-se que uma única CTH é capaz de reconstituir toda a população hematopoiética de um indivíduo (OSAWA *et al.*, 1996).

As CTH são produzidas no fígado fetal, baço, timo e principalmente na medula óssea, que é o maior produtor de células hematopoiéticas do adulto (DRIZE *et al.*, 2015). A proporção de CTH na medula óssea é baixa, em torno de 0,05-1 % do total de células e, em geral, proliferam pouco – em torno de 95 % das CTH são quiescentes (WILSON *et al.*, 2006). O principal marcador utilizado para CTH humanas é o CD34, que é exclusivo de células-tronco e de células progenitoras – ou seja, células hematopoiéticas completamente

diferenciadas não o expressam. Para caracterizar a população verdadeiramente tronco, as chamadas CTH *long-term,* que são capazes de reconstituir o sistema hematopoiético de mais de um receptor, o conjunto de marcadores CD34+ CD38- CD90+ CD45RA- CD49f+ é utilizado (NOTTA *et al.*, 2011). Em camundongos, utiliza-se, principalmente, o marcador linhagem negativo (Lin-) para células-tronco e progenitoras e, para populações mais enriquecidas em células-tronco *long-term,* utiliza-se Lin- Sca-1+ c-kit+ CD34- (WILKINSON *et al.*, 2019). A figura 6 ilustra a hierarquia das células hematopoiéticas e os principais marcadores em humanos e camundongos.

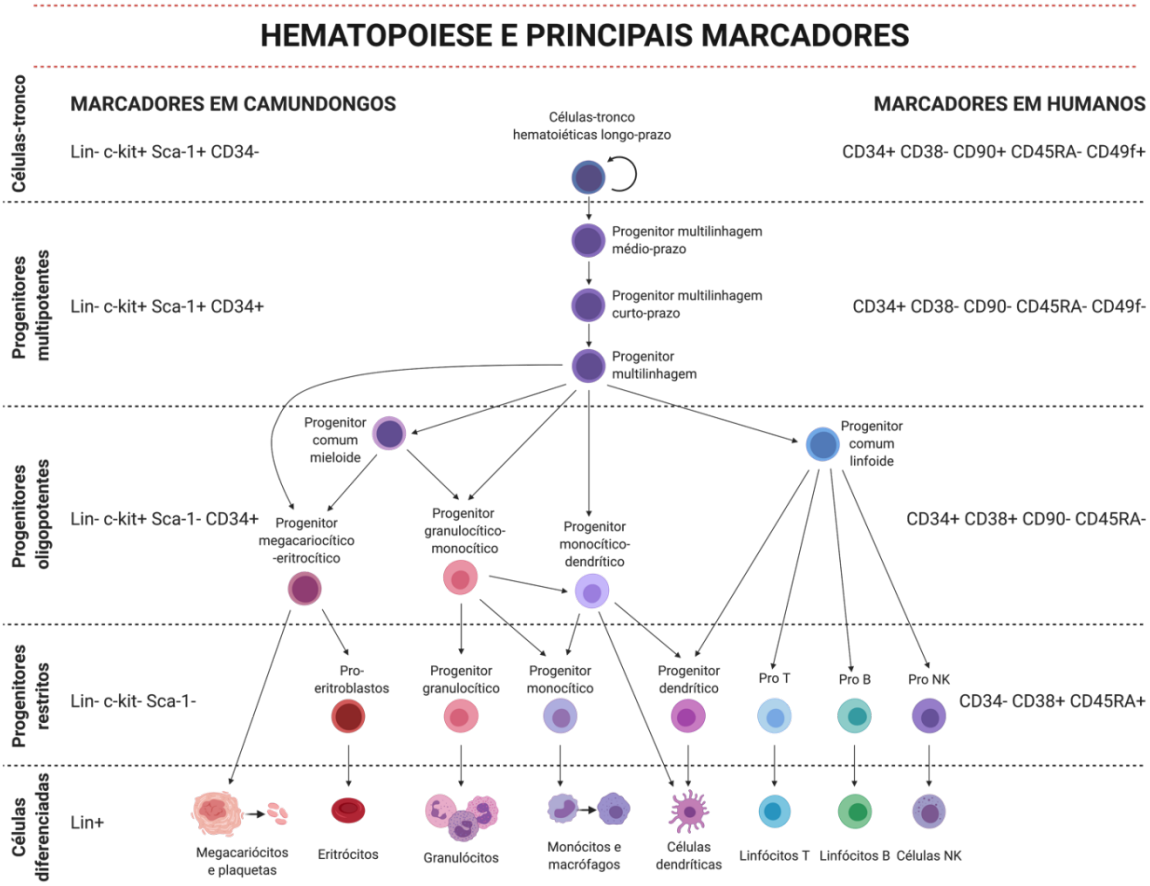

Figura 6. Esquema simplificado da hematopoiese e marcadores principais de células-tronco e células progenitoras hematopoiéticas. Células-tronco de longo prazo são as únicas com capacidade de auto renovação e proliferação indefinida. Conforme o processo de diferenciação avança, os marcadores de superfície que caracterizam cada estágio mudam. Baseado em (RIEGER *et al.*, 2012).

O transplante alogênico: benefícios e desvantagens

O transplante alogênico de CTH (alo-TCTH) é realizado, tecnicamente, desde 1957, quando o primeiro transplante de medula óssea foi reportado. Nesses últimos 60 anos, centenas de milhares de transplantes foram realizados e, embora muito tenha se avançado no entendimento do processo, muitos desafios ainda permanecem (SIMPSON *et al.*, 2019).

Para que as células-tronco transplantadas enxertem eficientemente na medula óssea do paciente receptor, é necessário submetê-lo a um regime de condicionamento, que pode ser de baixa intensidade (subletal) ou completamente mieloablativo (letal) (GYURKOCZA *et al.*, 2014). No condicionamento, as células da medula óssea são destruídas, permitindo que novas células enxertem e tenham sinalização adequada para proliferarem no nicho (ZHONG *et al.*, 2002). Em estudos pré-clínicos, pôde-se observar que as CTH conseguem enxertar em animais não condicionados, mas a eficiência é menor (WILKINSON *et al.*, 2019). Os principais regimes de condicionamento utilizados são irradiação de corpo inteiro (TBI, do inglês *total body irradiation*), agentes quimioterápicos alquilantes, como bussulfano ou treossulfano, e imunossupressores, como ciclosfofamida ou fludarabina que, inclusive, possuem ação sinérgica com os agentes alquilantes. A escolha do protocolo (que geralmente combina mais de um agente) depende da doença do paciente e das características do doador das células (GYURKOCZA *et al.*, 2014).

Além da possibilidade de as células transplantadas não enxertarem ou serem rejeitadas pelo hospedeiro, um dos principais riscos relacionados ao alo-TCTH é o desenvolvimento da doença do enxerto contra o hospedeiro (DECH), situação em que células T do doador atacam as células do indivíduo transplantado. A fisiopatologia da DECH inicia logo no condicionamento a que o paciente é submetido porque, além de destruir a medula óssea, também danifica outros tecidos. Com a extensa morte celular provocada no hospedeiro, há intensa liberação de citocinas pró-inflamatórias, que ativam expressivamente células apresentadoras de antígeno. Em resposta, as células T do enxerto são ativadas, proliferam e passam a atacar o hospedeiro. A depleção total de células T da população transplantada diminui a ocorrência de DECH, mas, em casos em que o transplante está sendo realizado para tratar câncer hematológico, a resposta imune do enxerto é desejada de forma controlada, para que possam combater possíveis células malignas remanescentes no paciente e di-

minuir reincidências, além de auxiliar no combate à infecções nos primeiros meses pós-transplante (FERRARA *et al.*, 2009; ZEISER, 2019). A DECH, especialmente a de manifestação aguda, é uma das principais causas de morte relacionada ao transplante (KEKRE *et al.*, 2014).

O uso de doadores com antígeno leucocitário humano (HLA, do inglês *human leukocyte antigen*) idêntico diminui a chance de DECH ou de rejeição. Entretanto, pacientes que dispõem de um doador HLA-idêntico são minoria e, frequentemente, o transplante é feito utilizando doadores haploidênticos (que compartilham apenas um alelo), geralmente irmãos ou parentes próximos (KANAKRY *et al.*, 2016). O risco de DECH em transplantes alogênicos com enxerto haploidêntico ainda é relativamente alto, mesmo fazendo-se uso de abordagens como depleção de linfócitos T ou imunomodulação pós-transplante. Entretanto, o condicionamento e a imunossupressão induzidos deixam os pacientes muito suscetíveis a infecções, sendo outra causa importante de mortalidade associada ao procedimento (KEKRE *et al.*, 2014).

Modelos animais de transplante

Para experimentação animal em transplante de células-tronco hematopoiéticas, tanto células murinas quanto humanas podem ser utilizadas, graças aos modelos murinos imunocomprometidos. Um deles é o modelo NSG (NOD.*Cg-Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ), que permite o transplante de células humanas sem que ocorra rejeição. Isso porque esses animais não produzem células de defesa maduras, como linfócitos B e T, células dendríticas, macrófagos e células NK. Assim, pela completa ausência de sistema imune, as células hematopoiéticas humanas podem enxertar nestes camundongos sem que ocorra rejeição (ISHIKAWA *et al.*, 2005; SHULTZ *et al.*, 2005). Entretanto, a hematopoese nos animais não é completa – uma vez que a sinalização presente no nicho é voltada às células murinas e não às humanas, as células de origem humana não são capazes de se diferenciar adequadamente em todas as células hematopoiéticas, como eritrócitos ou plaquetas, por exemplo (SHULTZ *et al.*, 2005). Para transplante de células de camundongos, qualquer modelo isogênico pode ser utilizado, embora os animais de linhagem C57BL/6 sejam os mais frequentes. Caso animais de linhagens diferentes sejam utilizados, há grande risco de rejeição ou de DECH (REDDY *et al.*, 2008).

Terapia gênica *ex vivo* com CTH

Diversos estudos estão em andamento e/ou já foram concluídos utilizando terapia gênica *ex vivo* com CTH para tratamento de doenças hematológicas, culminando na aprovação da comercialização de dois produtos na União Européia – um para imunodeficiência severa combinada e outro para beta-talassemia, ambos utilizando vetores virais para a modificação das CTH (TUCCI *et al.*, 2020).

Os estudos de terapia gênica *ex vivo* utilizando CTH iniciaram com doenças hematológicas, principalmente imunodeficiências e hemoglobinopatias, já que essas doenças acometem somente o sistema hematopoiético e a substituição das células deficientes por células saudáveis é resolutiva. Entretanto, doenças metabólicas hereditárias também têm sido alvo, principalmente as causadas por deficiências de enzimas lisossômicas e com acometimento neurológico. Para essas doenças, as CTH do paciente são modificadas a fim de expressarem níveis supra fisiológicos da enzima ausente. Os tecidos viscerais conseguem facilmente captar a enzima produzida por todas as células sanguíneas modificadas; já no sistema nervoso central (SNC), o mecanismo de ação é por diferenciação das células-tronco modificadas em monócitos que, por sua vez, são capazes de cruzar a barreira hematoencefálica (WILKINSON *et al.*, 2013). Uma vez no cérebro, os monócitos diferenciam-se em células tipo microglia, passando a residir no tecido e constituindo uma fonte permanente e constante de enzima no tecido nervoso (BIFFI, 2017a; b). Ademais, sabe-se que a patogênese de algumas doenças no SNC está diretamente ligada à ativação de microglia e aumento de inflamação local – assim, células tipo-microglia saudáveis derivadas do transplante podem contribuir no tratamento da doença por mais de um mecanismo de ação (TUCCI *et al.*, 2020).

O vetor de entrega de ácidos nucleicos mais utilizado para as CTH são os lentivirus, por terem se mostrado superiores em termos de eficiência em comparação com os demais vetores virais ou não-virais. A maioria dos estudos clínicos com CTH modificadas utilizam lentivirus para adição gênica às células (ou seja, inserção de promotor e cDNA). Nesta lista está, inclusive, um dos produtos de terapia gênica *ex vivo* aprovados na Europa, o Zynteglo® (SCHUESSLER-LENZ *et al.*, 2020). Contudo, considerando a possibilidade de mutagênese insercional e a formação de neoplasias hematológicas, alternativas potencialmente mais seguras devem ser consideradas.

Edição de células-tronco hematopoiéticas

Uma maneira de realizar terapia gênica *ex vivo* com CTH de forma mais segura é a utilização de ferramentas de edição genômica. Assim que protocolos para edição eficiente desse tipo celular foram desenvolvidos (BAK; DEVER*; et al.*, 2018), a técnica foi utilizada para aplicação em doenças hematológicas, como anemia falciforme (DEVER *et al.*, 2016) e imunodeficiência severa combinada ligada ao X (PAVEL-DINU *et al.*, 2019). Também foi aplicada à doenças lisossômicas, como doença de Gaucher (SCHARENBERG *et al.*, 2020) e mucopolissacaridose do tipo I (GOMEZ-OSPINA *et al.*, 2019). Além dos modelos murinos, as CTH editadas já se mostraram capazes de enxertar em primatas não-humanos (DEMIRCI *et al.*, 2020).

Ensaios clínicos utilizando CTH editadas já estão sendo conduzidos para anemia falciforme (NCT03745287 e NCT04443907) e para β-Talassemia (NCT03655678), com resultados iniciais demonstrando que as CTH editadas com CRISPR/Cas9 enxertam eficientemente e fornecem expressão contínua e persistente do transgene aos pacientes (FRANGOUL *et al.*, 2020). Todavia, a edição genômica para doenças metabólicas ainda se encontra em fase pré-clínica.

DOENÇAS LISOSSÔMICAS E AS MUCOPOLISSACARIDOSES

Dentre todas as doenças genéticas já descritas, as doenças lisossômicas compreendem aproximadamente 70 doenças monogênicas raras, mas que, em conjunto, possuem frequência mínima estimada de 1 a cada 5.000 nascidos vivos (PLATT *et al.*, 2018). A característica principal desse grupo de doenças é a alteração no catabolismo dos lisossomos, seja por deficiência de enzimas lisossômicas e seus ativadores/modificadores ou por ausência de proteínas de membrana e de transportadores (PLATT *et al.*, 2018). Quando há ausência de determinadas proteínas lisossômicas, há acúmulo gradual de substratos não degradados dentro do lisossomo que, além de aumentar de volume e alterar o funcionamento adequado da organela, leva à diversas consequências secundárias, como acúmulo de metabólitos, alteração de vias de sinalização, diminuição da autofagia, dano mitocondrial e aumento de espécies reativas de oxigênio, alteração da homeostase do retículo endoplas-

mático e complexo de Golgi, superexpressão de proteases e aumento de inflamação (GAFFKE *et al.*, 2019; PIERZYNOWSKA *et al.*, 2020; PLATT, 2018; PLATT *et al.*, 2012; PSHEZHETSKY, 2015). A consequência de tantas alterações celulares é a manifestação clínica progressiva das doenças que, frequentemente, levam à morte dos pacientes nos primeiros anos de vida.

Mucopolissacaridoses: características moleculares e fisiopatologia

As mucopolissacaridoses (MPS) são um grupo de doenças lisossômicas caracterizadas pelo acúmulo progressivo dos glicosaminoglicanos (GAGs) sulfato de condroitina, sulfato de dermatana, sulfato de heparana, sulfato de queratana e hialuronan (ou ácido hialurônico). Os GAGs são polissacarídeos lineares compostos de uma hexosamina e um ácido urônico (ácido D-glucorônico ou ácido L-idurônico) ou uma galactose. Com a exceção do hialuronan, os GAGs se ligam covalentemente a um núcleo proteico, formando proteoglicanos, cuja funções celulares são diversas – atuam desde receptores e modificadores biológicos de fatores de crescimento até a composição da matriz extracelular, conferindo estrutura e propriedades visco-elásticas aos tecidos (IOZZO *et al.*, 2015; LINDAHL *et al.*, 2015). O catabolismo dos GAGs é contínuo e é realizado principalmente no lisossomo, com a remoção dos grupos sulfatos e a quebra das ligações entre os dissacarídeos de forma sequencial, o que envolve 10 enzimas diferentes. Quando há deficiência em alguma dessas enzimas, há acúmulo lisossômico de GAGs não degradados, resultando nas mucopolissacaridoses do tipo I ao tipo IX, dependendo da enzima ausente (NEUFELD, 2001). As diferentes MPS estão descritas na Tabela 1, e as MPS dos tipos I e II serão apresentadas em maior detalhe a seguir.

Tabela 1. Mucopolissacaridoses e suas principais características

Tipo	Síndrome	Gene	Enzima	GAGs acumulados
MPS I	Hurler e Scheie	*IDUA*	α-L-iduronidase	HS, DS
MPS II	Hunter	*IDS*	Iduronato-2-sulfatase	HS, DS
MPS III A	Sanfilipo A	*SGSH*	Heparan-N-sulfatase	HS
MPS III B	Sanfilipo B	*NAGLU*	α-N-acetil-glicosaminidase	HS
MPS III C	Sanfilipo C	*HGSNAT*	Acetil-CoA: α-glicosaminideo acetiltransferase	HS
MPS III D	Sanfilipo D	*GNS*	N-acetilglicosamina 6-sulfatase	HS
MPS IV A	Morquio A	*GALNS*	Galactose-6-sulfatase	KS, CS
MPS IV B	Morquio B	*GLB1*	β-galactosidase	KS, CS
MPS VI	Marateaux-Lamy	*ARSB*	N-acetilgalactosamina 4-sulfatase	DS, CS
MPS VII	Sly	*GUSB*	β-glicuronidase	HS, DS, CS
MPS IX	Natawicz	*HYAL1*	Hialuronidase	hialuronan

GAGs: glicosaminoglicanos; HS: sulfato de heparana; DS: sulfato de dermatana; KS: sulfato de queratana; CS: sulfato de condroitina. Adaptado de (NEUFELD, 2001).

A mucopolissacaridose do tipo I (MPS I) foi a primeira MPS descrita e é causada pela deficiência da glicosidase alfa-L-iduronidase (IDUA; EC:3.2.1.76), responsável pela hidrólise dos resíduos de ácido L-idurônico dos GAGs sulfato de heparana e sulfato de dermatana (NEUFELD, 2001). A deficiência enzimática é causada por mutações no gene *IDUA*, sendo que o padrão de herança da doença é autossômico recessivo. Embora algumas mutações sejam mais frequentes, como as variantes p.Trp402* e p.Pro533Arg – que correspondem a 50% dos alelos mutados encontrados em pacientes brasileiros –, há grande heterogeneidade alélica no gene *IDUA*, com mais de 200 variantes patogênicas já descritas (POLETTO *et al.*, 2018). A prevalência de MPS I no Brasil é estimada em, aproximadamente, 0,24 casos a cada 100.000 nascidos vivos (FEDERHEN *et al.*, 2020), podendo chegar a 7 casos a cada 100.000 (BORGES *et al.*, 2020).

A mucopolissacaridose do tipo II (MPS II) é causada pela deficiência da enzima iduronato-2-sulfatase (IDS; EC:3.1.6.13), responsável pela remoção do grupo sulfato do ácido L-idurônico na primeira etapa da degradação dos GAGs sulfato de heparana e sulfato de dermatana (NEUFELD, 2001). Diferentemente da MPS I, a MPS II é uma doença ligada ao X, cujo gene responsável, *IDS,* também possui alta heterogeneidade alélica – mesmo que as mutações mais prevalentes nos pacientes sejam mutações de ponto, também são recorrentes deleções totais do gene e inversões, em razão da existência de um pseudogene (*IDS2*) de alta homologia que favorece eventos de recombinação (BRUSIUS-FACCHIN *et al.*, 2014). A incidência de MPS II é ligeiramente mais alta que a de MPS I no Brasil: são 0,37 casos a cada 100.000 nascidos vivos (FEDERHEN *et al.*, 2020).

Manifestações clínicas e diagnóstico das MPS I e II

Ambas MPS I e MPS II possuem diversas manifestações clínicas, que vai desde formas atenuadas até formas mais graves da doença. Classicamente, as formas distintas de apresentação da MPS I foram nomeadas de acordo com os médicos que as descreveram acreditando serem doenças de causas diferentes: a forma grave é conhecida como Síndrome de Hurler (MIM#607014); a forma atenuada é a Síndrome de Scheie (MIM#607016) e há, ainda, uma forma intermediária, chamada de Síndrome de Hurler-Scheie (MIM#607015). Embora exista esta classificação histórica, as manifestações clínicas compõem um espectro contínuo sem transição clara, o que torna a categorização muitas vezes complexa e imprecisa. Assim, prefere-se que os pacientes de MPS I sejam agrupados somente em formas atenuada e grave (GIUGLIANI *et al.*, 2010). A MPS II, ainda que também possua formas atenuada e grave, é conhecida historicamente somente por Síndrome de Hunter (MIM#309900).

O que determina a gravidade da doença, tanto para MPS I quanto para MPS II, é a presença de atividade enzimática residual (OUSSOREN *et al.*, 2013). Algumas mutações alteram o funcionamento da proteína de forma mais significativa do que outras; para MPS I, por exemplo, a variante patogênica mais frequente, p.Trp402*, gera um códon de parada prematuro que encaminha o mRNA transcrito diretamente à via de degradação mediada por mutação sem sentido, um sistema de proteção celular contra proteínas truncadas (MENON *et al.*, 1994). Assim, a proteína nem chega a ser traduzida e a atividade enzimá-

tica de IDUA é indetectável em pacientes homozigotos para essa alteração, resultando em fenótipo grave. Por outro lado, mutações de sentido trocado, que alteram aminoácidos em regiões distantes dos sítios ativo e de glicosilação da enzima, frequentemente geram apenas pequenas mudanças estruturais, levando à atividade enzimática residual suficiente para evitar grandes acúmulos de substrato nos tecidos. Entretanto, embora a correlação genótipo-fenótipo seja clara em alguns contextos, ela nem sempre é evidente e cada caso deve ser avaliado individualmente (CLARKE *et al.*, 2019; HEIN *et al.*, 2003). Ademais, recentemente têm-se sugerido que pacientes com as formas atenuadas desenvolvem dificuldades cognitivas ao longo dos anos, como déficit de atenção (YUND *et al.*, 2015), o que sugere que a patogênese da doença no SNC é complexa e pode ser causada por mecanismos ainda não conhecidos.

As manifestações clínicas das MPS I e MPS II são bastante similares, uma vez que os mesmos GAGs são acumulados em ambas as condições. As formas graves são caracterizadas pela presença de manifestações neurológicas, como atraso no desenvolvimento neuropsicomotor e prejuízo cognitivo e, na MPS II, somam-se também hiperatividade e comportamento agressivo. Além da progressão no SNC, os pacientes com o fenótipo grave, em geral, apresentam hepatoesplenomegalia, hérnias, insuficiência respiratória e cardíaca, alterações nas articulações e no esqueleto – como atraso no crescimento, rigidez articular, disostose múltipla e face infiltrada característica –, podem apresentar perda auditiva e, na MPS I, opacificação da córnea. A apresentação dos sintomas ocorre logo a partir do primeiro ano de vida; o diagnóstico é feito, em média, após o primeiro ano e a expectativa de vida do paciente se limita à primeira década de vida, se não tratado (CLARKE *et al.*, 2019; GIUGLIANI *et al.*, 2010; KUBASKI *et al.*, 2020; STAPLETON *et al.*, 2018; STAPLETON *et al.*, 2017). Já as formas atenuadas não possuem sintoma neurológico, uma vez que a atividade enzimática residual é suficiente para evitar o comprometimento desse tecido. As manifestações ficam restritas aos demais tecidos e os pacientes apresentam, principalmente, hepatoesplenomegalia, hérnia umbilical, rigidez articular e doença cardíaca, como valvulopatia. O crescimento e a estatura podem ser normais e o surgimento dos sintomas é notado, normalmente, após os 5 anos de idade; o diagnóstico, por sua vez, é efetuado mais tardiamente, em média aos 7 anos, com casos diagnosticados até mesmo aos 54 anos de idade (CLARKE *et al.*, 2019; KUBASKI *et al.*, 2020; STAPLETON *et al.*, 2018).

Mediante suspeita clínica, o diagnóstico das MPS é feito, primeiramente, pela quantificação e identificação de GAGs excretados na urina, que pode ser feita por eletroforese ou por cromatografia líquida acoplada à espectrometria de massas em tandem. Constatada a presença de GAGs na urina e a identificação dos tipos, é possível fazer o diagnóstico diferencial das MPS e direcionar o teste bioquímico para analisar as enzimas responsáveis pela degradação dos GAGs excretados. O ensaio de atividade enzimática pode ser empregado em amostras de papel filtro, plasma, leucócitos ou fibroblastos de pacientes, porém é considerado definitivo somente quando realizado em células. Além disso, a análise molecular dos genes responsáveis pode reafirmar o diagnóstico e, frequentemente, predizer o fenótipo do paciente – o que pode vir a ser um fator determinante na escolha do tratamento (KUBASKI *et al.*, 2020; STAPLETON *et al.*, 2018).

Tratamentos aprovados para MPS e seu mecanismo de ação

A maioria das enzimas lisossômicas, incluindo IDUA e IDS, recebem resíduos de manose-6-fosfato (M6P) durante seu processamento no complexo de Golgi. Esses resíduos são reconhecidos pelos receptores de M6P (M6PR), que são ubiquamente expressos na rede trans-Golgi e na membrana plasmática das células, e promovem o tráfego de enzimas do Golgi ou do meio extracelular aos endossomos e, finalmente, aos lisossomos, onde a enzima encontra pH ótimo e é ativa (SAFTIG *et al.*, 2009). Considerando esse processo fisiológico, os tratamentos voltados para as MPS I e MPS II baseiam-se, majoritariamente, neste mecanismo chamado de correção-cruzada – em que a enzima pode ser captada do meio extracelular por células deficientes e ter ação terapêutica no alvo.

Baseados nesse mecanismo, existem dois tratamentos disponíveis para as MPS I e II: a terapia de reposição enzimática (TRE) e o transplante alogênico de células-tronco hematopoiéticas (alo-TCTH). A primeira consiste na administração intravenosa, com frequência semanal ou quinzenal, das enzimas recombinantes laronidase e idursulfase alfa, aprovadas pela agência americana FDA em 2003 e 2006 para MPS I e para MPS II, respectivamente. No Brasil, a ANVISA aprovou a laronidase em 2005 e a idursulfase alfa em 2008 (GIUGLIANI *et al.*, 2010). Pacientes tratados com a TRE apresentam redução de GAGs urinários, melhora significativa nos sintomas respiratórios, no volume do fígado e do baço (SIFUENTES *et al.*, 2007; WRAITH *et al.*, 2004). Embora essa terapia melhore a

qualidade de vida dos pacientes, ela não tem ação significativa em valvas cardíacas, articulações e, principalmente, no SNC, uma vez que a enzima não é permeável à barreira hematoencefálica. Além disso, muitos pacientes desenvolvem anticorpos contra a enzima e apresentam efeitos adversos às infusões (CHEN *et al.*, 2019). Finalmente, a TRE é um tratamento extremamente custoso, tanto pelo valor do medicamento como pela dedicação do paciente e familiares para realizar as infusões frequentes por toda a vida (BITENCOURT *et al.*, 2015).

O alo-TCTH foi incorporado ao rol de tratamentos ofertados pelo Sistema Único de Saúde (SUS) para as MPS I e II recentemente, em 2018 (BRASIL, 2018a; b). O procedimento consiste em substituir a medula óssea do paciente por células-tronco de sangue de cordão umbilical ou provenientes de um doador saudável. Por serem células sem a doença, elas são capazes de produzir e distribuir a enzima deficiente a diversos tecidos, incluindo ao SNC. De fato, pacientes submetidos ao transplante apresentaram melhora em aspectos neurológicos e cardiovasculares (ALDENHOVEN *et al.*, 2015; TANAKA *et al.*, 2012), tecidos sobre os quais a TRE não tem efeito. Assim, o alo-TCTH é o tratamento de escolha para pacientes com as formas graves/neurológicas das MPS I e II, uma vez que é o único disponível com efeito no SNC. Contudo, o alo-TCTH também possui desvantagens importantes. Primeiro, o transplante deve ser realizando antes do comprometimento neurológico se estabelecer – assim, recomenda-se que o procedimento seja realizado antes dos dois a três anos de idade (SCARPA *et al.*, 2017); segundo, deve-se dispor de doadores compatíveis, que muitas vezes não são encontrados com a rapidez necessária. O uso de familiares como doadores, caso sejam heterozigotos para mutações nos genes responsáveis pelas doenças, diminui o sucesso do transplante, uma vez que as células transplantadas produzem apenas 50 % de enzima em relação a células com genótipo selvagem (ALDENHOVEN *et al.*, 2015). Por fim, deve-se considerar o alto risco de morbimortalidade relacionado ao procedimento: 17% dos pacientes desenvolvem DECH, que é altamente incapacitante, ou mesmo falecem em decorrência de complicações secundárias (KUBASKI *et al.*, 2017).

Independentemente do tratamento escolhido, este deve ser iniciado o quanto antes para que o usufruto do benefício seja máximo: sabe-se que o tratamento precoce retarda a progressão dos sintomas que, quando já estabelecidos, dificilmente são revertidos (AL-SANNAA *et al.*, 2015; PEREZ-LOPEZ *et al.*, 2018). Porém, uma vez que os tratamentos disponíveis não são completamente eficazes e ainda apresentam desvantagens muito signi-

ficativas, terapias alternativas estão em constante desenvolvimento para o tratamento da MPS I e da MPS II, como a terapia gênica e, mais recentemente, a edição genômica.

Terapia gênica e edição genômica para MPS

As doenças lisossômicas não possuem tratamentos de terapia gênica aprovados até o momento, embora sejam excelentes candidatas por possuírem algumas características facilitadoras: são monogênicas, geralmente não são reguladas por mecanismos complexos e a recuperação de níveis enzimáticos de apenas 2 % do normal já pode apresentar eficácia clínica, uma vez que pacientes com 1 % de atividade enzimática apresentam fenótipo atenuado sem manifestação neurológica significativa, como no caso da MPS I (OUSSOREN *et al.*, 2013).

Inúmeros estudos pré-clínicos e clínicos já foram desenvolvidos com terapia gênica para MPS I e II desde os anos 2000. A maior parte deles envolve a forma *in vivo*, principalmente com a administração sistêmica ou localizada de vetores AAV para a entrega de transgene completos (SAWAMOTO *et al.*, 2018). Em se tratando de terapia gênica *ex vivo*, a abordagem mais estudada é a utilização de células-tronco hematopoiéticas (CTH) como fonte de transplante autólogo. Nesse contexto, a ideia é coletar as células do indivíduo e corrigi-las *in vitro* para utilizá-las no transplante, eliminando a necessidade de um doador HLA-compatível, anulando a possibilidade de DECH e, por fim, diminuindo a necessidade de condicionamento intenso ou mieloablativo – ou seja, removendo as principais desvantagens do transplante alogênico convencional. A Figura 7 ilustra o mecanismo de ação clássico da terapia gênica *ex vivo* utilizando CTH para tratamento do SNC.

Estudos pré-clínicos utilizando CTH modificadas por lentivirus para MPS I (VISIGALLI *et al.*, 2016; VISIGALLI *et al.*, 2010) e para MPS II (MIWA *et al.*, 2020; WAKABAYASHI *et al.*, 2015) demonstraram aumento significativo de atividade enzimática nos tecidos, inclusive no SNC. Assim, um ensaio clínico foi registrado e está recrutando pacientes com MPS I (NCT03488394). Por outro lado, a edição genômica de CTH ainda é muito recente, com apenas um trabalho publicado em 2019. Gomez-Ospina e colaboradores editaram com CRISPR/Cas9 células humanas CD34+ e as transplantaram em um novo modelo murino de MPS I com *background* NSG para permitir o enxerto de células humanas. As células foram editadas com a inserção de um transgene (contendo o cDNA de

IDUA regulado por promotores fortes) no gene *CCR5,* que pode ser considerado como um *safe harbor* para edição genômica visto que sua anulação não causa efeitos deletérios nas células. Os resultados foram muito interessantes: as células editadas enxertaram eficientemente nos camundongos e produziram IDUA, resultando em aumento da atividade enzimática e redução de GAGs acumulados em diversos tecidos. Os camundongos também tiveram melhora em testes comportamentais e na doença óssea, observada por ressonância magnética (GOMEZ-OSPINA *et al.*, 2019). Todavia, a melhora bioquímica observada no SNC foi modesta, com aumento de apenas 7 % de atividade enzimática e sem redução significativa do acúmulo de GAGs. Esse estudo inicial demonstrou que essa é uma abordagem muito promissora, mas que deve ser aprimorado para que se tenha um tratamento curativo aos pacientes.

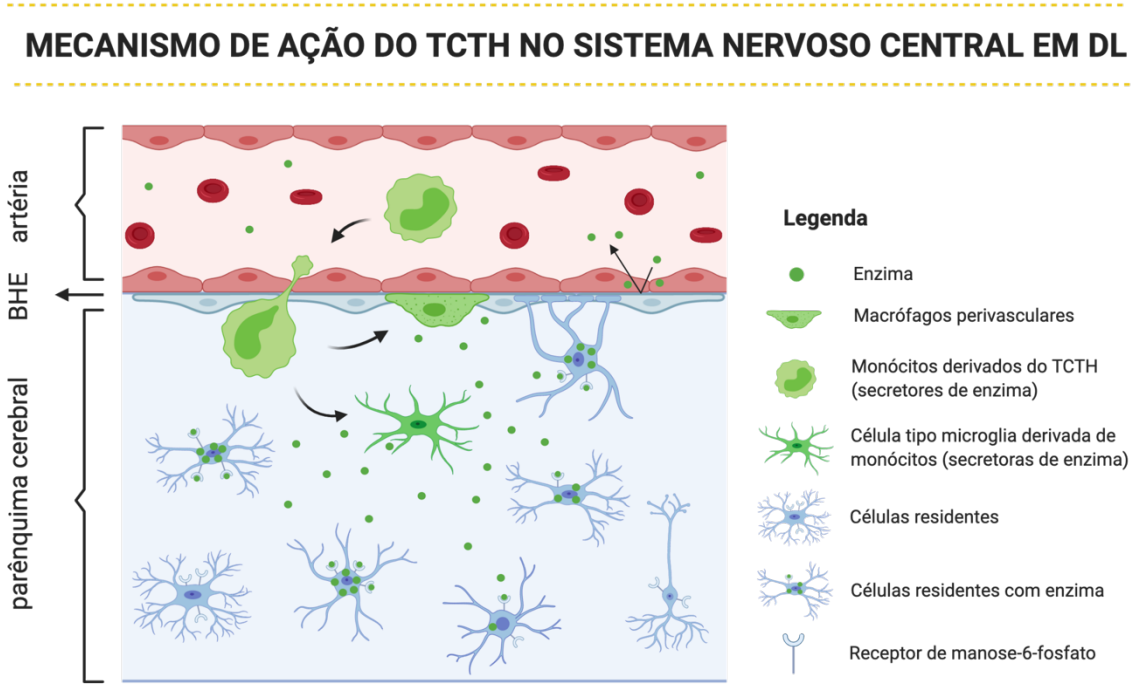

Figura 7. Mecanismo de ação clássico do transplante de células-tronco hematopoiéticas (TCTH) no sistema nervoso central (SNC) no contexto de doenças lisossômicas (DL). Os monócitos derivados das células-tronco hematopoiéticas transplantadas circulam pelo organismo, migrando para tecidos, inclusive para o parênquima cerebral por serem capazes de atravessar a barreira hematoencefálica (BHE). Alguns monócitos tornam-se macrófagos perivasculares, enquanto outros se diferenciam em células tipo microglia; ambas, por serem derivadas de células modificadas, são secretoras de enzima. As células residentes do tecido (como microglia, oligodendrócitos, astrócitos e neurônios) possuem receptores de manose-6-fosfato na membrana plasmática e conseguem captar a enzima do meio extracelular. Uma vez dentro das células residentes (que possuem a doença lisossômica), a enzima consegue reduzir o depósito de substrato. A enzima também está presente na circulação, mas ela não é capaz de permear a BHE.

Considerando os avanços observados em doenças hematológicas e o estudo pré-clínico promissor, porém limitado, desenvolvido para MPS I, a edição genômica tem muito potencial para vir a se tornar, de fato, uma terapia efetiva para os pacientes com mucopolissacaridoses. Contudo, aprimoramentos do protocolo são necessários, principalmente objetivando maior migração de células transplantadas e editadas ao SNC. Ademais, muitos mecanismos fisiopatológicos das MPS – e de doenças lisossômicas em geral – ainda são desconhecidos. O uso de edição genômica como ferramenta de estudo das MPS pode contribuir no desenvolvimento de novos alvos terapêuticos e, inclusive, pode eventualmente auxiliar no desenvolvimento de terapias gênicas mais efetivas. Assim, na presente tese, apresentamos o uso de edição genômica como ferramenta de estudo da fisiopatologia da MPS II, bem como no desenvolvimento de uma terapia para MPS I com ação significativa no SNC.

OBJETIVOS

OBJETIVO GERAL

O objetivo desta tese é a aplicação do sistema de edição genômica CRISPR/Cas9 para pesquisa das mucopolissacaridoses do tipo I e II, tanto voltado à geração de ferramentas de estudo de mecanismos fisiopatológicos quanto ao desenvolvimento de uma nova terapia gênica mais segura e eficaz que as disponíveis atualmente.

OBJETIVOS ESPECÍFICOS

- Desenvolver modelos celulares humanos relevantes das mucopolissacaridoses do tipo I e II;
- Avaliar a eficiência do processo de geração de nocautes em linhagens celulares por CRISPR/Cas9 em vetor plasmidial;
- Caracterizar os modelos celulares produzidos quanto à sequência genômica, atividade enzimática e ensaios morfológicos relevantes;
- Editar células-tronco hematopoiéticas humanas com o sistema CRISPR/Cas9 para superexpressão da enzima lisossômica IDUA e avaliar a eficiência da edição por atividade enzimática e integração do transgene;
- Transplantar células-tronco hematopoiéticas editadas em camundongos NSG-MPS I e avaliar a manutenção do potencial tronco das células por meio de análise de enxertia e composição dos tecidos hematopoiéticos;
- Avaliar a eficiência do tratamento por terapia gênica *ex vivo* utilizando transplante de células-tronco hematopoiéticas editadas em camundongos MPS I, analisando parâmetros bioquímicos;
- Definir o melhor protocolo de condicionamento e a via de administração para transplante de células-tronco e progenitoras em modelo animal murino que apresentem maior alcance das células transplantadas no sistema nervoso central.

RESULTADOS

Esta tese está dividida em duas partes. A Parte 1 descreve a utilização da ferramenta CRISPR/Cas9 para geração de modelos celulares de estudo. A Parte 2 apresenta a utilização da edição genômica na terapia gênica *ex vivo,* utilizando células-tronco hematopoiéticas.

PARTE 1

INTRODUÇÃO

A primeira parte desta tese está dividida em três capítulos e refere-se à utilização da ferramenta de edição genômica CRISPR/Cas9 para criação de modelos celulares, com enfoque nas mucopolissacaridoses do tipo I e do tipo II. A pesquisa básica dessas doenças é fundamentada, principalmente, na utilização de culturas primárias coletadas a partir de biópsias dos pacientes, como fibroblastos, ou utilizando células provenientes de modelos animais. Inegavelmente, células derivadas de pacientes foram, e ainda são, de muita valia como modelos *in vitro*; porém, dificuldades na obtenção, o tempo de cultivo limitado e a pouca variedade celular são limitações importantes. Células derivadas de animais, por sua vez, apresentam limitações inerentes à diferença entre espécies. Assim, a utilização de edição genômica cria a possibilidade de geração de modelos nocautes para diversos genes humanos, utilizando tipos celulares diversos, incluindo células do sistema nervoso cujo cultivo primário humano é, atualmente, impraticável.

O primeiro capítulo desta tese é composto do capítulo de livro "***Creating cell lines for mimicking diseases***", que integrará o livro *Reprogramming of the Genome: CRISPR-Cas-based Human Disease Therapy Volume 182*, parte da série *Progress in Molecular Biology and Translational Science*, da Elsevier. Neste capítulo de livro, todas as etapas da geração de um modelo celular nocaute são detalhadas, incluindo a escolha do tipo celular, vetores de entrega dos componentes de CRISPR/Cas9, avaliação da eficiência de edição e validação do modelo. Alguns exemplos de aplicações em doenças monogênicas e complexas também são apresentados.

O segundo capítulo da tese é composto do artigo "***Generation of cell models of mucopolysaccharidoses using CRISPR/Cas9***", que será submetido para a revista *Methods and Protocols*. Neste artigo, apresentamos o processo de criação de um modelo celular em formato de protocolo detalhado. Utilizando células HEK293 e tendo como alvo os genes *IDUA* e *IDS*, demonstramos o nocauteamos destes genes, os resultados esperados e a eficiência de cada etapa, que julgamos necessários uma vez que alguns processos são apresentados de forma demasiadamente simplificada na metodologia dos artigos.

O terceiro e último capítulo da parte I trata-se de uma *brief communication* a ser submetida à revista científica *Molecular Genetics and Metabolism*, intitulada "***Impaired autophagy, mitophagy and reduced neurite density in a MPS II neural cell model***".

Neste trabalho, criamos um modelo celular de mucopolissacaridose tipo II utilizando a linhagem celular imortal SH-SY5Y. Essas células, provenientes de neuroblastoma, podem ser diferenciadas *in vitro* em neurônios funcionais e são ótimos modelos neuronais, sendo utilizadas amplamente no estudo de doenças neurodegenerativas, como doenças de Parkinson e Alzheimer. No modelo de MPS II, observamos alterações significativas na morfologia e homeostase celular em comparação com células normais. Observamos aumento importante do número de autolisossomos e da massa mitocondrial, além de modesto aumento de espécies reativas de oxigênio. Estas modificações podem estar implicadas na diminuição da autofagia celular nesse modelo. Ao diferenciarmos as células em neurônios, também verificamos a diminuição significativa da densidade de neuritos formados. Algumas destas alterações já foram descritas em outras doenças lisossômicas e corroboram a utilidade do modelo celular que desenvolvemos.

CAPÍTULO I.

CREATING CELL LINES FOR MIMICKING DISEASES

Creating cell lines for mimicking diseases

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Contents

Abstract

Cell lines can be good models for the disease they are derived from but can also be used to study general physiological and pathological processes. They can also be used to generate cell models of diseases when primary cultures are not available. Recent genome editing tools have been very promising tools toward creating cell models to mimic diseases in vitro. In this chapter, we highlight techniques used to obtain genome-edited cell lines, including cell line selection, transfection and gene editing tools available, together with methods of phenotype characterization and, lastly, a few examples of how in vitro disease models were created using CRISPR-Cas9.

1. Introduction: Concepts and characteristics of cell lines

A cell line is a population of cells that can proliferate indefinitely under appropriate culture conditions, i.e., they have infinite lifespan. Their use

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dates back to 1951, when the first one was described, the famous HeLa line.[1] Since then, many more from different origins and tissues were established; in 2020, the American Type Culture Collection (ATCC) offers more than 2000 human cell lines, from multiple tissues and distinct morphologies. As these cells are very accessible and easy to manipulate, they became one of the main research tools in laboratories worldwide, contributing significantly to our knowledge of basic cell and molecular biology, pathology and development of therapies. After genome editing tools became widely accessible in the past several years, studies of gene function increased drastically, as well as the generation of genetically engineered cell lines to model different genotypes and diseases. These novel cell models can be used in vitro for unlimited purposes, from basic research to drug screening.[1]

Cell lines can be derived from biopsies of tumorous tissues (like the HeLa cells), or they can be artificially immortalized from primary cultures. The first carries characteristics of cancerous cells, like aneuploidies, undifferentiated phenotypes, cell invasion properties and altered expression of multiple genes involved in cell cycle regulation.[1] Artificial immortalization, however, can generate cells with relatively consistent diploidy and good proliferative capacity, without tumorigenicity characteristics.[2] Immortalization can be mainly achieved by introduction of a viral oncogene that deregulates the cell cycle (as SV40 used in HEK293, which inhibits p53 and retinoblastoma protein pRB, for example[3]) or by activation of telomerase (hTERT) to prevent chromosomal shortening; often, both methods are used in combination, as it seems to produce more genetically stable cells. Some strategies for conditional immortalization had also been developed, as temperature and recombinase-based regulations (fully reviewed in[2]).

The majority of cell lines were obtained from tumorous tissues and, therefore, present features of cancer cells. Whereas these lines are fundamental in cancer research, as they provide insights about cancer variability and pathophysiology, and they are the first platform for testing anti-cancer drugs, they are also used as models for non-oncologic studies—epithelial lines, for example, can be good models of epithelial barriers, becoming a tool to study transporters and permeability.[4] Cells derived from tumors of patients with genetic diseases, as cystic fibrosis, can be used as a model of the genetic disorder and used to do basic research or gene therapy studies, at some extent regardless of the cancerous phenotype.[5,6] Undifferentiated cell lines, like

glioblastoma cells or myeloblasts, can be differentiated and studied in their final mature state, or have the differentiation process compared under various conditions—to compare if knocking out certain gene impacts any pathway required for differentiation, for example. Another good example of how broad is the use of cell lines is the characterization of cell susceptibility to viral infections, like ZIKV and DENV-2,[7] using multiple cell types available to represent various tissues. Finally, these cells are the easiest tool to study basic science of human cells, as the importance of mitochondrial subunits in energy metabolism and cell viability, for example.[8]

Besides their phenotype and intrinsic characteristics, cell lines can be genome edited for use as in vitro disease models of virtually any condition. Although they carry many undesired features, advantages of using cell lines to mimic diseases include accessibility, durability, low cost, reproducibility and easiness of maintenance. While cell lines can be purchased, easily modified once and stored indefinitely, primary cells have to be collected from a patient (which makes the collection of some cell types, like neurons, unviable), are considerably harder to modify (specially depending on the cell type) and have limited lifespan. Besides, primary cells have a lot of variability between different batches of cells depending on the donor, while cell lines have the enormous advantage of being isogenic, where one can induce various disease-causing mutations in the same line and compare the phenotypes without any other confounding factor due to genetic background differences that could be found in affected and control primary cells.[9]

At some extent, induced pluripotent stem cells (iPSC) overcome most of the primary cells' limitations; however, the use of iPSC has some caveats of its own—it is very expensive to culture, requires experienced personnel and the differences in genetic background can still be an issue.[10] Therefore, cell lines are a great resource for basic science and proof-of-concept studies.

Besides the election of the cell type, the process for creating a disease model using genome-edited cell lines comprises the choice of the genome editing components, the delivery method of these components to the cell, the selection of the modified cells and the characterization of the newly created cell model. In this chapter, we discuss these processes in the following sections, together with examples of creation of cells lines to mimic diseases, from monogenic to multifactorial disorders.

2. Genome editing of cell lines: The basics

2.1 The CRISPR-Cas9 system

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)—CRISPR Associated (Cas) system is currently the easiest and the most used genome editing tool. The system was engineered to consist of two components only: the Cas9 nuclease and a guide RNA (gRNA). The gRNA is composed of two sequences, the tracrRNA and the crRNA: the first is the invariable part which interacts with the Cas9; the second is the customizable component of the guide, thus, the sequence that should be designed according to the target. Briefly, the Cas9, guided by the gRNA, recognizes the target and cleaves the double-stranded DNA as long as there is an NGG sequence—called Protospacer Adjacent Motif (PAM)—immediately downstream of the recognition site. Upon a double-strand break in the genome, the cellular repair machinery can fix the lesion by non-homologous end joining (NHEJ) or by homologous recombination (HR). The first normally causes small mutations in the editing site and is widely used for generation of knockouts; the second, in turn, is used to induce precise modifications. For precise HR to occur, there has to be a DNA template (or donor template) containing the intended modification, flanked by sequences homologous to the targeted site in the genome (homology arms). After the repair is done, if the HR occurred successfully, the genome will contain the exact sequence located between the homology arms, inserting or deleting from few nucleotides to whole genes.[11] This donor vector can be a small sequence of single-stranded oligodeoxyribonucleotides (ssODNs), a plasmid, or be delivered in viral vectors.[12]

The simple two-piece system of Cas9 + gRNA can be delivered to the cells in three different ways: (1) as a single plasmid that expresses both Cas9 and gRNA; (2) as a mRNA molecule of Cas9 and gRNA separately or (3) as a ribonucleoprotein (RNP) complex, where Cas9 (as a protein) is complexed to the gRNA molecule. The first is the cheapest method and has good efficiency in cell lines, but not in some primary cells—in hematopoietic stem cells, for example, editing is practically zero.[13] Also, as the expression of the plasmid might last for a long time, Cas9 seems to accumulate within cells overtime, increasing frequency of off-targets.[14]

The RNP, on the other hand, is more costly, but the system is active immediately after introduction in the cell, and is undetectable in less than

a couple of days,[14] resulting in reduced off-target activity. Although highly dependable on the cell type, the RNP also seems to be more effective at editing,[15] especially if chemically modified gRNAs are used,[13] since in vitro transcribed gRNAs cause interferon release and cell death.[16] Using RNP is also a better option when engineering multiple sites.[14,17] The mRNA method normally stands in between the others in both efficiency and off-target activity[14]; however, an important remark is that Cas9 mRNA promotes an important global transcriptional repression that is not seen in other methods.[18] Another interesting note is that, even though the mRNA's sequence length is smaller than the plasmid's, its total size is much larger due to the linearized structure, which might limit the options for transfection.[19] Fig. 1 illustrates the three methods and

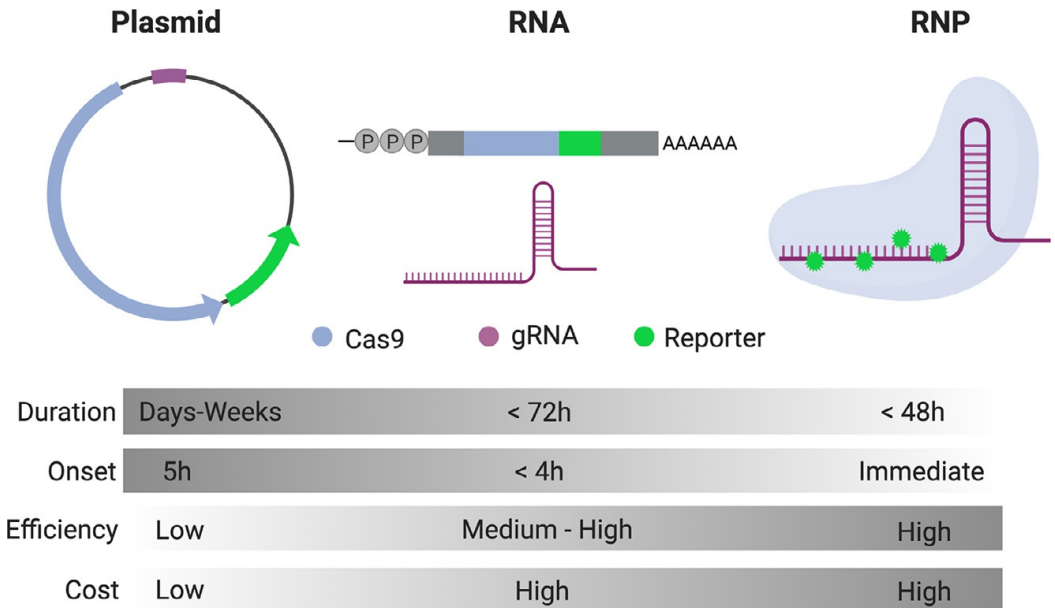

Fig. 1 Methods for delivery of CRISPR components gRNA and Cas9. Three methods are used: plasmid, RNA and RNP (ribonucleoprotein complex). Plasmid: a single plasmid encodes the Cas9 and the gRNA; frequently, a reporter is included (such as GFP) downstream of the Cas9 sequence, so it is expressed under the same promoter and it reports which cells were transfected. Plasmids are expressed after about 5 h inside the cell and can last for days; editing efficiency and cost are both low. Delivery of gRNA with mRNA for Cas9 can also include the sequence of a reporter; the editing can start in about 1 h after delivery and becomes undetectable before 72 h. The efficiency varies with cell type, but can be high, as well as expensive. Lastly, delivery of Cas9 as protein complexed with the gRNA confers the highest efficiency (but this is dependable on the cell type) and the least off targets, as the complex starts the editing as soon as it enters the cell and lasts for only several hours; however, this system is the most expensive. Fluorescent molecules can be conjugated to the gRNA to label cells that received the complex.

summarizes their main characteristics. Reviews previously published fully characterize the CRISPR system.[19–21]

2.2 Transfecting cells with the CRISPR/Cas9 machinery

Transferring nucleic acids into cells is an essential step toward genome editing. This can be done either by transduction or by transfection. The first uses viral vectors for transference and is normally a highly efficient process, indicated to be used mainly for primary cultures or stem cells as they are classically more resistant to transfection (i.e., non-viral methods). Many cell lines, however, are particularly susceptible to genetic transfer without the use of viral vectors, being able to internalize exogenous DNA by transfection in satisfactory levels. These cells are referred to as transfection hosts in the ATCC catalog. Table 1 shows most of the human transfectable cell lines available in the ATCC catalog, along with their characteristics.

Classical and emerging transfection methods are reviewed extensively elsewhere.[21,22] The most used ones are electroporation, lipid-mediated cell transfection (or lipofection) and cationic polymers[23] (Fig. 2). Electroporation applies an electric field in the cell solution, causing instability in the cell membrane and forming pores that allows the diffusion of molecules, including proteins and nucleic acids.[22] On the other hand, cationic lipids or polymers rely on their positive charge. As they interact with negatively charged proteoglycans in the cell membrane, they can induce endocytosis and enter the cell.[24] Even though Cas9 is positively charged, its association with the gRNA turns the complex overall sufficiently negative to be carried by cationic-based methods.[25] For transfection of either plasmid, mRNA or RNP, the protocol should be optimized for the highest editing efficiencies for every cell type used. Parameters as buffer, voltage and electrical pulse (for electroporation) and the ratio of reagent/cargo (for cationic reagents) vary substantially depending on the cell type.

It is important to note that, when using plasmid vectors, the goal is to reach the nucleus of the cell, where the plasmid must be transcribed. Transfection methods deliver nucleic acids only to the cytosol and further entrance into the nucleus depends on binding to specific DNA-binding proteins[26] or to cell division, when the nuclear membrane is dissociating. One way to increase the efficiency of this process is adding transcription factors or nuclear localization signals (NLS) to the plasmid, which will facilitate its transport to the nuclear compartment through nuclear pores.[19,21] In contrast, it has been suggested that addition of a NLS to the Cas9 in its protein

Table 1 Transfection hosts in the ATCC catalog.

Area of study	Tissue collected	Disease	Name	ATCC #	Characteristics and main uses	Cell type	Morphology	Culture	Gender
Bone and cartilage	Bone	Osteosarcoma	Saos-2	HTB-85	Can be differentiated into osteoblast and osteoclast		Epithelial	A	F
Bone and cartilage	Bone marrow	Chondrosarcoma	SW 1353	HTB-94	Mimic chondrocytes		Fibroblast	A	F
Bone and cartilage	Bone	Osteosarcoma	U-2 OS	HTB-96	High transfection efficiency. Attach more strongly than HEK293		Epithelial	A	F
Brain	Bone marrow	Neuroblastoma	SH-SY5Y	CRL-2266	Can be differentiated into neuron-like cells	Neuroblast	Epithelial	Mixed	F
Brain	Brain	Neuroepithelioma	SK-N-MC	HTB-10	Can be differentiated into neuron-like cells		Epithelial	A	F
Brain	Brain	Neuroblastoma	IMR-32	CCL-127	Can be differentiated into neuron-like cells, model of Alzheimer's disease	Neuroblast	Fibroblast	A	M
Brain	Brain	Glioblastoma	U-87 MG	HTB-14	Can be differentiated into cholinergic neuron-like cells, astrocytes, oligodendrocytes and ependymal cells		Epithelial	A	M

Continued

Table 1 Transfection hosts in the ATCC catalog.—cont'd

Area of study	Tissue collected	Disease	Name	ATCC #	Characteristics and main uses	Cell type	Morphology	Culture	Gender
Brain	Testis	Malignant pluripotent embryonal carcinoma	NTERA-2 cl.D1 [NT2/D1]	CRL-1973	Can be differentiated into dopaminergic neuronal precursor cells and few oligodendrocyte-type cells		Epithelial-like	A	M
Connective tissue	Connective tissue	Fibrosarcoma	HT-1080	CCL-121			Epithelial	A	M
Cystic fibrosis	Colon	Ductal adenocarcinoma; cystic fibrosis	SW480	CCL-228	Cells from cystic fibrosis patient		Epithelial	A	M
Eye	Eye	Retinoblastoma	WERI-Rb-1	HTB-169	Can be differentiated into retinal neuron-like cells	Retinoblastoma	Clusters of round cells	S	F
Eye	Eye	Normal	ARPE-19	CRL-2302	Model of epithelial barrier and expression of transporters	Epithelial	Epithelial	A	M
Gastroenteral	Colon	Colorectal adenocarcinoma	HT-29	HTB-38	Can be differentiated into enterocytes		Epithelial	A	F

Gastroenteral	Colon	Colorectal adenocarcinoma	Caco-2	HTB-37	Used as monolayer for absorption, cell invasion and lipid transport studies	Epithelial	Epithelial	A	M
Gastroenteral	Colon	Colorectal carcinoma	T84	CCL-248	Model of epithelial barrier		Epithelial	A	M
Hematopoietic	Bone marrow	Chronic myelogenous leukemia (CML)	K-562	CCL-243	Erythroid and monocyte/ macrophage differentiation		Lymphoblast	S	F
Hematopoietic	Peripheral blood	Acute lymphoblastic leukemia	HL-60	CCL-240	Can be differentiated into granulocytes and monocytes	Promyeloblast	Myeloblast	S	F
Hematopoietic	Bone marrow	Acute lymphoblastic leukemia	KG-1	CCL-246	Can be differentiated into macrophages	Macrophage	Myeloblast	S	M
Hematopoietic	Bone marrow	Chronic myelogenous leukemia (CML)	MEG-01	CRL-2021	Can be differentiated into megakaryocytes and produce platelets	Megakaryoblast	Lymphoblast	Mx	M
Hematopoietic	Lymphoblast	Burkitt's lymphoma	Raji	CCL-86		B lymphocyte	Lymphoblast	S	M

Continued

Table 1 Transfection hosts in the ATCC catalog.—cont'd

Area of study	Tissue collected	Disease	Name	ATCC #	Characteristics and main uses	Cell type	Morphology	Culture	Gender
Hematopoietic	Peripheral blood	Acute lymphoblastic leukemia	THP-1	TIB-202	Can be differentiated into macrophages	Monocyte	Monocyte	S	M
Hematopoietic	Peripheral blood	Malignant non-Hodgkin's lymphoma	NK-92	CRL-2407	Cells can recognize and kill tumor cells	Natural killer cell	Lymphoblast	S	M
Hematopoietic	Peripheral blood	Acute T cell leukemia	J.RT3-T3.5	TIB-153	T cell model that do not express T cell receptor (TCR)	T lymphocyte	Lymphoblast	S	M
Kidney	Kidney	Normal	HEK 293	CRL-1573	High transfection efficiency	Epithelial	Epithelial	A	F
Kidney	Kidney	Adenocarcinoma	A-704	HTB-45			Epithelial	A	M
Liver	Liver	Hepatocellular carcinoma	Hep G2	HB-8065	Model of polarized human hepatocytes		Epithelial-like	A	M
Liver	Liver	Hepatocellular carcinoma	Hep 3B	HB-8064	More differentiated mature hepatocytes than HepG2; p53 null		Epithelial	A	M
Lung	Lung	Normal	WI-38, MRC-5 and IMR-90	CCL-75, CCL-171 and CCL-186	Diploid, but limited lifespan	Fibroblast	Fibroblast	A	F, M, F

Lung	Lung	Carcinoma	A549	CCL-185	Model of alveolar type II pulmonary epithelium	Epithelial	Epithelial-like	A	M
Lung	Lung	Normal	BEAS-2B	CRL-9609	Characteristics of mesenchymal stromal cells	Epithelial	Epithelial	A	
Lung, cystic fibrosis	Lung	Adenocarcinoma	Calu-3	HTB-55	Studies of barrier integrity, chloride ion secretion, pulmonary drug delivery	Epithelial	Epithelial	A	M
Mammary gland	Mammary gland	Adenocarcinoma	MCF7, MDA-MB-231	HTB-22, HTB-26		Epithelial	Epithelial	A	F
Pancreas	Pancreas	Adenocarcinoma	BxPC-3	CRL-1687	High expression of angiogenic factors		Epithelial	A	F
Pancreas	Pancreas/ duct	Epithelioid carcinoma	PANC-1	CRL-1469	Can be differentiated into insulin-producing cells	Epithelial	Epithelial	A	M
Pancreas, cystic fibrosis	Pancreas	Ductal adenocarcinoma	CFPAC-1	CRL-1918	Characteristic of pancreatic duct cells, homozygote for CFTR ΔF508	Epithelial	Epithelial	A	M
Pancreas, cystic fibrosis	Pancreas	Adenocarcinoma	Capan-1	HTB-79	Cells express CFTR and secrete gastric type mucins		Epithelial	A	M

Continued

Table 1 Transfection hosts in the ATCC catalog.—cont'd

Area of study	Tissue collected	Disease	Name	ATCC #	Characteristics and main uses	Cell type	Morphology	Culture	Gender
Reproductive system	Cervix	Adenocarcinoma	HeLa and HeLa S3	CCL-2, CCL-2.2		Epithelial	Epithelial	A	F
Reproductive system	Ovary	Adenocarcinoma	NIH: OVCAR-3	HTB-161		Epithelial	Epithelial	A	F
Reproductive system	Prostate	Adenocarcinoma/ Carcinoma	PC-3, DU 145 and LNCaP clone FGC	CRL-1435, HTB-81 and CRL-1740			Epithelial	A	M
Skin	Skin	Normal	BJ	CRL-2522	Diploid, longer lifespan than other normal fibroblasts	Fibroblast	Fibroblast	A	M
Skin	Skin	Melanoma	A2058	CRL-11147	Cells express Nerve Growth Factor. Highly invasive		Epithelial	A	M
Vessels	Aorta	Normal	T/G HA-VSMC	CRL-1999	Model of vascular smooth muscle	Smooth muscle	Fibroblast	A	F
Vessels	Liver	Adenocarcinoma	SK-HEP-1	HTB-52	Model of liver sinusoidal endothelial cells	Endothelial	Epithelial	A	M
Vessels	Umbilical vein	Normal	HUV-EC-C	CRL-1730	Model of vascular endothelium	Endothelial	Endothelial	A	

A, adherent; S, suspension; M, male; F, female.

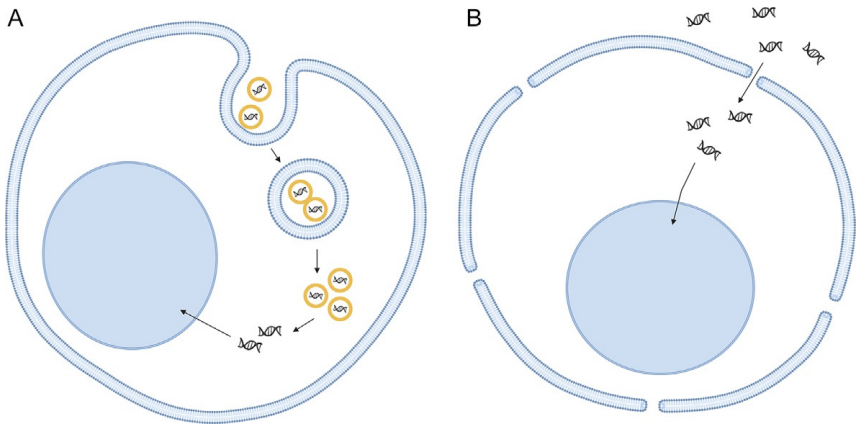

Fig. 2 Most common transfection methods. (A) Lipid-mediated cell transfection uses lipids (commonly liposomes) to complex with nucleic acids and induce endocytosis to enter the cell. Once in the cytoplasm, the liposome is released, and the nucleic acid dissociates from the lipid. (B) Electroporation creates an electric field in the solution containing the cells, creating pores in the membrane from where the nucleic acids can enter the cell. To reach the nucleus, both methods rely on cell division or transport mediated by specific proteins.

form (which also has to enter the nucleus where the editing occurs) does not increase targeting efficiencies, regardless of the NLS type or position.[27]

For analysis of transfection efficiency and quantification, a reporter is required. When using a fluorescent protein (hereon referred as GFP or RFP for simplification, although other proteins and fluorophores could be used), for example, it is possible to visualize transfected cells by fluorescent microscopy and quantify them by flow cytometry, allowing the comparison of different transfection protocols (Fig. 3).

All three options of CRISPR vectors allow the inclusion of a reporter to track the cells that received the CRISPR system. In a plasmid, the DNA sequence coding for GFP is inserted downstream the Cas9 and a cleavage signal (such as T2A or P2A), so that both are expressed under the same promoter. Similar strategy can be used for mRNA, where a single mRNA molecule can contain the sequences for both Cas9 and the GFP, resulting in translation of both in the targeted cells. Alternatively, it has been reported[28] the fluorescent labeling of the gRNA, which allows for visualization of RNP complexes in efficiently transfected cells (Fig. 1).

Besides reporters, one could use genes for antibiotic resistance. These resistance genes can be included in the plasmid or in the mRNA, conferring resistance to transfected cells as soon as they are expressed. It is important to note, however, that expression of the reporter or the resistance indicates cells

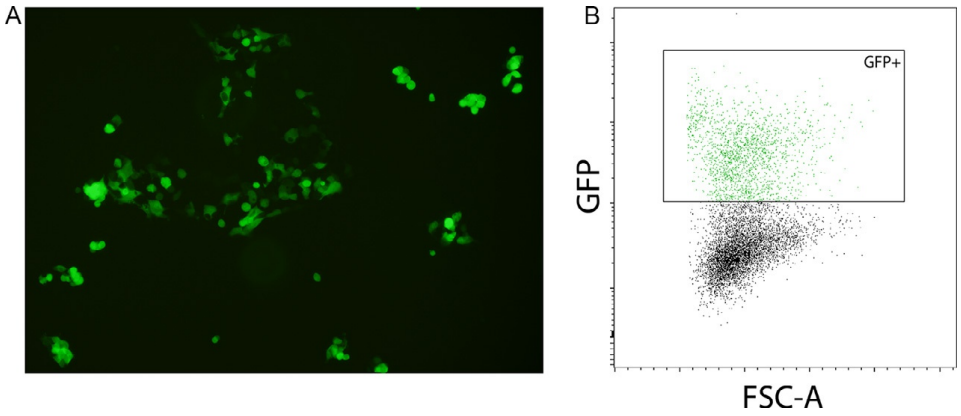

Fig. 3 HEK293 cells transfected with GFP-expressing plasmid. (A) Fluorescence microscopy showing GFP+ cells. (B) Example of flow cytometry plot showing GFP+ cells. *Source: personal archive.*

that have been efficiently transfected, but not necessarily edited, and this is only transient while the sequences remain in the cells.

2.3 Selection of modified cells and clonal expansion

One way of selecting cells that had certainly undergone editing is the inclusion of a reporter in the donor template that will be used for HR, so that the reporter is permanently integrated in the genome of edited cells. The strategy can be to insert the reporter alone in the genome to generate a knockout, or to place it downstream of a sequence of interest (like a cDNA for a missing gene, for example). This way, only cells that had at least one edited allele will be fluorescent, becoming easily identifiable at any point. Alternatively, antibiotic resistance genes can be used, and cells can be selected using antibiotic in the culture.

For selection of bi-allelic edited cells, one has to use a dual donor template, where each one of the donor vectors contain the sequence for a different and distinguishable fluorophore—GFP and RFP, for example. This way, cells that express both colors surely have two alleles targeted and can be easily isolated all at once (Figs 4 and 5A).[17]

If inserting a reporter in the genome is not an option, one can still enrich the edited population by selecting transfected cells. This selection must occur when the expression of the reporter is at its highest—for plasmids, this is usually around 24–72 h; for mRNA, this can be done at 12–24 h post-transfection and for labeled gRNA, 6 h should be enough.[28] The selection of transfected cells is particularly useful for cells that normally have low

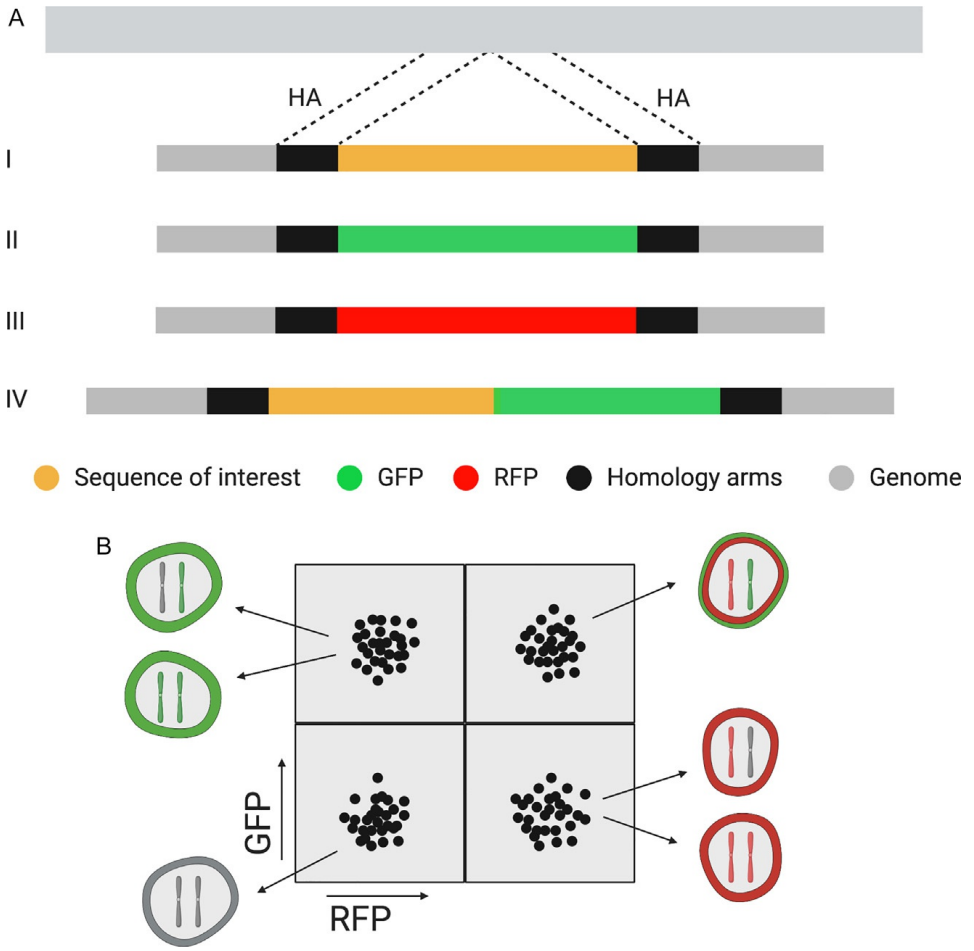

Fig. 4 Homologous recombination with different reporters. (A) Donor vectors for homologous recombination must contain homology arms according to the site of the genome where the editing will occur. Donor vectors can carry a sequence of interest alone (I) or a reporter alone, like GFP (II) or RFP (III). Alternatively, both sequence of interest and reporter can be designed in the same donor vector, so that both are integrated in the genome. (B) When two donor vectors with different reporters are used simultaneously, cells can have basically four outcomes, easily distinguishable in the flow cytometry, as illustrated: lower left panel—not-fluorescent and not-edited; upper left panel—green-fluorescent cells edited in either one or both alleles; lower right panel—red-fluorescent cells edited in either one or both alleles; and upper right panel—cells double-positive for both green and red fluorescence, thus edited in both alleles (each one containing a different reporter). HA: homology arms.

transfection efficiencies or for cells that might undergo a negative selective pressure after editing, meaning they can be rapidly lost as the unedited cells overgrow the edited ones. Fig. 5 illustrates most common methods of enriching and/or isolating modified cells.

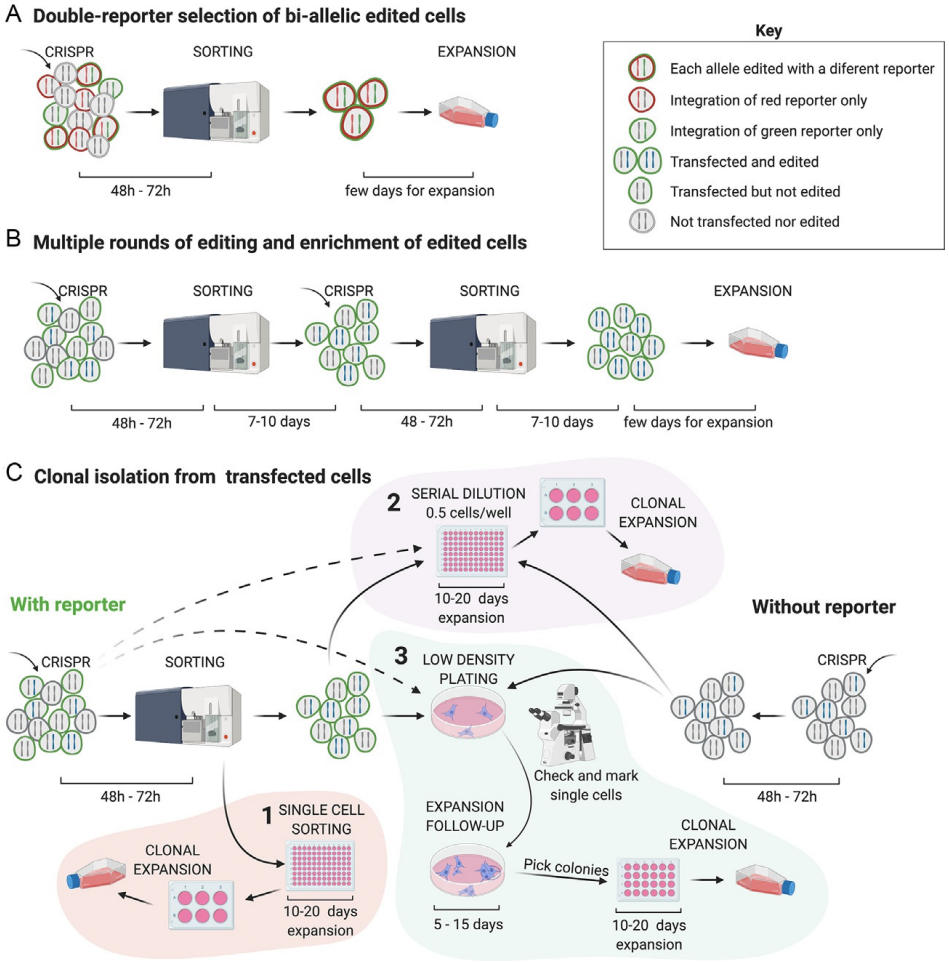

Fig. 5 Enrichment and isolation of edited cells. (A) Selection of bi-allelic edited cells can be done using two constructs with distinct reporters for integration into the genome. Cells can be selected by FACS based on the presence of double fluorescence and be expanded soon after. (B) For cells that struggle to expand from single cells, multiple rounds of editing can be done. After editing with a reporter, cells are sorted based on fluorescence, which will include cells that were transfected, but not edited. When the reporter is not expressed anymore (around 10 days), cells are edited again and submitted to the whole process again for as many rounds as needed. At the end of the process edited cells are enriched, but there might have few unedited in the population and the mutations will be variable. (C) For isolation of clones with the same variant, three approaches can be done. With a reporter, cells can be sorted as single cells (1) into 96-well plates, expanded for several days to bigger containers; alternatively, cells can be sorted as reporter positive population, diluted and seeded as 0.5 cells/well, followed with clonal expansion (2) or plated in low density (3), in which cells have to be monitored for single cells and colony formation. When colonies are visible, they are picked and expanded in 24-well plates. Finally, in the absence of a reporter or without a sorter, clonal isolation can be done with either (2) serial dilution or (3) low-density plating and colony picking.

Transfected cells with a reporter gene can be sorted by Fluorescence Activated Cell Sorting (FACS) into transfected (GFP+) and non-transfected (GFP−) populations. Transfected cells (which might or might not be edited) can be submitted to additional rounds of targeting to increase the proportion of edited cells (Fig. 5B).[29] If the goal is to obtain a highly enriched population of edited cells regardless of the mutation provoked, this can be done without further selection, but few unedited cells might still be present. Alternatively, multiple targeting rounds can be used to increase targeting efficiency and, later, cells can still undergo clonal expansion for isolation of different mutations.

Isolation of single cells for clonal expansion can be done in different ways. One of them is by FAC-sorting single cells into 96-well plates; the chances of expanding edited clones in GFP+ sorted cells is higher than if sorting the bulk population. In cases where the protein of interest to be knocked out localizes in the cell surface, like a cluster of differentiation molecule (CD), one could stain the bulk transfected population (containing edited and non-edited cells) with an antibody specific for the knocked out protein and sort only the negative fraction—assuming all cells normally express the protein in the membrane—obtaining only knockouts at the end.[30]

If a sorter is not available, one could do serial dilution with the cell suspension—guaranteeing that the cells are very well detached one from another and in a single cell suspension—and plate the volume equivalent to 1 or 0.5 cells per well, in a 96-well plate. If cells are adherent, this process can be done in liquid media; if using cells that grow in suspension, it is advised to use a semi-solid media, so that they can be spotted by light microscopy. For both, wells should be checked for single cells only, and any well with two or more cells should be discarded. It is always useful to seed one well with higher number of cells, so that it will be easier to focus the cells under the light microscope, as a single cell might be easy to miss (Fig. 6A).

Lastly, colony picking can be done to isolate clones. In this case, cells are plated in bigger wells or plates at very low densities—optimized by cell type and area—in a way that single cells attach far apart. Under observation in the microscope, single cells can be identified and followed for expansion. Once a cell clump is visible, the colony can be detached from the plate and seeded somewhere else for expansion. Collecting the colony might be tricky depending on the cell type and should be optimized—one could use a P10 pipette with few microliters of trypsin, a cloning cylinder with trypsin in it or simply scraping the colony to detach it from the plate (Fig. 6B).

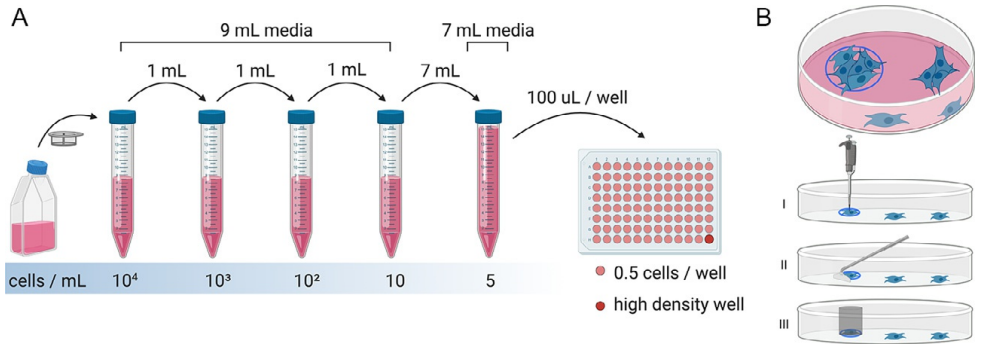

Fig. 6 Methods for cell isolation without sorting. (A) Starting from bulk population of transfected cells, a single cell suspension is done by pipetting up-and-down and/or using cell strainers and serial diluted starting from 10^4 cells/mL up to 5 cells/mL. At this final concentration, plating 100 μL of cell suspension into each well of a 96-well plate results in 0.5 cells per well, minimizing the risk of having two cells in the same well. At least one well should be seeded with more cells so that focusing of the light microscope is easier. (B) After identifying a colony that started from a single cell and that is relatively far from other colonies, one can pick the colony by (I) pipetting up-and down trypsin in a 10 μL tip at a 90° angle; (II) scraping the bottom of the plate with a cell scraper exactly where the colony is; (III) placing a cloning cylinder around the colony and adding trypsin to detach the cells.

Sorting cells might be too stressful for some cell types, especially as single cells—thus one should test which approach works best with each cell type. Moreover, cells might proliferate slowly or not at all[31] without the physical contact to other cells and in the absence of growth factors secreted by neighboring cells in the media. Use of conditioned media can be a useful resource in assisting cell expansion.[32] Conditioned media can be obtained by collecting media that has been for 24 h in a 50–70% confluent cell culture. After collection, media has to be filtered through a 0.22 μm filter to exclude any cells that will lead to contamination of the single-cell culture and diluted to at least 1:1 ratio with fresh media.

2.4 How efficient is the process?

The simplest way one can measure targeting efficiency is by tracking indels decomposition (TIDE, https://tide.deskgen.com).[33] To do this, DNA from the pool of edited cells should be amplified in the target locus and sequenced by Sanger method. TIDE compares two Sanger sequencing reactions (one wild-type and one edited, from bulk population of cells) and quantifies the different sequence traces obtained, giving a percentage of different sequences identified (targeting efficiency) and also highlighting

the predominant indels in the targeted sample. This method shows the proportion of alleles modified by CRISPR, but not necessarily edited by HR. TIDE is also a good method to compare different gRNAs and loci, aiming for the ones with highest targeting efficiencies. Lastly, one can also use TIDE in the bulk population before plating single cells, in an attempt to estimate how many clones should be selected to obtain one mutant or if another round of editing should be conducted to increase the efficiencies.[9]

The overall efficiency to obtain a mutated cell clone varies depending on every step of the process. First, the efficiency of all steps is primarily subject to the cell type. One should consider if the cell chosen is a good transfection host, if it proliferates well (as cell division facilitates HR, for example), if it is resistant to the stress of cell sorting and if this cell type expands well from single cells. Human beta cell line EndoC-βH, for example, do not expand from single-cells, thus isolation of clonal cell lines is not possible.[31] In this case, these cells would be good models to study knockout effects rather than specific mutation analyses.

Regarding the editing with CRISPR, efficiency varies significantly depending on the sequence of the gRNA used and the locus in the genome; thus, trying different guides beforehand could be a good time-saving strategy. Wieser et al. for example, observed allele targeting efficiencies of 7% and 66% with two adjacent guides for the same gene,[30] highlighting the substantial difference two guides can have. Moreover, the platform chosen and the delivery method also influences directly, as RNP is normally more efficient than mRNA or plasmid[14] and some delivery methods have poor transfection rates, compromising all downstream steps. If a key cell for creating a model is hard to transfect, then viral vectors should be considered for delivery—as exemplified by Chung et al. where all tests were performed in HEK293 cells, obtaining 80% transfection rates, but when using T84, transfection decreased to 10% and the authors decided to switch to a lentiviral vector instead.[34] In conclusion, one should choose carefully each step of the process.

2.5 Validation of cell models: Confirming genotype and phenotype

After the transfection (and supposedly the editing) and the clonal isolation, one should confirm the knocking in or out of the cells to validate the new model. This has to be done both at the DNA and at the protein level, as sometimes the variant induced in the genome is silent and/or it does not decrease protein

expression significantly to consider it a knockout model. There is not a single order of how these experiments should be done—if starting with DNA or with protein analysis. One should choose one assay (probably the most accessible and/or the easiest) to do the initial screening, select few clones based on that and, only then, conduct the full characterization.

Initial screening from DNA samples can be done in few different ways, although Sanger sequencing is the most definite method and should be performed eventually to identify the exact genome change caused by CRISPR. Before sequencing, however, Skakic et al. screened the cells using RFLP, as the target site was designed in a restriction enzyme site. Monoallelic and biallelic alterations were identified this way and later were confirmed by sequencing.[35] A simple PCR can also be useful to check for insert integration[36] or deletions[31] in the target site when the difference in the amplicon size is big enough to be identifiable in a gel. However, screening by DNA sequencing is more commonly used.[37–40]

Since Sanger sequencing screens for the presence of variants but not necessarily for functional knockouts, some authors prefer to screen by Western blot,[9,41] as the absence of protein represents a true knockout. The same is valid for knock ins, where the protein expression means that the insert is being successfully expressed. The screening at the protein level can also be done by flow cytometry[30]—especially when it is a membrane protein—or by specific functional assays.[41]

Following the confirmation that the selected clone is indeed edited and—in the case of knockout models—the protein is not being produced, the newly created cell model has to undergo functional assays to be fully validated. For metabolic diseases lacking enzymes, for example, enzyme activity assay and analysis of substrate accumulation (direct or indirect) are normally done. Frequently, both are equally important, as reduction in enzyme activity might not be drastic enough and still be sufficient to clear the substrate, thus not fully mimicking the disease phenotype.[9,36,39] For diseases lacking specific transporters, the metabolite can be measured in different compartments to prove absent transport, like insulin in a diabetes-related model[31] or chloride in cystic fibrosis.[34] Finally, light microscopy with specific staining—like Prussian blue for visualization of siderotic granules[38]—and/or immunofluorescence can aid the validation of the model and the evaluation of any undesired phenotypical change.[30]

Fig. 7 Summarizes the workflow for creating a cell line for in vitro disease modeling.

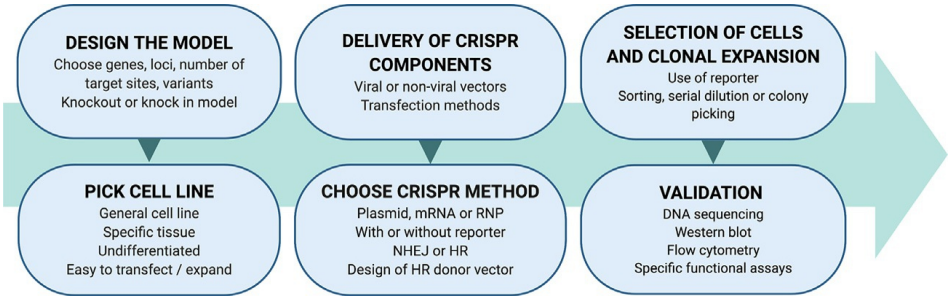

Fig. 7 Workflow for creation of cell line for disease modeling. Designing the first steps carefully can increase the efficiency of the whole process and provide models with multiple possible downstream studies.

3. Edited cell lines can mimic diseases in vitro: From diabetes to lysosomal diseases

Both monogenic and complex diseases can be studied using cell line models. For monogenic diseases, knocking out the gene that causes the disease is usually enough for developing the model. For complex diseases, however, there is not a single gene that can be knocked out; instead, researchers use lines from disease-relevant cell types to characterize novel variants and test modulator genes, among other approaches. Below we describe briefly some disease models created using cell lines and CRISPR-Cas9. Table 2 summarizes the methods used by these studies, regarding CRISPR platform, delivery method and isolation of clones.

3.1 Cell lines for monogenic diseases

3.1.1 Inborn errors of metabolism

Gaucher disease is characterized by deficiency of the enzyme glucocerebrosidase, leading to accumulation of glucosylceramide and glucosylsphingosine mainly in macrophages. Most of the disease symptoms are resultant of the infiltration of engorged macrophages, known as Gaucher cells, and some patients also have neurological manifestations. Considering the main cell types involved in the pathogenicity of the disease (macrophages and neural cells), Pavan et al.[9] developed two new Gaucher cell models inducing *GBA1* mutations in two cell lines—the monocytic line THP-1 and the glioblastoma line U87. Besides increased substrate accumulation and nearly absent enzyme activity, the glial model also had increased apoptosis and cell death, possibly

Table 2 Methods used for cell line generation.

Cell	CRISPR	Transfection	Enrichment transfected cells	Clone isolation	Efficiencies mentioned	References
BV-2	Plasmid	Electroporation	Antibiotic selection	Single-cell sorting	12/52 Clones had undergone HR	36
HAP1	Plasmid	Lipofection	Antibiotic selection	Serial dilution	6/15 Clones edited	39
HEK293	Plasmid	Lipofection	Reporter GFP	Single-cell sorting		42
HEK293	Plasmid	Lipofection	Antibiotic selection	Serial dilution	27/215 monoallelic clones, 2/215 biallelic knock ins; 13.5% HR	35
HEK293	Plasmid	Lipofection		Single-cell sorting	80% transfection efficiency	34
HEK293	Plasmid	Electroporation	Reporter GFP	Colony picking		41
HEK293, THP-1, U87	RNP	Lipofection		Single-cell sorting	3/38 Clones with low expression in the western blot (possible knockouts)	9
HeLa	Plasmid	Lipofection	Reporter GFP	No isolation		29
Immortalized human podocytes	Plasmid	Lipofection	Reporter mRFP, antibiotic selection	Single-cell sorting, colony picking	~4% Transfection efficiency	43
K562, HUDEP2	Plasmid	Electroporation		Serial dilution		38
RPTEC/ TERT1	Plasmid	Lipofection	Antibiotic selection, enrichment for membrane protein expression	Colony picking	1/7 Clones with 1 allele modified and 2/3 clones with homozygous mutations, depending on the guide	30

RNP, ribonucleoprotein complex; HR, homologous recombination.

induced by ubiquitin-proteasome system activation, neuroinflammation and α-synuclein accumulation, mechanisms also seen in patient-derived cells, thus confirming the phenotype observed in the disease.

Glycogen storage disease type Ib (GSD Ib) is caused by deficiency of the glucose-6-phophate translocase (coded by the *SLC37A4* gene) in the endoplasmic reticulum's membrane. Its main role is to translocate glucose-6-phosphate to the lumen of the reticulum for conversion to glucose, ultimately maintaining the glucose/energy homeostasis. The novel variant c.248G>C in *SLC37A4*, observed in two unrelated GSD Ib patients, was induced in HEK293 cells with CRISPR-Cas9 to determine how this variant could affect the functionality of the protein and modulate the disease. In this cell model, besides the expected decrease in SLC37A4 expression that characterizes the disease, it was also possible to observe persistent endoplasmic reticulum stress and increased apoptosis in the GSD Ib cells, which might give more insights about the disease pathogenesis.[35]

X-linked adrenoleukodystrophy is a neurodegenerative disease caused by impaired β-oxidation of fatty acids in the peroxisome, leading to their accumulation throughout the body. Targeting both the *Abcd1* and *Abcd2* genes in the murine microglial cell line BV-2 allowed for the creation of a disease model in vitro.[37] The same approach was used to generate a cell model for another peroxisomal disorder, the acyl-CoA oxidase deficiency, caused by mutations in the *Acox1* gene.[36] Both cell models presented slower growth rate, increased number of peroxisomes and mitochondria, distinct lipid droplet distribution and, most importantly, altered microglial polarization and phagocytic ability when compared to control cells, proving to be valuable models for peroxisomal disorder studies.

Niemann-Pick disease type C (NPC) is mostly caused by mutations in the *NPC1* gene, causing lysosomal accumulation of unesterified cholesterol and glycolipids. Patient-derived fibroblast are normally used for disease studies, but the majority are compound heterozygotes, making difficult the study of molecular mechanisms for each variant individually. To address this, the HAP1 cell line was submitted to genome editing with CRISPR-Cas9. This cell line was selected as these cells are nearly haploids, which facilitates the knockout induction and the posterior selection and characterization. Additionally, to induce precise modifications, the authors also used base editing, creating missense, nonsense and splice site mutations, modeling variants seen in patients and recapitulating the primary phenotype observed in patient-derived cells. They found out that there are different mechanisms of pathogenicity across the 19 different variants, emphasizing the need for analysis of every mutation.[39]

Lastly, Fabry disease is a lysosomal storage disease caused by accumulation of globotriaosylceramide (Gb3), a result of α-galactosidase A deficiency encoded by the *GLA* gene. For drug screening and pharmacokinetic studies, a new cell line model of Fabry disease was created. HEK293 cells were edited using CRISPR-Cas9 to knockout the *GLA* gene, generating cells that have no expression of α-galactosidase A. Knockout cells were treated with recombinant human α-galactosidase A with or without the proteostasis modulator MG132, resulting in increased substrate reduction when both therapies were applied together.[41] Even though HEK293 cells are kidney-derived epithelial cells, they do not represent a good model for kidney disease observed in Fabry patients. Kidney podocytes, however, have unique architecture and are specialized in glomerular filtration; in Fabry-derived cells, they have more and larger Gb3 inclusions than other renal cells, suggesting they play an important role in the disease. Thus, another model was created from immortalization of human podocytes with Fabry disease to study the disease mechanisms in relevant renal cells.[43] Using this model, a high-throughput screening was conducted and several candidate signaling pathways were identified to be disrupted in the disease.

3.1.2 Cystic fibrosis

Cystic fibrosis is caused by mutations in the *CFTR* gene, responsible for expressing an ion channel in the cell membrane, leading to alterations in the epithelia of lungs, intestine, colon, reproductive system and salivary and sweat glands. The pancreatic cell line CFPAC-1 is derived from a cystic fibrosis patient with pancreatic cancer; this line harbors the most common *CFTR* pathogenic mutation, ΔF508, and is frequently used for diverse studies along with another pancreatic line, Capan-1, as control.[6] However, both are derived from different patients and, consequently, have distinct backgrounds. Therefore, an epithelial cell line was created to study the mechanisms of the ΔF508 mutation without the genetic background bias.[34] Human colon T84 epithelial cells were chosen due to the high expression of CFTR in this tissue, providing an appropriate in vitro model for drug screening and molecular mechanisms of the most common mutation observed in cystic fibrosis patients.

3.1.3 Huntington's disease

Huntington's disease is caused by increased CAG repeats in the huntingtin (*HTT*) gene, resulting ultimately in death of brain cells and severe neurological impairment. Using HEK293 cells, Morozova et al.[40] selected clones

with three different *HTT* mutations: a deletion, a frameshift mutation and an insertion of 100 and 150 CAG repeats, and conducted numerous morphometric analyses. They observed that mutant clones had increased apoptosis and decreased proliferation than controls. Additionally, the increased CAG number caused disturbances in the mitochondrial organization, increased number of autolysosomes, vacuolation and disturbances in the integrity of the membranes, while such changes were not seen in knockout clones. These findings were consistent with what was previously described for patient-derived iPSCs, ratifying that the cell line created is a good model for in vitro studies of Huntington's disease.

3.1.4 X-linked sideroblastic anemia

Mutations in the *ALAS2* gene cause X-linked sideroblastic anemia, a disease in which ringed sideroblasts are produced rather than erythrocytes. The mechanisms underlying sideroblast formation are still not fully understood. Animal models for this condition do not present the characteristic ring in the sideroblasts or are lethal in the embryonic stage. The *ALAS2* gene has an enhancer containing a GATA1 binding site and mutations in this site resulted in knockdown of *ALAS2*, also causing sideroblastic anemia. Considering this, a cell model was established by *ALAS2* knockdown via GATA1 binding site mutations, using human umbilical cord blood-derived erythroid progenitor-2 (HUDEP-2) cells. HUDEP-2 cells can be differentiated into erythrocytes, making them a great choice for modeling this disease. The model presented significant reduction in *ALAS2* expression, resulting in efficient formation of rings in sideroblasts. Upon observation by electron microscopy, the rings formed in this model had the same morphological properties as the ones observed in patient-derived cells, proving to be a good in vitro system to study this disease.[38]

3.2 Cell lines for complex/multifactorial diseases

3.2.1 Kidney disease

Kidney disease is frequently associated with the complement system and renal proximal tubular epithelial cells (RPTECs) seem to be involved in this process. In order to study the complement system in renal cells, Wieser et al. knocked-out the *CD46* gene in immortalized RPTECs.[30] *CD46* knockout cells kept their typical cell morphology and expression surface markers, proving to be a suitable model for further studies.

Polycystic kidney disease is an inherited cause of end-stage renal disease. This condition is caused by mutations in the *PKD1* and *PKD2* genes,

encoding for polycystin 1 and 2, respectively, leading to changes in the energy metabolism. Another gene, *PKHD1*, encodes for fibrocystin/polyductin, and is also involved in bioenergetic pathways. Therefore, Chumley et al. sought to knockout both *PKHD1* and *PKD2* genes to mimic truncating mutations found in patients to further understand the outcomes of their deficiency. The HEK293 line was chosen due to its proliferation rate, as other renal epithelial cells do not expand well from a single clone. The model showed different phenotypes, some of them contrasting with what was described previously in the literature; however, it proved to be a simple and efficient method for initial studies.[42]

3.2.2 Type 2 diabetes

Type 2 diabetes is a complex disease with more than 240 loci associated with disease risk, creating a robust need of in vitro models for functional studies of different variants and disease mechanisms. To address this, the human beta cell line EndoC-βH1 was used to generate different knockouts of variants that needed characterization, targeting the genes *PAM*, *IDE*, *INS*, *SLC30A8* and *NEUROD1*.[31] Parameters such as insulin secretion in response to glucose, insulin content and susceptibility to diazoxide and tolbutamide were analyzed, making them good models for the disease, although not all knockouts behaved equally. For example, NEUROD1 knockout cells presented increased levels of endoplasmic reticulum stress and apoptosis markers compared to the others, making the cells unviable and depleting all edited cells within few passages.

3.2.3 Cancer

CRISPR-Cas9 genome editing is an important tool to study the mechanisms underlying cancer. Cancerous cells are known to accumulate innumerous mutations that are believed to be important in the oncogenic process, conferring resistance to drugs and increased invasiveness, for example. Genome editing can help in the characterization of such variants, ultimately enlightening some of the pathogenic mechanisms. Detailed cancer studies are reviewed somewhere else.[44]

4. Conclusions and future remarks

In this chapter we presented few examples of how in vitro disease models can be created using CRISPR-Cas9 and cell lines. Genome editing with CRISPR-Cas9 is very simple, but the efficiency of the entire process can vary significantly depending on the tools chosen for each step of the process.

Some cell lines are easily transfected; Table 1 is a summary of all transfection hosts in the ATCC catalog. Other lines not considered as transfection hosts could be susceptible to transfection too, however the efficiencies might be lower. Starting with a cell line that has high transfection rates makes all the downstream processes easier and possibly more efficient. Cells that have the capacity of expanding from single cells are also desirable, as this is the easiest way to isolate different clones and characterize different variants. Finally, designing the model carefully can yield cells that are useful to study multiple aspects of the disease—some precursor cell lines, for example, can be differentiated into more than one cell type in the same tissue—like neurons, astrocytes and oligodendrocytes from U87 cells, or K-562 cells that can become erythroid cells or monocytes. There are numerous options of different cell lines from many tissues to choose from to design the best model for a given disease.

Although some of the models described in this chapter present few caveats—like the cell type chosen not being exactly relevant for the disease, for example, they are still valuable tools for proof-of-concept studies. Initial tests and screenings can be done in these cells for later validation in more relevant models, once cell lines are extremely easy to manipulate, are normally resistant to gross media changes and also expand relatively fast. Having a disease model in a cell line background can be a great time- and resource-saving strategy, especially for laboratories with limited structure.

Even though the number of genome-edited cell lines have increased considerably in the past years, there is still room for developing many more. Drug screening or therapy development studies, for example, would strongly benefit from different cell types engineered to have the same disease-causing genotype, so that the effect of each treatment is seen in different tissues. Alternatively, a single cell type can be edited in the same gene to compare different sequence variants, in order to understand if the genotype influences the response to a given experimental therapy. The possibilities are virtually endless, and the creation of different models will speed basic research and the development of new therapies, regardless of the disease.

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CAPÍTULO II.

GENERATION OF CELL MODELS OF MUCOPOLYSACCHARIDOSES USING CRISPR/CAS9

CAPÍTULO III.

IMPAIRED AUTOPHAGY, MITOPHAGY AND REDUCED NEURITE DENSITY IN A MPS II NEURAL CELL MODEL

PARTE 2

INTRODUÇÃO

A parte II da presente tese refere-se à aplicação da metodologia de edição genômica por CRISPR/Cas9 ao tratamento da doença genética mucopolissacaridose do tipo I (MPS I). As terapias disponíveis atualmente para MPS I contribuem significativamente para a qualidade de vida dos pacientes. Porém, algumas limitações são muito importantes, como a ineficácia no sistema nervoso central – no caso da terapia de reposição enzimática – ou os riscos relacionados ao procedimento – referente ao transplante alogênico de células-tronco hematopoiéticas que, além de necessitar de doador compatível, ainda apresenta alto risco de desenvolvimento de doença do enxerto contra o hospedeiro, causa importante de morbimortalidade entre os pacientes. Para superar essas limitações, uma alternativa é o transplante autólogo de células-tronco hematopoiéticas editadas, ou terapia gênica *ex vivo*. Com essa abordagem, as células do paciente são coletadas, editadas *in vitro* para superexpressarem a enzima deficiente e transplantadas de volta ao paciente. Nesse contexto, o risco de rejeição e de doença do enxerto contra o hospedeiro é virtualmente nulo e a terapia tem potencial de atuação no sistema nervoso central. Portanto, nesta parte, são exploradas possíveis terapias que as ferramentas de edição podem criar para as mucopolissacaridoses, bem como o desenvolvimento e otimização de uma terapia baseada em transplante autólogo de células-tronco hematopoiéticas editadas.

O capítulo IV desta tese é composto do artigo de revisão publicado: Poletto E, Baldo G, Gomez-Ospina N. ***Genome Editing for Mucopolysaccharidoses***. *International Journal of Molecular Sciences*. 2020; 21(2):500, que apresenta as abordagens utilizadas atualmente para terapia gênica das mucopolissacaridoses utilizando ferramentas de edição genômica. Dentre os estudos revisados, apresentamos ensaios pré-clínicos com CRISPR/Cas9 utilizando vetores não-virais e administração *in vivo*, abordagens *ex vivo* utilizando células-tronco hematopoiéticas e, por fim, ensaios clínicos conduzidos em pacientes com MPS I e MPS II utilizando vetores virais *in vivo* para entrega de componentes de edição por nucleases dedo de zinco.

O capítulo V consiste no manuscrito em preparação intitulado "***Improved therapeutic efficacy in the brain by human genome edited hematopoietic stem cells with busulfan-based myeloablation*****"**, que será submetido à revista científica *Nature Communications.* Neste manuscrito, apresentamos um estudo pré-clínico de terapia gênica *ex vivo* de-

senvolvido utilizando transplante de células-tronco hematopoiéticas editadas por CRISPR/Cas9 em camundongos adultos MPS I pré-condicionados com bussulfano. Neste estudo, células-tronco hematopoiéticas foram editadas para superexpressarem a enzima IDUA. A manipulação das células não influenciou seu potencial de enxertia ou de diferenciação, sendo observado altos níveis de quimerismo humano em camundongos transplantados. Ademais, os animais tiveram melhora significativa em todos os parâmetros bioquímicos avaliados, inclusive no sistema nervoso central. Demonstramos por PCR digital que há migração de células humanas para o córtex de animais transplantados. Por fim, a inclusão de um marcador de seleção clinicamente relevante impacta negativamente na eficácia geral do tratamento. Com esses dados, sugerimos que esta terapia é potencialmente eficaz e segura para ser traduzida aos pacientes.

O último capítulo, capítulo VI, é composto do manuscrito "***Biodistribution of transplanted hematopoietic precursor cells injected through different administration routes in newborn mice*",** publicado na revista *Human Gene Therapy*. Este trabalho aborda a utilização do transplante de células hematopoiéticas em camundongos neonatos, avaliando sua distribuição quando transplantadas por vias de administração distintas. Visto que pacientes com MPS I devem ser tratados o quanto antes for possível, preferencialmente no período neonatal, objetivamos desenvolver um modelo pré-clínico para estudos futuros. Baseado na literatura ausente no tema, comparamos a distribuição de células hematopoiéticas murinas transplantadas pelas rotas intravenosa via seio venoso retro-orbital, intravenosa via veia temporal e intraperitoneal. Avaliamos a presença de células transplantadas GFP+ por imunohistoquímica e por PCR quantitativo nos diferentes tecidos, incluindo cérebro, e a facilidade e sucesso das injeções, uma vez que o tamanho diminuto dos animais consiste num obstáculo importante para execução da técnica.

CAPÍTULO IV.

GENOME EDITING FOR MUCOPOLYSACCHARIDOSIS

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Review

Genome Editing for Mucopolysaccharidoses

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Abstract: Genome editing holds the promise of one-off and potentially curative therapies for many patients with genetic diseases. This is especially true for patients affected by mucopolysaccharidoses as the disease pathophysiology is amenable to correction using multiple approaches. Ex vivo and in vivo genome editing platforms have been tested primarily on MSPI and MPSII, with in vivo approaches having reached clinical testing in both diseases. Though we still await proof of efficacy in humans, the therapeutic tools established for these two diseases should pave the way for other mucopolysaccharidoses. Herein, we review the current preclinical and clinical development studies, using genome editing as a therapeutic approach for these diseases. The development of new genome editing platforms and the variety of genetic modifications possible with each tool provide potential applications of genome editing for mucopolysaccharidoses, which vastly exceed the potential of current approaches. We expect that in a not-so-distant future, more genome editing-based strategies will be established, and individual diseases will be treated through multiple approaches.

Keywords: genome editing; gene therapy; mucopolysaccharidoses; lysosomal storage disease; CRISPR/Cas9; Hurler; Hunter; Zinc Finger Nucleases; viral vectors; non-viral vectors; hematopoietic stem cell transplantation

1. Introduction: Therapeutic Principles in Mucopolysaccharidoses

The mucopolysaccharidoses (MPSs) are a group of genetic disorders caused by deficiencies in lysosomal enzymes, whose function is to degrade glycosaminoglycans (GAGs). Depending on the missing enzyme, the degradation of specific GAG species is blocked, generating a distinctive constellation of clinical symptoms, characteristic of each MPS form. Most MPSs are inherited as autosomal recessive disorders, except MPSII, which is X-linked [1].

Most MPSs, except MPSIIIC [2], result from deficiencies in soluble lysosomal enzymes that are secreted continuously into the extracellular space and blood, where they are taken up by adjacent cells and tissues. Consequently, cells with restored enzymatic capacity can "cross-correct" cells with the deficiency, a property known as cross-correction that forms the basis for most established and experimental therapies for MPSs [3]. This process of cross-correction explains why enzyme, when delivered systemically (such as in enzyme replacement therapy, or "ERT") can improve symptomatology in some organs. Furthermore, cross-correction predicts that endogenous enzyme depots that could persistently produce enzymes could be effective at treating multiple affected tissues. Accordingly, cross-correction also explains why hematopoietic stem cell transplantation has been successful in some MPS diseases and why most viral and genome editing-based approaches aim to create such enzyme depots by targeting different organ systems [4].

An important property of MPSs is the relatively low therapeutic threshold, which is an essential consideration when developing gene therapy/gene editing-based therapeutic approaches for these disorders. Phenotypic characterization from healthy individuals with partial enzymatic activity and patients with mild phenotypes inform the target levels of enzymatic correction necessary for symptomatic relief. Though specific for every MPSs, restoration of enzyme level to approximately 10% of normal can be sufficient in most MPSs to produce clear benefit [5].

This ability to cross-correct the phenotype by targeting specific cell populations and organs, along with the low therapeutic threshold, has spurred development of a variety of therapeutic approaches for MPSs, and support the idea that in vivo and ex vivo gene editing approaches can be effective in this group of diseases. Genome editing, unlike ERT, has the promise of providing a one-time, definitive therapy for various genetic diseases. Compared to non-integrating viral therapy such as adeno-associated viruses (AAV), genome editing ensures that modifications are cemented into the genome, without risk of dilution with organ growth. In addition, ex vivo genome editing could circumvent some of the potential immunological complications of AAV. Finally, compared to randomly integrating viruses, e.g., lentiviruses, genome editing provides more specific (and therefore, more predictable) genetic modifications, and maintains the ability to preserve endogenous regulation of the corrected gene if desired.

2. Gene Editing: The basics

In this section, we present a brief description of the currently available genome editing platforms with a focus on those being developed for MPS. The discovery, evolution, and more technical aspects of each platform are not discussed here, as these have been extensively reviewed. A more in-depth discussion of each tool and its potential uses in MPS is provided elsewhere [6].

2.1. Genome Editing Platforms

Different genome editing platforms have been developed in the last decade [7]. The most relevant for therapeutic purposes are based on programmable nucleases and include the clustered regularly interspaced short palindromic repeat (CRISPR)–Cas9 system, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the more recently described CRISPR-Cas9-based editors and prime editing (Figure 1). To date, only ZFNs and CRISPR–Cas9 have been used to develop therapies in MPSs beyond proof-of-concept studies and only ZFNs have reached the clinical stages of testing in human patients.

At the core of these nuclease-based editing technologies is the ability to create double-strand DNA breaks (DSB) at specific DNA sequences or genomic locations. Different platforms vary primarily in the mechanism by which the target DNA sequence is recognized (Figure 1). Engineered nucleases like ZFNs are customizable endonucleases consisting of an engineerable and sequence-specific DNA-binding domain, and the nuclease domain of a restriction enzyme, FokI [8] (Figure 1a). ZFNs require dimerization to create DSBs, resulting in laborious and expensive protein engineering for each potential target site, which has consequently prevented its widespread adoption in basic research and limited its development to a single commercial entity.

The field of genome editing made a significant advance in the earlies 2010s with the description of the bacterial Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated system (Cas), in which a nuclease (Cas9) is guided to the target DNA sequence using RNA [9]. To achieve DSB at specific locations in eukaryotic genomes, the platform was engineered as a simple two-component system encoding an RNA element (called guide RNA or gRNA) and a Cas9 nuclease that has been codon-optimized for expression in eukaryotic cells [10–13]. The most widely utilized Cas9 in basic research and therapeutics are derived from *Streptococcus pyogenes* and *Staphylococcus aureus* [14]. DNA target recognition requires both complementarity to a 20 bp sequence in the gRNA and the presence of an adjacent short sequence (i.e., protospacer adjacent motif or PAM) in the DNA

changes in the gRNA, a cheap and simple process that has driven the widespread adoption of this technology for basic research and therapeutic applications.

CRISPR-mediated base editing is a recent addition to the genome-editing toolkit. It does not rely on DSBs, even though it is based on the CRISPR/Cas9 system. This technology employs catalytically inactive Cas9 (not cut) or Cas9 nickase (cuts one of the two DNA strands) to target base-modifying enzymes, such as cytosine deaminase [15] or adenosine deaminase [16], to specific locations in the genome. Adenine and cytidine deaminases convert C·G to T·A base pairs, or vice versa, within a narrow window of the binding site (Figure 1d). This platform is, therefore, limited to pathogenic variants involving C or A residues in the vicinity of the PAM sequence required for Cas9 binding, so it is mutation-specific and not generalizable in diseases with many known causative mutations, such as MPSs. On the other hand, CRISPR-mediated base editing has the theoretical advantage of decreasing the probability of creating DSBs in unintended locations, commonly referred to as off-target sites.

The newest addition to the CRISPR tool kit is referred to as prime editing [17]. As with CRISPR-mediated base editing, prime editing does not rely on DSBs. Prime editors use a reverse transcriptase fused to a Cas9 nickase and a prime editing guide RNA (pegRNA) (Figure 1e). This pegRNA is a two-part RNA containing (a) a sequence complementary to the target site that directs Cas9 to its target sequence and (b) an additional sequence spelling the desired sequence changes. Once the RT-Cas9 protein is targeted to the genomic site and a nick in one of the DNA strands is created, the reverse transcriptase produces DNA complementary to the sequence in the pegRNA, which gets inserted at one of the cut ends and replaces the original DNA sequence. This technology has several advantages over the existing tools. Compared to the CRISPR-mediated base editing, prime editing can perform all transversion mutations (C→A, C→G, G→C, G→T, A→C, A→T, T→A, and T→G) as well as targeted deletions and insertions. Compared to tools that rely on DBSs, where NHEJ and HDR are competing repair processes resulting in varied outcomes, the editing outcomes are more precise and efficient, as they do not rely on exogenous donor DNA repair templates. In the absence of DSBs, this tool is potentially less genotoxic. Prime editing is predicted to correct up to 89% of known genetic variants associated with human diseases [17] though its specificity and potential for off-target modifications remains to be studied.

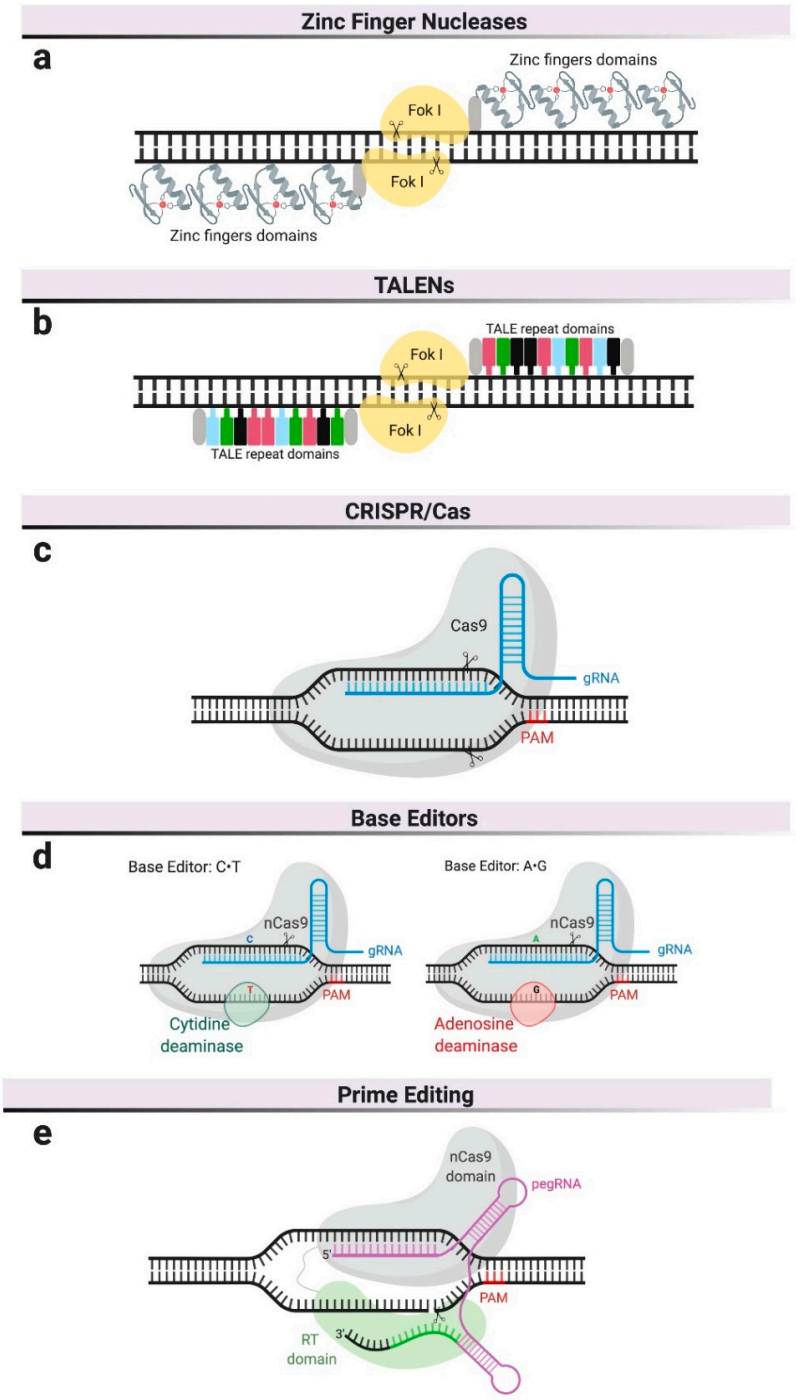

Figure 1. Genome editing platforms. (**a**) Zinc finger nucleases—zinc finger domains are fused to the restriction endonuclease, FokI, and require dimerization. (**b**) Transcription activator-like effector nucleases (TALENs) also use FokI as endonuclease but their DNA-binding domain is composed of repeats derived from transcription activator-like effectors (TALEs). (**c**) Clustered, regularly interspaced, short palindromic repeat (CRISPR)/Cas9 system uses a guide RNA (gRNA) to recognize specific DNA sequences and a Cas9 nuclease to cleave both DNA strands. DNA cleavage only occurs if the gRNA is adjacent to a Protospacer Adjacent Motif (PAM). (**d**) Base editors use inactive Cas9 or Cas9 nickase (nCas9), complexed with base-modifying enzymes and a gRNA. (**e**) Prime editors use a Cas9 nickase fused to reverse transcriptase and are complexed with pegRNA, which serves as gRNA and as template for reverse transcription.

2.2. Multiple Genetic Modifications and Their Therapeutic Applications

Once introduced into the cell, the ZNFs and Cas9/gRNA complexes translocate to the nucleus and cleave DNA at the intended sequences, generating a DSB, which triggers DSB-break repair mechanisms, primarily non-homologous end joining (NHEJ) or homologous recombination (HR) (Figure 2). NHEJ can result in imprecise repair, leading to small deletions or insertions (indels) at the break site (Figure 2). The therapeutic application of NHEJ-based genome editing is limited, particularly in diseases resulting from loss-of-function alleles and in which many pathogenic mutations have been reported, as in the MPSs disorders. Most commonly, NHEJ is used for the disruption of coding or regulatory sequences (Figure 2). Notably, this approach has reached clinical testing for hemoglobinopathies, such as sickle cell disease and beta-thalassemia, in which NHEJ-based genome editing is used to disrupt a regulatory sequence, to selectively turn off the expression of a repressor. This increases production of an alternative form of hemoglobin (fetal hemoglobin), which can ameliorate the phenotype [18]. In very specific circumstances, NHEJ can be used to create insertions or deletions of 1, 2, or 3 nucleotides that can restore the reading frame in a mutant gene (Figure 2). The efficacy of this approach depends on the frequency of the intended indel, compared to other potential indels and has been primarily aimed at targeting specific mutations in Duchenne muscular dystrophy [19,20], but not yet in MPSs.

In addition to NHEJ, repair of the DSB can be achieved by homology-directed repair (HDR). This type of repair is favored when the cell is supplied with an exogenous template containing the intended sequence changes and homology around the cut site (Figure 2). HDR allows for precise genetic changes with therapeutic potential, depending on the design of the exogenous homologous template. A common use of HDR is for single nucleotide variant (SNV) correction. This approach is particularly relevant in diseases with a common pathogenic mutation, and it is the most efficient HDR-based mechanism [21,22]. HDR can also be used to insert entire coding sequences under the control of endogenous promoters, thereby providing a single platform for all pathogenic variants. Coding sequences can also be integrated under alternate regulatory regions or under exogenous promoters, allowing for modifications to the temporal and spatial patterns of expression that might add therapeutic value [23]—an approach that is usually referred to as a "safe harbor" approach. Another therapeutic use of HDR is to perform partial cDNA insertions containing an abbreviated functional protein, or coding regions with mutational hot spots, a strategy that can be used in cases where the cDNA is long and full replacement is not feasible.

For most therapeutic applications, the desired outcome is for repair to be directed by a template DNA, resulting in precise edits. For genome editing that relies on DSBs (not CRISPR-mediated base editing or prime editing), outcomes of genome editing can have multiple byproducts, resulting in a mixture of NHEJ and HDR. In most cells, these processes are competing and NHEJ is generally the most efficient. This observation has been explained, at least in part, by the activation of DNA-damage responses such as the p53 activation resulting in cell cycle arrest or even apoptosis [24,25]. Much effort over the past few years has focused on shifting this balance from NHEJ to HDR [26–34]. Alternative strategies to achieve precise and efficient editing have relied on moving away from DSBs by using catalytically inactive Cas9 or Cas9 nickase, as in CRISPR-mediated base editing or prime editing. Both of these tools have a great therapeutic potential, but proof-of-concept studies of efficacy and safety are still lacking, particularly in MPS diseases.

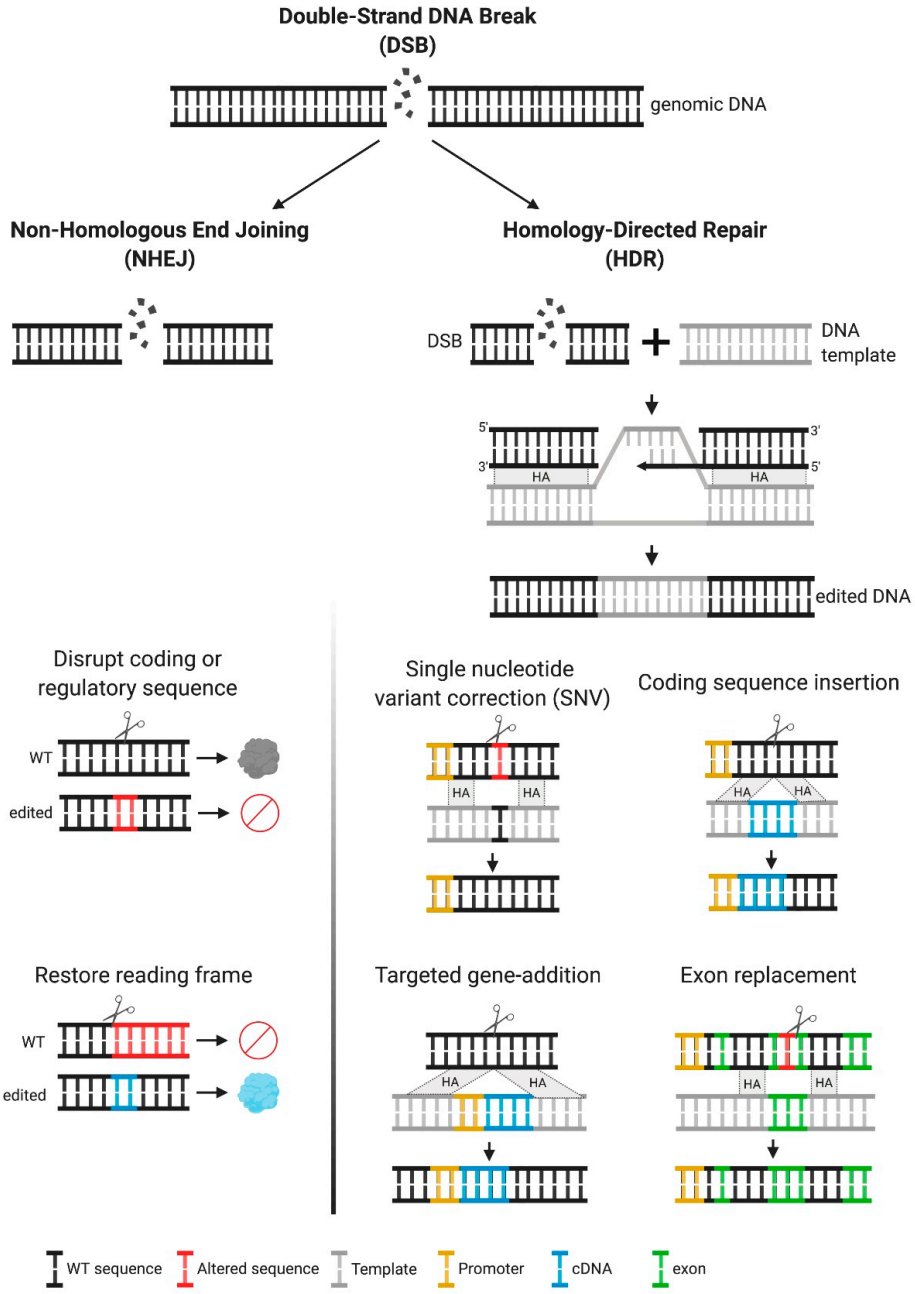

Figure 2. Multiple genetic modifications and their therapeutic applications. Upon a double-strand break (DSB), DNA can be repaired by two mechanisms, non-homologous end joining (NHEJ) or homology directed repair (HDR). The first mechanism frequently results in insertions or deletions (indels). Inducing indels can be used for disruption of coding sequences or to restore the reading frame, for frameshift mutations. For HDR to occur, a DNA template containing the desired modification, with homology arms flanking the target site is required. This approach is recommended when specific DNA modifications are intended, as this is an error-free repair mechanism. HDR can result in the following modifications, depending on the template used—single nucleotide variant (SNV), insertion of coding sequences under their own endogenous promoter control or alternative endogenous promoters, addition of genes (promoter + cDNA) in safe harbor loci, and replacement of partial coding sequences (one or multiple exons).

2.3. Delivery Platforms: Ex Vivo vs. In Vivo Genome Editing

There are two primary approaches for targeting the genome editing components to the intended cells or tissues. In vivo approaches deliver the genome editing tools directly in the live organism, while in ex vivo delivery cells from the patient, suitable donor, or cell bank are modified outside of the body (Figure 3). With the in vivo approach, the correction of specific cells in the relevant organs depends highly on the tropism of the delivery vector used, the route of administration, and the physical as well as genomic accessibility of the target organ for genome editing. In the ex vivo approach, target cells with a regenerative potential must be isolated or be available for transplantation. Upon transplantation, these corrected cells could replace organs or migrate to the affected tissues (such as the brain). The choice of approach generally depends on the target organ. Advantages of the ex vivo approach include the control over which cells are targeted and the ability to fully characterize the editing outcomes, both intended (on-target) and unintended (off-target), in the targeted population. This approach has been extensively used in the hematopoietic system, where the isolation, culture, and transplantation of these cells is now routine [35,36]. However, not all organ functions can be replaced by transplantation of genome edited cells modified ex vivo. In these organs, e.g., the musculoskeletal and central nervous systems, an in vivo approach can be more effective.

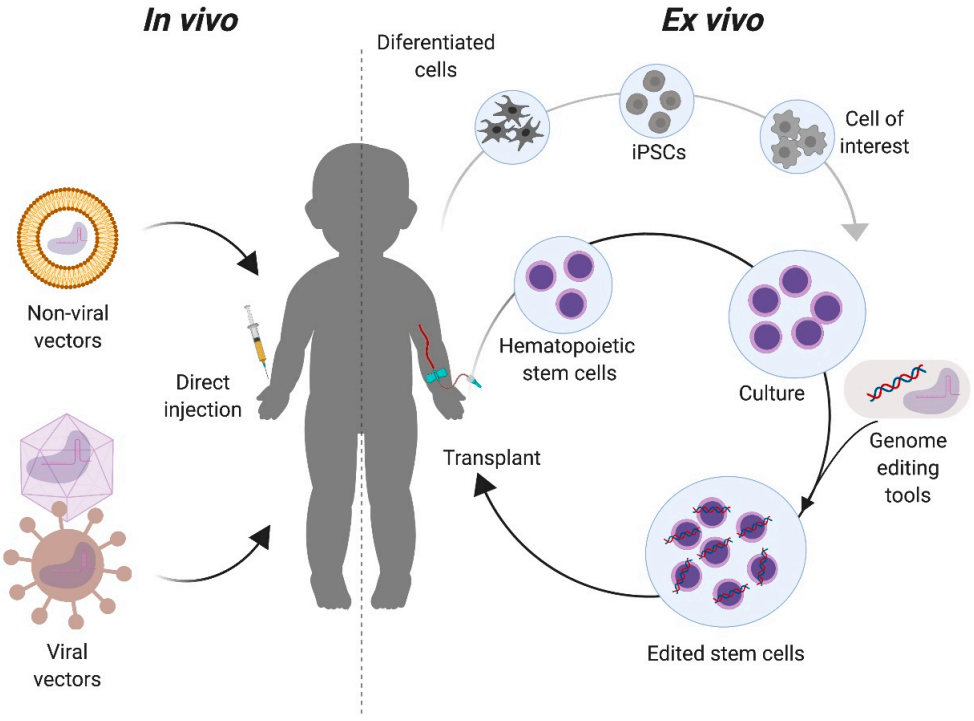

Figure 3. In vivo and ex vivo approaches for genome editing of Mucopolysaccharidoses. For in vivo strategies, the components required for the genome editing are complexed to delivery vectors that will be directly injected to the patient. These vectors can be non-viral, such as liposomes, or viral, such as adeno-associated virus. For ex vivo, cells with a regenerative potential, such as tissue stem cells (e.g., hematopoietic stem cells) or induced pluripotent stem cells (iPSCs) are collected from the patient and modified with the genome editing tools in vitro. Modified cells are then transplanted back to the patient for either autologous or allogeneic transplantation, depending on the cell source.

3. Genome Editing vs. Other Therapeutic Approaches in MPS Disorders

3.1. Enzyme Replacement Therapy (ERT)

Early studies in cultured skin fibroblasts derived from MPS patients, serendipitously showed that a mixture of fibroblasts derived from patients with MPSI (Hurler syndrome) and MPSII (Hunter

syndrome) had normal glycosaminoglycan metabolism, suggesting that cells with different lysosomal deficiencies could "cross-correct" each other [3]. Subsequent studies elucidated that lysosomal enzymes were secreted and could be internalized into cells via mannose-6-phosphate (M6P) receptors [3,37]. Based on these pivotal findings, recombinant lysosomal enzymes containing the M6P signal have been developed as therapies for MPSI [38], MPSII [39], MPSIVA [40], MPSVI [41,42], and MPSVII [43].

In MPS diseases, ERT has significant limitations in efficacy. Current formulations involve the intravenous administration of recombinant enzyme, most of which ends up in the reticuloendothelial cells of the liver and spleen, limiting its uptake in the affected tissues [44]. Specifically, intravenously-delivered ERT is mostly ineffective against central nervous system (CNS) manifestations (as it does not cross the blood–brain barrier) and in connective tissues such as cartilage and bone (due to poor vascularization). In addition, immune reactions to the recombinant enzyme and the development of neutralizing antibodies can decrease bioavailability [45]. Approaches to improve tissue targeting by engineering enzymes to cross the blood–brain barrier [46,47] and through alternative routes of administration (intrathecal or intracerebroventricular) [48] have been under development, in order to improve its efficacy, particularly in the CNS. Despite these improvements, ERT is an onerous therapy for patients as it requires life-long weekly or bi-weekly infusions, and is expensive for healthcare systems [49].

Unlike ERT, genome editing promises a one-time therapy for MPS and depending on the delivery platform (in vivo or ex vivo), it has the potential to treat organs such as the brain and bone. Similar to the ERT, challenges remain for efficiently targeting the genome editing components to the affected organs and for potential immune reactions to the expressed enzyme in the edited tissues, particularly in patients with null alleles [50].

3.2. Substrate Reduction Therapy

An alternative approach to ERT is substrate reduction therapy (SRT), a strategy that uses small molecule inhibitors to reduce the synthesis of stored metabolites [51].The most successful agents in this class are Miglustat and Eliglustat tartrate, both glucosylceramide synthase inhibitors that are effective at ameliorating disease manifestations in Gaucher disease type 1, a common lysosomal storage disease [52,53]. For MPS, the ability of SRT to provide therapeutic benefit remains unknown. Miglustat did not reduce ganglioside levels and failed to improve or stabilize behavior in a randomized trial in MPS III [54]. Non-specific inhibitors such as rhodamine B and genistein could reduce lysosomal storage in MPS mouse models [55,56], but failed to show any clinical effect [57]. Even if successful, SRT will need to be chronically administered, which is a disadvantage compared to one-time approaches such as gene therapy or genome editing. Compared to gene replacement strategies, it might be less likely to elicit immunological complications but side effects due to non-specific inhibition could be considerable [58].

3.3. In Vivo Gene Therapy with Adeno-Associated Viruses (AAV)

Adeno-associated viruses (AAVs) are viruses that commonly infect humans and appear to lack significant pathogenicity [59]. Gene therapy vectors using AAV can transduce dividing and non-dividing cells, and persist mostly in an extrachromosomal state without integrating into the genome, making them attractive vectors for gene delivery [60]. These vectors are typically delivered in vivo where the targeting to the affected organs is achieved by choosing serotypes with the appropriate tropism. In vivo gene therapy with AAV for MPS is currently being tested in clinical trials for MPSI (NCT03580083), MPSII (NCT03566043), MPSIIIA (NCT02053064), MPSIIIB (NCT03315182), and MPSVI (NCT03173521). As AAV rarely integrates into the genome, this approach is best at targeting organs that are not undergoing expansion through cell division such as the central nervous system or adult tissues where cell division is not expected to dilute the therapeutic gene. This is quite different from genome editing, where modifications are cemented into the genome, without risk of dilution with

organ growth. Like all gene replacement strategies, AAV-mediated gene therapy and genome editing have the potential to elicit immune reactions to the delivery vectors or the transgenes.

3.4. Allogeneic Hematopoietic Stem Cell Transplantation

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) replaces enzyme-deficient bone marrow cells with donor-derived enzyme-competent cells. Compared to ERT, allo-HSCT results in better outcomes by providing an endogenous source of enzyme in the vasculature, and by replacing the phagocytic cells of the monocyte macrophage lineage (osteoclasts, macrophages, and histiocytes) in the affected organs [61–63]. It has been most successfully used to treat severe MPSI (Hurler) where it prolongs survival, delays cognitive decline, and leads to improvements in hepatosplenomegaly, airway disease, and hearing loss [63–65]. However, allo-HSCT has not demonstrated significant benefit on musculoskeletal involvement, growth, or valvular disease [66]. Other caveats of allo-HSCT include finding compatible donors (which delays treatment) and the morbidity resulting from conditioning, graft versus host disease, and immunosuppression. Given the significant morbidity and mortality of the procedure, allo-HSCT has been limited to patients with severe MPSI, before age 2, and with normal cognitive function at the time of evaluation [67]. In addition to MPSI, allo-HSCT has resulted in positive clinical outcomes in patients with MPSIV, MPSVI, and MPSVII, but compared to other available therapies, the risks have outweighed the benefits [40,68,69]. Allo-HSCT was not found to prevent neurological decline in patients with severe MPSII, and MPSIII, where it might actually worsen symptoms [70,71].

3.5. Ex Vivo Lentiviral Modification of Hematopoietic Stem and Progenitor Cells

Given the potential benefit of allo-HSCT in some types of MPS, ex vivo modification of the patient's own hematopoietic stem cells using integrating viruses or genome editing, to establish autologous transplantation, are being pursued. Such approaches have several advantages over ERT, SRT, and allo-HSCT—(1) achieving higher levels of enzyme expression, (2) eliminating the morbidity of graft rejection, graft-versus-host disease, and immunosuppression, and (3) earlier intervention.

To establish autologous HSCT in MPS, genetic modification of the patient's cells is needed to correct the biochemical defect and restore enzyme activity. One way to accomplish this is to use integrating viruses [72]. Proof of the therapeutic potential of this strategy for MPS has been accomplished with lentiviral vectors. Specifically, for MPSI, preclinical studies in mouse models demonstrated improved efficacy, compared to transplantation using unmodified cells expressing endogenous levels of the enzyme [73,74]. This strategy is currently being tested in patients with severe MPSI (NCT03488394) and preliminary data from lentiviral delivery in HSPCs in humans, suggesting that this approach is effective in the CNS [75].

Delivery of lysosomal enzyme using lentiviruses constitutes an "untargeted" gene addition approach. Integrations into the genome are random or semi-random, raising concerns about the potential for tumorigenesis [76]. Furthermore, because of the variability in the location and number of integration sites in different cells, untargeted gene addition can result in heterogenous expression of the enzyme. As genome editing allows for precise, more targeted genetic modifications, it can result in more predictable transgene expression and theoretically less chance of insertional mutagenesis. Unlike gene addition using lentiviruses, genome editing provides the opportunity to modify the enzyme locus to preserve endogenous regulation of the corrected gene if desired, or to combine gene addition with precise knockout of other genes, to enhance efficacy.

4. From Proof of Concept Studies in Animal Models to Clinical Trials

For a complete summary of all preclinical and clinical studies using genome editing for MPSs, see Table 1.

4.1. In Vivo Approaches

The first studies describing in vivo genome editing for MPSs used the ZFN platform. An initial study reported a general approach for in vivo protein production, using ZFNs [23]. In this approach, the authors tried to circumvent the low efficiency of in vivo genome editing by integrating into a locus with high transcriptional activity. For this, they designed ZFNs targeting the albumin locus, using it as a safe harbor site. The genome-editing components were delivered in vivo via intravenous injection of adeno-associated virus 8 (AAV8). Using this method, they were able to produce supraphysiologic levels of several proteins, including alfa-galactosidase A (the enzyme deficient in Fabry disease), factor IX (hemophilia), and alpha-L-iduronidase (or IDUA, the enzyme deficient in MPSI).

Studies were then conducted in the MPSII and MPSI mouse models by using the same approach for the targeted insertion of the respective enzymes, Iduronate-2-sulfatase (IDS) and IDUA, into the albumin locus. For MPSII, three dose levels of recombinant AAV2/8 were tested, ranging from 2.5×10^{11} to 1.2×10^{12} viral genomes per mouse. Plasma IDS activity increased in a dose-dependent manner, with supraphysiological levels being observed in the higher-dose group (up to 200-fold normal). The enzyme produced was captured by other tissues (including the spleen, the heart, and the lungs) and successfully normalized the GAG levels. At a higher dose, an increase in brain IDS activity was observed, but it was not enough to reduce the GAG levels in this organ, despite improvements in behavioral parameters [77]. In MPSI mice, a dose of 1.5×10^{11} vector genomes (vg) of AAV2/8 with the ZFN and 2×10^{12} vg of an AAV2/8 with the hIDUA construct were administered intravenously in young mice (from 4 to 10 weeks of age). As observed in the MPSII mice, serum IDUA levels were increased to 9-fold normal and remained steady for up to 4 months. Tissue enzyme activity was significantly increased, and the GAG levels were normalized in all analyzed tissues, except for the brain, despite behavioral improvements [78].

These promising results in animal models suggested that this approach could be effective in humans, and the strategy is being tested in two clinical trials in MPSI (ClinicalTrials.gov, NCT02702115) and MPSII (NCT03041324). It is important to note that both ZFN products are intended only for patients with mild forms of the diseases who have little or no CNS involvement, as the enzymes produced from the liver are not expected to cross the blood–brain barrier. CHAMPIONS, the first trial to attempt in vivo genome editing in humans, is an ongoing Phase 1/2 clinical trial to determine if dose escalation of ZFNs is safe and tolerable in patients with MPSII. The trials aim to test four different cohorts with ascending doses of the genome editing components (ZFN1, ZFN2, and IDS donor). Interim analysis of the clinical data for the first three cohorts showed no serious adverse effects related to the drug, and other adverse events were mild or moderate and were eventually resolved. An RT-qPCR assay was able to detect the integration in the mid-dose cohort, but a genomic-based test failed. No measurable increases in plasma IDS were detected (except in one patient with transaminitis). Patients were maintained on their ERT therapy during the initial studies. Additional clinical data is being collected after withdrawal of ERT. However, 1 subject in cohort 2 was planning to restart ERT after approximately 3 months, due to fatigue and concurrent increase in GAGs [79,80]. EMPOWERS is an ongoing phase 1/2 clinical trial of ZFNs for MPSI (NCT02702115). It aims to test three different cohorts with ascending doses of the genome editing components (ZFN1, ZFN2, and IDUA donor). Interim analysis of the first three subjects was reported [81]. The preliminary results suggest that the treatment is safe although its efficacy remains to be proven. Plasma IDUA activity was measured but has not significantly changed from pre-treatment values. Data collection is ongoing.

CRISPR-Cas9 has also been used for preclinical studies in mice for in vivo genome editing in MPSI. The correction strategy was based on preliminary studies in human MPSI fibroblasts, where an HDR-based SNV correction approach was implanted using a liposome as vector, plasmids encoded the Cas9 nuclease and the gRNA, and a donor repair template aimed to correct one of the most common mutations found in MPSI patients with severe disease (p.Trp402Ter) [82,83]. To demonstrate efficacy in an MPSI mouse model, an HDR-based safe harbor approach was used by inserting the IDUA cDNA into the ROSA26 locus. Mice were treated with a single injection of liposome-complexed

plasmids at 2–3 days of age. IDUA production was increased, compared to the untreated controls, with serum IDUA levels reaching 5–7% of wild-type mice for up to 6 months [84]. IDUA tissue levels were increased in all analyzed organs, except for the brain, followed by a similar pattern of reduction in the GAG levels. Despite low serum levels, the treated MPSI mice showed normalization of the cardiorespiratory function, which is the leading cause of death in patients. Some organs, such as the bones and the aorta, had partial improvements, with reduction in femur thickness and in elastin breaks. Organs traditionally known to be hard to correct, such as the heart valves and the brain, showed no improvement after therapy [85]. An additional study used CRISPR-Cas9 in vivo to target the common p.Trp402Ter mutation in a compound heterozygous mouse model of MPSI [86]. The strategy was shown to be partly efficacious in post-mitotic tissues like the heart.

4.2. Ex Vivo Approaches

Ideal candidate cells for ex vivo modification are tissue-specific stem cells. Among these, hematopoietic stem and progenitor cells have been heavily studied as clinicians and researchers have extensive experience with their isolation, ex vivo manipulation, and transplantation [35,36]. Furthermore, hematopoietic stem cell transplantation (HSCT) in the allogenic setting has been shown to be a feasible enzyme reservoir in several metabolic disorders and MPSs [87–89]. Specifically, for MPSI, allogeneic HSCT has been shown to expand life expectancy and halt neurological decline. It is also the standard of care for patients under two years of age, who have a severe form of the disease and show a normal developmental quotient at the time of the evaluation [67]. Accordingly, the first genome editing ex vivo approach using hematopoietic stem cells was first established for MPSI. The goal of this approach is to establish autologous transplantation of genetically corrected cells by targeting the patient's own hematopoietic stem cells and engineering them to produce high levels of the needed enzyme [90] (Figure 4). Compared to allogeneic HSCT, this overall strategy achieves higher levels of enzyme expression, eliminates the morbidity of graft-versus-host disease and immunosuppression, and can lead to earlier intervention by obviating the need for donor matching. Compared to ERT and the ZFN approaches described earlier, it provides enzymatic correction in the CNS.

In these studies, human hematopoietic stem and progenitor cells (HSPCs) were targeted using CRISPR-Cas9 to insert an IDUA expression cassette into the safe harbor locus, *CCR5* (Figure 4). The safe harbor allowed for expression of the enzyme outside the restrictions of the endogenous locus, as the goal was to engineer cells for supra-endogenous expression, which had been previously shown to enhance therapeutic potency [74]. A safe harbor also establishes a universal corrective approach for all patients with MPSI, as it circumvents the design for specific patient mutations. Finally, because the targeted locus does not change, the same genome-editing tools can be easily adapted to express other lysosomal enzymes, as there is no additional optimization of the gRNA and the donor repair template. To enhance the genome editing efficiency in human HSPCs, the researchers used gRNAs that were chemically modified with 2′-*O*-methyl 3′phosphorothioate [91] and *Streptococcus pyogenes* Cas9 protein complexed with the gRNA (RNP) and delivered into the cells by electroporation. The donor template for repair was delivered via adeno-associated virus 6 (AAV6) (Figure 4). When inserting an expression cassette where the IDUA expression was driven by the phosphoglycerate kinase (PGK) promoter, human HSPCs and the HSPCs-derived macrophages expressed 25-to-50-fold more IDUA than the unmodified cells. Serial transplantation studies showed that HSPCs modified in this manner retained the long-term repopulation and multilineage differentiation potential, confirming that this strategy can modify long-term stem cells and could constitute a one-time therapy for the disease. The efficacy of the edited HSPCs was examined in a model of MPSI capable of human cell engraftment. Transplantation of the edited cells led to a reconstitution of enzyme activity in serum, liver, spleen, and brain. GAG storage was also decreased in serum, liver, spleen, but not in brain. The transplanted mice demonstrated improvement in the bone pathology, neurobehavior (ambulation, short-term memory, and anxiety), and neuroinflammation. Together, this work provided specific evidence of safety and

efficacy to support the optimization and development of this strategy into a clinical protocol to treat patients with MPSI and a platform approach to potentially treat other lysosomal storage disorders.

Other cell sources are beginning to be investigated. Induced pluripotent stem cells (iPSCs) are pluripotent stem cells generated directly from somatic tissues of patients and have the capacity to give rise to various cell types in the body (neurons, cardiomyocytes, immune cells, hepatocytes, skeletal muscle, etc.). These cells have been targeted in vitro in cellular models of MPSI, though efficacy studies are still lacking [92].

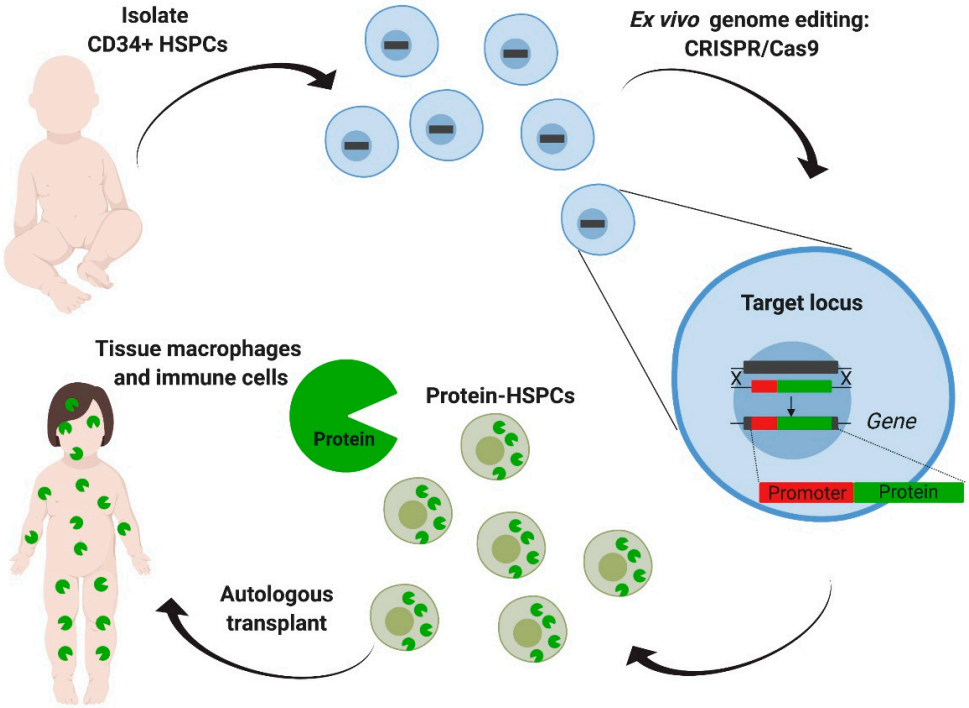

Figure 4. Ex vivo genome editing of hematopoietic stem cells targeting a safe harbor locus. Hematopoietic stem and progenitors cells (HSPCs) derived from the patient are targeted with CRISPR/Cas9 aiming the addition of the deficient enzyme's coding sequence, driven by an exogenous constitutive promoter in the *CCR5* safe harbor locus. After transplantation, modified HSPCs will eventually engraft, reconstitute the hematopoietic system, and generate tissue macrophages and other immune cells that can produce and deliver the enzyme. This strategy eliminates the need for donor matching and decreases the risk of graft-versus-host disease. Furthermore, a safe harbor extends its applicability to other lysosomal diseases.

Table 1. Genome editing studies for Mucopolysaccharidoses.

Pre-Clinical Studies in Cell Models							
Disease	**Affected Gene**	**Targeted Gene**	**Platform**	**Cell Type**	**Delivery Method**	**Genetic Modification**	**Reference**
MPS I	*IDUA*	*IDUA*	CRISPR/Cas9	Human fibroblasts	Plasmid-Liposome complex	SNV correction	[82]
MPS I	*IDUA*	*IDUA*	CRISPR/Cas9	Human fibroblasts	Plasmid-Liposome complex	SNV correction	[93]
MPS I	*IDUA*	*IDUA*	CRISPR/Cas9	mouse iPSCs	Plasmid-Liposome complex	Precise deletion	[92]

Pre-Clinical Studies in Murine Models							
Disease	**Affected Gene**	**Targeted Gene**	**Platform**	**In Vivo Vs. Ex Vivo**	**Cargo and Vehicle**	**Genetic Modification**	**Reference**
MPS I	*IDUA*	*CCR5*	CRISPR/Cas9	ex vivo	RNP/AAV6	Gene addition	[90]
MPS I	*IDUA*	*ROSA26*	CRISPR/Cas9	in vivo	Liposome and plasmid vectors, IV	Gene addition	[84]
MPS I	*IDUA*	*IDUA*	CRISPR/Cas9	in vivo	2 AAV9 vectors, IV	SNV correction	[86]
MPS I	*IDUA*	*ALB*	ZFNs	in vivo	3 AAV2/8 vectors, IV	Gene addition	[78]
MPS II	*IDS*	*ALB*	ZFNs	in vivo	3 AAV2/8 vectors, IV	Gene addition	[77]

Clinical Trials									
Disease	**Affected Gene**	**Targeted Gene**	**Platform**	**In Vivo vs. Ex Vivo**	**Cargo and Vehicle**	**Genetic Modification**	**Company**	**Trial Name**	**Clinicaltrials.gov Identifier**
MPS I	*IDUA*	*CCR5*	CRISPR/Cas9	ex vivo	RNP/AAV6	Gene addition	Stanford University	in the pipeline	
MPS I	*IDUA*	*ALB*	ZFNs	in vivo	3 AAV2/6 vectors, IV	Gene addition	Sangamo therapeutics	SB-318	NCT02702115
MPS II	*IDS*	*ALB*	ZFNs	in vivo	3 AAV2/6 vectors, IV	Gene addition	Sangamo therapeutics	SB-913	NCT3041324

IV: intravenous

5. Challenges to the Clinical Adaptation of Genome Editing in MPSs

Many potential challenges still exist in the future application of therapeutic genome editing for MPSs. Within the preclinical stages of therapy development, appropriate assessment of efficacy and safety remains a challenge. Efficacy must be examined within the parameters of the intended therapeutic threshold in humans, as this might differ from current animal models, which is particularly important in MPSs, where murine models appear to have a lower threshold for biochemical correction and have a more permissible bone and CNS pathology. Not surprisingly, approaches that were shown to be effective in these models have failed to show results in humans. Challenges are specific to the delivery platform. For in vivo approaches, efficacy can be limited by the biodistribution of the available vectors and whether the vector biodistribution would be replicated in humans. Furthermore, genetic correction strategies that rely on HDR are less likely to work in post-mitotic tissues such as neurons. For ex vivo approaches, intrinsic challenges lie in the ability to find cells with a regenerative potential to target disease pathology. While this has been easy to conceive in the hematopoietic system, it has more challenging to achieve in the musculoskeletal and central nervous system.

Perhaps more importantly, there is a significant challenge in assessing the safety of therapeutic genome editing before translation to humans. Several aspects of safety that need to be evaluated, include specificity, tumorigenicity, and immunogenicity. A lingering concern of genome-editing technologies is their potential to create modifications at unintended genomic sites that could ultimately result in tumorigenicity. Many methods have been described to assess, in an unbiased manner the frequency of off-target modification and potential chromosomal abnormalities induced during the genome-editing process [94–102]. While studies have shown that the use of short-lived Cas9 (as protein) and mutant Cas9 with improved fidelity can significantly decrease and sometimes abrogate this off-target problem [90,103–105], there is still no gold-standard test or threshold. Ultimate assessment of tumorigenicity relies on pathological studies in animal models, but whether these studies accurately predict tumorigenicity is debated. Immunogenicity of the delivery vectors and Cas9 is another critical concern, particularly in the in vivo setting. Currently, most in vivo approaches rely on AAV for delivery of the genome editing tools. Specifically, AAV-neutralizing antibodies can reduce AAV-transduction, while $CD8^+$ T cells directed to AAV capsid antigens can cause rejection of the AAV-transduced cells [59]. Similarly, preexisting antibodies and primed cytotoxic T cells have been found in healthy human donors to the *S. aureus* and *S. pyogenic* Cas9 [106,107]. For ex vivo approaches, immunological challenges lie in the indissoluble relationship between the origin of the cells and the organism. The therapeutic potential of edited human cells, the ultimate intended target, can only be faithfully assessed in the context of a human organism and immune system.

6. Conclusions and Future Directions

Gene editing holds the promise for precise, definitive, and sometimes curative therapies for patients affected with genetic diseases. This is especially true for patients affected by mucopolysaccharidoses where disease pathophysiology is highly amenable to correction. Preclinical studies have shown efficacy of in vivo and ex vivo approaches with different genome editing platforms. Though still not shown to be effective in humans, the fact that in vivo genome editing was first-in-humans for MPSs diseases is highly encouraging. While ex vivo modification of hematopoietic stem cells is still in preclinical stages, the strategy is based on 30 years of clinical and research experience in MPSI, supporting its potential use in treating CNS and skeletal symptoms in other MPSs disease. Autologous transplantation of genetically engineered cells will be safer than the current allogeneic option, and with additional engineering, its therapeutic potential could be enhanced so as to be a viable alternative for MPS patients who are not routinely considered to be transplantable. Challenges remain regarding the safety and efficacy of these tools for clinical translation. Many are not specific to genome editing, so the concurrent advancement of viral-based therapies will improve the therapeutic application of genome editing as well.

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CAPÍTULO V.

IMPROVED THERAPEUTIC EFFICACY IN THE BRAIN BY HUMAN GENOME EDITED HEMATOPOIETIC STEM CELLS WITH BUSULFAN-BASED MYELOABLATION

CAPÍTULO VI.

BIODISTRIBUTION OF TRANSPLANTED HEMATOPOIETIC PRECURSOR CELLS INJECTED THROUGH DIFFERENT ADMINISTRATION ROUTES IN NEWBORN MICE

HGT

Biodistribution of Transplanted Hematopoietic Precursor Cells Injected Through Different Administration Routes in Newborn Mice

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Hematopoietic stem cell transplantation has been studied for several decades now, mostly as a treatment for malignancies and hematological diseases but also for genetic metabolic disorders. Since many diseases that could be potentially treated with this approach develop early in life, studies of cell transplantation in newborn mice are needed, especially for gene therapy protocols. However, the small size of pups restricts the possibilities for routes of administration, and those available are normally technically challenging. Our goal was to test different routes of administration of Lin^- cells in 2-day-old mice: intraperitoneal, intravenous through temporal vein (TV), and intravenous through retro-orbital (RO) sinus. Routes were evaluated by their easiness of execution and their influence in the biodistribution of cells in the short (48 h) and medium (30 days) term. In either 48 h or 30 days, all three routes presented similar results, with cells going mostly to bone marrow, liver, and spleen in roughly the same number. RO injection resulted in quick distribution of cells to the brain, suggesting better performance than the others. Rate of failure was higher for the TV route, which was also the hardest to execute, whereas the other two were considered easier. In conclusion, TV was the hardest to perform and all routes seemed to demonstrate similar results for cell biodistribution. In particular, the RO injection results in quicker biodistribution of cells to the brain, which is particularly important in the study of genetic metabolic disorders with a neurological component.

Keywords: administration routes, hematopoietic stem cells, intraperitoneal, retro-orbital, temporal vein, newborn

INTRODUCTION

HEMATOPOIETIC STEM CELL TRANSPLANTATION (HSCT) has been very effective for several hematological and metabolic conditions, including those with neurological impairment. Owing to the capacity of cells to migrate throughout the body and, under certain conditions, to reach the central nervous system (CNS) and differentiate into microglia, HSCT is being extensively studied for inherited metabolic diseases.[1] In these conditions, early intervention is primal for good outcomes as disease progression can only be prevented, but not reverted by treatment.[2] Minding this issue, preclinical studies aiming at transplantation in the early stages of life have been increasing, mainly using mouse models of diseases.

One of the main limitations of mouse models of transplantation is the animal size, especially if the procedure has to be performed in the newborn period. Mice pups have ~3.5 cm and weight ~2 g on day 2 postnatal. This is a major issue when intravenous administrations have to be performed, as the volume of injection is limited, and the vessels caliber is very tiny. Considering all difficulties for administering cells in newborn mice, the literature is very heterogeneous about routes of administration.

Intraperitoneal (IP) route is widely used to inject pharmacological drugs and viral vectors for gene therapy,[3,4] though it is also frequently used to inject mesenchymal stem/stromal cells (MSCs).[5–7] The administration of cells in the peritoneum has results similar to intravenous administration, especially for cells with strong tropism for certain tissues or conditions—as MSCs that migrate to injured/inflamed tissues[6]—or cells that act by paracrine mechanisms.[7] This via tolerates well up 10–30 mL/kg

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body weight (although bigger volumes can be used in specific circumstances upon ethics committee approval).[8] IP injection is the easiest to perform but may cause significant animal distress if they are manually restrained.[9]

For intravenous routes of administration, options for pups are different from those for adults. Although unpractical in newborns, the lateral tail vein is commonly used for intravenous access in adult mice. It is an effective systemic route, though some studies[10–13] consider it relatively hard to access, requiring trained personnel to avoid multiple venepuncture attempts that may lead to animal discomfort and distress. If multiple administrations are required, scarring of the tail can occur, making the last injections even more difficult to perform.[12] Another option is the retro-orbital (RO) sinus—a venous complex situated in the retrobulbar space. Contrarily to the lateral tail vein, this route is accessible in newborn mice, even though the eyelids are shut in the first few days postnatal.[14] Comparison between these two routes normally favors the RO route, which is easier to perform and leads to equivalent results—although repeatedly being cited in the literature as aesthetically unpleasant to execute.[10–13] In pups, however, there is another intravenous access: the lateral temporal vein (TV), a small vessel that is visible in both face sides in the first postnatal days.[15] It is widely used for all sorts of different treatments,[16–18] although it seems to have more limitations—it is considerably harder to perform after 4 or 5 days postnatal as the skin thickens and pigmentation increases (especially in black strains), thus interfering in the visualization of the vessel. In addition, some techniques require two trained people.[19]

Up to date, there are few studies comparing administration routes considering cell biodistribution, easiness of manipulation, and animal discomfort,[5,7,10,20] but none performed on newborn mice. Upon difficulties faced by our research group in assessing the best route for hematopoietic cell transplantation in newborn mice aiming at future studies, here we describe our experience with three routes—IP, intravenous through RO sinus, and intravenous through lateral TV. Our goal was to determine which route was the easiest to perform while allowing effective cell distribution.

METHODS

Animals

This research project was approved by the ethics committee for the use of animals of Hospital de Clínicas de Porto Alegre under number #16-0260. C57BL6-GFP (herein referred as GFP) and 129/SV mice (herein referred as WT) were used as donors and recipients, respectively. The choice of a different strain (129/SV) was based on availability of mice in the animal facility. Animals from both genders were used. After weaning at 21 days of age, animals from the same litter were kept in appropriate cages with a maximum of five animals per cage in a controlled environment (temperature 20–24°C, 40–60% relative humidity, and air exhaust systems) with cycles of 12 hours of light and 12 hours of dark, standard commercial feed for the species, and water *ad libitum*.

Experimental design

Up to five animals per group were analyzed, as indicated in each figure legend. Recipient mice were assigned to three major groups according to the administration routes to be tested: IP, intravenous through RO sinus, and intravenous through lateral TV. Animals from the same litter were assorted randomly to each group and front paws were marked with subcutaneous injection of nontoxic ink for posterior identification. Since the goal was to provide an idea of cell distribution among various tissues (and not only hematopoietic organs), with special focus in the brain, and verify whether there would be a major difference between the routes tested rather than analyzing long-term engraftment, the endpoints chosen were 48 h and 30 days post-transplantation (Fig. 1A) (considered short term and medium term, respectively).[19] Other than in the brain, results are descriptive and are shown as the average and standard error of the mean from the three animals in each group. Another experiment following the same design was performed in mice without preconditioning with busulfan, and representative results from this experiment are shown in Supplementary Fig. S1. In addition, 10 animals were treated with busulfan only, without cell transplantation—3 mice were euthanized 48 h after busulfan injection for histological analysis of bone marrow (Supplementary Fig. S2) and 7 mice were kept for 30 days as controls for the conditioning treatment.

Bone marrow collection and Lin^- cells isolation

Hind legs were collected, and femurs and tibias were dissected from 6 to 8 week-old GFP+ mice. The bone marrow was flushed with Dulbecco's modified Eagle's medium containing 1% penicillin/streptomycin and 20% fetal bovine serum (all from Gibco), using a 27-gauge needle attached to a 3 mL syringe (BD). Flushed cells were homogenized by gentle up-and-down pipetting and filtered in a 70 μm Falcon cell strainer (Fisher Scientific) to remove cell clumps and bone particles. Cells were spun at 300 *g* for 5 min and then resuspended at a concentration of 1×10^6 cells/mL, plated in 6-well treated plates (Nest, China), and incubated in a humidified atmosphere at 37°C, 5% CO_2, for 2 h, so that adherent cells (as macrophages and mesenchymal stromal cells) could attach to the plate. Upon completion of incubation, nonadherent cells were collected, pelleted, and resuspended in MACS buffer (Miltenyi Biotec, Germany). Lineage positive cells were depleted using MiniMACS Lineage Cell Depletion Kit (No. 130-090-858; Miltenyi Biotec, Germany), according

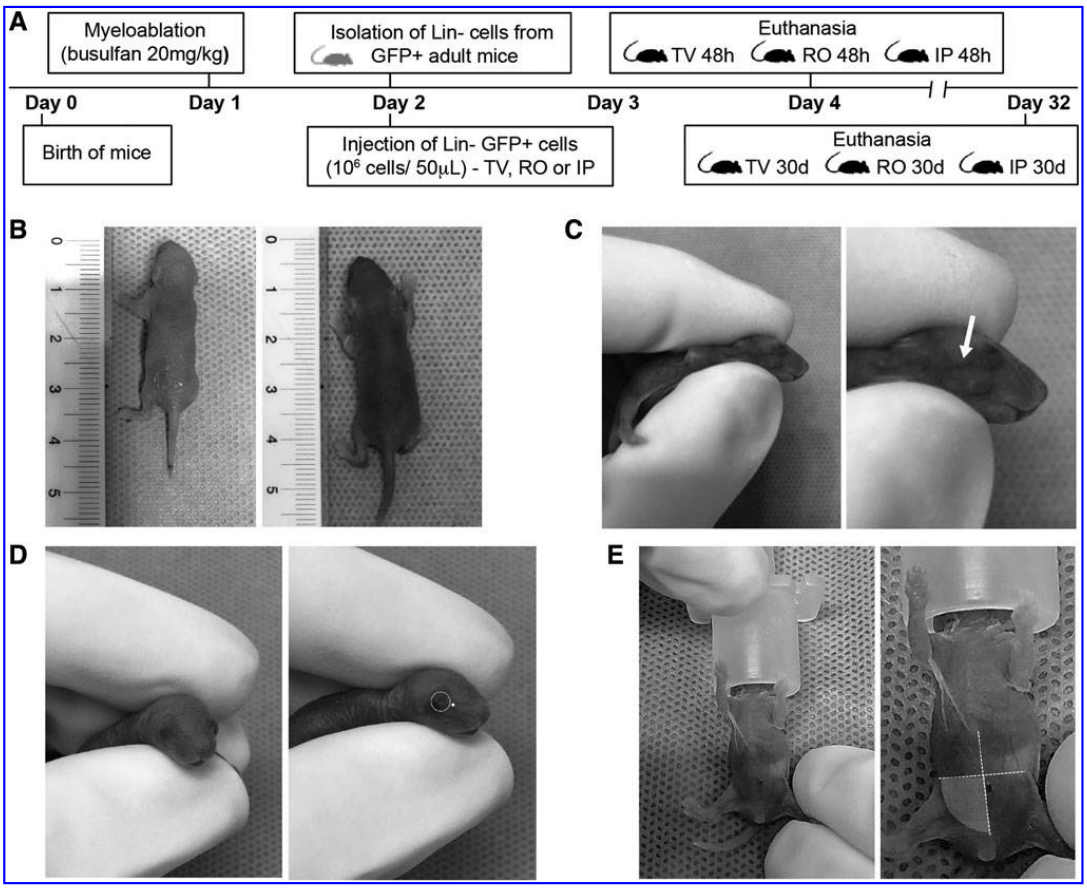

Figure 1. Experimental design and routes of administration. **(A)** Timeline of experimental design: at day 1 postbirth, animals were treated with busulfan for myelosuppression; 24 h after, transplantation of Lin^- cells was performed in one of three different routes and distribution of cells was analyzed at two endpoints, 48 h or 30 days after transplant. **(B)** Size of 2-day-old and 3-day-old mice. **(C)** Positioning used for TV injection, with *arrow* pointing to the site of injection. **(D)** Positioning used for RO injection, with eye globe highlighted (○) and injection site marked (*white dot*). **(E)** Positioning of pup for IP injection, with area of injection highlighted. IP, intraperitoneal; RO, retro-orbital; TV, temporal vein.

to manufacturer's protocol. For each donor adult mouse, the yield of Lin^- cells was approximately 5×10^5 (data not shown); therefore, for each pup, cells were collected from two donors, on average. Purity of lineage-depleted population was assessed by flow cytometry using antimouse Lineage Cocktail (BioLegend), which showed >90% of Lin^- cells in each cell preparation (data not shown).

Busulfan conditioning

Newborn mice ($\sim$24–48 h after birth, when weighting >1.5 g) were treated intraperitoneally with 20 mg/kg of busulfan (Busilvex, Patheon Manufacturing Services) in a maximum volume of 20 μL. This dose partially ablates the bone marrow, as determined by previous experiments from our research group (pilot study; data not published). The day chosen to initiate the treatment regimen was based on pup's size and weight (>1.5 g), as different litter sizes influence the pup's growth. After busulfan injection, pups were then placed back to the dam's cage.

Procedure for Lin^- cells transplantation

Pups were transplanted 24 h after busulfan administration, ultimately varying from 2 to 3 days postnatal. Each pup received 1×10^6 Lin^- cells suspended in 50 μL of saline solution[21] and cell suspension was kept on ice until the moment of injection. All procedures were performed in anesthetized mice, regardless of the route of administration. Newborns were removed from the dam's cage one by one and placed in a heated bench to induce vasodilation. Anesthesia was induced with isoflurane 3–4% (Isoforine; Cristalia, Brazil) vaporized in oxygen flow (300 mL/min) (Takaoka, Brazil). In the absence of motor reflex upon

digital pinching, isoflurane concentration was reduced to 1–2% to maintain absence of reflexes. In the TV and RO groups, isoflurane flow was temporarily interrupted while performing the injection (up to 15 s). All injections were performed with a 6 mm 31-gauge needle (BD Ultrafine II, Brazil).

For intravenous injections through TV, animals were positioned in lateral decubitus, with thoracic members kept close to the thorax (Fig. 1C). The needle was inserted in the TV in the cranial–caudal direction. Elapsed injection time was ~5 s. The injection was considered a success when no extravasation of solution was observed in the adjacent tissues and when the vessel recovered its dark color right after the end of the infusion (though before removing the needle). Hemostasis was achieved by light pressure with a cotton swab in the injection site upon needle removal.

For intravenous injections through RO sinus, pups were held in a vertical position, facing the executioner. Needle was inserted in the medial aspect of the shadow produced by the ocular globe (Fig. 1D), with the tip pointing to the orbit. The injections were considered successful when no extravasation of solution was observed in the adjacent tissues, no occurrence of ocular globe protrusion was observed, and nostrils were free of liquid.

For IP injections, we used the same procedure for injections in the peritoneal space for both cells and busulfan. Pups were placed in dorsal decubitus and the needle was introduced in the inferior right quadrant of the abdomen, in a 15° angle in an attempt to puncture skin and abdominal layers in different sites, thus reducing the reflux of liquid through the injection site upon needle's removal. In addition, mice were kept under anesthesia for 2 min after completion of injection for liquid settling. Liquid extravasation, including during the recovery of spontaneous movements, was considered as a failure of the procedure.

Animals were warmed and placed in oxygen flow after injection and only placed back to the dam's cage when spontaneous movements were fully recovered. In the few cases of failure of the procedure, mice were immediately euthanized by decapitation under deep anesthesia with isoflurane.

Euthanasia

Euthanasia was performed by isoflurane overdose and confirmed by cervical dislocation. In treated animals, blood was collected from heart puncture and stored at −20°C. Heart, liver, lungs, kidneys, spleen, cortex, and hind legs were also collected. For DNA extraction, one fraction of each tissue was frozen at −20°C, except for the bone marrow cells that were stored in phosphate-buffered saline solution collected by flush from the femur. For the immunohistochemistry (IHC) analysis, the remaining fraction of tissues and one hind leg were fixed in buffered formalin followed by paraffin processing according to routine techniques. The formalin fixed leg also went through 14% EDTA decalcification for 1 week immediately before being processed.

Immunohistochemistry

To evaluate the cell biodistribution and morphology, the IHC technique was performed using anti-GFP antibody to detect cells from the GFP+ donor. Collected organs were processed in paraffin blocks, cut in thin sections, and incubated for 1 h at 75°C. After dewaxing, antigen retrieval was performed by incubation with 10 mM citrate buffer pH 6 for 35 min at 94°C. Slides were incubated with primary anti-GFP antibody (1:600 dilution; rabbit polyclonal IgG from Santa Cruz Biotechnology) overnight at 4°C in a dark camera. Finally, slides were incubated with peroxidase-conjugated goat antirabbit IgG (1:200; Santa Cruz Biotechnology) secondary antibody for 90 min at room temperature on a dark camera and then developed through the DAB Kit (Dako) through chromogen 3-3′-diaminobenzidine (DAB). After IHC, slides were analyzed using ImageJ software, by means of the Color Deconvolution plugin, resulting in percentage of area occupied by GFP+ cells in the field, analyzing three fields of each organ. GFP+ tissues for positive controls and WT tissues and secondary antibody only for negative controls were run in all experiments.

Quantitative PCR

Genomic DNA was extracted from frozen tissues by the salting out method: small tissue fragments were homogenized with sterile pestles and incubated overnight in a bath shaker at 65°C, 350 rpm with 5 μL Proteinase K (10 mg/mL; Invitrogen) and 400 μL of digestion buffer (100 mM TRIS pH 8.3, 10 mM EDTA pH 7.5, 200 mM NaCl, 1% SDS). Upon complete dissolution of tissue, 100 μL of 5 M potassium acetate was added. Samples were spun 18,000 g at 4°C for 20 min, supernatant was collected, and 250 μL of ice-cold isopropanol was added. DNA was pelleted after 10 min centrifugation at 18,000 g and washed with 1 mL of ethanol 70%. Samples were resuspended in 49 μL of TE buffer and 1 μL of RNase A (ThermoFisher Scientific), incubated at 37°C for 1 h and quantified by Nanodrop (ThermoFisher Scientific). Purity ratios 260/280 and 260/230 were ~1.8/2.0 for all samples.

Reactions in standard mode were carried out using PowerUp SybrGreen MasterMix (ThermoFisher Scientific), primers forward 5′ TGACGGGAACTACAA GACGC 3′ and reverse 5′ CCTCCTTGAAGTCGATG CCC 3′ (RefSeq NC_025025.1) at 0.2 μM each, with the following cycling conditions: 50°C 2 min; 95°C 2 min; 40 × 95°C 15 s, 55°C 15 s, 72°C 1 min; followed by dissociation curve in QuantStudio 3 and StepOne equipments (Applied Biosystems). Samples were run in triplicates, with 100 ng of DNA each.

In addition, amplification of the reference gene β-actin was conducted to control DNA input in brain samples. Primers used were forward 5′-CAAGATCATTGCTCCT CCTGAG-3′ and reverse 5′- GACTCATCGTACTCCTG CTTGC-3′.

For the absolute quantification, the 5.3 kb pIRES2-EGFP plasmid (Clontech) was used to create the standard curves, where a regression line was obtained plotting the logarithm of the number of copies versus the Ct. Correlation coefficients R^2 were >0.97 in all reactions. As positive and negative controls, samples from GFP+ and WT mice, respectively, were analyzed. No amplification was detected in WT samples neither in nontemplate controls, whereas amplification was positive in all GFP+ samples. Melting curves were obtained and confirmed the presence of single amplicons, and amplification efficiency was determined at every reaction, being $\sim$90% in all experiments. Absolute number of GFP molecules was calculated using the linear equation obtained from the standard curve. Each sample was analyzed in triplicates.

RESULTS

Cell distribution

The presence of transplanted cells was assessed 48 h and 30 days after injection, both by IHC and quantitative PCR (qPCR) (Figs. 2–5). qPCR was used as a quantitative method, whereas IHC was used mostly to evaluate the localization of cells within the tissue and their morphology, as the last is not as sensitive as the first. Since cells are supposed to engraft in the bone marrow, results are presented both using raw numbers (absolute number of GFP copies in qPCR or percentage of GFP+ area per field in IHC sections) or as relative numbers, considering the bone marrow at each time point as 100%.

At 48 h, in the IP group, GFP copies were seen mainly in the bone marrow, whereas in other tissues, the number of copies was very low (Fig. 4). By IHC, only the bone marrow presented positive cells (Fig. 2), reinforcing the result obtained by qPCR. This may suggest that cells migrate rapidly to the bone marrow when using the IP route. On the contrary, cells injected through the RO and TV routes were observed in tissues such as the spleen, lungs, and specially the liver, not only in the bone marrow (Fig. 2).

As expected, after 30 days, qPCR results showed that most cells migrate to the bone marrow, followed by spleen and liver. All three routes performed similarly in the medium-term assays. More importantly, all organs presented increased number of GFP copies when compared with 48 h results, which could be an indication that the cells were able to proliferate in the recipient.

In summary, the organs that retain most transplanted Lin^- cells in 48 h are the liver and the bone marrow. In 30 days, cells home to the bone marrow and seem to be proliferating there, as the GFP+ detection increased in both qPCR and IHC. Following bone marrow and liver, cells also migrate to spleen and lungs, where the proportion of cells compared with bone marrow decreases over time (except for IP, where it increases from almost null to 30% in the spleen). Heart, kidney, and blood present similar number of cells and the proportion is roughly kept throughout time. Although the number of cells in blood seemed to increase, the proportion reduced in all routes at 30 days, which was expected as cells probably homed to the hematopoietic organs.

Brain had undetectable GFP+ cells in IHC assay for most samples (except from one mouse each time point from RO and one from TV 30 days). In the mouse from RO 30 days, GFP+ cells had the aspect of glial cells, not macrophages nor monocytes (Fig. 3), which could suggest engraftment and differentiation of transplanted hematopoietic cells. In qPCR at 48 h, number of GFP copies was lower in TV and IP than in the RO route ($p = 0.023$ for IP and $p = 0.085$ for TV) (shown in Fig. 4 for comparison with other organs and detailed in Fig. 5). Accordingly, at 30 days, mice transplanted through RO also presented higher number of copies than the others. Surprisingly, all five mice transplanted IP had detectable levels of GFP, with mean number of copies even higher than the TV route, though statistical significance was not observed. Overall, this could suggest that the RO route addresses the brain quickly and more efficiently than the others, although the variability was high in all groups (Fig. 5).

Easiness of procedure

All transplantations were performed by two veterinarians and one nurse. All three professionals are very experienced with injections in adult mice (ranging from 5 to 15 years of experience), but none had expertise in handling newborn. All performed injections by the three routes and here we describe their impressions exclusively regarding the injection procedures.

The TV route is the hardest to perform, as the hand has to be perfectly steady, requiring more and continuous practice. If the volume of administration is high (in case of hydrodynamic injections, *e.g.*), the difficulty increases as the syringe's piston is farther and another person is required to push it. In contrast, it is the only route that the executioner is completely sure of success, as it is possible to observe the administered solution inside the vessel and the recovery of blood flow when the injection procedure is over.

The RO route does not require as much training as the TV route. However, the executioner must estimate the needle's deepness and the site of puncture, requiring anatomy knowledge. It is possible to assess the success of injection if there is no swelling in the applied region or presence of liquid in the nostrils, although it is not as perfectly visible as for TV route.

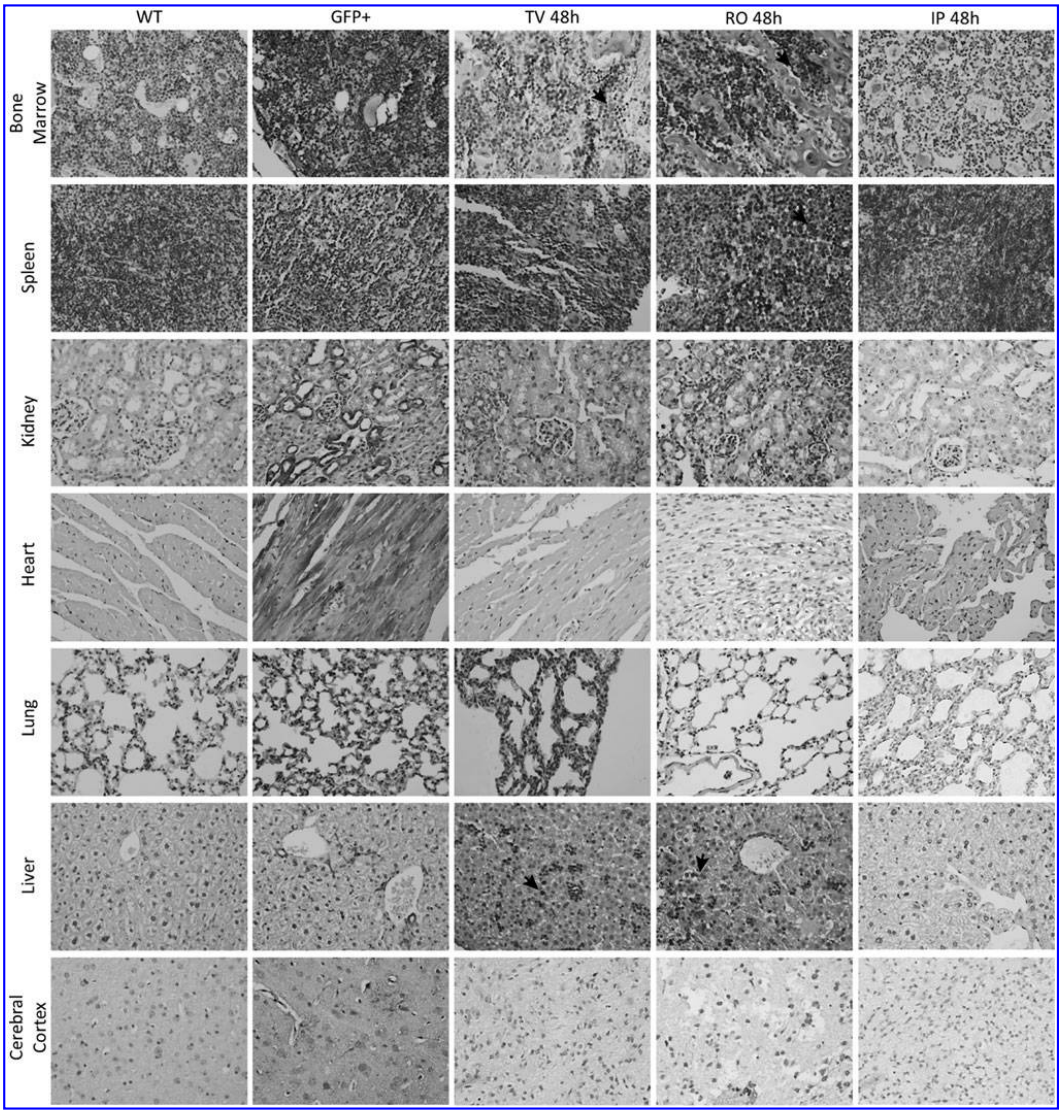

Figure 2. IHC for GFP+ cells in all organs analyzed at 48 h post-transplant of Lin^- cells. Histological sections from positive and negative controls were obtained from adult mice. In 48 h, bone marrow, liver, lungs, and spleen were the tissues that presented more GFP+ cells in the TV route and in the RO route. Heart and kidney were almost negative, and few cells could be found in the brain of one mouse in the RO group. IP route had negative sections for all organs analyzed. Magnification 400×. $n=3$ per group. IHC, immunohistochemistry.

Finally, the IP route is by far the easiest and does not require prolonged training. The needle has to be very thin, 30-gauge or more, otherwise the liquid will flow back through the puncture site and the pup has to be anesthetized during and after the injection to avoid reflux caused by their movement. In neonates, the skin is considerably translucent, and it is possible to visualize when viscera are pushed by the injected liquid without evident lesions. Therefore, veterinarians considered this route as easier to determine the success of the procedure compared with RO route, but not as well as TV route in this parameter.

The mortality rate solely due to the injections was null, although the success of the procedure varied between groups. TV showed ∼a 20% failure, mostly due to liquid extravasation in the subcutaneous skin layer, whereas no adverse events or consequences were observed in

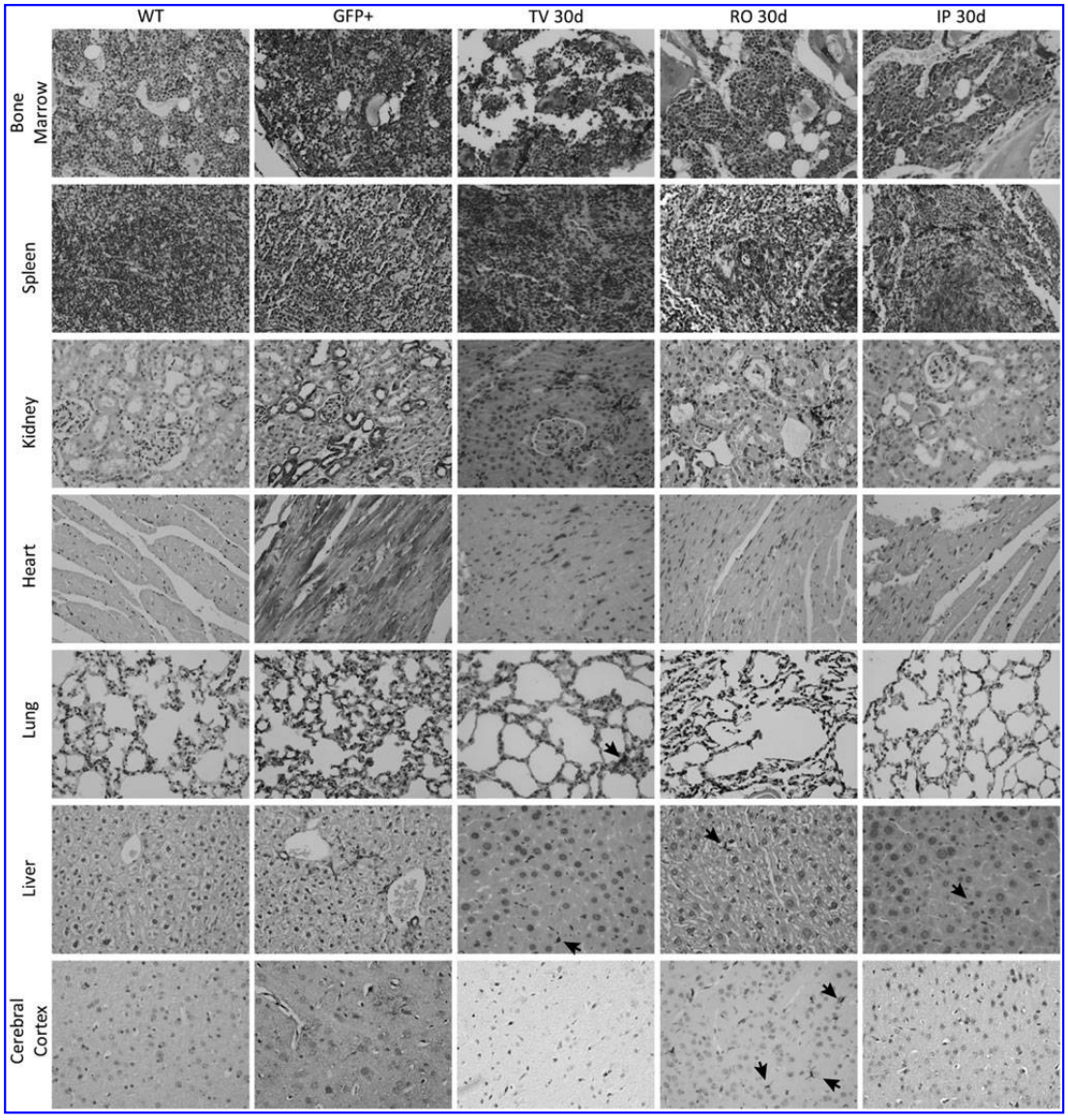

Figure 3. IHC for GFP+ cells in all organs analyzed at 30 days post-transplant of Lin^- cells. Cells were found mainly in the hematopoietic organs bone marrow and spleen, but also in lungs, liver, heart, and kidney. In the brain, only the RO route presented few cells, with morphological characteristics of microglia cells. Representative GFP+ cells are pointed by *black arrows*. *Arrows* point to Kupfer cells in the liver, alveolar macrophages in lungs, and microglia-like cells in the brain. Negative and positive controls are the same as used in Figure 2. Magnification 400×. $N=3$ per group.

successful TV injections. For RO, one case of edema occurred immediately after injection, considered as failure. For IP, failure (liquid reflux) occurred in 10% of injections. Adverse events after successful injections occurred for RO and IP only. From all 12 animals injected through the RO route, we had one case of abscess in the injected site (Supplementary Fig. S3A) and one case of corneal clouding. In the IP route, we had one case of microphthalmia and growth retardation (Supplementary Fig. S3B), possibly due to complications of the transplant (as graft rejection or acute graft-versus-host disease) rather than due to the injection. As myeloablation control, seven pups were injected with busulfan only and were accompanied for 30 days. Five of these fully recovered from the partial myeloablation whereas the other two died at 48 h and 20 days after busulfan injection.

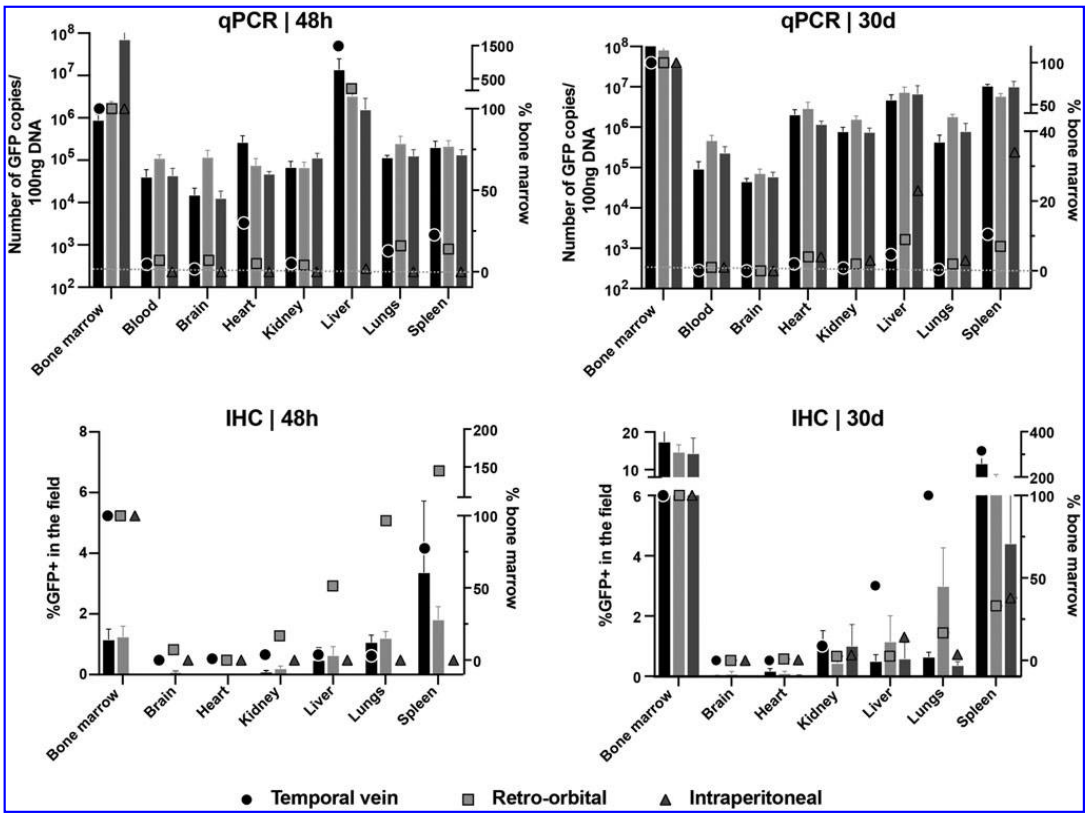

Figure 4. Quantitative results of GFP content in different tissues. Each *color* represents a different route of administration. *Bars* were plotted according to the *left y*-axis and represent the absolute number of GFP copies (qPCR: *top plots*) or GFP+ area (IHC: *bottom plots*); whereas *symbols* were plotted in the *right y*-axis and represent the percentage of GFP in the organ comparing with the bone marrow at each time point, assuming the latter is 100%. Standard error mean is shown above each bar. $N=3$ per group in all organs but the brain, which is 5 per group. qPCR, quantitative PCR.

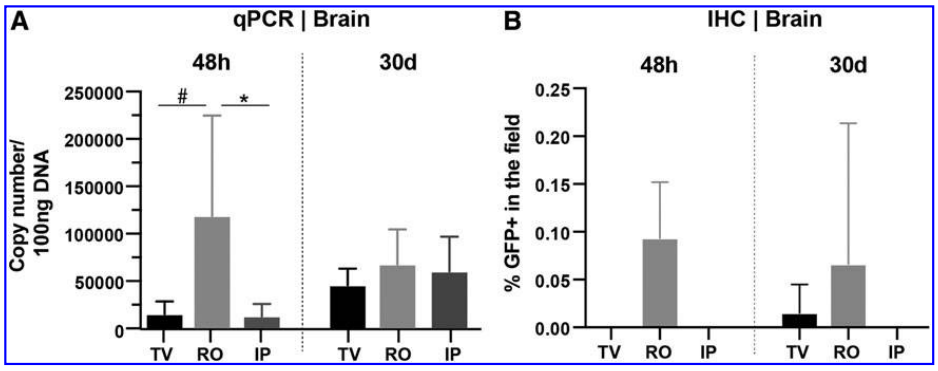

Figure 5. GFP quantification in brain. **(A)** Number of GFP copies in the brain, assessed by qPCR. Five mice per group were analyzed and DNA input was controlled using a reference gene. $^{*}p=0.023$; $^{\#}p=0.085$; Kruskal–Wallis test. **(B)** GFP content in brain tissues by IHC. Mean ± SD shown. These data are the same shown for brain results in Figure 4, but in a dedicated graph to highlight differences among routes in this particular tissue.

DISCUSSION

When planning a project that involves newborn treatment, one might not consider how challenging it could be to administer a treatment to a mouse pup. Without experience, our research group has failed many times when trying to perform the first intravenous injections in 2-day-old mice, and some of these times were particularly frustrating as the treatments were expensive and/or laborious. The literature does have several systemic administration routes described, such as TV,[15] jugular vein,[19] RO sinus,[14] and so on, but evaluation of distinct routes using hematopoietic cells in newborn mice was not performed before. Most studies about administration routes of hematopoietic cells only evaluate engraftment in hematopoietic organs, as bone marrow and spleen, while we were interested in seeing cell distribution as a whole, including the CNS, as this is an important target for several conditions that could be treated with HSCT.[1,2] Intrahepatic injection is, possibly, the most common route for transplantation of hematopoietic cells in newborn pups, especially human CD34+ in humanized mouse models.[22,23] However, there is evidence that cells transplanted in the liver do not reach the brain[24]—thus our interest for systemic routes.

The ability of hematopoietic stem cells to home and engraft can vary considerably depending on the source of the cells. In humans, it is well known that cells derived from umbilical cord blood, bone marrow, or mobilized peripheral blood engraft differently—umbilical cord blood cells have the highest risk of graft failure, followed by bone marrow and peripheral blood.[25] Besides the cell source, age-related differences in engraftment have also been described: for example, hematopoietic cells derived from young mice (up to 4 months old) can prevent aging-related processes, whereas cells from older donors cannot.[26,27] The transplant outcome also depends on the recipient—cells derived from adult mice engraft better in adults than in newborns.[28] In this study, we used 6–8 weeks old mice as cell donors for newborn recipients, which may have influenced the engraftment and distribution of cells, although this diminished activity of adult cells occurred to all experimental groups, making the observations still valid.

Both qPCR and IHC techniques were used as they complement each other. The absolute quantification method detects the presence of DNA, which is independent from GFP expression; whereas IHC provides the localization and morphology of GFP+ cells in the tissue, thus making it possible to observe whether there are donor-derived round-shaped monocytes, differentiated ramified microglia-like cells, or tissue macrophages, for example. IHC also excludes the possibility of positive cells detected by qPCR being located only in remnants of blood in tissue vessels.

IHC results were all based on percentage of GFP+ area in the field. They could not be compared with their correspondent GFP+ control tissues because the expression and intensity of GFP largely varies between different organs, possibly due to different promoter activation.[29] Even though tissues were cut symmetrically upon collection (half for each method) and multiple fields were analyzed per slide, IHC results are still not representative of the whole. This may explain why many tissues had absent GFP staining while presenting high number of GFP copies by qPCR. As results from both techniques showed high variability, we calculated the correlation between them (Supplementary Fig. S4). Correlation scores were $R^2 > 0.4$ for most tissues, which is an indication that there is some correlation. Nevertheless, IHC was very useful to analyze the morphology of transplanted cells in different tissues. Despite the rare possibility of differentiating in other cell types (including hepatocytes,[30] *e.g.*), the majority of Lin^- cells resembled monocyte-derived cells, as Kupffer cells in the liver, alveolar macrophages in the lungs, and microglia-like cells in the brain (Figs. 2 and 3).

At first (based on 48 h results), Lin^- transplanted cells go mainly to the liver when using RO and TV routes. This could be explained by high vascularization and/or because the liver is the primary source of hematopoietic cells during the embryonic development, with the last colony-forming progenitor cells migrating to the bone marrow in the first few days after birth.[31] Besides the liver, in 48 h, a good portion of cells home to the bone marrow, where they seem to engraft and proliferate, as they were also detected at 30 days in even higher numbers in all routes (as seen in both IHC and qPCR). It is important to highlight, however, that this configuration is not necessarily final, as the transplanted cells only achieve a steady state 8 weeks post-transplant, and the effect of reconstitution performed by long-term hematopoietic stem cells can only be analyzed after 16 weeks *in vivo* and after serial transplantations.[32] Furthermore, spleen showed the third biggest GFP content; other organs, such as heart, kidney, lungs, and brain had fewer cells and in comparable number (Fig. 6).

At 30 days, cell distribution resembled that observed at 48 h, except that in the later point the bone marrow had much higher GFP+ copies. In all tissues analyzed, it is possible that cells are proliferating, as the absolute number of GFP copies and/or GFP staining increased in all 30 days groups when compared with 48 h groups, even though the content of GFP in some tissues may seem lower if compared proportionally with the content in the bone marrow. This ability to migrate to and/or proliferate in different organs together with the thorough distribution of cells in systemic administrations (as in the RO route that even seems to facilitate cell migration to the CNS, at least in one mouse) is extremely helpful when one thinks about long-term treatments.

Hematopoietic cells migration activity is increased to the CNS in cases of injury and neuropathology,[1] which was not the case and could explain why not many cells were seen in this tissue. Busulfan conditioning also is supposed to help cell engraftment in the CNS,[33,34] but it may not occur when partial myeloablation is achieved as compared with full myeloablation with lethal doses. It has been suggested that few transplanted cells migrate to the

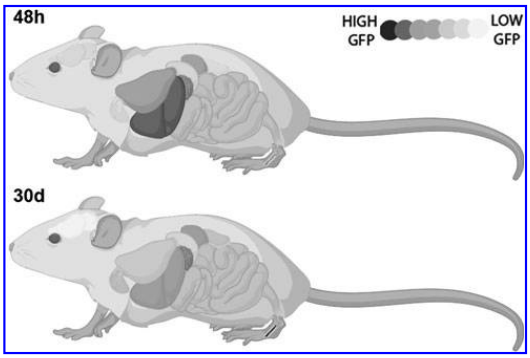

Figure 6. Distribution of Lin^- cells in mouse. Summary of distribution of Lin^- cells considering all three routes of administration and both qPCR and IHC techniques. In 48 h post-transplant, cells are mainly found in liver, but bone marrow, lungs, and spleen also have a moderate number of cells. Kidney, heart, and brain present very few cells. At 30 days post-transplant, however, cells home to the bone marrow, where they engraft and proliferate. Spleen and liver have significant number of cells, followed by kidney and heart. Very few cells migrate to the brain and presumably engraft there, being barely detectable.

brain soon after conditioning, as the myeloablative regimen affects resident microglia cells and facilitates the influx of circulating myeloid cells to the brain. However, this is not correlated with the bone marrow engraftment and only few cells do engraft in the brain parenchyma, proliferating in the tissue if necessary, rather than recruiting more cells from circulation.[33] Nevertheless, we observed positive cells in the brains of few mice, two of them from the RO route. IHC from 48 h did not show well-defined cells, but qPCR presented 10-fold more copies than other individuals in other groups. Moreover, the mouse analyzed at 30 days presented several microglia-like cells in IHC assay, with multiple visible ramifications (Fig. 3). As targeting the brain was one of our main goals, we performed the analysis in five mice per group and included a DNA input control in the qPCR, by amplifying the reference gene β-actin in every run. In all samples analyzed, variations in β-actin amplification were <5% between samples, confirming that the robust variation observed in number of GFP copies is solely due to the treatment (Supplementary Fig. S5).

Although the small number of mice does not allow us to reach robust conclusions on this subject, better performance of the RO route over the TV route aiming at the CNS was already described by Gruntman *et al.*,[21] when they compared both routes for adeno-associated viral vectors injection and observed higher transduction of brain and retina with the RO administration—although the authors discussed that the volume administered (50 μL) might be too much for this route, which could cause a hydrodynamic effect and increase efficiency. Surprisingly, the IP route performed similarly to the RO route at 30 days (Fig. 5), although this should be further investigated with larger sample size and longer time periods.

The mouse that developed microphthalmia and had growth retardation was transplanted through IP and was euthanized at 30 days (Supplementary Fig. S3B). This mouse had the highest content of GFP in all organs seen in both qPCR and IHC, compared with the others in the same group. These manifestations are characteristic of acute graft-versus-host disease, in which the transplanted immune cells proliferate indefinitely and attack the host's tissues. The acute response in mouse models typically arises at 14–28 days post-transplantation and usually deceases the mouse within few days.[35] Unfortunately, phenotypical analysis of immune cells was not performed to confirm this hypothesis.

If we consider the easiness of execution and the less distress caused to animals, the IP route would definitely be the unanimous choice. It had fewer failures and only one adverse event, although it was not related to the procedure itself but probably due to graft-versus-host disease. The possibility of local effects at the site of injection was not analyzed in our study, but if present, they do not seem to last long term.[36] Lin^- cells were distributed in a similar manner in visceral organs in IP as other routes, with even better results in the brain than TV route. RO injections were also easy to perform but unpleasant; it was the route that seemed to yield higher GFP content in most organs and, more importantly, the route that reached the CNS the most in our sample. Lastly, the TV route was the hardest to master, with many failures and discarded animals throughout the study. As a bright side, this route seemed to have the most homogenous results among groups—possibly because it was easier to notice the failure of injections than it was in the others. Finally, the best route of administration of Lin^- cells depends on the goal, personnel, and resources available.

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AUTHOR DISCLOSURE

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SUPPLEMENTARY MATERIAL

Supplementary Figure S1
Supplementary Figure S2
Supplementary Figure S3
Supplementary Figure S4
Supplementary Figure S5

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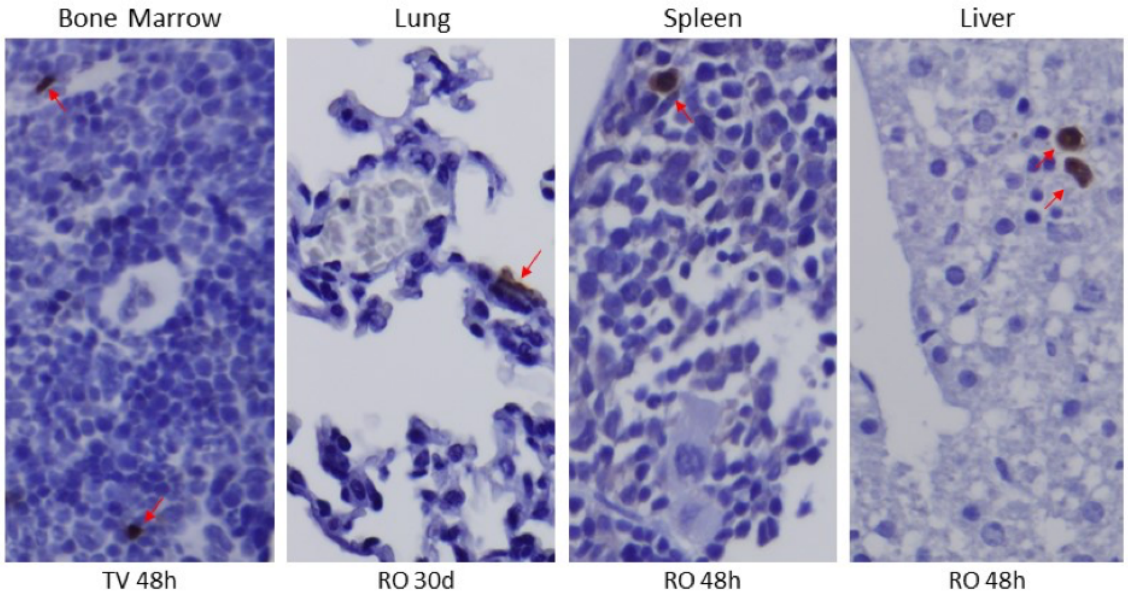
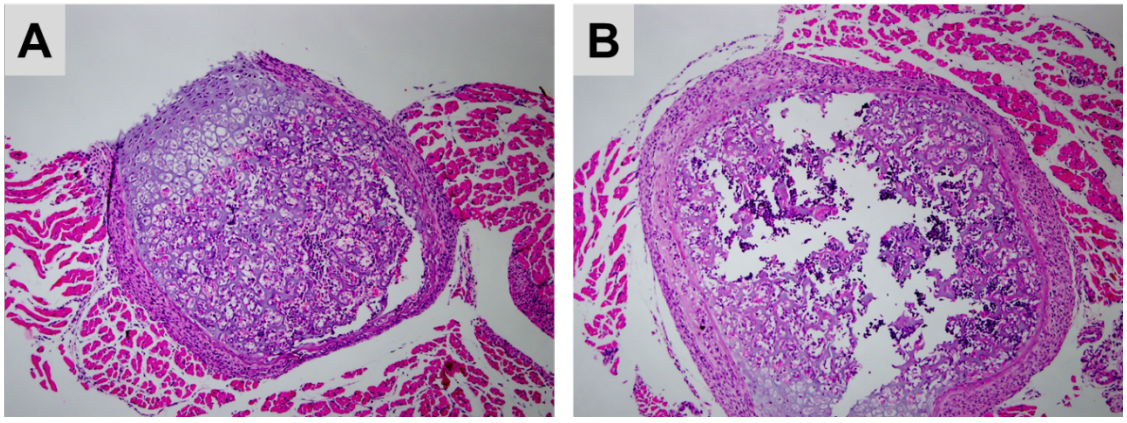
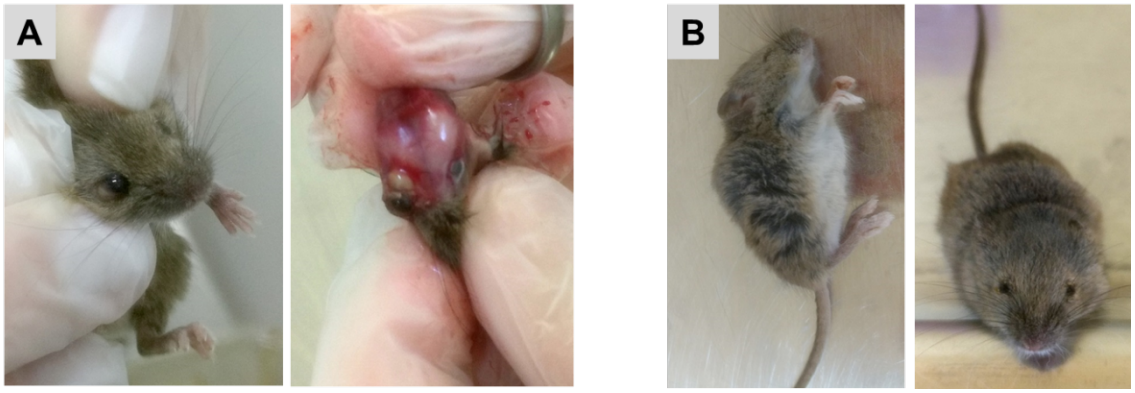
SUPPLEMENTARY DATA



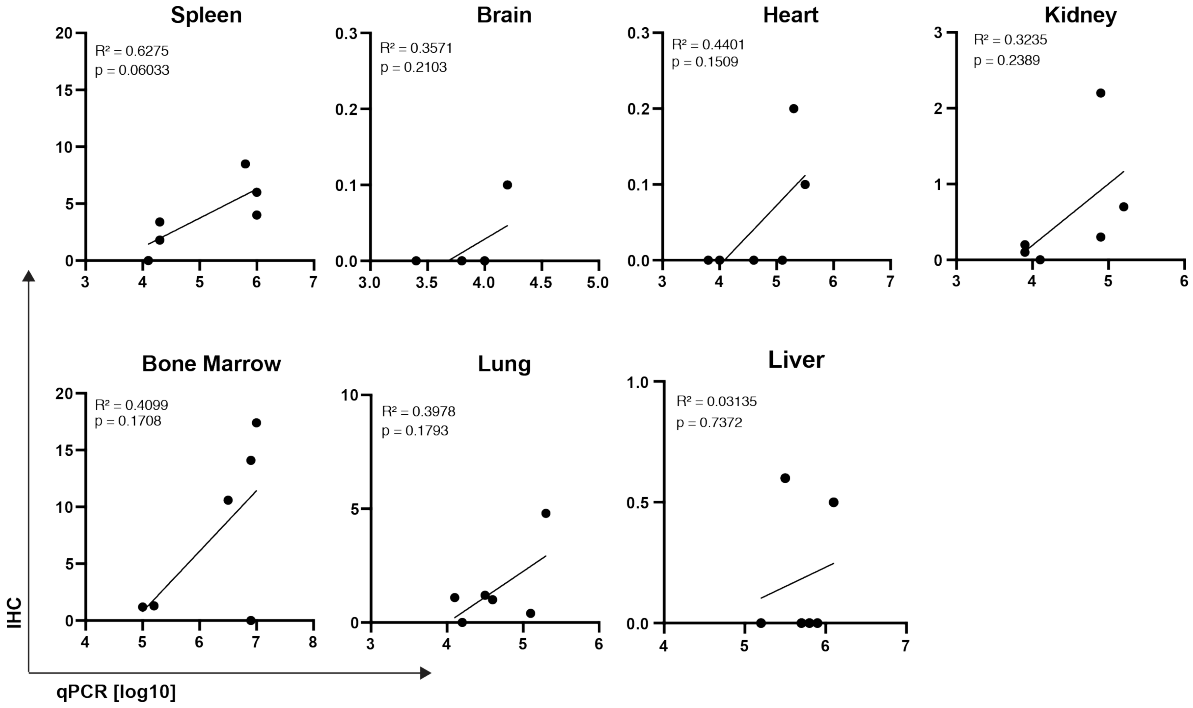
Supplementary Figure 1. Representative immunohistochemistry results of transplanted mice without myeloablation. Engraftment was very poor in animals transplanted without conditioning regimen, independently of the time analyzed. We found most tissues to be negative for GFP cells in the immunohistochemistry, with very few cells (red arrows) only in bone marrow, lung, spleen and liver. Only 10^5 Lin- cells were transplanted in these series of experiments. Magnification 400x.



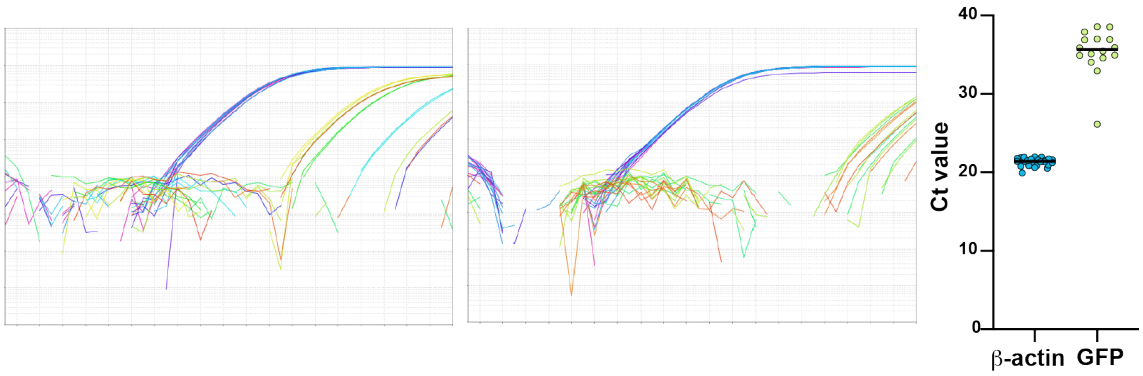
Supplementary Figure 2. Histological sections of bone marrow from femur partially ablated with busulfan. Three mice were treated with 20 mg/Kg of busulfan intraperitoneal at day 2 after birth and were euthanized 48 h after conditioning. Hind legs were collected, fixed, decalcified and processed for histological analysis of the bone marrow in the femur. **A)** Control mouse, without conditioning treatment. **B)** Busulfan treated mouse, showing empty spaces in the bone marrow. Hematoxylin and eosin, 100 x magnification.



Supplementary Figure 3. Adverse events observed in transplanted mice. A) Mouse injected via retro-orbital that developed an abscess at the site of injection, easily seen upon dissection. **B)** Intraperitoneally injected mouse that presented growth retardation and microphthalmia at 30 days post-transplant.



Supplementary Figure 4. Correlation between immunohistochemistry and quantitative PCR for GFP detection. Immunohistochemistry quantitative results were plotted in the y-axis and log GFP copies obtained by qPCR were plotted in the x-axis. R^2 values are depicted in each plot, as p values for each correlation analysis.



Supplementary Figure 5. Representative amplification plots from qPCR assays. Example of two runs amplifying the β-actin reference gene (blue/purple curves on the left) and amplifying the GFP sequence (set of curves on the right). Reference gene amplified in all samples had less than 5% variation in Ct value between samples, as observed in the plot on the right.

DISCUSSÃO GERAL

As técnicas de edição genômica desenvolvidas recentemente possibilitaram inúmeros estudos na área biomédica, da pesquisa básica ao desenvolvimento de novas terapias. O método de CRISPR/Cas9, especialmente, democratizou a geração e o uso de modelos *knockout* e *knockin*, uma vez que utiliza um sistema simples, barato e eficiente, ampliando o acesso à edição genômica a laboratórios sem conhecimento prévio na área.

O sistema CRISPR/Cas possui inúmeras aplicações desenvolvidas, baseadas no reconhecimento eficiente de locais específicos do genoma pelo complexo Cas-gRNA. Além do sistema clássico usado em estudos de função de genes, geração de modelos de doença e terapia gênica, variações da técnica permitem fazer edição de RNA, diagnóstico de doenças e *screening* genômico (XU *et al.*, 2020).

Iniciamos estudos utilizando a técnica de CRISPR/Cas9 em 2014, pouco após a publicação original descrevendo a técnica (JINEK *et al.*, 2012). Para prova de conceito, adquirimos um plasmídeo comercial, de sequência protegida, codificando a enzima Cas9 e um gRNA, que foi desenhado para reconhecer o gene *IDUA*, no local da variante patogênica p.Trp402*, a mais frequente em pacientes com MPS I. O objetivo era utilizar o sistema para corrigir a variante em fibroblastos de pacientes. Embora com baixa eficiência de edição (em torno de 5 % dos alelos), as células editadas passaram a produzir e a secretar a enzima (DE CARVALHO *et al.*, 2018). Esse resultado fomentou estudos subsequentes utilizando a técnica para terapia gênica *in vivo* (SCHUH *et al.*, 2019; SCHUH *et al.*, 2018). Além da aplicação terapêutica, decidimos explorar as possibilidades da edição genômica para estudos além da terapia, como na geração de modelos celulares de MPS para reduzir a utilização de fibroblastos de pacientes que, embora tenham valor imensurável, possuem limitações importantes, cujas principais são o tempo de cultivo limitado, a dificuldade de obtenção e a impossibilidade de coleta de tipos celulares diferentes.

Diversos modelos celulares utilizando edição genômica por CRISPR/Cas9 já foram desenvolvidos, inclusive para doenças lisossômicas como Fabry (SONG *et al.*, 2019), Gaucher (PAVAN *et al.*, 2020), adrenoleucodistrofia ligada ao X (RAAS *et al.*, 2019), Niemann Pick tipo C (ERWOOD *et al.*, 2019), entre outras. Para MPS, entretanto, nenhum modelo utilizando linhagens celulares editadas foi descrito até o momento.

A primeira tentativa de geração de linhagens nocaute para *IDUA* deu-se com a utilização do mesmo vetor utilizado para a edição dos fibroblastos de pacientes, porém com o gRNA específico para células normais (5’ GCTC**TAG**GCCGAAGTGTCGC 3’ > 5’

GCTC**TGG**GCCGAAGTGTCGC 3'). Essa mudança de sequência se fez necessária uma vez que se sabe que incompatibilidades na sequência do gRNA podem causar reduções drásticas de eficiência (JIANG *et al.*, 2017). Inicialmente, utilizamos células HEK293 e HepG2: a primeira pela alta eficiência de transfecção, e a segunda pela disponibilidade no laboratório. O protocolo de transfecção utilizado havia sido previamente otimizado utilizando um plasmídeo que expressa proteína verde fluorescente (GFP), pIRES-eGFP.

Os testes iniciais de isolamento de clones foram feitos usando métodos variados, independentes de FACS. Em um primeiro momento, plaqueamos 0,5 células por poço, em placa de 96 poços. Com esse método, observamos que muitos poços continham clones de crescimento muito rápido em comparação com os demais, possivelmente por terem iniciado com mais de uma célula. Esta ocorrência era particularmente mais frequente nas células HepG2, que crescem aderidas umas às outras formando grumos e são relativamente difíceis de serem obtidas em suspensão de células únicas. Por outro lado, muitos clones eram visivelmente derivados de uma única célula, porém possuíam crescimento demasiadamente lento. Em face à incerteza de obter clones únicos e à demora de crescimento em geral, decidimos testar o isolamento das células por plaqueamento em baixa densidade e seleção de colônias. Com este método, clones únicos e distantes uns dos outros recebem sinalização de seus vizinhos, o que dá suporte ao crescimento. De fato, selecionamos muitas colônias dessa maneira, porém a carga de trabalho era inegavelmente muito alta, visto que as células deveriam ser monitoradas e marcadas diariamente para garantia de que as colônias permaneciam distantes umas das outras e livres de contaminação cruzada.

Embora tenhamos selecionados muitos clones por esses métodos e sequenciado o DNA de 37 deles, não encontramos nenhum com mutações em homozigose, somente alguns poucos heterozigotos. A baixa eficiência pôde ser explicada por um somatório de fatores de cada etapa, que só foram esclarecidos posteriormente. Primeiro, a baixa eficiência de transfecção que, mesmo atingindo até 70 % em HEK293 utilizando pIRES-eGFP, na prática era possivelmente menor, já que o plasmídeo CRISPR possui o dobro do tamanho. Em segundo, a taxa de clivagem do gRNA utilizada é baixa, diminuindo a quantidade de eventos passíveis de ocorrência mutação. Por fim, os métodos de plaqueamento utilizados são sujeitos à contaminação cruzada por outras células, que podem ser um fator de confusão na análise do DNA – um cromatograma contendo duas sequências distintas não neces-

sariamente corresponde a um heterozigoto, pois pode significar uma população mista em que parte das células é mutada e parte não.

Identificados os pontos falhos do projeto, adquirimos um novo vetor CRISPR contendo um repórter GFP para selecionarmos somente as células transfectadas. Além disso, buscamos por um equipamento de FACS para realizarmos plaqueamento em *single-cell*. Para auxiliar a expansão dos clones, fizemos uso de meio condicionado com alto teor de soro fetal bovino. Assim, com os novos ajustes no protocolo, foi possível obter células HEK293 nocautes para *IDUA* e para *IDS*. As células HepG2, porém, não expandiram a partir de células únicas e foram desconsideradas.

Na ocasião em que estes experimentos foram conduzidos, a diferença de 10× de eficiência de edição entre os genes nos surpreendeu muito. De fato, com a literatura crescente sobre o assunto nos últimos anos, já foi demonstrado que gRNAs podem ter eficiências de reconhecimento e clivagem do alvo muito variadas, mesmo que sejam em locais adjacentes. Fatores como sequência e ordem dos nucleotídeos influenciam na atividade do sistema, assim como mecanismos epigenéticos (HINZ *et al.*, 2015; LIU *et al.*, 2016). Dados de atividade de clivagem de cada gRNA não foram coletados; porém, com as ferramentas disponíveis (como o TIDE, por exemplo), pretendemos repetir os experimentos de transfecção, quantificar a atividade de clivagem e, se possível e interessante, testar novos gRNAs para o gene *IDUA*. Além disso, também pretendemos avaliar o acréscimo de eficiência após múltiplas rodadas de transfecção.

Convertemos todo o aprendizado deste projeto em um capítulo de livro explicando o processo como um todo, além de um protocolo detalhado de geração de modelos nocaute, apresentando as dificuldades e as eficiências de cada etapa. O objetivo destes trabalhos é auxiliar pesquisadores que queiram criar modelos celulares e que não possuam experiência com edição genômica.

Uma vez que obtivemos o modelo nocaute inicial, buscamos por uma linhagem celular humana que fosse relevante para estudos de fisiopatologia das MPS. Optamos pela linhagem SH-SY5Y, muito utilizada em estudos de neurodegeneração por serem passíveis de diferenciação em neurônios funcionais. Embora os níveis de transfecção tenham sido baixos (~10 % de células GFP+ em cada experimento), a possibilidade de selecionar e isolar somente células GFP+ aumentou em muito a eficiência do processo como um todo, resultando na obtenção de células mutadas em homozigose sem grandes dificuldades com

o novo protocolo. Escolhemos o clone de MPS II contendo a variante c.259insA, por ser uma variante simples e em homozigose que se confirmou posteriormente gerar o nocaute do gene, já que a atividade enzimática de IDS foi nula no extrato celular.

As células SH-SY5Y mutadas para IDS (SH-MPS II) foram analisadas preliminarmente utilizando principalmente citometria de fluxo e marcadores de organelas. O objetivo inicial foi verificar, de modo abrangente, se algum desses marcadores se encontrava alterado e se o modelo desenvolvido apresentava diferenças morfológicas em relação às células normais para que, posteriormente, análises dirigidas pudessem ser executadas. Embora tenha sido possível realizar apenas um único experimento para cada marcador, alguns ensaios mostraram grandes diferenças entre os grupos. A quantidade de autolisossomos, por exemplo, foi quatro vezes maior nas células SH-MPS II, e a massa mitocondrial, duas vezes. A melhora observada com o tratamento das células SH-MPS II utilizando a enzima recombinante confirma que as alterações morfológicas encontradas são em decorrência da indução da variante e do fenótipo de MPS II, e não devido a uma eventual seleção de um clone previamente distinto.

Algumas alterações na autofagia já foram descritas no cérebro de animais MPS IIIB (LOTFI *et al.*, 2018), MPS IIIC (MARTINS *et al.*, 2015; PSHEZHETSKY, 2016) e MPS VI (TESSITORE *et al.*, 2009). Em nosso estudo, os dados obtidos por estes experimentos iniciais indicam claramente que o processo de autofagia está alterado também em nosso modelo celular. Contudo, por termos utilizado uma marcação estática com laranja de acridina, não é possível concluir quais etapas estão disfuncionais. Considerando a literatura no assunto, é provável que a sinalização para autofagia esteja aumentada, com aumento da formação de autofagossomos e autolisossomos; porém, a conclusão do processo autofágico possivelmente não ocorre, por dificuldades na degradação do conteúdo nos autolisossomos (PIERZYNOWSKA *et al.*, 2020). Inclusive, o aumento do pH lisossomal em decorrência de outros processos poderia estar contribuindo para o déficit autofágico. Seguindo neste raciocínio, a marcação de lisossomos que observamos com Lysotracker pode ter sido subestimada, já que o marcador não fluoresceria em compartimentos menos ácidos (HEATON *et al.*, 2020). De todo modo, prevemos a repetição destes experimentos e a inclusão de outros ensaios, como quantificação de autofagossomos e o *clearance* dos autolisossomos, a fim de clarificar especificamente quais etapas da autofagia estão alteradas. O uso de marcadores acídicos específicos para faixas de pH restritas (YAPICI *et al.*, 2015)

também pode esclarecer se ocorre aumento de pH lisossomal e/ou se o número de lisossomos é distinto nas células SH-MPS II.

A autofagia é um processo de degradação de várias macromoléculas, inclusive de organelas. Para que o processo ocorra eficientemente, o lisossomo precisa estar funcionando adequadamente. Nas doenças lisossômicas, cujo funcionamento do lisossomo é afetado, a autofagia é inevitavelmente comprometida. Uma vez que a reciclagem dos materiais não é completa, há acúmulo de substratos e de organelas, como mitocôndrias. Aumento de mitocôndrias disfuncionais já foi relatado em diversas doenças lisossômicas (IVANOVA *et al.*, 2019; RYAZANTSEV *et al.*, 2007; STEPIEN *et al.*, 2020), e acredita-se que isso impacte em múltiplos processos celulares, contribuindo para a patogênese das doenças. Observamos duas vezes mais massa mitocondrial e um modesto aumento de espécies reativas de oxigênio nas células SH-MPS II. O aumento de mitocôndrias pode ser em decorrência – além da mitofagia alterada – de outros mecanismos falhos, como deficiência de coenzima Q10, por exemplo (MARTINS *et al.*, 2015). Pretendemos executar experimentos de colocalização entre autolisossomos e mitocôndrias para averiguar se, de fato, as mitocôndrias estão acumulando devido à autofagia ineficiente. Ensaios para medição de níveis de antioxidantes também poderão ser feitos para observar se há mecanismos compensatórios do estresse oxidativo.

A escolha da linhagem SH-SY5Y se deu pelo potencial de diferenciação em células neuronais. Assim, um teste preliminar de diferenciação das células foi realizado, no qual o desenvolvimento de neuritos foi acompanhado. Os neurônios derivados de SH-MPS II apresentaram significativamente menos neuritos do que neurônios derivados de células normais. Neuritogênese alterada e/ou redução da arborização neuronal já foi descrita em modelo neuronal de MPS I (LITO *et al.*, 2020) e em outras doenças lisossômicas (LANGE *et al.*, 2018), e pode constituir um dos mecanismos de morte celular, já que a comunicação entre neurônios é fundamental para sua sobrevivência (PARA *et al.*, 2020). A avalição de proteínas sinápticas também pode vir a esclarecer se a comunicação neuronal está alterada nesse modelo.

Outros modelos celulares para as MPS já foram desenvolvidos, utilizando principalmente células-tronco pluripotentes induzidas (iPSCs, do inglês *induced pluripotent stem cells*) diferenciadas em células-tronco neurais (NSC, do inglês *neural stem cell*). Muitas das alterações descritas nesses modelos se sobrepõem entre as diferentes MPS, o que suge-

re que os mecanismos fisiopatológicos são similares – especialmente para as MPS com acúmulo de sulfato de heparana, considerado o responsável pelas manifestações neurológicas. A Tabela 2 abaixo apresenta as principais alterações descritas nestes modelos baseados em iPSCs.

Tabela 2. Principais alterações descritas em modelos neuronais de MPS criados a partir de iPSCs.

	MPS I	**MPS II**	**MPS IIIB**	**MPS IIIC**	**MPS VII**
Apoptose		aumentada			
Atividade neuronal				reduzida	reduzida
Autofagia	Expressão gênica aumentada	aumentada			Fusão com lisossomos prejudicada
Autofagossomos	Fusão com lisossomos prejudicada	aumentado			
Biogênese de GAGs	alterada				
Complexo de Golgi		alterado	alterado		
Comunicação neuronal				reduzida	reduzida
Expressão de GFAP		aumentada			aumentada
Lisossomos	aumentado	aumentado			
Matriz extracelular (expressão gênica)	alterada		alterada		
Migração celular	alterada				
Neuritogênese	reduzida				
Retículo endoplasmático		alterado			
Referências	(LITO *et al.*, 2020; SWAROOP *et al.*, 2018)	(KOBOLAK *et al.*, 2019)	(LEMONNIER *et al.*, 2011)	(CANALS *et al.*, 2015)	(BAYO-PUXAN *et al.*, 2018)

MPS: mucopolissacaridose; GAGs: glicosaminoglicanos; GFAP: proteína glial fibrilar ácida.

Combinando as alterações observadas nos modelos celulares, modelos animais e tecidos cerebrais de pacientes, o modelo SH-MPS II que desenvolvemos apresentou alterações morfológicas condizentes com as já descritas nas MPS. Infelizmente, as análises realizadas no nosso modelo foram todas realizadas uma única vez, em decorrência do crescimento limitado das células. Após alguns meses em cultivo, as células SH-MPS II cessaram

sua proliferação e os estoques preservados em nitrogênio líquido não possuíam viabilidade adequada para expansão das células e continuidade/reprodução dos experimentos. Neste contexto, possuímos algumas hipóteses para a queda da proliferação das células SH-MPS II: a) o acúmulo crescente de substrato no interior das células, com autofagia limitada, pode ser tóxico e explicar porque as células expandiram normalmente no início e cessaram a expansão progressivamente; b) seleção inicial de um clone contendo outras variantes prévias não identificadas ou c) técnica de cultivo inadequada, com repiques frequentes em baixa confluência de células.

A perspectiva para conclusão desse projeto é a geração de novos modelos, incluindo para MPS I, e a seleção de mais clones com mutações diversas, para que eles possam ser comparados entre si quanto à capacidade de proliferação e o tempo de cultivo. Uma vez obtidas e selecionadas as células, a criação de um grande estoque de células criopreservadas logo nas primeiras passagens pode garantir seu uso por períodos maiores. Ademais, também pretendemos testar a indução de mutações específicas utilizando oligonucleotídeos doadores da sequência mutada, para que ela seja inserida por recombinação no momento do reparo da quebra do DNA. Assim, poderemos mimetizar o fenótipo de variantes específicas encontradas nos pacientes, como as geradoras de fenótipo grave (como p.Trp402*) ou atenuado (como p.Arg89Gln) (POLETTO *et al.*, 2018). Uma vez obtidos novos modelos, todos os experimentos serão refeitos, a fim de verificar se as alterações observadas se reproduzem em outros modelos portadores de mutações distintas e se o mesmo ocorre na MPS I.

Mesmo com poucos experimentos executados neste primeiro momento, o modelo que desenvolvemos demonstrou alterações fenotípicas importantes, condizentes com o observado em outros modelos de MPS ou doenças lisossômicas. Inclusive, considerando que os mecanismos fisiopatológicos parecem ser muito similares entre as MPS, o entendimento de uma contribui significamente para o estudo das demais, até mesmo no desenvolvimento de novas terapias.

A edição genômica por CRISPR/Cas9 não possui potencial somente como ferramenta de pesquisa básica, mas também para o desenvolvimento de produtos de terapia avançada. Para tratamento de câncer, por exemplo, a edição genômica é usada tanto em protocolos de terapia gênica *in situ*, com aplicação diretamente no tumor, como no desenvolvimento de células T quiméricas do receptor de antígeno (CAR, do inglês *chimeric an-*

tigen receptor) para aplicação *ex vivo* (MOLLANOORI *et al.*, 2018). Embora muitos estudos tenham sido desenvolvidos para doenças monogênicas utilizando a modalidade *in vivo* – inclusive em nosso grupo de pesquisa (SCHUH *et al.*, 2019; SCHUH *et al.*, 2018) – umas das opções mais interessantes e potencialmente eficazes do uso deste tipo de ferramenta é visando a terapia gênica *ex vivo* por meio do transplante autólogo de células-tronco hematopoiéticas. Essa abordagem apresenta vantagens importantes, como a ausência de imunogenicidade – efeito adverso já descrito para terapias *in vivo,* em que o sistema imune combate o vetor administrado, diminuindo a eficácia e podendo, inclusive, gerar hepatotoxicidade (COLELLA *et al.*, 2018). Além disso, a modalidade *ex vivo* já está sendo aplicada na clínica, o que corrobora seu potencial (FRANGOUL *et al.*, 2020).

Os primeiros estudos utilizando o modelo de transplante autólogo e células editadas foram destinados às doenças hematológicas, por apresentarem fisiopatologia conhecida e geralmente afetarem somente o tecido sanguíneo. É o caso das beta-hemoglobinopatias anemia falciforme e beta-talassemia, por exemplo. Embora ambas sejam causadas por mutações no gene *HBB* que codifica a subunidade beta da hemoglobina, as duas doenças possuem fisiopatologia distintas; porém, a indução da produção de hemoglobina fetal suprime o fenótipo patológico de ambas (YE *et al.*, 2016). A estratégia de edição utilizada em ensaio clínico foi a indução de mutações para reprimir o *enhancer BCL11A,* resultando na reativação da síntese de hemoglobina fetal nas células. *In vivo*, observou-se que as células produtoras de hemoglobina fetal possuem vantagem seletiva em relação às células não produtoras dos pacientes, o que torna o tratamento ainda mais eficiente. Os pacientes apresentaram melhora clínica significativa, eliminando a necessidade de transfusões de sangue (FRANGOUL *et al.*, 2020).

Embora o estudo supracitado seja o único realizado em pacientes que já apresenta resultados preliminares publicados, muitos ensaios pré-clínicos foram realizados para outras doenças monogênicas, conforme apresentados na Tabela 3.

Tabela 3. Estudos pré-clínicos para doenças monogênicas utilizando edição genômica em células-tronco hematopoiéticas

Nome da doença	Gene editado	Referência
Anemia de Fanconi	*FANCA*	(DIEZ *et al.*, 2017)
Anemia Falciforme	*HBB*	(ANTONIANI *et al.*, 2018; DEVER *et al.*, 2016; DEWITT *et al.*, 2016; HOBAN *et al.*, 2016; PARK *et al.*, 2019; YE *et al.*, 2016)
Anemia Falciforme / Beta-Talassemia	*BCL11A/ HBG1/ HBG2*	(CHANG *et al.*, 2017; DEMIRCI *et al.*, 2020; HUMBERT *et al.*, 2019; LI *et al.*, 2018; METAIS *et al.*, 2019)
Ataxia de Friedreich	*FXN*	(ROCCA *et al.*, 2020)
Beta-Talassemia	*HBB*	(ANTONY *et al.*, 2018; PATSALI *et al.*, 2019; XU *et al.*, 2019)
Doença de Gaucher	*CCR5*	(SCHARENBERG *et al.*, 2020)
Doença Granulomatosa Crônica ligada ao X	*CYBB*	(DE RAVIN *et al.*, 2017)
Epidermólise Bolhosa Distrófica Recessiva	*COL7A1*	(OSBORN *et al.*, 2018)
Imunodeficiência Severa Combinada ligada ao X	*IL2RG*	(PAVEL-DINU *et al.*, 2019)
Mucopolissacaridose do tipo I	*CCR5*	(GOMEZ-OSPINA *et al.*, 2019)
Síndrome de desregulação imune, poliendocrinopatia e enteropatia ligada ao X	*FOXP3*	(GOODWIN *et al.*, 2020)
Síndrome da Hiperimunoglobulina M ligada ao X	*CD40LG*	(KUO *et al.*, 2018)
Síndrome de Wiskott-Aldrich	*WAS*	(GUTIERREZ-GUERRERO *et al.*, 2018; RAI *et al.*, 2020)

Até 2019, nenhum estudo para doenças lisossômicas havia sido publicado utilizando terapia gênica *ex vivo*. A partir do sucesso obtido na edição das linhagens celulares e nos estudos *in vivo* realizados no laboratório, objetivamos utilizar essa ferramenta para edição de células-tronco hematopoiéticas (CTH) visando o tratamento da MPSI.

Entretanto, nossas tentativas de transfecção de CTH foram todas infrutíferas. Foram testados protocolos baseados em lipofecção e em eletroporação – o primeiro resultou em transfecção nula, enquanto o segundo induziu morte celular muito pronunciada. Uma possível explicação para a baixa eficiência de transfecção das CTH é pela resposta imune inata – células imunes e suas progenitoras apresentam um mecanismo de reconhecimento de moléculas e de DNA exógenos, mediado por receptores tipo Toll (TLR, do inglês, *toll-like receptor*). Células CD34+ humanas, particularmente, expressam TLR3 (que reconhece RNA dupla-fita), TLR4 (expresso na membrana plasmática), TLR7 e TLR8 (que reconhe-

cem RNA fita simples) e, finalmente, TLR9, que é expresso em endossomos e é responsável por reconhecer DNA dupla-fita não metilado (PIRAS *et al.*, 2020). Uma vez que a lipofecção é mediada por endocitose, o DNA plasmidial dupla-fita transfectado é reconhecido pelo TLR9 no endossomo, que ativa uma cascata de sinalização resultando em morte celular e/ou diferenciação enviesada em células mieloides. Por consequência, o DNA transfectado não chega ao núcleo e/ou as células perdem viabilidade e seu fenótipo indiferenciado (ESPLIN *et al.*, 2011). O fracasso da eletroporação, por outro lado, pode ser explicado por parâmetros de voltagem e de pulso inadequados, já que esse método é utilizado atualmente para entrega de proteínas e RNAs às CTH (BAK; DEVER*; et al.*, 2018).

Em 2018, porém, foi publicado por um grupo da Universidade de Stanford, nos Estados Unidos, um protocolo de edição de CTH com alta eficiência, utilizando eletroporação para entrega da Cas9 e do gRNA complexados e transdução das células por vetor recombinante viral adenoassociado (rAAV) para entrega do DNA molde para edição por recombinação homóloga (BAK; DEVER*; et al.*, 2018). Em seguida, foi publicado um trabalho com a aplicação do protocolo para MPS I, utilizando células humanas editadas e transplantadas em um novo modelo de camundongo imunocomprometido com MPS I, desenvolvido especialmente para estes estudos (GOMEZ-OSPINA *et al.*, 2019). Em face aos resultados promissores apresentados, iniciamos uma colaboração com o grupo para aperfeiçoamento da terapia desenvolvida.

O método de edição utilizado previamente foi mantido. Fizemos uso do mesmo gRNA com alvo no gene *CCR5*, uma vez que este guia já havia demonstrado alta atividade de clivagem no alvo e caracterização completa quanto à segurança – identificação e quantificação de *off-targets*, análise da via p53 em células editadas e autópsias em mais de 200 animais transplantados. O uso do gene *CCR5* como um *"safe harbor"* é uma estratégia interessante para que mais doenças possam ser tratadas utilizando um único sistema, o que elimina a necessidade de caracterização completa para cada alvo. O gene *CCR5* codifica para o receptor de quimiocina C-C tipo 5, expresso em linfócitos e conhecido por ser o receptor utilizado pelo vírus HIV para infecção. Embora haja indícios de maior susceptibilidade a infecções pelo vírus do Nilo Ocidental (LIM *et al.*, 2006), a inativação do gene não possui efeitos deletérios ao indivíduo e confere imunidade ao vírus HIV (LIU *et al.*, 1996), o que, inclusive, é alvo de estudos de edição genômica (XU *et al.*, 2017).

O estudo inicial demonstrou bons níveis de atividade enzimática no cérebro de animais transplantados, mas que não se traduziu em diminuição significativa dos glicosaminoglicanos (GAGs) acumulados no tecido (GOMEZ-OSPINA *et al.*, 2019). Objetivando o aumento da correção bioquímica no SNC, fizemos uso de outro agente condicionante, bussulfano. Alguns trabalhos na literatura já apresentam o bussulfano como uma opção mais eficiente à migração e enxertia de células transplantadas no SNC em comparação com irradiação (CAPOTONDO *et al.*, 2012; WILKINSON *et al.*, 2013). O mecanismo ainda não é completamente conhecido, mas sugere-se que o bussulfano estimule a liberação de citocinas envolvidas no recrutamento de monócitos (CAPOTONDO *et al.*, 2012; WILKINSON *et al.*, 2013). Por outro lado, a irradiação, comumente utilizada em protocolos animais, induz forte inflamação tecidual e rompimento temporário da barreira hematoencefálica (KIERDORF *et al.*, 2013; LAMPRON *et al.*, 2012). Recentemente, demonstrou-se que irradiação também causa diminuição de células precursoras neuronais, o que diminui a neurogênese, além de diminuir sinapses em longo prazo e prejudicar a locomoção (HOHSFIELD *et al.*, 2020). Sendo assim, a utilização de bussulfano como agente condicionante se torna mais assertiva para aumentar o efeito do TCTH no SNC. Deveras, utilizando bussulfano, obtivemos atividade enzimática cerebral muito superior ao estudo anterior, com dois animais apresentando níveis acima de 50 % do normal. Este aumento pronunciado resultou em redução significativa de GAGs, embora sem uma normalização completa dos seus níveis.

Embora tenhamos demonstrado melhora bioquímica evidente e presença de DNA humano na microglia total isolada dos animais transplantados, não obtivemos sucesso na imunofenotipagem das células humanas enxertadas. Inicialmente, tentamos analisar microglia isolada dos animais por citometria de fluxo, de modo semelhante ao utilizado para análise da medula óssea. O painel consistiu em marcadores hematopoiéticos (hCD45, mCD45) e marcadores mieloides/monocíticos (m/hCD11b, hCD14, hCX3Cr1). Em todas as tentativas, o número de células humanas (hCD45+) era inferior a 0,5 %, o que não corresponde aos dados obtidos por PCR digital para quantificação de DNA humano, que chegou a 14 % em relação ao DNA total da amostra. Experimentos paralelos realizados com células humanas e murinas GFP+ transplantadas intravenosas ou intracerebroventricular também resultaram em porcentagens baixas na avaliação por citometria de fluxo. Testamos, também, marcação imunofluorescente com hCD11b em cortes histológicos, mas os

animais MPSI possuem autofluorescência importante em decorrência do acúmulo de substrato generalizado, o que prejudicou a identificação adequada de potenciais células positivas. Por fim, testamos imunohistoquímica para proteína IDUA, que resultou em marcação positiva inespecífica difusa no córtex e, principalmente, nas células de Purkinje no cerebelo. Vale ressaltar que a disponibilidade de tecido é muito pequena e poucos experimentos podem ser realizados por animal. Considerando como prioridade a avaliação bioquímica, destinamos metade do cérebro para esse fim, restando apenas a outra metade para as demais análises, o que nos limitou à escolha de análise histológica ou isolamento de microglia e citometria de fluxo, não podendo ser possível a realização dos dois no mesmo animal.

Neste estudo também não foi possível realizar testes comportamentais para avaliarmos a melhora fenotípica dos animais. Na nossa coorte, a maioria dos animais teve alto quimerismo humano na medula óssea, acima de 50 %, o que pode resultar em anemia nos animais após 16 semanas do transplante. Isso porque, apesar de ocorrer eritropoiese completa na medula óssea, eritrócitos maduros humanos não são encontrados na periferia em número adequado (BEYER *et al.*, 2017), sendo sequestrados e destruídos no fígado murino (SONG, Y. *et al.*, 2019). Com o alto quimerismo humano, há menos células-tronco murinas e a produção de eritrócitos não é suficiente. Portanto, os animais permanecem saudáveis por até ~120 dias, período de vida dos eritrócitos maduros produzidos antes do transplante (HIGGINS, 2015). Após esse prazo, os animais podem apresentar anemia severa, definhando rapidamente. Inclusive, dois animais da coorte tiveram sua eutanásia antecipada da data prevista por questões éticas e humanitárias. Por conseguinte, a análise comportamental é inevitavelmente comprometida, podendo ser realizada somente em animais com quimerismo intermediário, como ocorreu no estudo anterior – cujos testes foram realizados somente em animais com até 50 % de quimerismo humano. Em testes futuros, transfusões de sangue frequentes aos animais podem ser feitas para aumentar a expectativa de vida e permitir a realização de testes comportamentais, mesmo em animais com alto quimerismo.

Além da migração aumentada de células ao SNC, sugerimos que o bussulfano também permitiu maior enxertia de células editadas em comparação com a irradiação, como visto na medula óssea. Entretanto, para confirmação desta hipótese e comparação direta entre os regimes condicionantes, experimentos devem ser conduzidos com a utilização do mesmo doador de células, já que neste trabalho os transplantes foram realizados com doa-

dores independentes, que podem apresentar grande variabilidade entre si (DE RAVIN *et al.*, 2017).

A aplicabilidade dessa abordagem é grande para doenças lisossômicas. Em um estudo paralelo, editamos CTH humanas para o tratamento da doença de Gaucher, que também apresenta manifestações neurológicas nas formas graves. O gene alvo também foi o "*safe harbor" CCR5,* porém a abordagem foi diferenciada: inserimos o cDNA da proteína glicocerebrosidase (GBA) sob o comando do promotor CD68, cuja expressão é específica em células mieloides/monocíticas. Considerando que os macrófagos deficientes de GBA são os principais envolvidos na fisiopatologia da doença (chamados de células de Gaucher) e que a expressão de GBA em CTH demonstrou-se possivelmente tóxica, encontramos no promotor linhagem-específico uma maneira de direcionar a expressão do transgene nas células mais importantes para a doença. Na ausência de um modelo animal de Gaucher que permitisse a enxertia de células humanas, transplantamos as células modificadas em animais NSG para avaliação da expressão do transgene e confirmamos que as CTH editadas são capazes de se diferenciar em células mieloides e que a enzima GBA só é expressa nessa fração celular (SCHARENBERG *et al.*, 2020).

Com esses estudos, demonstramos que as CTH editadas são seguras e são eficazes para expressão da enzima deficiente em modelos de transplante autólogo. Inclusive, a expressão pode ser controlada por promotores específicos e direcionadas às células de interesse. Poucas questões devem ser respondidas antes de avançar para a clínica, e a principal delas é a resposta imune do organismo. Em primatas não humanos, foi constatado que o sistema imune combateu CTH modificadas que produziam GFP, uma proteína não congênita. A resposta pode ser controlada com condicionamento prévio adequado que inclui uso de imunossupressores, mas mais testes devem ser conduzidos (DRYSDALE *et al.*, 2020).

Uma das perspectivas para esse estudo é a reprodução da terapia utilizando CTH murinas editadas transplantadas em modelo animal imunocompetente. Dessa forma, seria possível avaliar a) a resposta imune ao transgene, b) a reconstituição hematopoiética adequada, por haver a sinalização necessária e c) a eficácia no sistema nervoso central quando as condições são ideais, isto é, com as citocinas apropriadas da espécie.

Considerando que o TCTH para doenças neurodegenerativas deve ser realizado o quanto antes a fim de evitar a progressão irreversível do dano tecidual, desenvolvemos um projeto para determinação da melhor via de distribuição de células hematopoiéticas muri-

nas em camundongos imunocompetentes, visando a aplicação futura de protocolos de transplante de CTH editadas, com especial interesse na migração das células para o sistema nervoso.

Este projeto fez-se necessário em razão da dificuldade que tínhamos com injeções intravenosas em camundongos neonatos, cujo principal acesso é pela veia temporal. Em estudos anteriores do grupo, muitos animais foram perdidos em decorrência da administração falha. De fato, esta é uma via muito desafiadora, pois a veia temporal de um neonato possui o calibre similar ao da agulha utilizada na injeção. Ademais, modelos murinos com pelagem escura dificultam a visualização da veia sob a pele. Assim, nos propomos a testar diferente rotas de administração de células precursoras hematopoiéticas e a avaliá-las quanto à facilidade de execução, desconforto do animal, sucesso de injeção e distribuição das células nos tecidos, incluindo ao SNC.

A escolha da via retro-orbital se deu como alternativa intravenosa à veia temporal, enquanto a via intraperitoneal foi utilizada como um "controle não intravenoso". Utilizando animais GFP+ como doadores para permitir o rastreamento das células, testamos inicialmente a distribuição de células hematopoiéticas Lin- sem condicionamento prévio da medula óssea dos receptores. Conforme esperado, a ausência de condicionamento resultou em baixo quimerismo e detecção de poucas células em alguns animais apenas. Em seguida, introduzimos condicionamento com bussulfano em dose considerada de baixa intensidade, pré-definida em estudo piloto. Com o novo desenho experimental, pudemos observar enxertia das células nos tecidos hematopoiéticos e, inclusive, no cérebro de alguns animais.

Ainda que tenha sido apenas um animal em cada tempo analisado, somente a via retro-orbital apresentou animais com número importante de células GFP+ no cérebro. Teoricamente, as duas vias intravenosas deveriam apresentar o mesmo resultado; porém, a performance superior da via retro-orbital já foi descrita em outros estudos com animais adultos (GRUNTMAN *et al.*, 2017; LEON-RICO *et al.*, 2015).

Este estudo possui limitações importantes que devem ser endereçadas. Primeiro, o tamanho amostral é, infelizmente, muito pequeno e impede análises mais robustas. A variabilidade intragrupo permitiu somente a observação de tendências. Estudos de TCTH em camundongos adultos realmente apresentam variabilidade muito grande – inclusive em nosso estudo de MPS I, cujo quimerismo humano variou de 25 a 91 %, mesmo entre animais da mesma ninhada que foram transplantados com o mesmo *pool* de células.

Outra limitação importante foi a utilização de animais de linhagens diferentes, o que pode gerar resposta imune com desencadeamento de rejeição ou de doença do enxerto contra o hospedeiro (DECH). A escolha da linhagem 129SV como receptora de células se deu pela disponibilidade destes animais na unidade de experimentação. Como o período máximo de avaliação foi de 30 dias, observamos somente um caso de possível DECH aguda, desenvolvida aproximadamente 25 dias pós-transplante e manifestada como retardo no crescimento, microftalmia e sinais de sofrimento do animal. Em geral, mesmo em modelos animais de DECH, o desencadeamento da doença ocorre de 20 a 50 dias após o transplante (SCHROEDER *et al.*, 2011). Ainda assim, a utilização de animais incompatíveis pode ter alterado os resultados obtidos.

No momento da idealização do projeto, não tínhamos expertise em modelos de transplante e por isso não conseguimos avaliar completamente a distribuição das células, como por meio de citometria de fluxo, por exemplo. Alguns testes em sangue periférico e medula óssea foram feitos, mas os eritrócitos não foram removidos das amostras. A presença deste tipo celular inviabilizou a análise porque, além de apresentarem autofluorescência acentuada (SWENSON *et al.*, 2007) que dificultou a definição de células verdadeiramente positivas, os eritrócitos não expressam GFP (SPANGRUDE *et al.*, 2006), tornando o número de eventos GFP+ em relação ao total de eventos muito variável. Assim, os controles GFP+ (coletados de animais GFP) não foram reprodutíveis e optamos por não utilizar estes dados.

Mesmo com as limitações mencionadas, fomos capazes de observar células no cérebro, inclusive com morfologia de células tipo microglia, o que é de grande valia para estudos posteriores. Além disso, definimos que a via de utilização para estudos futuros será a retro-orbital, pela facilidade de execução e menor número de insucessos, o que diminui o consumo de animais desnecessários na pesquisa. Os dados gerados neste estudo serão importantes em projetos de transplante de CTH editadas murinas que serão desenvolvidos posteriormente no grupo de pesquisa.

O transplante de CTH autólogo, utilizando células editadas com CRISPR/Cas9, tem muito potencial clínico, por remover as limitações importantes do transplante de CTH alogênico (alo-TCTH) a apresentar as mesmas vantagens. O alo-TCTH é realizado há décadas e apresenta resultados muito bons para algumas doenças metabólicas. O mecanismo de ação clássico descrito é via correção cruzada, em que a enzima é secretada pelas células

transplantadas e captada pelas células hospedeiras deficientes, inclusive no sistema nervoso central (SNC). Entretanto, alguns estudos sugerem que a ação do TCTH é mais complexa e que a correção cruzada não é a única responsável pela melhora clínica. De fato, foi demonstrado recentemente em modelo de MPS II que neurônios não captam enzima extracelular de forma eficiente, acumulando metabólitos progressivamente (RYBOVA *et al.*, 2018). No último ano, foi sugerido que enzimas expressas por células da microglia não são captadas por outros tipos celulares, mesmo que eles expressem receptores de manose-6-fosfato (M6PR). Inclusive, a endocitose de enzimas em microglia parece ser mediada por outros receptores que não o M6RP. Ou seja, a melhora clínica observada pode não ser por correção cruzada de neurônios, astrócitos e oligodendrócitos, mas por outros mecanismos (KAMINSKI *et al.*, 2020).

A correção cruzada independente de M6PR pode estar ocorrendo por transferência de lisossomos entre as células. A transferência de organelas foi descrita pela primeira vez em 2004 (RUSTOM *et al.*, 2004), e foi demonstrada em modelo animal da doença lisossômica cistinose, causada por deficiência de uma proteína transmembrana. Em experimentos *in vitro* e *in vivo*, observou-se que macrófagos derivados de TCTH geram nanotubos de membrana, que atuam como pontes intercelulares capazes de penetrar matrizes extracelulares densas e realizar trocas de vesículas e organelas inteiras, como os lisossomos (NAPHADE *et al.*, 2015).

Este mecanismo pode explicar também por que o TCTH parece ser eficaz em Ataxia de Friedreich, uma doença neurodegenerativa causada por deficiência de uma proteína mitocondrial, a frataxina. Foi demonstrado que proteínas mitocondriais são transferidas das células transplantadas diferenciadas em microglia aos neurônios residentes, sendo que 50 % dos neurônios continham frataxina transferida (ROCCA *et al.*, 2017). O mecanismo exato ainda é desconhecido, mas pode ser que esteja ocorrendo a transferência de mitocôndrias inteiras pelos nanotubos de membrana, assim como já demonstrado para lisossomos.

A microglia também possui um papel fundamental no mecanismo de ação do TCTH. Sabe-se que a microglia derivada de monócitos (transplantados ou não) são diferentes da microglia residente, tanto transcricionalmente e morfologicamente, quanto epigeneticamente. Essas células possuem, porém, meia-vida longa, similar às células residentes, e atividade fagocítica aumentada (HOHSFIELD *et al.*, 2020), o que auxilia ainda mais no contexto de doenças lisossômicas ou neurodegenerativas.

Na adrenoleucodistrofia ligada ao X, por exemplo, a doença é causada pela deficiência de um transportador de colesterol expresso nos peroxissomos, o ALDP, gerando acúmulo de ácidos graxos de cadeia muito longa em tecidos e fluidos (TURK *et al.*, 2020). Esse acúmulo é particularmente importante no SNC, onde se concentra na mielina, gerando desmielinização acentuada. A fagocitose de mielina ocorre em condições normais e é responsável por polarizar macrófagos, ativando um estado anti-inflamatório que auxilia a regeneração tecidual. Nesta doença, a indução do estado anti-inflamatório é prejudicada e os macrófagos se mantêm em estado pró-inflamatório constante, inclusive aumentando progressivamente de tamanho pelo acúmulo de moléculas não degradadas e piorando o quadro de desmielinização. Na condição do TCTH, os monócitos/macrófagos saudáveis possuem sua atividade fagocítica intacta e possuem ação anti-inflamatória e regenerativa, com manutenção da remielinização (WEINHOFER *et al.*, 2018). Neste caso, portanto, a ação terapêutica do transplante se dá pela restauração da função de macrófagos e da microglia.

O TCTH também apresenta resultados positivos para leucodistrofia metacromática, causada por deficiência da enzima lisossômica arilsulfatase A, que gera acúmulo de esfingolipídios. Nessa doença também ocorre desmielinização importante, com diminuição significativa de oligodendrócitos – células responsáveis pela produção da mielina – pelo acúmulo de debris de mielina. Uma vez realizado o TCTH, macrófagos derivados migram ao SNC e fagocitam debris de mielina, o que permite a ativação de oligodendrócitos e a produção de mielina nova. De uma maneira geral, o transplante contribui para a manutenção e sobrevivência dos oligodendrócitos, uma vez que pacientes não transplantados apresentaram diminuição pronunciada desse tipo celular (WOLF *et al.*, 2020). Assim, neste caso, a melhora clínica observada pode ser em decorrência do processo de remielinização estimulado por células transplantadas, ao invés da correção cruzada clássica sugerida.

A Figura 8 resume os mecanismos de ação alternativos do TCTH, bem como as alterações morfológicas observadas nas MPS e em algumas doenças lisossômicas.

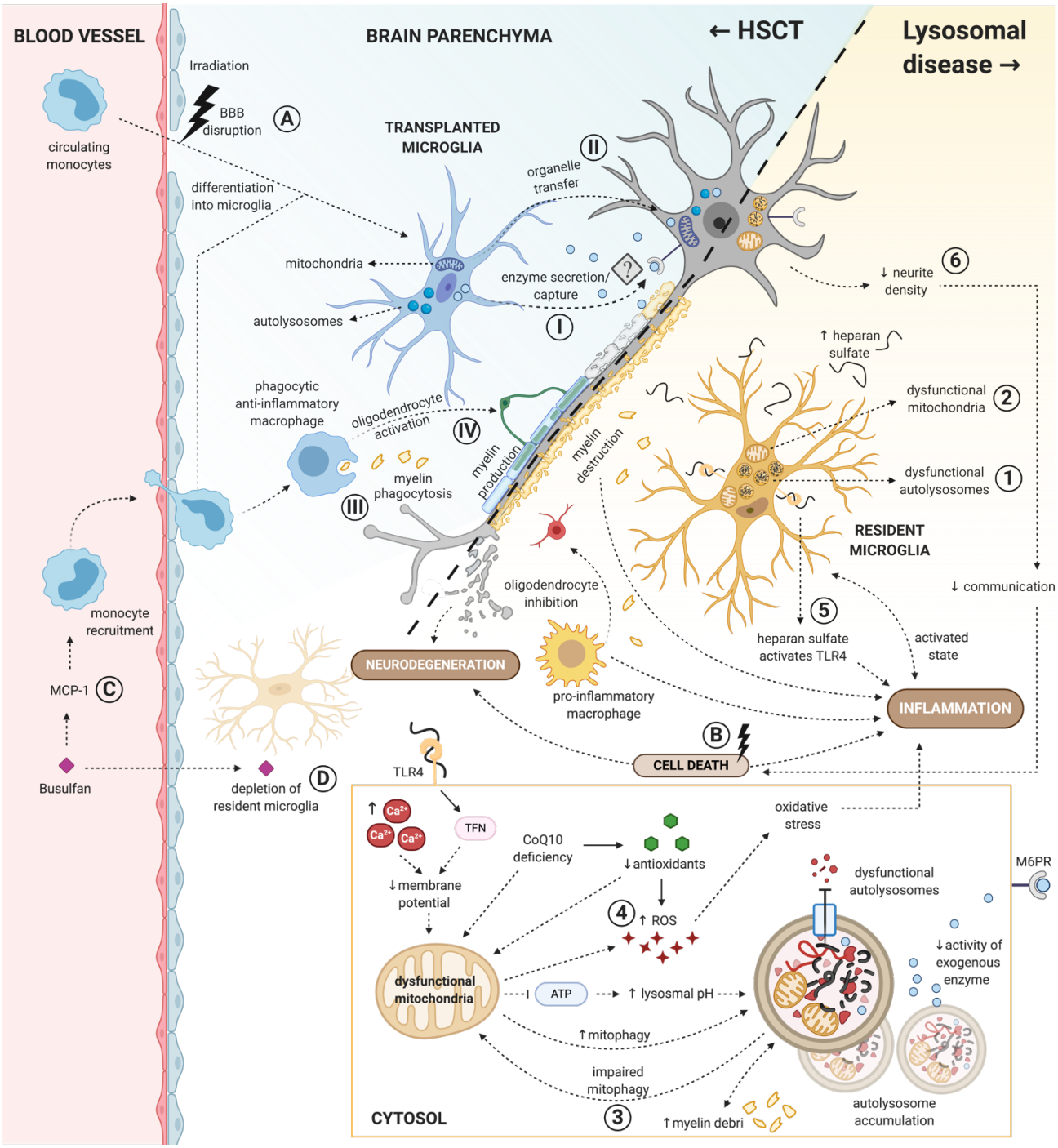


Figura 8. Resumo das alterações morfológicas observadas em modelo neuronal de mucopolissacaridose (MPS), possíveis mecanismos de ação alternativos do transplante de células-tronco hematopoiéticas (TCTH) e ação de agentes condicionantes no sistema nervoso central. As células com MPS possuem (1) aumento de autolisossomos não-funcionais, repletos de macromoléculas e organelas não degradadas em seu interior, impedindo o fluxo autofágico. Isso gera um acúmulo de mitocôndrias não-funcionais (2), possivelmente por redução da mitofagia (3), mas também por alteração de potencial de membrana, por deficiência de coenzima Q10 (CoQ10) e por diminuição de antioxidantes, conforme já descrito. Mitocôndrias defeituosas produzem menos ATP, o que aumenta o pH lisossômico e retroalimenta o processo patológico. Além disso, gera mais espécies reativas de oxigênio (ROS) (4), que contribui para o estresse oxidativo, aumentando a inflamação. Outro mecanismo descrito é a ativação de receptores tipo Toll 4 (TLR4) em decorrência do acúmulo de sulfato de heparana (5), que também aumenta inflamação. Por fim, também observamos diminuição da densidade de neuritos (6), o que prejudica a comunicação neuronal e pode contribuir para

a morte celular. Por outro lado, o TCTH pode atuar no SNC pelos seguintes mecanismos: (I) Células derivadas de monócitos transplantados são secretoras de enzima que, por sua vez, pode ser captada por outras células pelo receptor de manose-6-fosfato (M6RP). Este mecanismo, entretanto, parece não ser completamente eficiente em neurônios e microglia parece receber a enzima por outro meio. (II) Organelas podem ser transferidas por nanotubos de membrana da microglia derivada de monócito ao neurônio residente. Estas organelas podem ser lisossomos contendo a enzima ou mesmo mitocôndrias funcionais. (III) Macrófagos transplantados conseguem fagocitar metabólitos acumulados e debris de mielina, além de manter o estado anti-inflamatório que auxilia na contenção da neuroinflamação. (IV) Macrófagos anti-inflamatórios ativam/estimulam oligodendrócitos a produzirem mais mielina, remielinizando os neurônios. Condicionamento mieloablativo com irradiação rompe a barreira hematoencefálica (A) e causa morte neuronal (B), enquanto o agente bussulfano induz liberação de citocinas que estimulam o recrutamento de monócitos (C), além de causar morte de microglia residente (D), o que aumenta a enxertia de células transplantadas no parênquima cerebral.

Neste contexto, estes mecanismos alternativos recentemente descritos atuariam de forma muito positiva para as MPS. A transferência de lisossomos de células transplantadas para neurônios residentes corrigiria o acúmulo de autolisossomos e contribuiria para o processo de autofagia, que auxiliaria na mitofagia e reduziria os danos causados pelo acúmulo de mitocôndrias alteradas. Adicionalmente, a transferência de mitocôndrias funcionais reduziria a produção de espécies reativas de oxigênio, contribuiria para a manutenção do pH lisossomal e, de modo geral, auxiliaria na restauração da homeostase celular. Com atividade fagocítica aumentada, microglia derivada de monócitos transplantados podem reduzir o acúmulo de materiais não degradados no tecido, inclusive agregados proteicos – como alfa-sinucleína e placas amiloides, já relatadas em MPS (MARTINS *et al.*, 2015). Por fim, macrófagos transplantados seriam capazes de adotar um perfil anti-inflamatório e contribuir com a renovação da mielina e regeneração tecidual e, principalmente, com a diminuição do estado de neuroinflamação generalizado que ocorre nestas doenças.

Em conjunto, apresentamos na presente tese que a edição genômica com CRISPR/Cas9 é uma ferramenta muito útil para estudo e para tratamento de doenças monogênicas, especialmente doenças lisossômicas. Fazendo uso da edição genômica, pudemos desenvolver um modelo celular relevante para o estudo da MPS II, identificando possíveis mecanismos patogênicos da neurodegeneração nesta doença. Este modelo permitirá que mais estudos *in vitro* sejam conduzidos e que o entendimento da fisiopatologia seja ampliado. Utilizando edição genômica, também desenvolvemos um novo produto de terapia, baseado em transplante autólogo de células-tronco hematopoiéticas editadas, com resultados importantes no sistema nervoso central. Com condicionamento ablativo usando bussulfano, observamos atividade enzimática aumentada no cérebro de animais com MPS I, com consequente redução significativa de substrato acumulado, algo não observado ante-

riormente. Adicionalmente, estudamos diferentes vias que podem aumentar ainda mais a migração de células transplantadas para o cérebro dos animais. Uma vez que os processos fisiopatológicos parecem se sobrepor entre as MPS, estes estudos podem ser complementares, mesmo que tenham sido desenvolvidos para MPS diferentes.

Combinando estes estudos, fornecemos uma terapia potencialmente eficaz para MPS e, também, uma ferramenta importante de estudo. Esta pode, inclusive, ser utilizada para auxiliar no entendimento do mecanismo de ação completo do TCTH, incluindo os novos mecanismos propostos, podendo otimizar ainda mais a eficácia do tratamento na doença neurológica.

CONCLUSÃO

A conclusão geral desta tese é que edição genômica por CRISPR/Cas9 pode ser aplicada tanto na geração de modelos celulares de estudo de fisiopatologia quanto no desenvolvimento de novas terapias, como pudemos aplicar às mucopolissacaridoses.

- Foram desenvolvidos modelos celulares de MPS I e MPS II utilizando a linhagem celular HEK293, cujo processo foi descrito em forma de capítulo de livro e de artigo de métodos.
- Foi possível desenvolver um modelo celular relevante de MPS II utilizando a linhagem SH-SY5Y, que pode ser utilizada para estudos de fisiopatologia da doença neurológica observada nos pacientes.
- A eficiência do processo pôde ser avaliada e comparada entre os dois modelos nas etapas de transfecção, plaqueamento, expansão e edição, cujo resultado demonstrou que houve uma diferença de 10x na eficiência de nocauteamento entre os dois modelos, ressaltando a variabilidade do processo.
- Os modelos criados apresentaram mutações em homozigose e níveis de atividade enzimática não detectáveis, caracterizando o fenótipo básico das MPS.
- As células SH-SY5Y com MPS II apresentaram alterações morfológicas importantes, como aumento de autolisossomos e mitocôndrias, além de diminuição na densidade de neuritos formados quando diferenciadas em neurônios.
- Células-tronco hematopoiéticas editadas para superexpressarem IDUA enxertaram eficientemente em camundongos NSG-MPSI e mantiveram seu potencial tronco, como pôde ser observado pela presença de populações mieloides e linfoides na medula óssea 16 semanas pós-transplante e pelo alto quimerismo humano observado em transplante secundário.
- O transplante de células-tronco hematopoiéticas editadas conferiu correção bioquímica em todos os tecidos viscerais analisados, com altos níveis de atividade enzimática e normalização do acúmulo de GAGs. Inclusive, no sistema nervoso central, a atividade enzimática foi significativamente maior do que o apresentado em estudos anteriores, com redução significativa nos níveis de GAGs.
- O condicionamento com bussulfano apresenta melhor desempenho no que se refere à enxertia de células transplantadas no sistema nervoso central, como constatamos pela melhora pronunciada nos parâmetros bioquímicos avaliados em comparação com a

irradiação de corpo inteiro. Bussulfano também parece ter maior ação na enxertia de células editadas em comparação com a irradiação.

- Para o transplante de células hematopoiéticas em camundongos neonatos imunocompetentes, concluímos que a melhor via de administração é a via retro-orbital, por ter sido a que apresentou células no cérebro em maior quantidade e mais rapidamente, além de ter maior chance de sucesso na aplicação.

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ANEXOS

ANEXO I.

EFFECTS OF GENE THERAPY ON CARDIOVASCULAR SYMPTOMS OF LYSOSOMAL STORAGE DISEASES

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Review Article

Effects of gene therapy on cardiovascular symptoms of lysosomal storage diseases

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Abstract

Lysosomal storage diseases (LSDs) are inherited conditions caused by impaired lysosomal function and consequent substrate storage, leading to a range of clinical manifestations, including cardiovascular disease. This may lead to significant symptoms and even cardiac failure, which is an important cause of death among patients. Currently available treatments do not completely correct cardiac involvement in the LSDs. Gene therapy has been tested as a therapeutic alternative with promising results for the heart disease. In this review, we present the results of different approaches of gene therapy for LSDs, mainly in animal models, and its effects in the heart, focusing on protocols with cardiac functional analysis.

Keywords: Lysosomal storage disease, gene therapy, cardiovascular disease, animal models, heart.

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Introduction

Lysosomal storage diseases (LSDs) are a group of inherited disorders characterized by impairment of lysosomal function due to accumulation of undegraded or partially degraded metabolites. There are more than 50 disorders mainly caused by deficient lysosomal enzymes, but also by decreased function of membrane proteins or non-enzymatic soluble proteins, ultimately resulting in substrate accumulation, reduced lysosomal trafficking and cellular dysfunction (Platt *et al.*, 2012; Boustany, 2013).

Even though LSDs are monogenic, the phenotypes may vary considerably among individuals with the same disease depending on the mutation profile – while some alleles produce proteins with residual activity, others result in complete loss of function. Hence, the clinical spectrum amongst LSDs varies largely accordingly to the residual protein function in addition to the type of metabolite stored (Segatori, 2014).

LSDs are multisystemic and progressive, normally non evident at birth but leading to premature death if not treated (Platt *et al.*, 2012). Common symptoms shared by most of them are organomegaly, cognitive impairment, skeletal defects and coarse facial features, all at variable degrees (Ortolano *et al.*, 2014). Some have cardiovascular involvement, with cardiac failure generally being one of the main causes of death (Braunlin *et al.*, 2011).

In this context, defects in the glycogen, lipid or glycosaminoglycan metabolism lead to disturbed energy production, altered cellular homeostasis and consequent cardiomyopathy (Guertl *et al.*, 2000). Diseases such as glycosphyngolipidoses, mucopolyssacharidoses, and of glycogen storage, normally present significant cardiovascular manifestations, including hypertrophic and dilated cardiomyopathy, coronary artery disease, and valvular disease (Linhart and Elliott, 2007) (Table 1).

Current treatments for LSDs, such as intravenous enzyme replacement therapy (ERT) and hematopoietic stem cell transplantation (HSCT), normally have satisfactory response in some visceral organs such as the liver and spleen, but are insufficient to correct specific tissue manifestations, such as in brain, bones and some cardiovascular symptoms. The central nervous system (CNS) is hard to reach due to the blood brain barrier, which is mostly impermeable to exogenous enzymes and most drug therapies that use conventional administration routes (Parenti *et al.*, 2013). Bones normally respond poorly to treatments due to low vascu-

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Table 1 - Lysosomal storage diseases with cardiovascular involvement.

Disease	Enzyme	OMIM	Cardiovascular involvement	References
Fabry	α-Galactosidase A	301500	Left ventricular hypertrophy, myocyte hypertrophy and vacuolation, myocardial fibrosis, prominent papillary muscles, valve thickening and insufficiency and arrhythmias	(Linhart and Elliott, 2007)
Galactosialidosis	Cathepsin A	256540	Moderate mitral insufficiency, valve thickening, ventricular hypertrophy, myocardial tissue is thickened and vacuolated	(Senocak *et al.,* 1994) (Bursi *et al.,* 2003)
Gaucher disease	Glucocerebrosidase	2310000	Calcification of cardiac structures, mitral and aortic stenosis, thickened mitral and aortic valves and cardiomegaly	(Guertl *et al.,* 2000)
GM1-gangliosidosis	β-Galactosidase	230500	Cardiomegaly and congestive cardiomyopathy with decreased contractility	(Brunetti-Pierri and Scaglia, 2008) (Morrone *et al.,* 2000) (Guertl *et al.,* 2000)
GM2-gangliosidosis (Sandhoff)	β-Hexosaminidase	268800	Thickening of valves, valvular regurgitation, cardiomyopathy, intimal coronary artery thickening	(Venugopalan and Joshi, 2002) (Guertl *et al.,* 2000)
GSD IIa (Pompe)	α-Glucosidase	232300	Cardiomyopathy, cardiomegaly and heart failure, inrceased aortic stiffness, broad high voltage QRS complexes and short PR interval on ECG.	(Linhart and Elliott, 2007) (Hicks *et al.,* 2011) (Wens *et al.,* 2014)
GSD IIb (Danon)	LAMP-2	309060	Hypertrophic cardiomyopathy, severe conduction abnormalities	(Cheng and Fang, 2012)
MPS I (Hurler, Scheie)	α-Iduronidase	252800	Present in 60-100% cases - valve thickening, mitral and aortic regurgitation and/or stenosis, coronary artery disease, dilation of the ascending aorta and markedly reduced aortic elasticity, systemic hypertension due to arterial narrowing	(Braunlin *et al.,* 2011)
MPS II (Hunter)	Iduronate sulfatase	309900	Present in 60-100% cases - valve thickening, mitral and aortic regurgitation and/or stenosis, coronary artery disease, systemic hypertension due to arterial narrowing, conduction abnormalities and sinus tachycardia	(Braunlin *et al.,* 2011)
MPS IVA (Morquio A)	N-acetylgalactosamine-6 sulfatase	253000	Aortic and mitral valve dysplasia, coronary artery disease, moderate mitral and aortic regurgitation and valve thickening	(Braunlin *et al.,* 2011) (Rigante and Segni, 2002) (Hendriksz *et al.,* 2013)
MPS VI	arylsulfatase B	253200	Valve stenosis and/or insufficiency, cardiomyopathy and fibroelastosis	(Braunlin *et al.,* 2011) (Valayannopoulos *et al.,* 2010)
MPS VII (Sly)	β-glucuronidase	253220	Coronary artery disease, aortic dilation , thickened and stenotic aortic valve leaflets, intimal thickening of the aorta and muscular arteries, left ventricular hypertrophy	(Braunlin *et al.,* 2011) (Gniadek *et al.,* 2015)
Mucolipidosis II/III	N-acetylglucosamine-1-phosphotransferase	607840	Cardiomegaly, mitral and aortic valve thickening	(Cathey *et al.,* 2010)
Niemann Pick type A and B	Acid sphingomyelinase	607608	Mild mitral insufficiency, coronary artery disease (due to atherogenic lipid profile) , cardiomegaly with thickened left ventricular wall	(Senocak *et al.,* 1994) (McGovern *et al.,* 2013) (Guertl *et al.,* 2000)
Sialidosis	Sialidase	256550	Valve disease, ventricular hypertrophy	(Senocak *et al.,* 1994)

larization, preventing the therapy to reach the affected area (Clarke and Hollak, 2015). Accordingly, as paradoxical as it may seem, cardiovascular structures as heart valves and aorta are also poorly vascularized, making them less responsive to treatments as well (Ma *et al.*, 2007; Brands *et al.*, 2013).

Most therapeutic approaches for LSDs are based on the event of cross-correction, in which cells can uptake extracellular lysosomal enzymes – administered exogenously or secreted by other cells – via mannose-6-phosphate (M6P) receptor and route them to the lysosomes, where the pH is acid and they can work normally (Sands and Davidson, 2006). In addition, it has been suggested that a recovery of only about 10% of enzyme activity would be enough to prevent or even revert most clinical manifestations (Leinekugel *et al.*, 1992; Sands and Davidson 2006). Hence, modifying few cells would be enough to achieve a satisfying therapy for some LSDs and gene therapy is an approach that meets this idea.

Gene therapy

The sole purpose of gene therapy is to genetically modify targeted cells (Cotrim and Baum, 2008). Gene therapy is typically performed to correct mutated genes or to add functional copies of a required sequence, although it can also be used to provide new functions to the cells or even to silence overexpressing genes. Its different approaches depend on the pathological condition and targeted tissue and studies have been conducted for genetic, psychiatric, immune and cardiovascular diseases and cancer (Collins and Thrasher, 2015).

For the LSDs, gene therapy seems to have great potential. As opposed to ERT, the promise of gene therapy is that a good gene transfer method could bypass the blood-brain barrier and modify brain cells, which in turn would produce the missing enzyme and possibly brake the CNS manifestations (Zhang *et al.*, 2011).

There are different ways to deliver the gene of interest into cells: using viral or non-viral vectors and also performing gene transfer *in vivo* or *ex vivo*. The simplest way to deliver a gene is cloning it on a plasmid, together with regulatory sequences that ensure its transcription, and then inject it into a cell – this, however, is poorly efficient. Although non-viral vectors are very safe, due to their episomal conformation and very low immunogenicity, their main drawback is the low transfection efficiency. Many strategies have been developed to enhance non-viral gene delivery (Wang *et al.*, 2013; Jayant *et al.*, 2016), as electroporation, gene gun, hydrodynamic injection, nanotechnology-based carriers (Schuh *et al.*, 2016), DNA minicircle (Osborn *et al.*, 2011), and transposons (Aronovich *et al.*, 2009), but all still present low efficiency of transfection and specific procedure limitations.

Viral vectors are recombinant viruses lacking sequences for auto replication, while preserving the ones required to transduce cells, plus the gene of interest. Therefore, they still express surface proteins specific to certain cell types, making the transgene uptake possible through cellular endocytosis, but they fail in replicating and lysing cells (Wang and Gao, 2014). The process of transducing cells is normally highly efficient, making viral vectors the first choice of many studies. Currently, most used are retrovirus (RV), lentivirus (LV) adenovirus (AV), and adeno-associated virus (AAV).

Retroviral and lentiviral vectors are classicaly integrative vectors, i.e., they integrate the transgene into the genome of proliferating cells (and also quiescent cells in the case of lentivirus), which ensures persistent expression and stability. On the other hand, it also brings the risk of insertional mutagenesis by possibly activating oncogenes or disrupting tumor suppressor genes. Integration-deficient LVs are being developed to surpass this limitation, but its efficiency is yet to be determined (Alfranca *et al.*, 2018).

Alternatively, adenoviruses can transduce both quiescent and proliferating cells and do not integrate the sequence into the genome, residing episomally in the cell nucleus. Although this prevents insertional mutagenesis, the expression of the transgene is transient and eventually lost. Another disadvantage of AV is the very strong immunogenicity, leading to possible severe adverse effects in the host (Wold and Toth, 2013).

Adeno-associated viruses have strong tropism for certain cell types due to their capsid serotype, guaranteeing directed treatment to specific tissues (Wu *et al.*, 2006). The apparent lack of immunogenicity and the stable episomal conformation of the DNA, which promotes long-term transgene expression with minimal risk of insertional mutagenesis, are other vantages of this vector. Nevertheless, AAV vectors only enable the insertion of small transgene cassetes, limited to less than 5Kb (Salganik *et al.*, 2015).

Viral vectors are constantly engineered to develop safer versions, as by removing sequences that interact with neighbour genes (as the Long Terminal Repeat, LTR), or preventing the insertional mutagenesis by mutating the integrase or, yet, using gene editing tools to direct the integration site (Wang and Gao, 2014).

Gene therapy in LSD

There are many studies in the literature about gene therapy in LSDs with different types of vectors, administration protocols and/or auxiliary drugs. Since the cardiovascular system is one of the major systems affected in several LSDs and not responsive to current treatments, it has also been targeted in some gene therapy studies. Therefore, reports that address gene therapy effect on the cardiac system, most still in preclinical stage, are summarized over the next sections.

Glycogen Storage Diseases (GSD)

Pompe Disease (GSD Type II a)

Pompe disease, or Glycogen Storage Disease type II a (GSD-IIa), is caused by deficiency of lysosomal acid α-1-4-glucosidase (GAA). In this disorder, glycogen accumulates mainly within the heart and muscles, among other organs. Cardiac disease is characterized by hypertrophic cardiomyopathy, increased aortic stiffness (Wens *et al.*, 2014), broad high voltage QRS complexes, and short PR interval on electrocardiogram (ECG) (Linhart and Elliott, 2007).

Gene therapy for GSD-IIa has been extensively studied, with many different approaches focusing mainly on amelioration of skeletal and cardiac muscles. There is one study describing the use of lentivirus with positive results in the heart, as reduction of glycogen storage (Kyosen *et al.*, 2010), although the majority of published papers describe efforts to improve transduction efficiency and control of immune response using either adenovirus or AAV, as discussed further.

In 1998, a gene therapy protocol "was developed" using transmyocardial injection with adenoviral vector with GAA (AV-GAA) in newborn rats (Pauly *et al.*, 1998). GAA activity was measured in whole heart extracts at day 7, when the animals were euthanized, and resulted in 10-fold the value of the control groups (non-treated or mock treated). According to the authors, no deleterious effects were observed in any groups. Subsequently, analysis of vector sequences in different organs showed that the GAA activity observed in the heart was due to transduced cells in the liver, which would function as a factory of enzyme that could be uptaken by other organs (Pauly *et al.*, 2001). This result inspired other studies that focus on transducing efficiently the liver or a muscle to produce enough enzyme for the whole body.

Similar outcomes using adenovirus were also seen in posterior study using young (Ding *et al.*, 2001) and old (Xu *et al.*, 2005) GAA-knockout (GAA-KO) mice. In spite of the initial promising results, reduced glycogen clearance in muscles of older animals was noticed. Among other possibilities, one speculation is that the fusion of endosomes containing GAA with pre-existing lysosomes full of glycogen may impair the enzyme acitivity as the GAA-KO mice age, resulting in deficient activity of active and correctly processed GAA.

Regarding therapy with AAV vectors, newborn mice receiving the vector with cytomegalovirus promoter (CMV) AAV1-CMV-GAA intravenously resulted in initial supraphysiologic levels of GAA in hearts, with consistent drop in enzyme levels as mice aged (Mah *et al.*, 2005). Posteriorly, heart function was evaluated by electrocardiogram and AAV1-treated animals presented significant prolonged PR interval, with values in between untreated GAA-KO and wild-type mice. Moreover, left ventricular mass was very similar to the wild-type age-matched controls. Although biochemical, histological and functional analysis showed improvement in the cardiac tissue after AAV1 therapy, it only partially corrected the pathology (Mah *et al.*, 2007).

Many AAV vectors, with different promoters and serotypes, proved to be safe and efficient in increasing GAA activity and reducing glycogen deposits in hearts of GAA-KO mice (Table 2), as AAV2 (Fraites *et al.*, 2002), AAV7-MCK (MCK - Muscle Creatine Kinase Promoter) (Sun *et al.*, 2005a), AAV2/8 (Franco *et al.*, 2005; Sun *et al.*, 2005b; Wang *et al.*, 2014), AAV8/DC190 (DC190 - Human Serum Albumin Promoter) (Ziegler *et al.*, 2008) and AAV9-DES (DES - Desmin Promoter) (Falk *et al.*, 2015), this last resulting also in elongation of PR interval, increased ejection fraction and reduction in left ventricular mass. A combination between AV and AAV also showed to be effective in long-term GAA production in heart, even when administered in the gastrocnemius muscle (Sun *et al.*, 2003).

One of the major drawbacks of viral vector use is the immune response elicited against the vector itself or against the transgene, induced by the viral-mediated expression of GAA which compromises the effectiveness of the therapy, since anti-GAA antibodies probably inhibit cross-correction of peripheral tissues. Xu *et al.* (2004) illustrated this by using GAA-KO/SCID (SCID - Severe Combined Immunodeficiency) mice to analyse the response to AV-GAA therapy in an immunodeficient environment and concluded that the lack of anti-GAA antibodies found in GAA-KO/SCID mice resulted in higher GAA activity and glycogen clearance was maintained longer than previous studies using immunocompetent GAA-KO mice (Xu *et al.*, 2004). To work around this issue, many strategies have been used: pre-treatments with GAA to induce tolerization (Cresawn *et al.*, 2005) or with anti-CD4 to inhibit antibody formation (Han *et al.*, 2015); adaptations in the vector design, using codon-optimized GAA driven by nonviral promoters (Kiang *et al.*, 2006; Doerfler *et al.*, 2016). Interestingly, it has been shown that a pre-treatment with gene therapy using AAV2/8 may prevent IgG antibody formation later with ERT, acting as a pre-conditioning therapy that could enhance the effectiveness of the ERT (Han *et al.*, 2017).

Recently, the use of salmeterol, a β2-receptor agonist, as an adjuvant has been tested, since this drug may enhance the expression of cation-independent mannose-6-phosphate receptor (CI-M6PR) and therefore could improve the response to ERT or gene therapy in heart tissues (Han *et al.*, 2016). The treatment did enhance cardiac response to gene therapy, but further studies should be performed before adding the drug as adjunctive therapy.

Besides the comparison between different vectors or pre-treatment options, alternative administration routes are constantly being analysed as well. Intramyocardial (Pauly *et al.*, 1998; Fraites *et al.*, 2002), intramuscular (Sun *et al.*,

Table 2 - Effects of gene therapy on cardiovascular system in Glycogen Storage Diseases (Pompe Disease).

	Vector	Administration route	Model and age at administration	Endpoint (time post-injection)	Results in heart		Other remarks	Reference
					Increase in enzyme activity	Substrate reduction		
AV	AV-GAA	transmyocardial	rats, newborn	5-7 days	Yes	Yes	Transduction occured mainly in the liver	(Pauly *et al.,* 1998)
	[E12, polymerase2] AV-GAA	IV, retroorbital sinus	mice, 3 months	2-3 days up to 6 months	Yes	Yes	Treatment was more efficient in the first 50 days post injection	(Ding *et al.,* 2001)
			mice, 3 months	180 days	Yes	Yes	Compared GAA-KO/SCID mice with GAA-KO mice, the first had better results	(Xu *et al.,* 2004)
			mice, 12-14 and 17-19 months	17 days	Yes	Yes	No difference between age groups	(Xu *et al.,* 2005)
	HD-AV	balloon catheter occlusion to liver	Healthy baboon, 6 years	6 months	Yes	No	High levels of protein in the heart, treatment well tolerated	(Rastall *et al.,* 2016)
AV/AAV	hybrid AV-AAV	Intramuscular	Mice, 3 days	24 weeks	Yes	yes	Transduction of the heart rather than cross-correction from other tissues.	(Sun *et al.,* 2003)
AAV	AAV1-CMV	IV, superficial temporal vein	mice, 1 day	11 months	Yes	Yes		(Mah *et al.,* 2005)
	AAV2*	intramyocardial*	mice, 8 weeks	6 weeks	Yes	Yes		(Fraites *et al.,* 2002)
	AAV2/1-CMV	IV, superficial temporal vein	mice, 1 day	1 year	Yes	Yes	Elongation of PR interval, reduction of left ventricular mass, but mild improvement in correction of cardiac disease	(Mah *et al.,* 2007)
	AAV2/7-MCK*	IV, retroorbital sinus	mice, 12 weeks	24 weeks	Yes	Yes	GAA-KO and GAA-KO/SCID mice	(Sun *et al.,* 2005a)
	AAV2/8	IV, retroorbital sinus	mice, 12 weeks	24 weeks	Yes	Yes	GAA-KO/SCID mice. Restoration of normal myofiber structure	(Sun *et al.,* 2005b)
	AAV2/8-LSP	IV, tail vein	mice, 9–29 weeks	16 weeks	Yes	Yes	Proved to be safe and well-tolerated. Efficacy was higher in males and at later timepoints	(Wang *et al.,* 2014)
	AAV2/8-LSP*	IV, retroorbital sinus	mice, 12 weeks	12 weeks	Yes	Yes		(Franco *et al.,* 2005)
	AAV2/8-MHCK7*	hydrostatic isolated limb perfusion	mice, 3 months	18 weeks	Mild	Mild	Good results in skeletal muscles, but not in the heart	(Sun *et al.,* 2010)
	AAV2/8-LSPhGAA	IV	mice, adult	36 weeks	Yes	Yes	Defined the minimum effective dose; prevented IgG formation due to ERT.	(Han *et al.,* 2017)
	AAV2/9 -MHCK7*	IV, retroorbital sinus	mice, 3 months	18 weeks	Yes	Yes		(Sun *et al.,* 2008)
	AAV2/9-CB	IV	mice, 6 months	12 and 24 weeks	Yes	Yes	Pre-treatment with anti-CD4 mAb enhanced biochemical correction in the heart	(Han *et al.,* 2015)
	AAV2/9-CB	IV, tail vein	mice, 4 months	18 weeks	Yes	Yes	Daily treatment with salmeterol* enhanced biochemical correction observed with AAV treatment	(Han *et al.,* 2016)

Table 2 (cont.)

	Vector	Administration route	Model and age at administration	Endpoint (time post-injection)	Results in heart		Other remarks	Reference
					Increase in enzyme activity	Substrate reduction		
	AAV5- or AAV8-DHBV	IV, portal vein	mice, 10 weeks	16 weeks	Yes	Yes	Neonatal pre-treatment with human GAA resulted in greater cardiac correction in mice Ab- for GAA	(Cresawn *et al.,* 2005)
	AAV8-DC190	IV, tail vein	mice, 12 week	6 months	Yes	Yes		(Ziegler *et al.,* 2008)
	AAV9-DES	IV, jugular vein	mice, 3 months	3 months	Yes	Yes	Elongation of the PR interval, increased ejection fraction and reduction in left ventricular mass. In comparison, AVV9 treatment increased more GAA activity in the heart than ERT.	(Falk *et al.,* 2015)
	AAV9-DES*	intrapleural	mice, 3 months	6 months	Yes	Yes	Improved cardiac ejection fraction and stroke volume.	(Falk *et al.,* 2013)
	AAV9-CAG-hGAA	Intrathecal	Mice, 1 month	11 months	Yes	Yes	presented reduced thickness of the left ventricular wall, well arranged myofibrils and correction of vacuolation of cardiac fibers due to glycogen storage	(Hordeaux *et al.,* 2017)
	Co-packaging of AAV9-LSP with AAV9-DES*	IV, tail vein	mice, 4-6 weeks	8 weeks	Yes	Yes	Co-packaged AAV9 attenuated pre-existing humoral and cellular immune responses, enhancing biochemical correction	(Doerfler *et al.,* 2016)
LV	LV-CMV-GAA	IV, superficial temporal vein	mice, 1-2 days	24 weeks	Yes	Yes		(Kyosen *et al.,* 2010)
	Ex vivo lentivirus*	IV, retroorbital sinus	mice, 6-8 weeks	17 weeks	NA	No	HSCT using lentivirus modified HSC	(Douillard-Guilloux *et al.,* 2009)
	Ex vivo lentivirus*	IV, tail vein	mice, 8-12 weeks	up to 15 months	Yes	Yes	HSCT using lentivirus modified HSC. Decreased relative right and left ventricular mass with restoration of left ventricular wall thickness. Heart rate normalized. Still poor response compared to *in vivo* therapy.	(van Til *et al.,* 2010)

AV: adenovirus; AAV: adeno-associated virus; LV: Lentivirus; IV:intravenous; IM: intramuscular; BMT: bone marrow transplant; HSC: hematopoietic stem cell; HSCT: hematopoietic stem cell transplant; NA: not analysed; *Results showed restricted to the most effective protocol tested.

2003, 2010), intrapleural (Falk *et al.*, 2013) and intrathecal (Hordeaux *et al.*, 2017) administrations were already tested and showed transduction of various tissues. Of these, intramuscular administration was seen to be restricted to the injection site (Sun *et al.*, 2003, 2010), while intrathecal injection of AAV9 vector surprisingly resulted in reduction of substrate storage in the heart and consequently reduction of left ventricular wall thickness (Hordeaux *et al.*, 2017). Intrapleural administration of AAV9 also ameliorated cardiac symptoms, with improvement in ejection fraction and stroke volume (Falk *et al.*, 2013).

Contrarily to some other LSDs, hematopoietic stem cell transplant is not an effective approach in GSD-II (Watson *et al.*, 1986) unless hematopoietic stem cells (HSC) are modified *ex vivo* to produce supraphysiologic enzyme levels enough to cross-correct peripheral tissues (van Til *et al.*, 2010). Therefore, this strategy was tested in a couple of studies using lentiviral vectors, in which HSC were harvested from GAA-KO mice donors, modified *in vitro* and transplanted intravenously in GAA-KO mice recipients after sublethal irradiation. The first study (Douillard-Guilloux *et al.*, 2009) showed no significant improvement in the heart of treated animals though the latter (van Til *et al.*, 2010) presented amelioration of echocardiographic findings, with decrease in relative right and left ventricular mass, and reconstitution of GAA activity with robust glycogen reduction in the heart. Even though some improvement was observed, other strategies have proven to be more effective in the context of GSD-II.

Finally, to evaluate safety and efficacy in other animal models, Rastall *et al.* (2016) used baboons, which underwent balloon catheter administration of helper dependent adenovirus (HD-AV) expressing GAA. Both animals treated presented high levels of GAA in the heart, as observed in Western Blot analysis and enzymatic assay.

Mucopolysaccharidoses (MPS)

MPS Type I

MPS I (Hurler, Scheie or Hurler-Scheie diseases) is caused by mutations in the *IDUA* gene, resulting in deficiency of α-L-iduronidase (IDUA), an enzyme required in the degradation pathway of glycosaminoglycans (GAGs) heparan and dermatan sulphate. Common cardiovascular manifestations include dilated cardiomyopathy, cardiac valve abnormalities as valve thickening, stenosis and regurgitation, coronary artery disease due to diffuse intimal proliferation from GAG deposition and dilatation of the aorta with reduced aortic elasticity (Braunlin *et al.*, 2011). Moreover, cardiovascular complications, as heart failure, sudden death from arrhythmias and coronary occlusion, are the main cause of mortality (Braunlin *et al.*, 2011).

Numerous studies were conducted using gene therapy to treat murine, canine and feline models of MPS I (Table 3), which also present cardiac disease, although with small differences (Jordan *et al.*, 2005; Braunlin *et al.*, 2006; Sleeper *et al.*, 2008). However, most of these studies focus on the CNS and neurological manifestations, limiting cardiovascular analysis only to IDUA activity and GAG deposition in the heart. Some papers describe the use of lentivirus (Di Domenico *et al.*, 2005; Kobayashi *et al.*, 2005; Ou *et al.*, 2016), adeno-associated virus (Hartung *et al.*, 2004) and non-viral DNA minicircle vectors (Osborn *et al.*, 2011), *Sleeping Beauty* transposon (Aronovich *et al.*, 2009), and microcapsules containing recombinant cells overexpressing IDUA (Baldo *et al.*, 2012; Lizzi Lagranha *et al.*, 2017) to treat MPS I. Results from all of them show increased IDUA activity and reduced GAG storage in heart after the treatment.

Ma *et al.* (2007) treated six-week-old MPS I mice intravenously with an RV expressing canine IDUA under control of the liver-specific Human α_1-Antitrypsin Promoter (hAAT-cIDUA-WPRE) and the Woodchuck Hepatitis Virus Post-Transcriptional Regulatory Element (WPRE), resulting in mild improvement in cardiovascular structures in animals expressing stable enzyme activity in the serum. Lysosomal storage was significantly reduced in aortic valves and cardiac parenchyma, but not in the aorta. The aorta of treated mice remained dilated and with elastic fiber fragmentations (Ma *et al.*, 2007). Accordingly, in another study (Herati *et al.*, 2008), even though the enzyme activity was 37% of normal in the aorta of RV treated mice, GAG levels remained high in this tissue, and 75% of treated animals developed aortic insufficiency due to aortic dilatation.

On the other hand, a study using newborn MPS I dogs and RV hAAT-cIDUA-WPRE gene therapy showed great improvement in cardiac disease on the treated group, with reduction of aortic diameter, mitral valve thickening and elastic fiber fragmentation (Traas *et al.*, 2007). The enzyme activity detected in the heart and the aorta was 30% and 20% of normal, respectively, and the lysosomal storage was reduced in both tissues. Furthermore, in MPS I cats (Hinderer *et al.*, 2014), AAV8 vector expressing feline IDUA by a liver-specific promoter was administered. Aorta and myocardium exhibited total correction of storage lesions and aortic valves had near complete resolution in animals with constant enzyme expression. In addition, collagen structures of the fibrosa layer of the valves from treated cats were very similar to the normal group. One cat had a decline in IDUA activity and, consequently, had the worst outcome, but antibodies against IDUA were not found in ELISA tests.

In another study, MPS I mice treated with high-dose RV hAAT-cIDUA-WPRE showed promising outcomes: the echocardiograms in treated mice were completely normal – no significant changes in wall thickness, left ventricular mass index or end-diastolic left ventricular chamber size – and none had aortic dilatation or aortic insufficiency, features found on untreated MPS I mice or treated with

Table 3 - Effects of gene therapy on cardiovascular system in the Mucopolysaccharidoses.

Disease	Vector	Administration route	Model and age at administration	Endpoint (time post-injection)	Results in the heart		Other remarks	Reference
					Increase in enzyme activity	Substrate reduction		
MPS I	non-viral vectors	Hydrodinamic injection, subcutaneous	mice, adult	variable	Yes	Yes	Use of Sleeping Beauty transposon, DNA minicircle and microencapsulated cells	(Aronovich *et al.*, 2009; Lizzi Lagranha *et al.*, 2017; Osborn *et al.*, 2011)
	AAV	IV, temporal vein	mice, 1 day	5 months	Yes	Yes		(Hartung *et al.*, 2004)
		IV, cephalic vein	cats, 3-5 months	6 months	Yes	Yes	Correction of storage lesions in aorta and myocardium, amelioration of aortic valve disease	(Hinderer *et al.*, 2014)
	LV	IV, tail vein	mice, 8-10 weeks	1 month	Yes	Yes	Lentiviral vector elicited low immune response, increasing further at later time points	(Di Domenico *et al.*, 2005)
		IV, temporal vein	mice, 1 day	20 weeks	Yes	Yes	Newborn mice responded better to treatment	(Kobayashi *et al.*, 2005)
	RV	IV, temporal or tail vein	mice, 6 weeks	8 months-old #	Yes	Yes	Reduced GAG in aortic valves and heart, but not in the aorta. Most RV-treated mice had elastic fiber fragmentation and aortic dilatation. Aorta had slight increase in IDUA activity, but not enough to prevent aortic disease. 56% of RV treated mice had aortic insufficiency.	(Ma *et al.*, 2007)
		IV, temporal vein	mice, 6 weeks	8 months-old #	Yes	Yes	Aortas remained dilated, with marked GAG storage, and 75% of treated mice had aortic insufficiency.	(Herati *et al.*, 2008)
		IV, temporal vein	mice, 2-3 days	8 months	Yes	Yes	Prevented aortic dilatation and insuficiency. No significant changes in left ventricular wall thickness, mass index or end-diastolic chamber size. Fractional shortening was significantly greater in high-dose RV mice.	(Liu *et al.*, 2005)
		IV, jugular vein	dogs, 2-3 days	up to 21 months	Yes	Yes	Reduction of aortic diameter, reduced mitral valve thickening and reduced elastic fiber fragmentation of aorta.	(Traas *et al.*, 2007)
	ex vivo RV	IV, tail vein	mice, 6-8 weeks	8 months	Low	No	BMT with RV-modified cells. One mice presented restoration of left ventricular function and normalization of myocites storage vacuoles.	(Jordan *et al.*, 2005)
	ex vivo LV		mice, 2 months	6 months	Yes	Yes	BMT with LV-modified cells.	(Visigalli *et al.*, 2010)
MPS II	plasmid	electro gene transfer on quadriceps	mice, 12-16 weeks	5 weeks	No	No	Transduction was restricted to injection site, had no effect of the heart	(Friso *et al.*, 2008)
	AAV	IV, tail vein	mice, 2 months	1 and 7 months	Yes	Yes	(Cardone *et al.*, 2006)	

Table 3 (cont.)

Disease	Vector	Administration route	Model and age at administration	Endpoint (time post-injection)	Results in the heart		Other remarks	Reference
					Increase in enzyme activity	Substrate reduction		
		IV, tail vein	mice, 20 weeks	6 and 24 weeks	Yes	Yes	(Jung *et al.,* 2010)	
	AAV9	Intrathecal	Mice, 2 months	4 months	Yes	Yes	Complete correction of storage lesions in heart, but possibly due to cross-correction from the serum enzyme	(Motas *et al.,* 2016)
		ICV	Mice, 2 months	40 weeks	Yes	Yes	Pilot study compared different routes (intrathecal intravenous and intracerebroventricular).	(Laoharawee *et al.,* 2017)
		ICV	Mice, 2-3 months	3 weeks	NA	Partial		
	ex vivo LV	IV	mice, 9 weeks	24 weeks	Yes	Yes	BMT with LV modified cells	(Wakabayashi *et al.,* 2015)
MPS IVA	AAV	IV	mice, NS	12 weeks	Yes	NA		(Tomatsu *et al.,* 2012)
MPS VI	AAV	IV and IM	cats and rats, newborn	6 months (rat) and 1 year (cat)	Yes	Yes	Vector spread to heart after both IM and IV injections for both animal models	(Tessitore *et al.,* 2008)
		IV, temporal or femoral vein	rats 5 and 30 days	6-7 months	Yes	Yes	Pre-treatment with immunosupressionperformed. Heart valve GAG storage was reduced in pre-treated animals.	(Cotugno *et al.,* 2010)
		IV, jugular or cephalic vein	cats, 5 and 50 days	12 months	NA	NA	Reduced or normalized mitral valve thickening independent of age of treatment	(Cotugno *et al.,* 2011)
		IV, retro-orbital	mice, 30 days	6 or 12 months	NA	Yes	Reduced GAG storage in aortic valves and myocardium	(Ferla *et al.,* 2014)
		IV, retro-orbital	Mice, 30 days	6 months	Yes	Yes	Combined low vector dose with monthly ERT infusions	(Alliegro *et al.,* 2016)
		IV	Mice, adult	6 months	NA	Yes	Described safety of the therapy. Minimal GAG reduction in heart valves.	(Ferla *et al.,* 2017)
	RV	IV, jugular vein	cats, newborn	6 months to 8 years	Yes	Yes	Supraphysiologic ARSB levels on the bloodstream, but only 9-85% of normal in heart and aorta of treated cats. Treated cats had significant reduced mitral valve thickening, but still developed aortic dilatation, aortic valve regurgitation and thickened aortic valve leaflets.	(Ponder *et al.,* 2012)
MPS VII	AAV	IV, temporal vein	mice, 2 days	16 weeks	Yes	NA		(Daly *et al.,* 1999)
		Intrahepatic injection	mice, 7-8 weeks	24 weeks	Yes	Yes		(Sferra *et al.,* 2004)
	LV	IV, temporal vein	mice, 2 days	12 or 18 months	Yes	Yes	Used two MPS VII mouse strains.	(Derrick-Roberts *et al.,* 2014)
	LV	IV, tail vein	Mice, 4 months	2 months	Partial	Partial	GAG storage in heart only stabilized but not normalized after treatment.	(Derrick-Roberts *et al.,* 2016)

Table 3 (cont.)

Disease	Vector	Administration route	Model and age at administration	Endpoint (time post-injection)	Results in the heart		Other remarks	Reference
					Increase in enzyme activity	Substrate reduction		
	RV	IV, tail vein	mice, 5-7 weeks	3 months	Partial	No	Mice were pre-treated with AV-CMV-HGF in the quadriceps. Treatment increased only 5% of GUSB activity in heart	(Gao *et al.,* 2000)
		IV, jugular vein	dogs, 2-3 days	variable, up to 12 months	Yes	NA	Treated dogs had normal valve thickness, no aortic valve insufficiency, mild mitral regurgitation and aortic diameter within normal limits at 8-9 months of age	(Ponder *et al.,* 2002)
		IV, jugular vein	dogs, 2-3 days	24 months	Yes	Yes	Treated dogs had mild mitral regurgitation at 4-5 months of age, which improved over time. At 2 years of age, murmurs were absent and valve thickness was normal. Aortic diameter was within normal limits. Treated dogs had mild improvement in GUSB activity and GAG storage in the aorta.	(Sleeper *et al.,* 2004)
		IV, jugular vein	dogs, 2-3 days	variable, up to 8 years	Yes	NA	Aortic dilatation was delayed in RV treated dogs, but it did occur at late times even with stable serum GUSB activity. They presented reduced elastin fragmentation, reduced expression of MMP-12 and of cathepsins B, D, K and S, compared to the untreated group. RV sequences were not found in the aorta.	(Metcalf *et al.,* 2010)
		IV, jugular vein	dogs, 2-3 days	variable, up to 8 years	Yes	Yes	GAG content in the mitral valve of treated dogs at 8 years post injection was lower than untreated dogs, but still higher than the normal. GUSB activity was 25% of normal in the mitral valves. Treatment reduced total cathepsins activity and increased content of intact collagen.	(Bigg *et al.,* 2013)
		IV, temporal vein	mice, 2-3 days	6 months	Yes	Yes	Aorta GUSB activity in treated animals was 5-fold de value of normal mice and 325-fold de value of the untreated ones. GAG content reduced to 5% of untreated mice, although stil higher than normal. Reduced aortic dilatation but did not prevent it.	(Baldo *et al.,* 2011)

AAV: adeno-associated virus; LV: Lentivirus; RV: retrovirus; IV:intravenous; IM: intramuscular; ICV: intracerebroventricular; BMT: bone marrow transplantation; NA: not analysed; NS: not specified; #Age at analysis.

lower RV doses (Liu *et al.*, 2005). Additionally, lysosomal storage was absent from most regions in the aorta, mitral valve and myocardium of high dose RV treated animals, while it was present in the untreated group. High dose RV had 9-fold normal IDUA activity in heart and aorta. Hexosaminidase levels – which is a lysosomal enzyme with activity elevated due to MPS I secondary effects – was also normal, as did GAG levels in both organs. Interestingly, low-dose RV treated MPS I mice had 14% of normal IDUA activity in the aorta and it was insufficient to prevent cardiac disease, which may be explained by the unequal distribution of the enzyme throughout the structure (Liu *et al.*, 2005).

Finally, few studies have used HSCT together with gene therapy, modifying the cells *ex vivo* to overexpress IDUA using viral vectors. Initially, in a study using a retroviral vector containing IDUA, only one treated mouse out of ten had mild cardiac improvement (Jordan *et al.*, 2005). On the other hand, Visigalli *et al.* (2010) compared normal and modified HSC from MPS I donor to overexpress IDUA through lentivirus transduction. Plasma IDUA levels were much higher with modified HSC than in other groups and resulted in almost complete absence of lysosomal storage and other pathological conditions, while the transplant with normal HSC only offered mild improvement in comparison. This study highlights the fact that the complete resolution of the cardiac manifestations is very much dependent on the supraphysiologic levels of the enzyme, otherwise the treatment would not be as effective in hard-to-target organs (Visigalli *et al.*, 2010).

MPS Type II

MPS II (Hunter disease) is an X-linked recessive disease caused by iduronate 2-sulfatase (IDS) deficiency, leading to dermatan and heparan sulfate accumulation. Cardiac involvement is very similar to MPS I, but MPS II patients may also present conduction abnormalities and sinus tachycardia (Braunlin *et al.*, 2011).

Gene therapy research for MPS II started in the decade of 1990 with *in vitro* modification of cells to express IDS based on viral vectors (Braun *et al.*, 1993, 1996 Whitley *et al.*, 1996; Di Francesco *et al.*, 1997; Stroncek *et al.*, 1999) and non-viral approaches in 2002 (Tomanin *et al.*, 2002). Only in 2006 the first paper describing *in vivo* gene therapy of MPS II was published (Cardone *et al.*, 2006), following the creation of the mouse model. The transduction was directed to the liver, using AAV2/8-TBG-IDS vector. In the latter, increased IDS activity was observed in the heart, as was clearance of lysosomal GAG deposition. Other analyses regarding cardiovascular function were not performed. These findings were also seen in a posterior study using a different mouse model and a similar vector (Jung *et al.*, 2010). On the contrary, when a plasmid vector was administered in the quadriceps muscle followed by electro-gene transfer, IDS activity was not detected in visceral organs, including heart, remaining restricted to the injection area (Friso *et al.*, 2008). Three recent studies described CNS-directed administration of AAV9 in MPS II mice, resulting in increased IDS activity in heart and correction of storage lesions (Laoharawee *et al.*, 2017; Motas *et al.*, 2016) or partial reduction of GAG storage (Hinderer *et al.*, 2016). Since the organ did not present sufficient AAV copies, the occurrence of cross-correction of IDS deficiency by uptake of the enzyme from circulation was suggested.

Similar to an MPS I study already discussed (Visigalli *et al.*, 2010), Wakabayashi *et al.* (2015) have shown that *ex vivo* HSC gene therapy using lentiviral vector improves the biochemical abnormalities of MPS II mice, including heart, with increased IDS activity to 3-fold higher than normal and normalized lysosomal GAG content. Unfortunately, echocardiographic analysis was not performed (Wakabayashi *et al.*, 2015).

MPS Type IV A

MPS IV A (Morquio A disease) is caused by keratan and chondroitin sulfate deposition due to deficiency of the lysosomal enzyme N-acetylgalactosamine-6 sulfatase (GALNS). Clinical findings normally include cardiovascular involvement, with moderate mitral e aortic regurgitation and valve thickening (Hendriksz *et al.*, 2013). Heart failure is the most common cause of death amongst patients (Rigante and Segni 2002).

The first record of gene therapy for MPS IV A dates back to 2001, where patients' fibroblasts and other lineage cells were transduced *in vitro* by retroviral vector containing the GALNS cDNA and efficiently produced the missing enzyme from 5-fold to 50-fold higher than the baseline enzyme activity of normal non-transduced cells (Toietta *et al.*, 2001). Only one paper described the use of gene therapy *in vivo*, in which MPS IV A mice were treated with AAV vector carrying GALNS cDNA. Twelve weeks after treatment, GALNS activity was about 30% of wild-type in the heart (Tomatsu *et al.*, 2014).

MPS Type VI

MPS VI (Maroteaux-Lamy syndrome) is an autosomal recessive disease caused by mutations in the *arylsulfatase B* (*ARSB*) gene, resulting in reduced or absent enzyme activity of arylsulfatase B (ARSB), responsible for the breakdown of dermatan sulphate. Cardiac disease is frequent in MPS VI patients and is an important cause of morbidity and mortality (Braunlin *et al.*, 2011). Common features are valve stenosis and/or insufficiency, with mitral valve being affected in 96% of the patients (Valayannopoulos *et al.*, 2010).

Gene therapy for MPS VI is being developed mainly using AAV vectors designed to target the liver, trying to use the organ as a factory that secretes enough enzyme to be taken up by the whole body. Tests were performed in cats

and rats, and increased ARSB activity and GAG clearance was observed in heart from both animal models after intravenous or intramuscular AAV treatment (Tessitore *et al.*, 2008). However, the intramuscular treatment showed a positive response in visceral organs, probably due to leakage of vector to other tissues, thus transducing other cell types, rather than a cross-correction effect. In addition, it elicited a humoral immune response in rats, resulting in ARSB levels decay. Thereafter, in another study (Cotugno *et al.*, 2010), the same AAV2/8-TBG-hARSB vector, with the liver-specific promoter TBG (Thyroxine Binding Globulin), was administered intravenously in MPS VI rats, newborn and juvenile, concomitant with immunosuppressive drugs (IS) to minimize the possible drawback caused by the immune response previously reported. GAG storage in heart valves was similar in AVV treated and non-treated MPS VI rats whereas it was reduced in AAV+IS group. Nevertheless, results varied considerably and were not reproducible in the cat model, which can be explained by the mixed genetic background of these animal models and differences in the performance of the essays.

Cotugno *et al.* (2011) used the same AAV2/8-TBG-fARSB vector intravenously in 5 and 50-days-old MPS VI cats (newborn and juvenile groups, respectively). Soon after administration of high vector doses (6×10^{13} gc/kg) in 5-days-old kittens, serum ARSB activity levels were 30-fold higher than normal, but shortly dropped to normal range due to intense hepatocyte proliferation, resulting in vector dilution since AAV vector is not integrative. On the other hand, same vector doses administered in juvenile group resulted in stable high ARSB activity and maintenance above or within the normal range throughout the follow-up time, suggesting that late gene therapy with AAV2/8 vector may be beneficial for eventual clinical application, since MPS VI patients are not normally diagnosed at birth. In this study, echocardiographic analysis was performed in 9-12 month-old animals. Untreated cats presented important mitral valve thickening, while treated cats had the condition reduced or normalized (Cotugno *et al.*, 2011).

In mice, AAV2/8-TBG-hARSB was used with intravenous injection in 30 days-old MPS VI mice in comparison to ERT (Ferla *et al.*, 2014). Both treatments showed to be effective in reduction of GAG storage in myocardium and heart valves, although gene therapy provided stable ARSB levels (on average 17% of normal levels) without the peak-and-drop serum kinetics observed with ERT. On the other hand, since high vector doses may compromise liver function, association between gene therapy and ERT was tested using a single IV administration of low dose AAV2/8 ($< 2 \times 10^{12}$gc/kg) and monthly enzyme infusions, resulting in increased ARSB activity and GAGs reduction in heart valves and myocardium compared to ERT alone (Alliegro *et al.*, 2016).

Finally, the vector AAV2/8-TBG-hARSB seems safe and effective up to 180 post-administration in MPS VI mice, as observed in a safety study (Ferla *et al.*, 2017), showing the feasibility of a possible translation of the therapy to the clinic.

Retroviral vector was also tested in MPS VI animal models. MPS VI kittens were treated with RV vector hAAT-fARSB-WPRE within 4 days after birth. As a result, GAG deposition reduced drastically in all tissues analysed from treated cats compared to untreated. Conversely, serum ARSB activity in RV group ranged an average of 13-fold compared to homozygous normal cats and 60-fold compared to the untreated MPS VI group. However, the enzyme activity in visceral organs including heart and aorta were 9-85% of the values in unaffected cats, demonstrating that the enzyme is not being taken up to cells efficiently even though there are supraphysiologic levels on the bloodstream. Untreated MPS VI cats eventually developed aortic dilatation at the sinus of Valsalva and at the sinotubular junction, aortic valve regurgitation, thickened aortic valve leaflets and thickening of the mitral valve. These conditions were not present in normal cats and all but thickening of mitral valve were significantly reduced in RV treated animals. Hence, neonatal gene therapy seems to prevent aortic dilatation and aortic valve thickening, although this does not indicate resolution of cardiac disease (Ponder *et al.*, 2012).

MPS Type VII

Mucopolysaccharidosis type VII, or Sly syndrome, is caused by β-glucoronidase (GUSB) deficiency, resulting in lysosomal build-up of chondroitin, dermatan and heparan sulfate. Cardiac symptoms include coronary artery disease, aortic dilation, thickened and stenotic aortic valve leaflets, intimal thickening of the aorta and left ventricular hypertrophy (Braunlin *et al.*, 2011; Gniadek *et al.*, 2015).

Since the late 1990s, several studies have been published analysing the effect of gene therapy on the cardiovascular system in MPS VII animal models. Daly *et al.* (1999) injected intravenously an AAV vector with human GUSB cDNA in newborn MPS VII mice resulting in stable GUSB expression, higher than normal, for up to 16 weeks in the heart. Contrarily, the RV vector hAAT-GUSB with pre-treatment of AV-CMV-HGF (HGF - Hepatocyte Growth Factor) – which induces transient hepatocyte replication thus allowing the RV vector to transduce the dividing cells – increased GUSB activity in heart only slightly (about 5%) and lysosomal storage did not improve (Gao *et al.*, 2000).

The retroviral vector hAAT-cGUSB-WPRE had better outcomes, as presented in few studies using murine and canine MPS VII animal models. Three newborn MPS VII dogs were treated intravenously (Ponder *et al.*, 2002), none of which presented aortic valve insufficiency or mitral valve thickening at 8-9 months, common features of age-matched MPS VII dogs, and only one had mild mitral re-

gurgitation. Subsequently, Sleeper *et al.*(2004) published a neonatal treatment of dogs with the RV vector hAAT-cGUSB-WPRE, which resulted in improvement in echocardiographic analyses – tricuspid, aortic and pulmonary valves thickness were normal in all treated dogs, none had murmurs and aortic diameter was within normal limits. Two dogs presented insignificant mitral regurgitation at 9 to 11 months; and at 2 years of age, one had minimal mitral regurgitation, but the same happened to 3 out of the 7 normal dogs analysed. GUSB activity in the aorta and myocardium was around 17% and 19% of normal, respectively, and GAG content were reduced to 178% of normal in both aorta and myocardium.

Histologically, the aorta from RV-treated dogs had fusiform myocytes with minimal hypertrophy and vacuolated cytoplasm, in contrast to the rounded, severely hypertrophic and vacuolated muscle cells found in the aortas of untreated dogs. Also, untreated MPS VII dogs had important nodular thickening of the mitral valve, while only mild thickening was seen in the treated group. Although the treatment did not clear GAG storage completely, these results have shown that neonatal intravenous RV gene therapy can ameliorate cardiovascular abnormalities in dogs with MPS VII, with no adverse effects.

Additionally, MPS I and VII dogs were treated with the RV vector hAAT-cIDUA-WPRE and hAAT-cGUSB-WPRE, respectively, at 2 to 3 days after birth (Metcalf *et al.*, 2010). All MPS VII treated dogs had stable high serum GUSB activity throughout the evaluation period, which means up to 8 years for some dogs, albeit GUSB activity in aorta was relatively low, reflecting the poor diffusion of the enzyme in this structure. The aorta appeared normal in a 6 month-old RV-treated MPS VII dog with high enzyme activity in serum, however in an 8 year-old treated dog the aorta was dilated and the aortic valve was thickened, with reduced range of motion. The first dog had 148% of normal enzyme activity and the second had 52%, which may contribute for the difference in the outcome. Summarizing, aortas from treated dogs appeared normal in the first 5 years after gene therapy, statistically better than untreated group, but became dilated thereafter, at 8 years of age. Since GUSB expression remained stable, the treatment with gene therapy delayed the aortic pathology but did not prevent it.

Mitral valve disease progression in MPS VII dogs was also evaluated by the same group (Bigg *et al.*, 2013). The authors suggested that mitral regurgitation occurs due to reduced content of collagen and its abnormal structure in the mitral valve, as evaluated by Masson's trichrome and Picrosirius-red staining, respectively. Normal and treated dogs had higher collagen content than MPS VII animals, although in the treated group it was slightly less than normal. Regarding collagen structure, RV gene therapy improved the integrity of the fibers to 45% of normal and 5-fold the value in untreated MPS VII dogs at 6 months of age. Biochemical analysis showed almost complete GAGs clearance and reduced protease activity such as cysteine cathepsins in RV treated samples.

In mice, GUSB activity was statistically higher in the hearts of mice treated with hAAT-cGUSB-WPRE than in the untreated group and GAG storage was cleared in heart valves and aorta of RV groups with high GUSB circulating levels (Xu *et al.*, 2002). In another study with the same protocol, GUSB activity in aortas from treated animals was statistically higher, with 5-fold the value of normal mice and 325-fold higher than the untreated group. GAG levels were 111-fold normal in MPS VII mice aortas and significantly reduced to 5% in RV treated mice. Although biochemical parameters were improved, aortic diameter in treated animals was about 155% of normal at 10 months of age, thus aortic disease still developed and progressed, giving another evidence that this gene therapy protocol was not fully effective in aortic abnormalities (Baldo *et al.*, 2011).

Other studies were conducted without performing functional analysis of heart and using different approaches. Serotype 2 AAV vector expressing human GUSB were administered intrahepatically in MPS VII adult mice, resulting in GUSB activity at 15% of normal (Sferra *et al.*, 2004). Besides AAV vector, the lentiviral vector derived from the Human Immunodeficiency Vector pHIV-1EF1α-GUSB (1EF1α - Eukaryotic Translation Elongation Factor 1 α1) was tested, administered intravenously in newborn pups 2 days after birth. Mice from two different strains were used, $GUS^{tm(L175)Sly}$ and $GUS^{mps/mps}$, representing the attenuated and severe form of the disease, respectively. Transduction of heart myocytes were more efficient in $GUS^{tm(L175)Sly}$ animals, as was the GUSB activity, reaching about 60% of normal in the organ. GAG storage reduced, but was still present in animals from both strains (Derrick-Roberts *et al.*, 2014). When treating older mice, results were more modest (Derrick-Roberts *et al.*, 2016).

Sphingolipidoses

Fabry Disease

Fabry Disease is an X-linked disorder caused by deficiency of the lysosomal hydrolase α-galactosidase A (α-GalA) with consequent accumulation of globotriaosylceramide (Gb3) within lysosomes. Cardiac symptoms are very common in both men and women with the classic form or the cardiac variant. Moreover, these are main causes of morbidity and mortality. The symptoms include: cardiac hypertrophy associated with depressed contractility and diastolic filling impairment, coronary insufficiency, atrioventricular conduction disturbances, arrhythmias and valvular involvement (Linhart and Elliott, 2007)

The initial reports of gene therapy in Fabry used adenoviral vectors (Ziegler *et al.*, 1999) (Table 4). The AV-CMV-α-GalA vector was administered via tail vein in the mouse model. Enzyme activity and Gb3 levels were

Table 4 - Effects of gene therapy on cardiovascular system in the Sphingolipidoses.

Disease	Vector	Administration route	Model and age at administration	Endpoint (time post-injection)	Results in heart		Other remarks	Reference
					Increase in enzyme activity	substrate reduction		
Fabry Disease	Non-viral	IV, tail vein	mice, 4 to 6 weeks; injection repeated after 28 and 56 days	Up to 84 days	Yes	Yes	Cationic lipid–pDNA complex. Increase efficiency with dexamethasone treatment and multiple intravenous injections	(Przybylska *et al.*, 2004)
	Non-viral	IV, left renal vein	NS	Up to 4 weeks	Yes	Yes	Naked plasmid	(Nakamura *et al.*, 2008)
	AAV1	IV, tail vein	mice, 3 months	Up to 37 weeks	Yes	Yes	At least 40-fold increase in enzyme levels and Gb3 normalization	(Ogawa *et al.*, 2009)
	AAV1	IV, external jugular vein	mice, 2 days	Up to 25 weeks	Yes	Yes	At least 40-fold increase in enzyme levels and Gb3 normalization	(Ogawa *et al.*, 2009)
	AAV2	IV, portal vein	mice, 10 to 12 weeks	Up to 25 weeks	Yes	Yes		(Jung *et al.*, 2001)
	AAV2	IM, quadriceps	mice, 3 months	Up to 25 weeks	Yes	Yes	Less than 10% enzyme activity in 25 weeks, but normalization of Gb3 and significant decrease of cardiac hypertrophy.	(Takahashi *et al.*, 2002)
	AAV2	IV, tail vein*	mice, 11 to 12 weeks	Up to 24 weeks	Yes	Yes	No Gb3 storage and 3-fold normal level increase in 24 weeks	(Park *et al.*, 2003)
	AAV2	IV, tail vein	mice, 3 months	Up to 12 weeks	Yes	Yes	Reduction of Gb3 to basal levels in 8 weeks	(Ziegler *et al.*, 2004)
	AAV2	IV, tail vein	mice, 18 weeks	Up to 60 weeks	Yes	Yes	Normalization of enzyme up to 48 weeks and clearance of Gb3 up to 60 weeks.	(Choi *et al.*, 2010)
	AAV8	IV, tail vein	mice, 4 months	Up to 12 weeks	Yes	Yes	Normalization of enzyme levels and Gb3 storage from 4-12 weeks	(Ziegler *et al.*, 2007)
	AV	IV, tail vein	mice, 4 to 6 months	Up to 24 weeks	Yes	Yes	Injection of monoclonal antibody against MR1 facilitated readministration	(Ziegler *et al.*, 1999)
		IV, tail vein	mice, 4 months	28 days	Yes	Yes	Pretreatment with gamma globulins enhanced transduction	(Ziegler *et al.*, 2002)
		IN, pulmonary instillation	mice, 4 months	Up to 8 weeks	Yes	Yes	Transduction restricted to lungs.	(Li *et al.*, 2002)
	LV	IV, temporal vein	mice, neonatal	28 weeks	Yes	Yes		(Yoshimitsu *et al.*, 2004)
		Intraventricular injection	NS	Up to 52 weeks	Yes	Yes	~20% normal levels in 7 days No enzyme activity 30 days or 1 year post injection	(Yoshimitsu *et al.*, 2006)
		HSCT *	mice, 8 weeks	24 weeks	Yes	Yes	After a secondary HSCT mice also presented therapeutic levels of enzyme in heart.	(Yoshimitsu *et al.*, 2007)
	RV	IV, temporal vein	mice, neonatal	26 weeks	Yes	Yes		(Higuchi *et al.*, 2010)
		HSCT	NS	12 and 26 weeks	Yes	Yes	Mice that received a secondary transplantation still exhibit improvement in heart tissue.	(Takenaka *et al.*, 2000)

Table 4 (cont.)

Disease	Vector	Administration route	Model and age at administration	Endpoint (time post-injection)	Results in heart		Other remarks	Reference
					Increase in enzyme activity	substrate reduction		
		HSCT, tail vein	mice, 6-10 weeks	Up to 6 months	Yes	NA	Transductions were performed once a day for 5 days and transplanted in lethally irradiated mice. Enrichment of CD25+ cells enhanced enzyme activity.	(Qin *et al.,* 2001)
		HSCT, tail vein	mice, 7-10 weeks	Up to 26 weeks	Yes	NA	Transductions were performed twice a day for 3 consecutive days and cells CD25+ were enriched. Lethally irradiated mice had the highest activity.	(Liang *et al.,* 2007)
Galactosialidosis	AAV	IV, tail vein	mice, 30 days	Up to 16 weeks	NA	NA	AAV2/8. Complete resolution of swollen lysosomes in the heart	(Hu *et al.,* 2012)
	RV	IV, tail vein	mice, 3 to 6 weeks	Up to 10 months	Yes	Yes	BMT using modified BM cells.Increased cathepsin A activity detected in heart 10 months after treatment, but it decreases as the mice age	(Leimig *et al.,* 2002)
Gaucher Disease	LV	IV, portal and tail vein	mice, 7 weeks	Up to 16 weeks	Yes	NA	Increased glucocerebrosidase activity in both administration routes	(Kim *et al.,* 2004)
GM1 Gangliosidosis	AAV	IV, tail vein	mice, 6 weeks	variable	Yes	NA	AAV9. Treated animals presented increased lifespan	(Weismann *et al.,* 2015)
	AV	IV, superficial temporal vein	mice, 24h-48h	30 and 60 days	Yes	NA		(Takaura *et al.,* 2003)
Niemann-Pick Disease types A and B	*Ex vivo* RV	IV	mice, 2 days	5 months	No	No	BMT using modified BM cells. No detectable ASM activity or sphingomyelin reduction in the heart of treated animals	(Miranda *et al.,* 2000)
	Ex vivo RV	IV, superficial temporal vein	mice, 3 days	16 and 24 weeks	Yes	NA	BM and MSC modified *in vitro* with RV. After 30 days of BM injection, MSC was administered intracerebral. ASM activity in the heart increased slightly, but was only 2% of normal after 24 weeks	(Jin and Schuchman, 2003)
Sialidosis	AAV	IV, tail vein	mice, 16-month	4 weeks	Yes	NA	AAV2/8. Increased NEU1 and PPCA activity in the heart	(Bonten *et al.,* 2013)

AV: adenovirus; AAV: adeno-associated virus; LV: Lentivirus; RV: retrovirus; IV: intravenous injection; IN: intranasal; BM: bone marrow; BMT: bone marrow transplant; NS: not specified. NA: not analyzed; *Results showed restricted to the most effective protocol tested

normalized in several tissues, including heart. However, expression declined rapidly and there was reaccumulation of substrate after 6 months. Immunosuppression with a monoclonal antibody against CD40 ligand enhanced outcome of vector readministration. The same group also demonstrated that both depletion of Kupfer cells or pretreatment with gamma globulins could significantly increase AV transduction (Ziegler *et al.*, 2002) and that pulmonary instillation was an effective administration method (Li *et al.*, 2002).

Adeno-associated vectors were the most frequently used vector in reports that evaluated cardiac tissue. Jung *et al.* (2001) detected increased enzyme activity and reduced substrate accumulation in the heart of Fabry mice 25 weeks after injection of an AAV2 vector. Administration of another AAV2 vector in the quadriceps increased enzymatic activity for 30 weeks without development of antibodies (Takahashi *et al.*, 2002). Echocardiography showed that cardiac hypertrophy was significantly reduced. Although the authors detected clearance of cardiac Gb3, wall thickness was not normalized. Since animals were 3-month-old when injected, it is possible that irreversible structural changes were already established and the treatment cannot revert them. This type of vector proved to be very effective, since other authors also reported increased enzymatic activity to at least normal levels and/or complete clearance of Gb3 deposits for up to 60 weeks with the use of intravenously injected AAV1(Ogawa *et al.*, 2009), AAV2 (Park *et al.*, 2003; Ziegler *et al.*, 2004; Choi *et al.*, 2010), or AAV8 (Ziegler *et al.*, 2007) vectors.

Lentiviral vectors were injected in the temporal vein of neonatal Fabry mice (Yoshimitsu *et al.*, 2004; Higuchi *et al.*, 2010). After 26 to 28 weeks, both studies reported reduction in Gb3 and increase α-GalA activity in the heart. The latter study further demonstrated that fusion of α-GalA with the protein transduction domain (PTD) from HIV transactivator of transcription (Tat) protein could enhance the Gb3 reduction. Direct heart intraventricular injection of LV was also shown to reduce disease burden in the cardiac tissue for short-term period (Yoshimitsu *et al.*, 2006).

The combined use of gene therapy and HSCT was also evaluated. Takenaka *et al.* (2000) used α-Gal-deficient HSC from 10-week-old donor mice that were transduced four times with a retrovirus encoding α-GalA. Subsequently, cells were transplanted into sublethally and lethally irradiated α-GalA-deficient mice. After 26 weeks, increased α-GalA activity and decreased Gb3 storage were observed in the heart and other organs, particularly in the group lethally irradiated. Although in a smaller scale, these results were observed even after a secondary transplant was performed (Takenaka *et al.*, 2000). Qin *et al.* (2001) used a bicistronic retroviral vector that expressed α-GalA and a selectable marker (CD25 – Interleukin 2 Receptor α). They harvested HSC from 6- to 10-week-old male Fabry mice and transduced once a day for 5 days. Cells were then sorted by flow cytometry and immunoaffinity enrichment and were injected via tail veins into several groups, including lethally irradiated Fabry mice. Tissue analysis after 6 months indicated near normal α-GalA levels in the heart of these animals. Moreover, a second transplantation of cells collected from these primary transplanted mice was performed. From the 5 transplanted mice, 3 showed enzyme levels equal or higher to normal in plasma. No evaluation of GB3 was performed (Qin *et al.*, 2001). Later, the effects of different Reduced-intensity Conditioning Regimens, in addition to pre-selection, were also analysed. Liang *et al.* (2007) described that both limb irradiation with lethal dose of X-ray and treatment with Fludarabine and Cyclophosphamide increased enzymatic activity after transduction with the same RV as the previous study. However, it was a modest increase. Only mice lethally irradiated presented near normal levels of enzyme activity in the heart with clearance of Gb3. In the same year, another group used a LV to transduce bone marrow mononuclear cells and transplant into Fabry mice. Treatment was effective and resulted in supraphysiological levels of enzyme and total clearance of substrate in the heart and other tissues. After a secondary transplant, enzymatic levels were still elevated in several tissues when compared to untreated mice (Yoshimitsu *et al.*, 2007).

Finally, the use of non-viral vectors as naked plasmid and cationic lipid–pDNA complex was also reported (Przybylska *et al.*, 2004; Nakamura *et al.*, 2008). Although these are safer options, these vectors were not as effective as viral vectors and were unable to normalize the disease in the mouse model.

Galactosialidosis

Galactosialidosis (GS) is an autosomal recessive disease caused by mutations in the Cathepsin A gene (*CTSA)* that encodes the protective protein/cathepsin A (PPCA). Defective PPCA leads to secondary combined deficiency of β-galactosidase and neuraminidase, resulting in sialyloligosaccharides and glycopeptides accumulation. Patients present typical lysosomal disorder manifestations, such as coarse facies and skeletal deformities (Okamura-Oho *et al.*, 1994). Myocardial tissue is thickened and vacuolated and cardiovascular disease may occur, with mitral and aortic valve thickening leading to valve insufficiency (Bursi *et al.*, 2003) and left ventricular dilatation (Okamura-Oho *et al.*, 1994).

Initially, gene therapy studies focused on developing overexpressing PCAA transgenic mice to use as HSC donors to transplant PCAA-KO mice, which resulted in mild improvement in phenotype (Hahn *et al.*, 1998; Zhou *et al.*, 1995). Later on, an *ex vivo* gene therapy protocol was tested using RV MSCV-PPCA (MSCV - Murine Stem Cell Virus) modified PCAA-KO hematopoietic progenitor cells to transplant PCAA-KO mice. Although the number of PPCA

expressing cells varied between treated mice, systemic correction was observed in all MSCV-PPCA transplanted animals. In heart homogenate, cathepsin A activity was detected at higher levels than in untreated group for as long as 10 months (Leimig *et al.*, 2002).

The latest published study using gene therapy was using the vector AAV2/8-LP1-PPCA (LP1 - Liver Specific Promoter) to treat a cohort of sixty 30-day-old PCAA-KO mice, intravenously and with three vector doses. In addition to improved overall appearance and rescue of fertility, histological heart sections showed complete resolution of swollen lysosomes, as seen in other tissues (Hu *et al.*, 2012).

Gaucher Disease

Gaucher disease (GD) is the most common LSD, with an estimated worldwide incidence of 1:75.000 (Huang *et al.*, 2015). It is caused by deficiency of the enzyme glucocerebrosidase (GCase) and consequent deposition of the substrate glucocerebroside in liver, spleen and bone. There are three classical forms depending on the severity and onset of symptoms – type I (most common, without CNS involvement), type II (acute neuronopathic form) and type III (chronic neuronopathic form). The latter is characterized by a more attenuated phenotype, with mild neuronopathic features and visceral manifestations, including cardiac symptoms.

Patients with Gaucher disease type III (GD III) generally present widespread calcification of cardiovascular structures, such as ascending aorta, coronary and carotid arteries, and cardiac valves, leading to valvular stenosis, dilated cardiomyopathy and possibly congestive heart failure (Guertl *et al.*, 2000).

Most gene therapy studies for GD were developed in the 1990's, focusing mainly on protocols of *in vitro* HSC transduction for HSCT. After creation of GD murine models, some studies tried *ex* or *in vivo* gene therapy in mice, one of them resulting in increased GCase in the heart (Kim *et al.*, 2004). However, gene therapy for GD was not as successful as it seemed for other LSDs, probably because GCase is not normally secreted unless cells are expressing high levels of the enzyme, requiring very efficient vectors to deliver and induce significant gene expression (Marshall *et al.*, 2002).

GM1 Gangliosidosis

GM1 gangliosidosis is characterized by accumulation of GM1 gangliosides and related glycoconjugates in lysosomes due to β-galactosidase (β-gal) deficiency. Besides manifestations shared by most LSDs, one third of patients with GM1 gangliosidosis present congestive cardiomyopathy, regardless the classification of the disease (infantile, juvenile and adult) (Guertl *et al.*, 2000; Morrone *et al.*, 2000; Brunetti-Pierri and Scaglia, 2008).

Since cardiac involvement is not experienced by all patients, and neurological involvement is very pronounced, gene therapy protocols developed so far did not focus on heart manifestations but on the CNS. A couple of studies designed to target the CNS described an increase of β-gal activity in heart and other visceral tissues, using either AV vector (Takaura *et al.*, 2003) or AAV (Weismann *et al.*, 2015) when treating GM1 gangliosidosis mice.

Niemann-Pick Disease types A and B

Niemann-Pick disease (NPD) is caused by storage of sphingomyelin within lysosomes mainly of monocytes and macrophages. Niemann-Pick types A and B are associated with mutations in the Sphingomyelin Phosphodiesterase 1 gene (*SMPD1)* and type C is caused by mutations in the Intracellular Cholesterol Transporter genes *(NPC1* or *NPC2)*, thus presenting different clinical features. Both types A and B are a result of acid sphingomyelinase (ASM) deficiency, though NPD-A has neuronal involvement ,while NPD-B is only visceral (Schuchman and Wasserstein, 2015).

Early cardiovascular disease may occur in some patients with NPD-A and NPD-B, presenting valvar or coronary artery disease, which in turn is probably caused by the abnormal atherogenic lipid profile found in most patients. The mechanisms regarding valvar disease are still unknown (McGovern *et al.*, 2013).

The first paper describing gene therapy for NPD used 2-days-old ASM-KO mice injected with bone marrow cells previously modified with retroviral vector containing ASM (acid sphingomyelinase). ASM levels and sphingomyelin storage in the heart of treated animals were not statistically different than those found in the untreated mice. In other organs, the results were slightly better, suggesting improvement of disease manifestations. However, all transplanted mice eventually developed ataxia and died earlier than normal mice, which highlighted the need of further studies (Miranda *et al.*, 2000). Subsequently, a similar approach using two transplants – one with modified bone marrow cells intravenously at day 3 after birth and another with modified mesenchymal stem cells intracerebrally at day 30 – achieved improvement of neurological features, and ASM activity initially increased in the heart, though it decreased to only 2% of normal after 24 weeks (Jin and Schuchman, 2003). To our knowledge, none of published studies evaluated the efficiency of gene therapy on cardiac disease of NPD.

Sialidosis

Sialidosis is caused by progressive accumulation of sialylated glycopeptides and oligosaccharides due to neuraminidase 1 (NEU1) deficiency, also known as sialidase. General clinical manifestations include visceromegaly, coarse facies, dysostosis multiplex and mental retardation. Patients may also present cardiac anomalies, including val-

ve disease and increased ventricular wall thickness (Senocak *et al.*, 1994). Currently, no treatment is available.

Genetic transference of NEU cDNA to patients' fibroblasts has been tested to induce transient expression of the missing enzyme. NEU levels in fibroblasts were restored to normal range and increased further 10-fold when co-transfection with cathepsin A cDNA (PPCA, a chaperone required for lysosomal routing) was performed (Igdoura *et al.*, 1998); abnormal sialylglycoprotein deposits were reduced to normal levels as well (Oheda *et al.*, 2006). *In vivo* gene therapy using AAV2/8 vector to deliver the PPCA cDNA resulted in indirect increase in NEU activity from its residual levels, including in the heart. Further studies should be performed in order to better understand the disease mechanisms and more efficient approaches of therapy.

Conclusion and final considerations

Gene therapy is being tested along the last three decades for many diseases, and it seems very promising to treat LSDs. Since cardiovascular disease is an important feature of many of them – normally being one of the main causes of mortality among patients – it is important to evaluate how efficient this therapy is on the cardiovascular system and how it can impact on the patient's life.

Many gene therapy protocols have already been tested and, so far, they showed better results when performed in young and in immunodeficient/pre-conditioned animals. Intravenous injection is the most used route of administration because it is the one that distributes the vector more homogenously. Retrovirus and adeno-associated virus are the most used vectors and have not yet produced serious adverse effects in these animal models, being efficient and apparently safe.

The heart seems to be much beneficiated from most of gene therapy protocols, ameliorating or normalizing many of analysed parameters (Figure 1), such as wall thickness, electric conductance and heart rate. On the other hand, heart valves and aorta do not respond so well, probably due to poor and/or uneven vascularization, even when there are

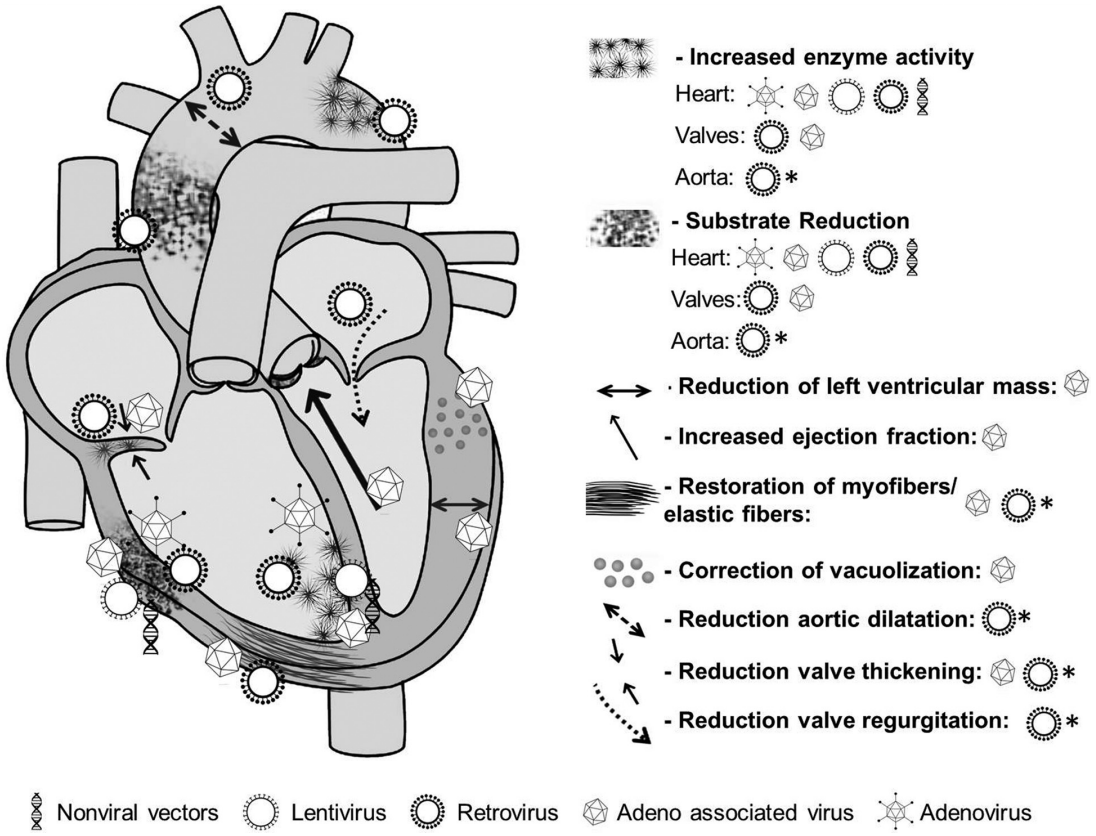

Figure 1 - Cardiovascular response to gene therapy depending on vector used, according to studies for lysosomal storage diseases. Schematic representation of the heart and the aorta showing the most prominent cardiovascular manifestations of lysosomal storage diseases (right) with the results obtained from *in vivo* gene therapy using different vectors (bottom). Retroviral and adeno-associated viral vectors resulted in better outcome for many aspects of the disease (although most studies using other vectors did not analyse thoroughly the effect of gene therapy on cardiovascular manifestations and there is no data available). Valves and aorta are most difficult to treat, as most vectors do not reach these structures as easily as the myocardium. Some features were only restored or prevented when treated in the first days of life (represented by * next to the vector symbol).

supraphysiologic levels of missing enzyme in the bloodstream. Aortic dilatation, valve thickening, valve regurgitation and valve insufficiency were delayed in most treated animals with MPS, but they still developed these conditions eventually. For Fabry and Pompe diseases, a similar outcome was observed – all treated mice had improvement in cardiac tissues, but the overall pathology was only partially corrected. For other diseases cited in this revierw, reduction of substrate and increase in enzyme activity was achieved after gene therapy, but this does not indicate cardiac disease resolution due to lack of more specific functional analysis.

Future directions

There are numerous studies using gene therapy so far, and this approach seems very promising, although more tests should still be performed, perhaps with combined treatments. The use of adjuvant drugs to modulate immune response or to increase enzyme uptake could be helpful, since only increasing serum levels does not seem suficient to have a complete correction of the phenotype.

Nanotechnology-based carriers designed specifically to target valve and aortic tissues, for example, could be a great adjuvant to current limited vectors. Possibly there will not be one perfect vector to fit all demands in an organism affected by a multisystemic disease, but an association of strategic vectors and adjuvant drugs could be the final solution.

Nonetheless, even if not completely corrective yet, stabilizing the disease or delaying the progression of important cardiovascular pathologies could make a big difference for the patient and thus should be continuously pursued.

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Conflict of interest

The authors declare absence of conflicts of interest.

Author contributions

EP and GP collected the data and wrote the manuscript; RG, UM and GB conceived the study and revised the manuscript. All authors read and approved the submitted version of the manuscript.

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ANEXO II.

ENGINEERING MONOCYTE/MACROPHAGE-SPECIFIC GLUCOCEREBROSIDASE EXPRESSION IN HUMAN HEMATOPOIETIC STEM CELLS USING GENOME EDITING

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Engineering monocyte/macrophage–specific glucocerebrosidase expression in human hematopoietic stem cells using genome editing

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Gaucher disease is a lysosomal storage disorder caused by insufficient glucocerebroside activity. Its hallmark manifestations are attributed to infiltration and inflammation by macrophages. Current therapies for Gaucher disease include life–long intravenous administration of recombinant glucocerebroside and orally-available glucosylceramide synthase inhibitors. An alternative approach is to engineer the patient's own hematopoietic system to restore glucocerebrosidase expression, thereby replacing the affected cells, and constituting a potential one-time therapy for this disease. Here, we report an efficient CRISPR/Cas9-based approach that targets glucocerebrosidase expression cassettes with a monocyte/macrophage-specific element to the CCR5 safe-harbor locus in human hematopoietic stem and progenitor cells. The targeted cells generate glucocerebroside-expressing macrophages and maintain long-term repopulation and multi-lineage differentiation potential with serial transplantation. The combination of a safe-harbor and a lineage-specific promoter establishes a universal correction strategy and circumvents potential toxicity of ectopic glucocerebrosidase in the stem cells. Furthermore, it constitutes an adaptable platform for other lysosomal enzyme deficiencies.

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Gaucher disease (GD) is genetic disorder caused by mutations in the *GBA* gene that result in glucocerebrosidase (GCase) deficiency and the accumulation of glycolipids in cell types with high-glycolipid degradation burden, especially macrophages[1]. GD encompasses a spectrum of clinical findings from a perinatal-lethal form to mildly symptomatic forms. Three major clinical types delineated by the presence (types 2 and 3) or absence (type 1) of central nervous system involvement are commonly used for determining prognosis and management[2]. In western countries, GD type 1 (GD1) is the most common phenotype (~94% of patients) and typically manifests with hepatosplenomegaly, bone disease, cytopenias, and variably with pulmonary disease, as well as elevated risk for malignancies and Parkinson's disease[3,4].

The pathophysiology in GD1 is thought to be driven by glucocerebroside-engorged macrophages that infiltrate the bone marrow, spleen and liver, and promote chronic inflammation, as well as low-grade activation of coagulation and complement cascades[5–7]. Current therapies for GD1 include orally available small-molecule inhibitors of glucosylceramide synthase (substrate reduction therapy or SRT) and glucocerebrosidase enzyme replacement (ERT) targeted to macrophages via mannose receptor-mediated uptake[8]. While ameliorative for visceral and skeletal disease manifestations, these therapies are chronically administered, life-long, and costly. Allogeneic hematopoietic stem-cell transplantation (allo-HSCT) has been applied successfully as a one-time treatment for GD1[9] and its therapeutic effect is achieved by supplying graft-derived GCase-competent macrophages. However, because of the significant transplant-related morbidity and mortality of allo-HSCT, ERT, and SRT are standard of care for patients with GD1[10,11].

The effectiveness of macrophage-targeted ERT and allo-HSCT for treating GD1 suggests that restoration of GCase function in macrophages alone is sufficient for phenotypic correction in GD1. Consequently, restoring GCase activity in the patient's own hematopoietic system to establish an autologous approach that averts many of the risks of allo-HSCT could be a safer and potentially curative therapy for this disease. Furthermore, unlike ERT and the best tolerated SRT, it could provide enzyme reconstitution in the brain that could benefit neuronopathic forms of the disease[9]. For these reasons, non-targeted gene addition into human hematopoietic stem and progenitor cells (HSPCs) have been explored, first using retroviruses[12–15] and later lentiviral vectors, and have yielded promising results in murine GD models[16–18]. Nevertheless, concerns remain about the potential for insertional mutagenesis and malignant transformation in viral gene transfer[19,20] stressing the need for the development of targeted gene addition strategies to generate genetically modified HSPCs for human therapy.

Modern genome-editing tools can achieve genetic modifications and integrations with single-base pair precision[21]. A highly engineerable platform derived from the bacterial CRISPR/Cas9 system has been optimized for gene editing in HSPCs[22–24]. This platform consists of two main components: (1) a sgRNA/Cas9 ribonucleoprotein complex (RNP) functioning as an RNA-guided endonuclease, and (2) a designed homologous repair template delivered using adeno-associated viral vector serotype six (AAV6). The RNP comprises a 100-bp, chemically modified, synthetically generated, single-guide RNA (sgRNA) complexed with *Streptococcus pyogenes* Cas9-endonuclase and delivered into the cells by electroporation[25]. In the nucleus, the RNP binds to the target sequence and Cas9 catalyzes a double-stranded break, stimulating one of two repair pathways: (1) non-homologous end joining (NHEJ), in which broken ends are directly ligated, often producing small insertions and deletions (indels); and (2) homology-directed repair (HDR), in which recombination with the supplied homologous repair template is used for precise sequence changes[21]. In human HSPCs, the AAV6 genome is an efficient delivery method for the homologous repair templates containing an experimenter-defined genetic change flanked by homology arms centered at the break site[22]. Accordingly, the HDR pathway can be leveraged not only to achieve single-base pair changes, but also to integrate entire expression cassettes into a non-essential safe harbor locus, thus enabling stable expression of tailorable combinations of regulatory regions, transgenes, and selectable markers[24,26]. One potential safe harbor locus is *CCR5*. This gene encodes the major co-receptor for HIV-1, and is considered a non-essential locus because of the high prevalence of healthy homozygous $CCR5^{\Delta 32}$ individuals in European populations (>10%)[27] and the observation that homozygous carriers of the Δ32 mutation are resistant to HIV-1 infection[28].

Here, we describe our generation and characterization of GCase-targeted human HSPCs, a crucial step towards establishing autologous transplantation of genome-edited cells for GD. We use the RNP/AAV6 platform to achieve efficient integration of GCase cassettes into the CCR5 safe harbor locus. By leveraging a lineage-specific promoter highly expressed in the monocyte/macrophage lineage, we achieve GCase expression in the affected cell lineages while also minimizing ectopic expression in hematopoietic stem and progenitor compartments. GCase-targeted HSPCs demonstrate the capacity for long-term engraftment and multi-lineage differentiation, including the generation of functional macrophages with supraphysiologic GCase expression in vivo.

Results

Efficient targeting of GCase to the *CCR5* locus in human HSPCs. We used the CRISPR/Cas9 and AAV system to target glucocerebrosidase (GCase) expression cassettes to the human *CCR5* safe harbor locus (Fig. 1a). The sgRNA targeting the third exon of *CCR5* was previously validated for high on-target activity in primary human HSPCs[24,29] and has excellent specificity as prior studies failed to reveal any detectable off-target activity using high-fidelity Cas9[24]. AAV donor repair templates were generated to drive GCase expression by two different promoters: (1) the Spleen Focus-Forming Virus (SFFV) promoter, which drives constitutive supraphysiologic expression; and (2) the CD68S promoter, a shortened derivative of the endogenous human CD68 promoter with expression restricted to the monocyte/macrophage lineage[30,31] (Fig. 1b). This lineage-specific promoter was chosen to minimize potential complications of GCase overexpression in the stem-cell compartment. The Citrine-containing vectors were designated SFFV-GCase-P2A-Citrine and CD68S-GCase-P2A-Citrine. A third AAV, CD68S-GCase, lacking the reporter protein, was developed as a more clinically relevant vector for in vivo studies (Fig. 1a).

The targeting efficiencies achievable for each vector were determined by the percent of Citrine-positive (Citrine+) cells and by the percent of *CCR5* alleles with on-target cassette integrations using molecular analysis (giving the cell and allele targeting frequencies, respectively). In the presence of both AAV and RNP, the SFFV-driven cassette resulted in approximately 51.5 ± 9.1% (mean ± SD) Citrine+ HSPCs 48-h post-targeting, while AAV alone produced 5.9 ± 4.2% dim Citrine+ cells, likely reflecting episomal expression (Fig. 1c, d). The fraction of *CCR5* alleles with on-target cassette integration in the unselected population was 29 ± 9% as measured by droplet digital PCR (ddPCR) (Fig. 1e and Supplementary Fig. 1a). To verify targeting in Citrine+ cells, these cells were sorted by FACS and the fraction of modified alleles measured (Fig. 1e and Supplementary Fig. 1a). The allelic modification frequency of HSPCs treated with the SFFV-GCase-P2A-Citrine vector that were Citrine+ (SFFV-GCase-Citrine+) was 65.9 ± 4.9%,

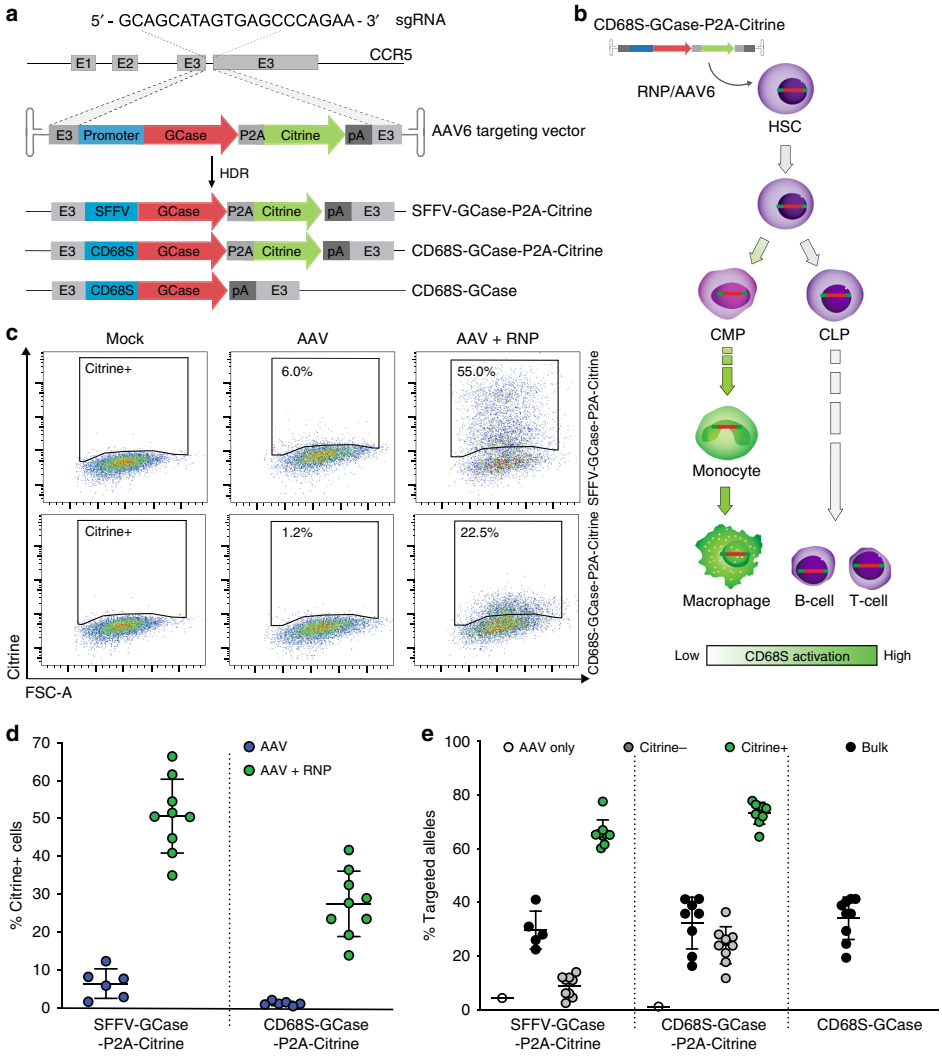


Fig. 1 Efficient targeting of GCase to the CCR5 locus in human HSPCs 48-hours post-modification. a Schematic of gene targeting mediated by sgRNA/Cas9 RNP and rAAV targeting vectors where E1-3 are *CCR5* exons. **b** Schematic of expected CD68S promoter activity. Green indicates activation. **c** Representative flow plots of Citrine expression versus forward scatter (FSC) for HSPCs without treatment (mock), treated with rAAV alone (AAV), and treated with RNP and rAAV (RNP+AAV). **d** Flow cytometric quantification of Citrine+ HSPCs targeted with SFFV-GCase-P2A-Citrine and CD68S-GCase-P2A-Citrine vectors in the presence (green circles) or absence (blue circles) of RNP ($n = 9$ biologically independent human donor samples). **e** Percent of *CCR5* alleles with integrated CD68S-GBA-P2A-Citrine and SFFV-GBA-P2A-Citrine cassettes in AAV only (white), bulk (black), FACS-enriched Citrine– (gray) and Citrine+ (green) HSPCs, and in bulk CD68S-GCase-targeted cells (black). Data shown as mean ± SD. Source data are provided as a Source Data file.

corresponding to 69% and 31% mono-allelically and bi-allelically targeted cells, respectively. Genotyping of single-cell-derived colonies corroborated that 98% percent of the Citrine+ HSPCs were targeted and, consistent with the ddPCR data, showed 67% mono-allelic and 33% bi-allelic targeting (Supplementary Fig. 1b–d).

We predicted that because the CD68S promoter should be lineage-specific, Citrine would not be highly expressed in stem and non-myeloid biased progenitor cells and therefore, Citrine expression in HSPCs would not reflect the true editing efficiency of the CD68S-P2A-GCase-Citrine vector (Fig. 1b). Consistent with this, we found that at 48-h post-modification, Citrine expression from HSPCs treated with the CD68S-GCase-P2A-Citrine AAV and RNP was dim (mean fluorescence intensity (MFI) was 24-fold lower than for the SFFV-GCase- Citrine+ cells) and the mean percentage of CD68S-GCase-Citrine+ HSPCs was 27.7 ± 8.5%, significantly lower than for the SSFV-driven construct despite having comparable *CCR5* allele targeting frequencies (32.3 ± 9.6%) (Fig. 1c–e). Most importantly, the allele targeting frequency within the CD68S-GCase-Citrine-negative population (CD68S-GCase-Citrine–) ranged from 11.8 to 36.4%, confirming the presence of targeted cells lacking Citrine expression (Fig. 1e). We reasoned that the subset of

CD68S-GCase-Citrine+ HSPCs likely comprise a subpopulation of granulocyte-monocyte-committed progenitors with increased CD68S promoter activation, while CD68S-GCase-Citrine– HSPCs contain the more primitive populations. Single-cell-derived colony genotyping confirmed that 96.5% of the Citrine+ cells had targeted cassette integrations and showed frequencies of mono-allelic and bi-allelic editing of 64% and 36%, respectively (Supplementary Fig. 1d). The allele targeting frequency of the CD68S-GCase vector lacking Citrine was 35.8 ± 7.9% in unselected cell populations corresponding to ~52% of cells having targeted integrations (Fig. 1e).

Generation of human GCase-macrophages from edited HSPCs. One mechanism by which HSCT is therapeutic in Gaucher disease is through the generation of GCase-expressing macrophages. To confirm the development of macrophages from GCase-targeted HSPCs, we first differentiated control human CD34+ HSPCs using a cytokine cocktail, including M-CSF, GM-CSF, SCF, IL-3, FLT3 ligand, and IL-6[32]. HSPCs differentiated in this manner exhibited characteristic ameboid morphology as well as expression of the monocyte/macrophage lineage markers CD14 and CD11b, with concurrent loss of the HSPC marker CD34 (Fig. 2a, b and Supplementary Fig. 2a). Following the same differentiation protocol, human HSPCs targeted with the SFFV-GCase-P2A-Citrine and CD68S-GCase-P2A-Citrine constructs, produced macrophages that exhibited Citrine expression, characteristic morphology, and normal phagocytosis of pHrodo-labeled *E. coli* (Fig. 2c). CD14 and CD11b marker expression in mock-treated, Citrine+ and Citrine– populations from these two constructs revealed comparable expression compared to unmodified cells in all conditions except in CD68S-GCase-Citrine+ cells, which had higher expression in both the standard HSPC and macrophage differentiation conditions (Fig. 2d, e and Supplementary Fig. 2b). These results indicate that GCase-targeted HSPCs can produce functional macrophages in vitro and suggest that CD68S-GCase-Citrine+ HSPCs are already primed for differentiation along this lineage.

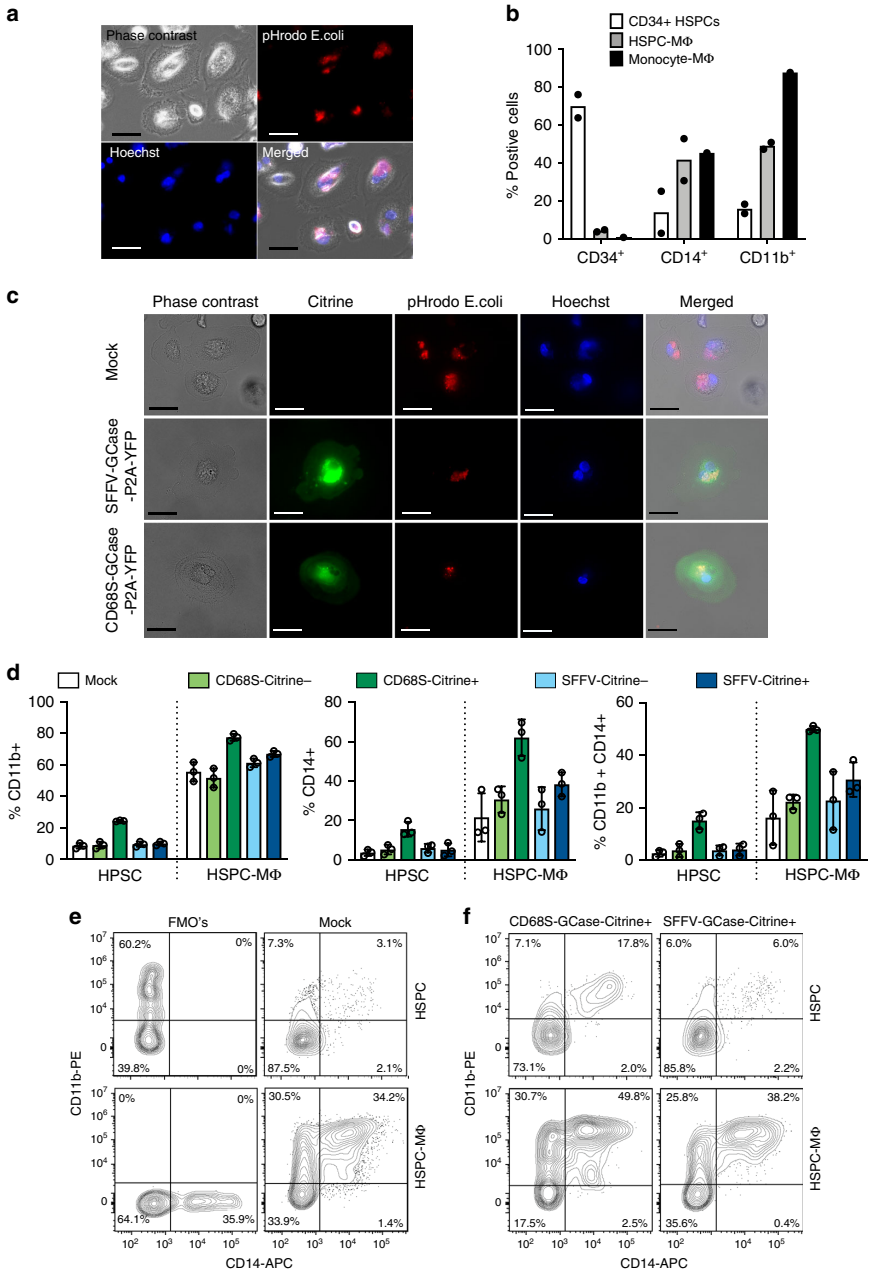
CCR5 is absent from HSPCs but becomes expressed with monocyte/macrophage differentiation. To examine the effect of our genome editing process on CCR5 expression we targeted human HSPCs, differentiated them, and quantified CCR5 protein by FACS (Supplementary Fig. 3). In the RNP alone condition, the efficiency of double-strand DNA break generation by our CCR5 RNP complex was estimated by measuring the frequency of insertions/deletions (Indel) at the predicted cut site. The mean indel frequencies in the undifferentiated and differentiated populations was 96.8% ± 1.2 and 96.4% ± 1.6, respectively, resulting in almost complete knock-down of CCR5 protein expression (Supplementary Fig. 3a). In the presence of both RNP and AAV, cells that successfully underwent HDR (Citrine+) lacked CCR5 expression, consistent with disruption of both CCR5 alleles by either bi-allelic integration of the cassette or mono-allelic with indel formation in the second allele (Supplementary Fig. 3b). In the presence of AAV, CCR5+ cells can be found in the population that did not undergo HDR (~20%), suggesting that AAV transduction decreases indel generation or exerts a small-negative selection in cells containing both AAV and RNP.

CD68S confines expression to the monocyte/macrophage lineage. The CD68S cassettes were designed to selectively express GCase in the monocyte/macrophage lineage in order to prevent potential toxicity to stem cells from ectopic GCase over-expression. To validate the lineage specificity of the CD68S promoter, CD68S-GCase-Citrine+ and SFFV-GCase-Citrine+ HSPCs were cultured with growth factors that promoted either HSPC maintenance (HSPC) or macrophage differentiation (MΦ) and Citrine expression was monitored for 20 days. As expected for a constitutive promoter, the fraction of SFFV-GCase-Citrine+ cells remained stable over time in both HSPC and MΦ cultures (>95%). An average of 9.2% and 16.3% of SFFV-GCase-Citrine– cells became positive in the HSPC and MΦ cultures, respectively, which was consistent with the presence of targeted *CCR5* alleles in this population based on ddPCR (Fig. 3a, b). When cultured long-term, the MFI of SFFV-GCase-Citrine+ cells decreased, but the drop in fluorescence intensity was seen exclusively in a subset of cells with very high Citrine expression (Supplementary Fig. 4a, b). Notably, the allele modification frequency did not differ throughout the culturing process, suggesting that the change in Citrine expression was due to regulation of transcription from SFFV promoter or translation but not to selection against the modified cells (Supplementary Fig. 4c). In contrast, the percentage of CD68S-GCase-Citrine+ cells decreased in the HSPC cultures but was maintained in the MΦ cultures (Fig. 3a, b). Moreover, there was a substantial increase (~30-fold) in Citrine MFI from CD68S-GCase-Citrine+ cells in the MΦ compared to the HSPCs culture over the 21-day differentiation (Fig. 3c).

As Citrine is only a proxy for GCase cassette expression, we also examined GCase protein expression directly by quantifying its enzymatic activity in HSPC and MΦ culture conditions. In HSPC cultures, SFFV-GCase-Citrine+ and CD68S-GCase-Citrine+ cells showed ~7.7 and 1.3-fold more GCase activity, respectively, compared to unmodified cells (mock-treated). The CD68S-GCase-Citrine– population showed the same activity as unmodified cells (1.0-fold) supporting the idea that there is no leakage GCase expression from the CD68S promoter in more primitive and non-myeloid HSPCs (Fig. 3d). Macrophages derived from CD68S-GCase-Citrine+ and SFFV-GCase-Citrine+ HSPCs expressed ~2-fold higher GCase than macrophages derived from mock-treated cells (Fig. 3e). In all but the SFFV-GCase-Citrine+ population, macrophage differentiation resulted in higher levels of GCase expression. This explains the decrease in fold expression in cells targeted with the SFFV-driven cassette with differentiation (from 7.7 to 2.3), as it reflects the marked increase in endogenous GCase (~4-fold) in the mock cells without a proportional change in exogenous GCase expression from the SFFV expression cassette (Supplementary Fig. 4d).

To examine the possibility that differential expression of the GCase cassette was due to changes in the targeted cell populations, we measured the allele targeting frequencies at the time of sorting and post-culture in the HSPC and MΦ cultures using ddPCR (Fig. 3f). We found that the percentage of alleles with on-target cassette integration within Citrine+ and Citrine– populations targeted with both cassettes did not differ between culturing conditions, thus confirming that the changes in expression were attributable to the lineage-specific activity of the CD68S promoter.

GCase-targeted HSPCs sustain long-term hematopoiesis. To examine the potential of GCase-HSPCs to become a one-time therapy for GD1, we tested their long-term repopulation capacity. We first assessed the colony-forming ability of the targeted HSPCs in vitro using the colony-forming unit (CFU) assay. We sorted mock, Citrine+ and Citrine– from SFFV and CD68S targeted populations as single cells in 96-well plates 48-h post-transplantation and assessed their phenotype 14 days later. Notably, SFFV-GCase-Citrine+ HSPCs produced the fewest colonies of all conditions and exhibited the highest variability in the distribution of colony phenotypes formed, suggesting that supraphysiologic GCase expression or other aspects of SFFV promoter physiology may have a toxic effect on HSPCs (Fig. 4a). As predicted by the model of restricted lineage expression of the



CD68S promoter, CD68S-GCase-Citrine+ HSPCs formed exclusively CFU-GM's (granulocyte/monocyte), while the cells that did not express Citrine (CD68S-GCase-Citrine–) produced a normal distribution of colony phenotypes (Fig. 4b). These results strongly support our earlier hypothesis that CD68S-GCase-Citrine+ cells in undifferentiated HSPCs represent granulocyte/monocyte primed progenitors and that bona fide CD68S-GCase–P2A-Citrine-targeted stem cells reside within the CD68S-GCase-Citrine– population.

To test in vivo engraftment potential, GCase-targeted HSPCs were serially transplanted into NOD-scid IL2Rgamma (NSG) mice. Cell doses varied from 2.5×10^5 to 2×10^6 HSPCs and were dependent on the CD34+ cell yield per human donor. We focused our long-term engraftment experiments on the CD68S-GCase-P2A-Citrine and CD68S-GCase vectors because of the potential detrimental effect of the SFFV promoter, its observed drop in expression, and its barriers to clinical translation. Targeted cells were transplanted without selection intrafemorally or intrahepataicaly into sub-lethally irradiated NSG mice. Primary human engraftment was quantified after 16 weeks as the percentage of cells expressing human CD45 within the total hematopoietic

Fig. 2 Generation of human GCase-macrophages from genome-edited HSPCs. a Representative images showing phase contrast, phagosomes visualized by pHrodo-labeled *E.coli* (red), and nuclei (blue) in mock-treated human HSPCs after 20 days in macrophage differentiation media for one of the two samples analyzed in **b**. Scale bar 10 µm. **b** Human CD34, CD14, and CD11b marker expression in HSPC-derived macrophages (HSPC-MΦ) and human monocyte-derived macrophages (Monocyte- MΦ) after in vitro differentiation compared to undifferentiated cells ($CD34^+$ HSPCs) ($n = 2$ biologically independent human donor samples). **c** Representative images showing phase contrast, Citrine expression (green), phagosomes visualized by pHrodo-labeled *E.coli* (red), and nuclei (blue) in mock-treated, SFFV-GCase-P2A-Citrine, and CD68S-GCase-P2A-Citrine targeted macrophages for one of the three samples analyzed in **d**. Scale bar 20 µm. **d** Human CD14, and CD11b marker expression in mock-treated (white), CD68S-GCase-P2A-Citrine targeted (Citrine−: light green; Citrine+: dark green), and SFFV-GCase-P2A-Citrine targeted cells (Citrine−: light blue; Citrine+: dark blue) with and without macrophage differentiation. Left graph: CD11b+. Middle graph: CD14+. Right graph: CD11b+/CD14+ ($n = 3$ biologically independent human donor samples). **e** Representative FACS plots of Fluorescence Minus One controls (FMO's) and Mock samples showing CD11b and CD14 expression in HSPC maintenance or Macrophage differentiation media. **f** Representative FACS plots showing CD11b and CD14 expression in CD68S-GCase-Citrine+ and SFFV-GCase-Citrine+ cells in HSPC maintenance or macrophage differentiation media. Data shown as mean ± SD. Source data are provided as a Source Data file.

population (mouse CD45+ and human CD45+, Supplementary Fig. 5).

Transplantation of GCase-targeted HSPCs resulted in substantial human cell chimerism. In the bone marrow, the median human cell chimerism was 23.2% (min: 0.17%; max: 91.5%) and 50.6% (0.53%; 91.7%) in CD68S-GCase-targeted and CD68S-GCase-P2A-Citrine-targeted cells, respectively (Fig. 4c). Similar engraftment numbers were seen in the spleen: 20.4% (0.14%; 79.3%) for the cassette lacking Citrine and 35.8% (0.38%; 89.6%) for the cassette having Citrine (Fig. 4d). To determine the proportion of engrafted cells derived from targeted HSPCs, the targeted allele frequency of the engrafted hCD45+ population in the bone marrow was measured using ddPCR in cell preparations that included mouse and human CD45+ cells as the ddPCR assay recognizes only human alleles (Fig. 4e and Supplementary Fig. 6a). The median allele targeting frequencies of the engrafted cell populations were 4.4% (min: 0.23%; max: 51.0%) and 4.2% (0.73%; 34.6%) for the CD68S-GCase and CD68S-GCase-P2A-Citrine cassettes, respectively; however, allele targeting frequency varied highly across human cell donors and mice. The allele targeting frequency of the engrafted cells tended to be lower compared to the transplanted HSPCs, with an observed drop ranging from 1.9 to 12.5-fold (Supplementary Fig. 6b). As cell doses of transplantation varied in the mice targeted with the Citrine-containing construct, the mice were colored-coded and tracked for engraftment and targeting efficiency in engrafted cells. This suggested a correlation between higher cell dose and higher engraftment of modified cells, a finding that is not surprising as there are likely more targeted long-term stem cells available for engraftment.

Serial engraftment studies are the gold standard to determine self-renewal capacity of hematopoietic stem cells. Secondary transplants were performed by isolating human CD34+ cells from bone marrow in eight 16-week mice (seven from CD68S-GCase and one from CD68S-GCase-P2A-Citrine targeted cells) and transplanting them (without pooling) into eight NSG recipient mice. Human engraftment and allele targeting frequency were assessed 16 weeks later (32 weeks post-modification) as previously described (Supplementary Fig. 7). The median human cell chimerism of all transplants was 10% (Range: 0.04%–48.9%) (Fig. 4f). Droplet digital PCR analysis of the engrafted cells from mice with human cell chimerism >1% ($n = 5$) showed a median allele targeting frequency of 21.9% (min: 1.3%; max: 40.5%), compared to 6.3% in the cells prior to transplantation (Fig. 4g). We reason that this increase in allelic targeting pre-to-post transplantation in secondary transplants reflects that targeted HSPCs that undergo primary engraftment in an NSG recipient have high engraftment potential and confirms the presence of long-term repopulating hematopoietic stem cells in the genome-edited population that are capable of long-term engraftment in vivo.

In vivo differentiation of GCase-targeted HSPCs. To examine the multi-lineage differentiation potential of GCase-targeted HSPCs in vivo we measured lymphoid and myeloid engraftment by the expression of the cell surface markers hCD19 (B-cells) and hCD33 (pan-myeloid), respectively. We included only mice with human engraftment >1% as these have sufficient cell numbers to reliably measure myeloid and lymphoid reconstitution. In primary engraftment studies, the median percentage of myeloid cells and B-cells in the bone marrow was 27.4% and 65.9%, respectively, for the mice transplanted with CD68S-GCase-targeted HSPCs, and 19.3% and 70%, respectively, for the mice transplanted with CD68S-GCase-P2A-Citrine-targeted HSPCs (Fig. 5a). In general, B-cell production was higher than myeloid and consistent with what has been previously reported for unmodified cells[33,34]. We similarly found myeloid and lymphoid cell production in secondary engraftment mice in five of the eight mice with bone marrow chimerism >1% (Fig. 5b). Mice with low human cell chimerism (<1%), have low cells numbers making the quantitation of targeted human alleles and human subpopulations less reliable.

To assess the lineage specificity of the CD68S promoter in vivo, we compared Citrine expression in the B-lymphoid and myeloid compartments in primary engraftments studies of CD68S-GCase-P2A-Citrine-targeted HSPCs that had robust engraftment of targeted cells (allele modification fraction >10%). As expected, expression of the CD68S-GBA-P2A-Citrine cassette was restricted to the myeloid (CD33+) and monocyte lineages (CD14+), with more frequent expression seen in monocytes (Fig. 5c, d). Despite robust modification in the bone marrow, three mice did not show Citrine expression in monocytes, which could be due to incomplete differentiation along this lineage since the human cells are lacking the appropriate cytokines or expression that is below our rigorous gating strategy. As the generation of GCase-expressing macrophages is critical to addressing Gaucher disease pathophysiology, it was also important to verify that engrafted, GCase-targeted HSPCs have the capacity to produce human macrophages with heterologous GCase expression. Towards this end, human CD14+ monocytes were isolated via FACS from the bone marrow of transplanted mice 16 weeks post-transplantation and differentiated by adding human macrophage colony stimulating factor (M-CSF). This step was performed in vitro because mouse M-CSF, a cytokine required for macrophage differentiation, does not have activity on human cells[35]. Human macrophages differentiated in this manner showed expression of the lineage marker CD68, as well as Citrine (12.3 ± 4.5% of human CD68+ cells), verifying that engrafted, targeted HSPCs can produce macrophages that express the therapeutic GCase cassette (Fig. 5e and Supplementary Fig. 8).

To improve engraftment and differentiation of myeloid lineages of our modified HSPCs in vivo, we performed transplantation experiments in NSG-SGM3 mice. These are

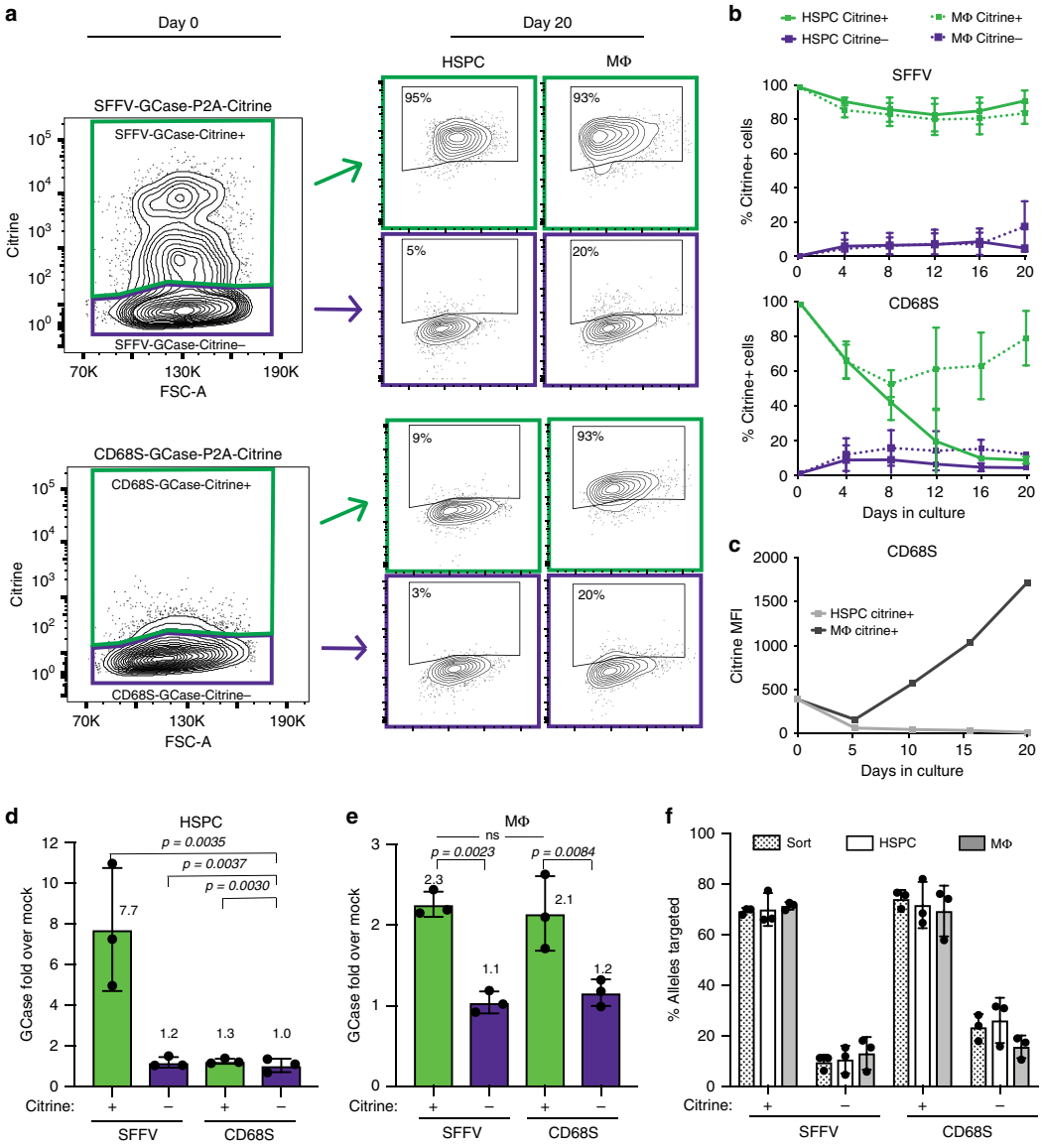


Fig. 3 CD68S promoter confines GCase expression to the monocyte/macrophage lineage. a Representative flow plots showing Citrine+ and Citrine- populations at the time of sort (day 0, 48-h post-modification) and after 20 days in HSPC maintenance (HSPC) or macrophage differentiation (MΦ) cultures. **b** Citrine expression expressed as %Citrine+ cells over time in HSPC and MΦ cultures. HSPC Citrine+ (solid green line), HSPC Citrine- (solid purple line), MΦ Citrine+ (dotted green line), MΦ Citrine- (dotted purple line) ($n = 3$ biologically independent samples). **c** Representative Citrine expression expressed MFI over time in HSPC (gray) and MΦ (black) cultures in the CD68S-GCase-P2A-Citrine-targeted cells. **d** Fold GCase activity in HSPC and **e** MΦ cultures in targeted cells compared to unmodified (mock-treated) cells ($n = 3$ biologically independent samples). Comparisons between groups were performed using one-way ANOVA test and post-hoc comparisons were made with the Tukey's multiple comparisons test. **f** Percent of targeted *CCR5* alleles at the time of sort (dotted) and after 20 days in HSPC (white) and MΦ (gray) cultures ($n = 3$ biologically independent samples). Data shown as mean ± SD. Source data are provided as a Source Data file.

NSG mice expressing human interleukin-3 (IL-3), human granulocyte/macrophage-stimulating factor (GM-CSF), and human Stem Cell Factor (SCF or KIT-ligand), cytokines that support the engraftment and differentiation of human-myeloid lineages[36,37]. At 16 weeks, transplantation of CD68S-GCase-P2A-Citrine-targeted cells resulted in median human cell chimerism of 17.7% (min: 5.1%; max: 39.6%), 61.7% (min: 22.1%; max: 85.8%), and 33.6% (min: 1.8%; max: 72%) in the bone marrow, spleen and peripheral blood, respectively (Fig. 6a). The median allele targeting frequencies of the engrafted cell populations were 15.6% (min: 12%; max: 20%), 20.4% (min: 16%; max: 25%), 5.0% (min: 2%; max: 29%) in the same tissues (Fig. 6b). The observed

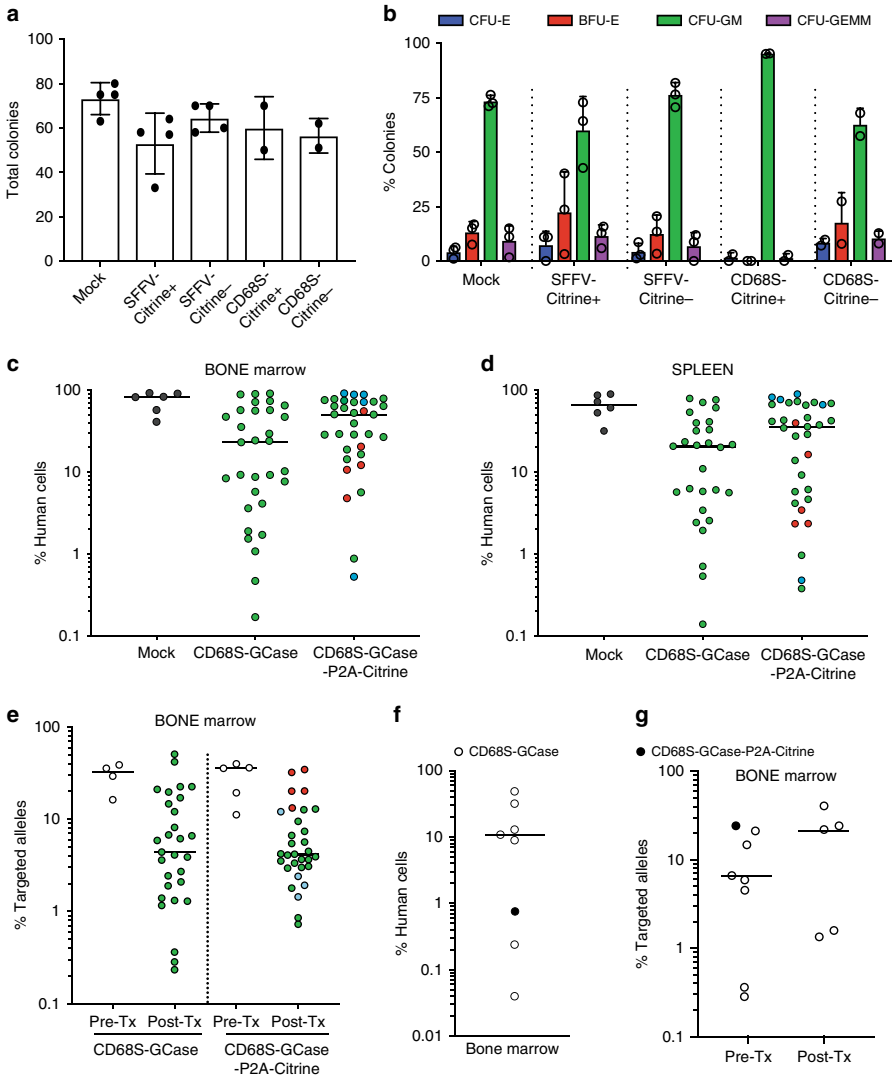


Fig. 4 GCase-targeted HSPCs sustain long-term hematopoiesis. a Total number of colonies formed from mock, Citrine+ and Citrine- SFFV and CD68S-driven constructs ($n = 4$ biologically independent human donor samples for Mock and SFFV and $n = 2$ for CD68S). **b** Distribution of phenotypes of colonies formed. Erythroid progenitors (burst-forming unit-erythroid or BFU-E (red)) and colony-forming unit-erythroid or CFU-E (blue), granulocyte-macrophage progenitors (CFU-GM, green), and multi-potential granulocyte, erythroid, macrophage, megakaryocyte progenitor cells (CFU-GEMM, purple) ($n = 4$ biologically independent human donor samples for Mock and SFFV and $n = 2$ for CD68S). **c** Primary human engraftment (16 weeks) in the bone marrow in transplants using CD68S-GCase-targeted and CD68S-GCase-P2A-Citrine-targeted cells (blue circles: 0.25E6, green: 1E6, and red: 2E6 cells transplanted; $n = 31$, 33 mice). **d** Primary human engraftment in the spleen. **e** Targeted allele frequency in CD68S-GCase- and CD68S-GCase-P2A-Citrine-targeted cells before transplantation (Pre-Tx) and 16-weeks post-transplantation (Post-Tx) in engrafted human cells in the bone marrow of mice with human chimerism >1% ($n = 29$, 31 mice). **f** Secondary human engraftment (32 weeks) in the bone marrow ($n = 8$, black: CD68S-GCase-P2A-Citrine, white: CD68S-GCase). Note, three mice have chimerism <1%. **g** Targeted allele frequency before (Pre-Tx) and after transplant (Post-Tx) in the bone marrow cells of secondary mice. **a**–**b** Data shown as mean ± SD. **c**–**g** Median shown. Source data are provided as a Source Data file.

drop in modified engrafted cells relative to the pre-transplant level (43%) was 2.7-fold in the bone marrow, consistent with but in the low range of studies in NSG mice (Fig. 4e). We observed B, myeloid, and monocyte development with less preponderance of B-lymphoid population compared to NSG mice. As before, Citrine+ cells were seen exclusively in the myeloid and monocyte cells (Fig. 6c). Tissue macrophages were extracted from liver and lung using an enzymatic method and peritoneal macrophages were obtained by analysis of peritoneal fluid. We found robust human cell populations that were CD45+ or CD45/CD11b+ as well as Citrine+ in these macrophage cell preparations (Fig. 6d–f). Samples with high cell numbers that allowed enrichment of live human-myeloid-Citrine+ for enzymatic analysis were sorted and the GCase activity measured.

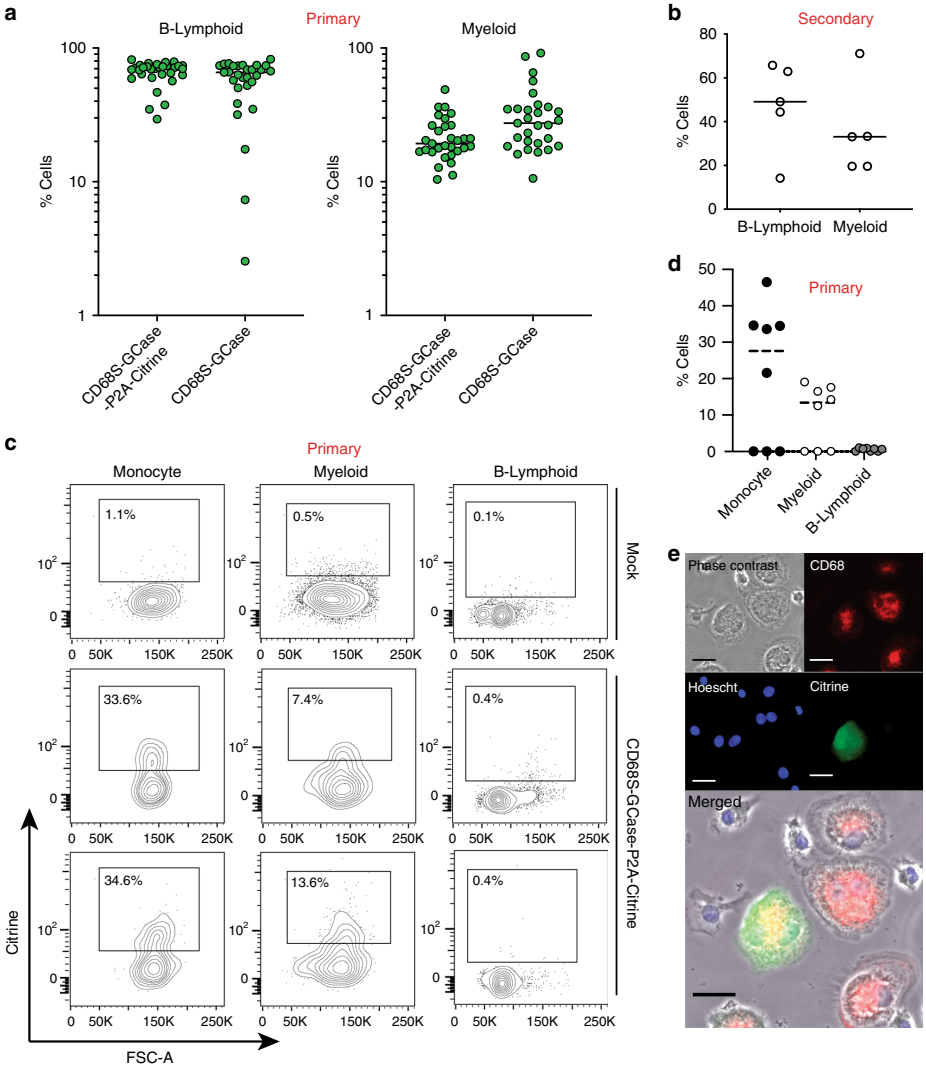


Fig. 5 In vivo monocyte/macrophage lineage differentiation of GCase-targeted HSPCs. a Distribution of B-lymphoid and myeloid lineage cells within the engrafted human cell population in the bone marrow from mice transplanted with CD68S-GCase and CD68S-GCase-P2A-Citrine-targeted HSPCs ($n = 29$, 31 mice). **b** Distribution of B-lymphoid and lineage cells within the engrafted human cell population from secondary transplants. Empty: vector without Citrine ($n = 5$ mice). **c** Representative FACS plots showing Citrine expression in human CD33+ (Myeloid), CD14+ (Monocyte) and CD19 (B-cells). **d** Percent Citrine-positive cells in monocyte (black), myeloid (white), and B-cell (gray) populations in mice with human CCR5 allele modification fraction >10%. **e** Representative epifluorescence microscopy images of human CD68S-GCase-P2A-Citrine-targeted macrophages differentiated from human CD14+ cells sorted from mice bone marrow and peripheral blood ($n = 10$ mice, additional examples in Supplementary Fig. 8). Images depict morphology (brightfield), nuclei (Hoechst, blue), CD68 protein (red), and Citrine (green). Scale bar is 10 µm. **a**, **b**, **d** Median shown. Source data are provided as a Source Data file.

Consistent with our studies of HSPCs differentiated in culture, the Citrine+ cells expressed 2.0 (bone marrow), 2.1 (spleen), and 1.6-fold (lungs) higher GCase than Citrine– cells (Figs. 3e and 6g). Analysis of targeted *CCR5* alleles from sorted cells populations, including bone marrow, lung, spleen, liver, and peritoneal macrophages show enrichment of targeted alleles in the Citrine+ cells compared to Citrine– cells confirming that the observed Citrine expression is from targeted cells (Fig. 6h).

Discussion

Gaucher disease is currently treated using enzyme replacement therapy (ERT) and substrate reduction therapy (SRT). Both approaches have been shown to be effective at addressing hematological and visceral manifestations[38,39] and can reduce, but not eliminate, bone complications in this disease[40,41]. Neither ERT, not the best tolerated form of SRT (eliglustat), are expected to impact neuronopathic forms of GD (GD2 and GD3) or the increasingly recognized neurological symptoms in GD1[42,43]. ERT

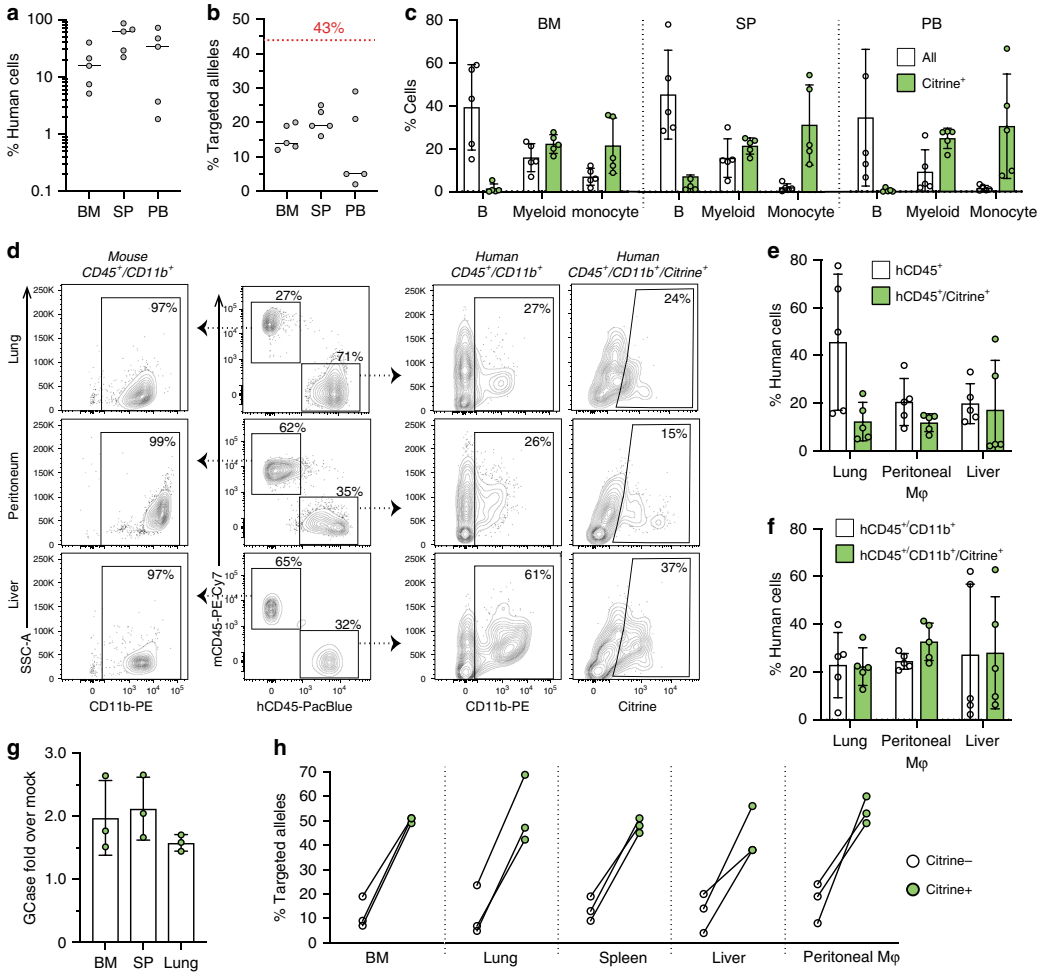


Fig. 6 Improved macrophage differentiation of GCase-targeted HSPCs in NSG-SGM3 mice. a Human cell engraftment 16-weeks post-transplantation in the bone marrow (BM), spleen (SP), and peripheral blood (PB) in transplants using CD68S-GCase-P2A-Citrine-targeted cells ($n = 5$ mice). **b** Modified allele frequency from engrafted CD68S-GCase-P2A-Citrine-targeted cells in the same tissues ($n = 5$ mice). **c** Percent human B-cell ($CD19^+$), myeloid ($CD33^+$), and monocyte ($CD14^+$) populations in BM, SP, and PB shown in white. Citrine-positive cells in each population are shown in green ($n = 5$ mice). **d** Representative FACS plots showing gating strategy for mouse and human $CD45^+$, $CD45^+/CD11b^+$, and $CD45^+/CD11b^+$/Citrine populations in macrophage preparations from lung, peritoneum, and liver. **e** Percent human $CD45^+$ and human $CD45^+$/Citrine cells in the same preparations ($n = 5$ mice). **f** Percent human $CD45^+/CD11b^+$ and human $CD45^+/CD11b^+$/Citrine cells in the same preparations ($n = 5$ mice). **g** Fold GCase activity in human Citrine$^+$ cells compared to human Citrine$^-$ cells in BM, SP, and lung ($n = 3$ mice). **h** Modified allele frequency in human Citrine$^+$ cells (green) compared to human Citrine$^-$ cells (white) in BM, SP, and lung in the same cells. **a**, **b** Median shown. **c**, **e**–**g** Data shown as mean ± SD. Source data are provided as a Source Data file.

involves life-long, bi-weekly infusions, and the development of antibodies can, in some cases, decrease enzyme bioavailability and impact clinical outcome[44,45]. Approved SRTs (miglustat and eliglustat) also require life-long administration, repeated dosing (three and two times per day, respectively) and, particularly for miglustat, significant side effects due to non-specific inhibition of other enzymes[46]. Both modalities are very costly with estimated annual cost of $300,000 to $450,000 (estimated life-time cost of ~ $6 to $22 million dollars) limiting their availability worldwide[47,48]. In the past, allo-HSCT was used effectively and led to rapid improvement in the hematological and visceral parameters as well as regression of skeletal disease, but given its significant morbidity and mortality, its use has been reserved for individuals with neurologic or progressive disease unresponsive to ERT and SRT[49–52]. Specifically, allo-HSCT has shown potential to halt neurological progression in patients with GD type 3 (D3) when treated at a young age and early in the disease process[53–56].

Given the potential for HSCT to constitute a one-time therapy for GD1 and its likely beneficial effect in the central nervous system (CNS), improving the safety of HSCT for GD would be a significant development. The use of autologous HSPCs is safer because it eliminates the morbidity of graft-versus-host disease, results in faster engraftment, and can lead to earlier intervention by obviating the need for donor matching. For this reason, non-otargeted lentiviral-mediated delivery of constitutively expressed GCase is being explored in HSPCs and has yielded

promising results in murine GD models where transplantation of these cells achieved normalization of GCase levels, reduced Gaucher cell infiltration, and lowered glucocerebroside storage[16–18]. However, because of the pseudorandom integration of the viral genomes, concerns remain about its potential for tumorigenicity[19,20]. Genome editing, as a more precise genetic tool, decreases the chance of random integration and ensures more predictable and consistent transgene expression. In addition to the hematopoietic system, the liver has also been considered as potential enzyme replacement depot and in vivo liver-directed approaches using zinc finger nucleases have also been investigated in mouse models[57]. However, it is not clear the liver-secreted GCase would have the proper glycosylation to cross-correct affected cells or that it could cross into the CNS. Transplantation of ex vivo genome-edited HSPCs can provide direct replacement of pathological cells and leverages the ability of graft-derived macrophages that can migrate to the brain[14] and bone. Therefore, autologous transplantation of gene-corrected cells, if coupled with safer conditioning regimens, could be a promising therapy for GD patients regardless of disease subtype.

To begin the development of autologous transplantation of genome-edited hematopoietic stem cells, we established an efficient application of CRISPR/Cas9 to target a functional copy of GCase into human CD34+ HSPCs. Here, we use sgRNA/Cas9 and AAV6-mediated template delivery to target GCase to the *CCR5* locus, a gene previously used for the insertion and expression of therapeutic genes[24,26]. *CCR5* is considered a safe harbor because germline deletions in this gene are common (up to 10% in the Northern European population) and have no overt developmental phenotype[27]. Germline *CCR5* loss might be beneficial as it provides protection against HIV[28], and possibly smallpox[58], although it also appears to reduce protection against influenza[59] and West Nile virus[60]. Compared to genetic correction of the affected locus, the use of a safe harbor is a universal therapy for all patient mutations and has greater designability as regulatory and GCase protein sequences can be engineered with enhanced therapeutic properties. For targeting Gaucher disease specifically, it circumvents the design of genetic tools for the *GBA* locus, which can be non-specific given the presence of *GBAP*, a pseudogene with 96% sequence homology to the *GBA* gene.

To express GCase from the *CCR5* locus, we used a previously characterized derivative of the CD68 promoter and confirmed through in vitro and in vivo differentiation protocols that it achieves monocyte/macrophage-specific expression of GCase[30,31]. We reasoned that because the primary manifestations of Gaucher disease are due to pathology in monocyte/macrophage lineage cells, enzyme reconstitution in this lineage should be sufficient to provide phenotypic correction in this disease. Furthermore, our studies with the SFFV promoter did not consistently result in sustained GCase and reporter expression in human HSPCs, suggesting that high and sustained GCase in the stem and progenitor compartment might have detrimental effects. This would not be surprising, as negative impact in long-term engraftment by lysosomal enzyme overexpression has been seen previously for galactocerebrosidase[61]. Furthermore, transplantation using retrovirally transduced CD34+ HSPCs in human where GCase was driven by the LTR promoter failed to show long-term reconstitution[13]. While several reasons can explain this observation, including insufficient cell dose and lack of conditioning, one explanation is that constitutive GCase expression by the LTR had a detrimental effect in the repopulating stem cell.

We examined the ability of the targeted human HSPCs to engraft and differentiate in serial transplantation studies in immunocompromised mice and demonstrate that our approach can modify cells with long-term repopulation potential and preserves multi-lineage differentiation capacity. We re-demonstrated a reduced repopulation capacity of the edited HSPC population in primary engraftment studies reported previously for engineered HSPCs in viral-mediated gene addition and gene-editing contexts[24,62,63]. However, the enhanced allele modification frequencies in the secondary transplants suggest that this initial decreased capacity is due to a reduced number of targeted long-term repopulating stem cells (LT-HSCs) compared to targeted shorter-lived progenitors and not to detrimental effect on engraftment per se. Interestingly, the allele targeting frequency of the engrafted cell population increased in some cases, suggesting that the variability in targeted HSPC engraftment may be accounted for by stochastic engraftment dynamics driven by oligoclonal reconstitution[64]. Even though these experiments do not achieve 100% human cell chimerism, transplantation outcomes in humans and mice indicate that low level chimerism could be sufficient to provide symptomatic relief[65,66]. Specifically, in mice, 7% wild type cell engraftment was shown to be sufficient to reverse disease pathology[67]. In our primary engraftment studies, the median allele modification frequency of the engrafted cells was ~4%, which corresponds to 4–8% of targeted cells (depending on the ratio bi-allelic or mono-allelic modification in the engrafted cells) and an 8–16% unmodified cell dose (given that our cells express twofold more GCase). Future experiments in an immunocompromised models of GD to allow engraftment and proliferation of human cells will establish the potential of these cells to correct the phenotype. Regardless of the outcome, future efforts aimed at increasing the permissiveness of long-term HSCs to undergo homology-dependent genome editing will be important for the therapeutic application of these cells.

Herein, we report the use of a genome editing to target a safe harbor to create lineage-specific expression of proteins. This approach is highly flexible and could serve as a platform to restore the expression of lysosomal enzymes and potentially other secreted proteins with therapeutic potential, provided the therapeutic cassettes are within the packaging capacity of AAV. These studies exemplify a specific use for this approach for the expression of human glucocerebrosidase as a potential intervention for the definitive treatment of GD and support further preclinical development of this strategy.

Methods

rAAV vector plasmid construction. The CCR5 donor vectors have been constructed by PCR amplification of 500 bp left and right homology arms for the *CCR5* locus from human genomic DNA. SFFV and wild-type GBA sequences were amplified from plasmids. The CD68S sequence was obtained from Dahl et al.[68] and was cloned from a gblock Gene Fragment (IDT, San Jose, CA, USA). Primers were designed using an online assembly tool (NEBuilder, New England Biolabs, Ipswich, MA, USA) and were ordered from Integrated DNA Technologies (IDT, San Jose, CA, USA). Fragments were Gibson-assembled into a the pAAV-MCS plasmid (Agilent Technologies, Santa Clara, CA, USA). Constructs were planned, visualized, and documented using Snapgene 4.2 Software.

rAAV production. rAAV was produced using a dual-plasmid system as described in Khan et al.[69]. Briefly, HEK293 cells were transfected with plasmids encoding an AAV vector and AAV rep and cap genes. HEK293 cells were harvested 48-h post-transfection and lysed using three cycles of freeze-thaw. Cellular debris was pelleted by centrifugation at 1350 × *g* for 20 min and the supernatant collected. Active rAAV particles were purified using iodixanol density gradient ultracentrifugation, dialyzed in phosphate-buffered saline (PBS), and stored in PBS at −80 °C. rAAV vectors for in vivo applications were ordered from Vigene Biosciences (Rockville, MD, USA). Viral titers were determined using droplet digital PCR with the following primer/probe combination: F: GGA ACC CCT AGT GAT GGA GTT, R: CGG CCT CAG TGA GCG A, P: /56FAM/CAC TCC CTC/ZEN/TCT GCG CGC TCG/ 3IABkFQ/.

HSPC isolation and culturing. Human CD34+ HSPCs mobilized from peripheral blood were purchased frozen from AllCells (Almeda, CA, USA) and thawed per manufacturer's instructions. Human Cord blood was obtained through The Binns Program for Cord Blood Research Program and not by the investigators themselves. The Program was approved by Stanford's IRB. Eligible donors were

expectant mothers scheduled to deliver at Lucile Packard Children's Hospital who provided informed consent prior to collection. Briefly, mononuclear cells were isolated by density gradient centrifugation using Ficoll Plaque Plus density gradient medium followed by two platelets washes. CD34+ mononuclear cells were positively selected using CD34+ Microbead Kit Ultrapure (Miltenyi Biotec, San Diego, CA, USA) per manufacturer's instructions. Purity of the isolation was assessed by staining cells with APC-conjugated anti-human CD34+ (Clone 561; Biolegend, San Jose, CA, USA) and analyzing the fraction of APC+ cells using an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA). Cells were cultured in media consisting of StemSpan SFEM II (Stemcell Technologies, Vancouver, Canada) supplemented with SCF (100 ng/ml), TPO (100 ng/ml), Flt3-Ligand (100 ng/ml), IL-6 (100 ng/ml), UM171 (35 nM), and StemRegenin1 (0.75 mM).

Gene editing in HSPCs. An sgRNA targeting *CCR5* exon 3 (sequence; 5′-GCAGCATAGTGAGCCCAGAA-3′) was purchased from TriLink Biotechnologies (San Diego, CA, USA) with the chemical modification 2′-O-methyl-3′-phosphorothioate[25]. Cas9 and Hifi Cas9 were purchased from Integrated DNA Technologies (IDT, San Jose, CA, USA Catalog #1081058 and #1081060). The editing procedure was performed as follows: sgRNA and Cas9 protein were complexed at a molar ration of 1:2.5 (sgRNA:Cas9) at room temperature for 5 min. The RNP was electroporated into human CD34+ HSPCs 48 h after thawing using the Lonza 4D nucleofector with the following conditions: pulse code: DZ100; cell density: 1×10^6 cells in 100 µl; [Cas9]: 30 µg; [sgRNA]: 15 µg. Following electroporation, cells were immediately rescued with HSPC culture media pre-warmed to 37 °C. rAAV6 was applied to cells at a MOI of 10,000–20,000. The frequency of indel formation was quantified using Tracking Indels by Decomposition (TIDE)[70]. CCR5 expression was quantified by flow cytometry using anti-human CCR5-APC antibody (BD Biosciences, #556903).

Measurement of cassette integration using ddPCR. Genomic DNA was extracted from selected or unselected cell populations using QuickExtract DNA Extract Solution and digested using AFIII (New England Biosciences). Two detection probes were used in the assay to simultaneously quantify wild-type *CCLR2* reference alleles gene targeted *CCR5* alleles. The ratio of detected *CCLR2*/*CCR5* events gave the fraction of targeted alleles in the original cell population. The *CCR5* detection assay was designed as follows: F:5′- GGG AGG ATT GGG AAG ACA-3′, R: 5′-AGG TGT TCA GGA GAA GGA CA-3′, labeled probe: 5′- FAM/AGC AGG CAT/ZEN/GCT GGG GAT GCG GTG G/3IABkFQ-3′. The reference assay was designed as follows: F:5′-CCT CCT GGC TGA GAA AAA G-3′, R: 5′-CCT CCT GGC TGA GAA AAA G-3′, and probe: /5HEX/TGT TTC CTC/ZEN/CAG GAT AAG GCA GCT GT/3IABkFQ/. Primer and probes final concentrations were 900 and 250 nM, respectively. Twenty microliters of the PCR reaction was used for droplet generation. Forty microliters of droplets was used in a PCR reaction with the conditions: 95 °C for 10 min, 45 cycles of melting at 94 °C for 30 s, annealing at 57 °C for 30 s, and extension at 72 °C for 2 min, with a final extension at 98 °C for 10 min. All steps were performed with ramping of 2 C/s and reactions were stored at 4 °C covered from light until droplet analysis. Analysis was performed on a Qx200 Droplet Reader (Bio-Rad) detecting FAM and HEX-positive droplets. Control samples included Mock (non-modified) genomic DNA and no-template control. Data analysis was performed using Quantasoft analysis software v1.4 (Bio-Rad).

Colony-forming unit assay and clonal genotyping. Colony-forming Unit assays were performed using Methocult methylcellulose (StemCell Technologies) as per the manufacturer's protocol. Briefly, CD34+ HSPCs were single-sorted into 96-well flat-bottom plates (Corning) pre-filled with 100 µl Methocult. Cells were cultured for 14 days at 37 °C, 5% O_2 and 5% CO_2. Colonies were quantified and characterized morphologically by color, size, and shape as burst-forming unit—erythroid (E-BFU), colony-forming unit—erythroid (E-CFU), colony-forming unit—granulocyte/monocyte (CFU-GM) or colony-forming unit—granulocyte/erythroid/macrophage/megakaryocyte. Colonies were genotyped by extracting genomic DNA in QuickExtract DNA Extraction Reagent (Lucigen, QE09050) and performing a 3-primer in-and-out PCR to amplify both wild-type *CCR5* alleles and *CCR5* alleles with targeted integrations. The 3-primer in-and-out PCR utilized a forward primer out the left *CCR5* homology arm (5′-CACCATGCTTGACCCA GTTT-3′), a forward primer binding the poly-adenylation signal in the cassette (5′-CGCATTGTCTGAGTAGGTGT-3′), and a reverse primer binding inside the right homology arm (5′-AGGTGTTCAGGAGAAGGACA-3′). Accupower pre-mix (Bioneer, Oakland, CA) was used for the PCR with cycling parameters: 95 °C for 5 min, and 35 cycles of 95 °C for 20 s, 72 °C for 60 s. DNA fragments were detected by agarose gel electrophoresis. Wild-type and targeted CCR5 alleles yielded bands of 590 base-pairs and 1100 base-pairs, respectively.

Macrophage differentiation and flow cytometry. CD34+ HSPCs were seeded at a density of 2×10^5 cells/ml in non-treated 6-well plates in differentiation medium (SFEM II supplemented with SCF (200 ng/ml), Il-3 (10 ng/ml), IL-6 (10 ng/ml), FLT3-L (50 ng/ml), M-CSF (10 ng/ml) and penicillin/streptomycin (10 U/ml)). After 48 h, non-adherent cells were removed and reseeded in a new non-treated 6-well plate at 2×10^5 cells/ml in differentiation medium. Adherent cells were maintained in the same dish in maintenance medium (RPMI supplemented with FBS (10% v/v), M-CSF (10 ng/ml) and penicillin/streptomycin (10 U/ml)). After 2 weeks, adherent macrophages were harvested by incubation with 10 mM EDTA in PBS. For phenotypic analysis, 1×10^5 cells per condition were harvested and resuspended in 100 µl staining buffer comprises PBS supplemented with 2% FBS and 0.4% EDTA. Non-specific antibody binding was blocked (5% v/v TruStain FcX, BioLegend, #422302) and cells were stained with 2 µl of each fluorophore-conjugated monoclonal antibody (30 min, 4 °C, dark). Antibodies used were hCD34-APC (BioLegend, #343509), hCD14-BV510 (BioLegend, #301842) or hCD14-APC (Invitrogen, #17-0149-41), and hCD11b-PE (BioLegend, #101208). Propidium Iodide (1 µg/ml) was used to detect dead cells and cells were analyzed on a BD FACSAria flow cytometer.

Phagocytosis assay. pHrodo Red *E.coli* BioParticles conjugate for Phagocytosis were purchased from ThermoFisher, USA and reconstituted to 1 mg/ml in 10% FBS-containing media. Reconstituted Bioparticles were added at a final concentration of 0.1 mg/ml to IDUA-HSPC-derived macrophages and incubated at 37 °C for 1 h. The cells were then washed and bathed in imaging media (DMEM Fluorobright, 15 mM HEPES, 5% FBS). Imaging followed using the appropriate absorption and fluorescence emission maxima (560 and 585 nm, respectively) with a BZ-X710 Keyence fluorescence microscope. Images were quantified using ImageJ 1.51.

Transplantation of CD34+ HSPCs into NSG Mice. Targeted HSPCs (unselected) were transplanted 48 h post-targeting into sub-lethally irradiated NSG recipients. Primary transplants were performed by intrahepatic injection into newborn pups or by intrafemoral injection at 6–8 weeks of age. Approximately 1×10^6 cells were transplanted into each mouse for all primary transplants. For secondary transplants, human CD34+ HSPCs were isolated from transplanted 16-week-old-mice at the time of primary engraftment analysis using CD34+ Microbead Kit Ultrapure (Miltenyi Biotec, San Diego, CA, USA) and transplanted without pooling into a second sub-lethally irradiated NSG recipient. Secondary transplants were performed by intrahepatic injection into newborn pups.

Assessment of human cell engraftment. Sixteen weeks post-transplantation, peripheral blood, bone marrow and spleen were harvested from transplanted mice. The tissues were passed through 100 µm filters to achieve a single-cell suspension and red blood cells were lysed with ammonium chloride (RBC lysis buffer). Non-specific antibody staining was blocked with Trustain FX (BioLegend, #422302) for 10 min at room temperature. For primary engraftment studies cells approximately one million cells were stained with 1 µl of the following antibodies: mTer119–PE-Cy5 (Invitrogen, #15-5921-83); mCD45–PE-Cy7 (Invitrogen, #25-0453-82), and 2 µl of hCD45–PacificBlue (Biolegend, #368540); hCD19–APC (BD Biosciences, #555415); hCD33–PE (BD Biosciences, #555450); hCD14–BV711(BD Biosciences, #563373). Dead cells were detected using Blue Reactive Dye (ThermoFisher #L34961) and excluded from analysis (Supplementary Fig. 5). For secondary engraftment studies, isolated bone marrow cells were stained with the following antibodies: mTer119–PE-Cy5 (Invitrogen, #15-5921-83); mCD45–PE-Cy7 (Invitrogen, #25-0453-82); hCD45–PacificBlue (Biolegend, #368540); HLA-ABC–APC-Cy7 (Biolegend, #311426); hCD19–APC (BD Biosciences, #555415); hCD33–PE (BD Biosciences, #555450). Dead cells were detected using Propidium Iodine and excluded from study (Supplementary Fig. 7). Analysis was performed by flow cytometry on a BD FACSAria II using FACSDiva v8.0.1 software. Human engraftment was defined as the percentage of hCD45 among all (mouse or human) CD45+ cells. Analysis of all flow cytometry data was done using FlowJo v10.6.

Glucocerebrosidase activity assay. To facilitate comparisons between different conditions, cells were FAC-sorted prior to quantification of enzyme activity and cell number ranged from 2×10^4 to 1×10^5 cells. Protein was extracted by lysing cells in 200 µl of deionized water with a Branson Sonicator with probe, centrifuging lysates at 17,000 × *g* for 10 min at 4 °C, and collecting the supernatant containing the soluble proteins. Protein concentration in the supernatants was measured by Bradford assay kit with BSA standard curve ranging from 0.25–0.5 mg/ml (Thermo Scientific). To prepare the GCase assay working reagent, the fluorogenic substrate 4-methylumbeliferyl-β-D-glucopyranoside (Sigma, #M3633) was dissolved to a final concentration of 5 mM in citrate/phosphate buffer (pH 5.5) supplemented with 15% (w/v) sodium taurocholate. To perform the GCase assay, 25–50 µg protein extract (50 µL) was mixed with 100 µL of working reagent and incubated for 1 h at 37 °C covered from light. Reactions were stopped with 200 µL stop buffer (0.2 M glycine/carbonate, pH 10.7). Fluorescence of 4-methylumbeliferone (4MU) liberated by GCase enzyme cleavage was measured using a Molecular Devices SpectraMax M3 multi-mode microplate reader with SoftMax Pro 7 software at excitation and emission wavelengths of 355 and 460 nm, respectively (top read). A standard curve for 4MU was established using 4MU sodium salt (Sigma) in assay buffer.

Immunocytochemistry and imaging. Cells were seeded on coverslips 24–48 h prior to analysis. All washes were performed with D-PBS (+calcium, +magnesium). Cells on coverslips were washed, fixed with 4% PFA in PBS for 30 min, permeabilized with 0.1% Triton-X in PBS for 10 min and blocked in 10% normal

goat serum (NGS; Gibco) containing 0.25% Triton X-100 for 30 min at 25 °C. After washing, coverslips were incubated in primary antibodies: mouse anti-CD68 (Biolegend, #333801; 1:100 dilution) and rabbit anti-GFP (Abcam, ab290; 1:500 dilution) overnight at 4 °C. Primary antibodies were thoroughly washed and coverslips were incubated with secondary antibodies (Alexa Fluor 488 donkey anti-rabbit IgG (Biolegend, #406416, and Alexa Fluor 568 goat anti-Mouse IgG (H+L) (Invitrogen/ThermoFisher, A-11004) at 1:1000 dilution for 1 h covered from light. Coverslips were washed once more and mounted on glass coverslips with mounting media containing Hoechst die. Cells were imaged on a BZ-X710 Keyence fluorescence microscope. Images were quantified using ImageJ 1.51.

Mice. NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice were developed at The Jackson Laboratory. NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl} Tg (CMV-IL3,CSF2,KITLG) 1Eav/MloySzJ were described in Wunderlich et al.[37] and Billerbeck et al.[36] and obtained from The Jackson Laboratory. Mice were housed in a 12-h dark/light cycle, temperature- and humidity-controlled environment with pressurized individually ventilated caging, sterile bedding, and unlimited access to sterile food and water in the animal barrier facility at Stanford University. All experiments were performed in accordance with National Institutes of Health institutional guidelines and were approved by the University Administrative Panel on Laboratory Animal Care (IACUC 20565 and 33365).

Tissue macrophage isolation. Peritoneal macrophages were isolated as single-cell suspension by injection of 6 ml of ice-cold PBS 1x in the peritoneal cavity, followed by aspiration of 4 ml of the peritoneal fluid, using syringe and 21 G needle. Liver and lung were dissected from mice after perfusion, minced and digested with 500 µg/ml Liberase TM (Roche, #05401119001) and 400 µg/ml DNase in RPMI media for 30 min at 37 °C. After incubation, tissues were passed through 100 µm filters and washed twice. Liver samples were further processed by centrifugation in 33% Percoll Plus (GE Healthcare) for 15 min at 700 × *g*, with brakes off. Red blood cells were lysed from cell pellets and a single-cell suspension was prepared. For flow cytometry, non-specific antibody binding was blocked with TruStain FcX (Biolegend, #422302) and Cd16/cd32 anti-mouse (2.4G2, BD Biosciences, #553142). Cells were stained with hCD45–PacificBlue (Biolegend, #3685340), mCD45–PE-Cy7 (Invitrogen, #25-0453-82), mTer119–PE-Cy5 (Invitrogen, #15-5921-83), and h/mCD11b-PE (BioLegend, #101208). Dead cells were detected with Blue Reactive Dye (ThermoFisher #L34961).

Statistical analysis. All statistical test including paired and unpaired *t*-tests, and one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test was performed using GraphPad Prism version 7 for Mac OS X, GraphPad Software, La Jolla California USA. Data was reported as means when all conditions passed three normality tests (D'Agostino & Pearson, Shapiro–Wilk, and Kolmogorov–Smirnov (KS) normality test).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All flow cytometry datasets in this study are available in Flowrepository, experiment number FR-FCM-Z2LQ. The authors declare that the other data that support the findings of this study are present within the paper, its Supplementary Information files, or are available from the corresponding author upon reasonable request. The source data underlying Figs. 1d–e, 2b, d, 3b–f, 4a–g and 5a, b, and as well as Supplementary Figs. 1d, 3a, 4b–d, 6a–b, and 8b are provided as a Source Data file.

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Author contributions

S.G.B. collected data, performed most experiments, carried out the analyses, and participated in manuscript preparation and figure design. E.P. collected data, performed experiments, carried out the analyses, and participated in manuscript preparation and figure design. K.L.L. assisted with mouse colony management, engraftment analysis, immunocytochemistry, and imaging. P.C. collected data and performed experiments. A.S. obtained and purified CD34+ HSPCs from donated cord blood and assisted with secondary transplants. T.J.M. participated in experimental design, provided funding and assisted with manuscript preparation. M.P.H. contributed to experimental design, and manuscript preparation. N.G.-O. conceived and directed the project, collected data, designed and cloned vectors, performed experiments and analysis with S.G.B. and E.P., and participated in manuscript preparation and figure design.

Competing interests

M.H.P. declares that he is a consultant and has equity interest in CRISPR Tx and Allogene Tx, and he states that neither company has had input or opinions on the subject matter described in this manuscript. The other authors declare no competing interests.

Additional information

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