Universidade Federal do Rio Grande do Sul (UFRGS)

Terapia gênica *ex vivo* e *in vivo* em modelo murino de mucopolissacaridose do tipo I: potencialidade de vetores retrovirais aplicados pela via intraventricular

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"... you shall above all things be glad and young/ For if you're young, whatever life you wear"

E.E Cummings (*You shall all above things be Glad and Young*)

v

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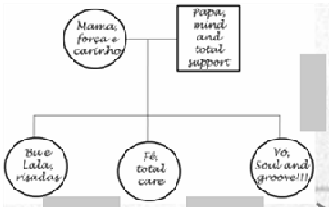

A vó dançarina, haha

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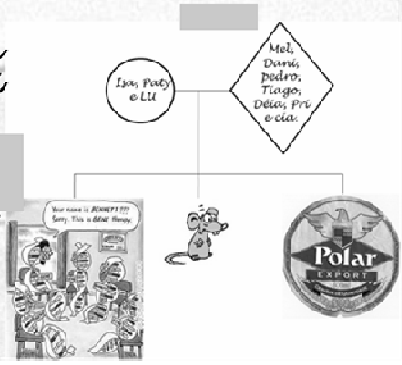

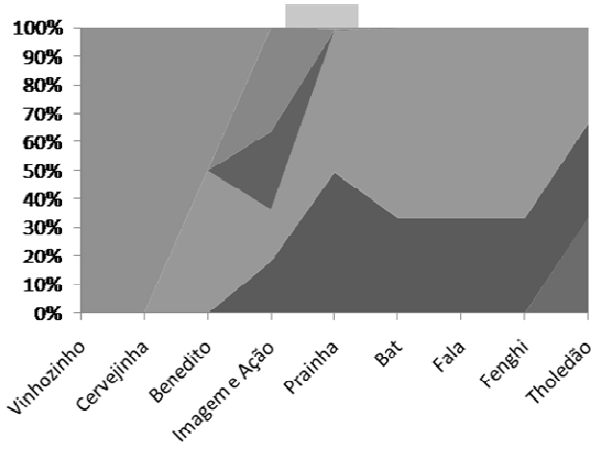

Lukets
Igor
Edu
Renan
Nessa
miga Fran
MALZBIER
lasagnaaaaaaaa

Ví

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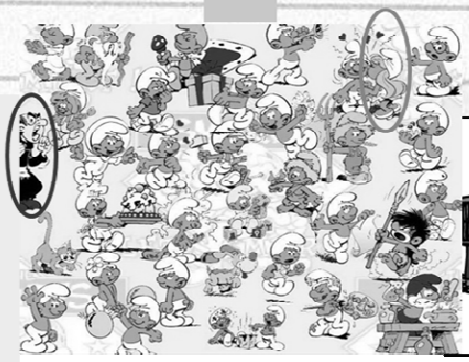
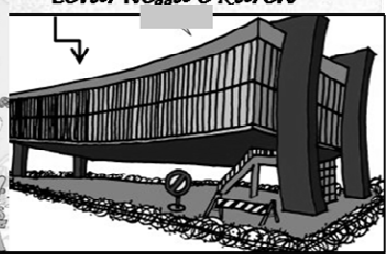
Entre as coisas mas lindas que eu conheci
Só reconheci suas cores belas quando eu te vi
Entre as coisas bem-vindas que já recebi
Eu reconheci minhas cores nela então eu me vi / E as coisas lindas são mais lindas Quando você está Hoje você está Onde você está As coisas lindas são mais lindas Porque você está Onde você está
Hoje você está Nas coisas tão mais lindas

nmol, nmol, ou µmol????

A profa. Vânia e aos colegas do LEIM, pela super colaboração científica e pelas festinhas... GRAZIE DI CUORE!!!!...



Levar Nessa e Karen



Sumário

Lista de abreviaturas e termos relevantes

4MU – *4-methylumbelliferyl*

4MU-I – *4-methylumbelliferyl-alpha-L-iduronide*

AAV – vetor adenoassociado ou *adenoassociated virus vector*

AAV5-IDUA – *adenoassociated vector sorotype 5 containing IDUA transgene*

Ad - adenovírus

ADA - adenosina deaminase

BS – *blasticidin S deaminase gene*

CFU – *colony formating unit*

CMV – citomegalovirus ou *cytomegalovirus*

CNS – *central nervous system*

CTM - Células-tronco mesenquimais

DAL - Doenças de acúmulo lisossomal

DS/HS – *dermatan and heparan sulfate*

DMEM - *Dulbecco's Modified Eagle's Medium*

ERT – *enzyme replacement therapy*

Ex vivo – nas células-alvo para posterior reintrodução das mesmas no organismo alvo

FIV – *Feline Immunodeficiency virus*

GAGs – glicosaminoglicanos ou *glycosaminoglicans*

Gene exógeno – ver "Transgene"

GFP - *green fluorescent protein*

gfp – *green fluorescent protein gene*

HIV - *Human Immunodeficiency Virus*

HIV GFP – vetor retroviral baseado em *Human Immunodeficiency Virus* contendo o transgene *gfp* ou *HIV vector containing gfp transgene*

HIV IDUA - vetor retroviral baseado em *Human Immunodeficiency Virus* contendo o transgene *IDUA* ou *HIV vector containing IDUA transgene*

IDUA – enzima alfa-L-iduronidase ou *alpha-L-iduronidase enzyme*

IDUA - gene da alfa-L-iduronidase ou *alpha-L-iduronidase gene*

In situ – no local

In vivo – no organismo alvo

IRES - *internal ribosomal entry site*

iv - *intravenous*

KO – *knockout*

KO/GFP – animal *knockout* tratado com CMT transduzidas com MLV GFP ou *knockout mice treated with MLV GFP transduced MSC*

KO/IDUA – animal *knockout* tratado com CMT transduzidas com MLV IDUA ou *knockout mice treated with MLV IDUA transduced MSC*

LSD – *lysosomal storage disorders*

LTR - *long terminal repeates*

MLV IDUA – vetor retroviral baseado no *Murine Leukemia Virus* contendo o transgene *IDUA* ou *Murine Leukemia Virus containing IDUA transgene*

MLV – vetor retroviral baseado no *Murine Leukemia Virus* ou *Murine Leukemia Virus*

MLV GFP – vetor retroviral baseado no *Murine Leukemia Virus* contendo o transgene *gfp* ou *Murine Leukemia Virus containing gfp transgene*

MOI – multiplicidade de infecção ou *multiplicity of infection*

MPS – Mucopolissacaridose ou *Mucopolysaccharidosis*

MPSI - Mucopolissacaridose I ou *Mucopolysaccharidosis type I*

MPSI MSC – *mesenchymal stem cells from MPSI mice*

MPSIIIB – Mucopolissacaridose tipo IIIB

MPSVII - Mucopolissacaridose tipo VII ou *Mucopolysaccharidosis type VII*

MSC – *mesenchymal stem cells*

MSC IDUA – células-tronco mesenquimais geneticamente modificadas com o gene terapêutico *IDUA ou genetic modified mesenchymal stem cells expressing therapeutic gene IDUA*

MSCV BS - vetor retroviral baseado no *Murine Stem Cell Virus* contendo o transgene *blasticidin S deaminase* ou *Murine Stem Cell Virus containing the blasticidin S deaminase transgene*

MSCV IDUA – vetor retroviral baseado no *Murine Stem Cell Virus* contendo o transgene *IDUA* ou *Murine Stem Cell Virus containing the IDUA transgene*

MSCV – vetor retroviral baseado no *Murine Stem Cell Virus* ou *Murine Stem Cell Virus*

PBS – *phosphate buffered saline*

PCL - *packaging cell line*

PCR - *polymerase chain reaction*

PGK – *phosphoglicerate kinase*

RE - Reposição enzimática

SCID – imunodeficiência severa combinada

SNC – sistema nervoso central

Síndrome de Hurler – forma grave de MPSI, com comprometimento neurológico progressivo

Síndrome de Hurler-Scheie e Scheie – formas branda de MPSI

Terapia *ex vivo* com CTM IDUA *in situ* - terapia baseada na reintrodução de células-tronco mesenquimais geneticamente modificadas contendo o gene exógeno/transgene IDUA no local desejado (órgão específico) do organismo alvo

TG - terapia gênica

Transgene – gene exógeno transferido via vetor

TU – *transducing unit*

UI - unidade de infecção ou *unit of infection*

WT – *wild type*

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Artigo 1

Resumo

A mucopolissacaridose tipo I (MPSI) é uma doença monogênica autossômica recessiva, devido a mutações no gene da alfa-L-iduronidase (*IDUA*). Seu caráter sindrômico compromete todas as funções vitais do organismo, inclusive a neurocognição. Atualmente, como formas disponíveis de tratamento existem apenas os transplantes de medula óssea/célula tronco hematopoiética e a reposição enzimática (RE). Os transplantes dependem da disponibilidade dos doadores e da idade em que o diagnóstico é comprovado. A RE é um procedimento semanal e contínuo por toda a vida do paciente, invasivo e de alto custo. Esse melhora consideravelmente as doenças visceral e articular/óssea; contudo, não apresenta o mesmo efeito sobre a neuropatologia, principalmente na fase mais avançada. No presente trabalho, células-tronco mesenquimais transduzidas com o vetor MLV contendo o transgene *IDUA* foram injetadas intraventricularmente no cérebro de camundongos MPSI adultos de duas faixas etárias (12 e 25 semanas). O estudo foi concluído quando os animais atingiram 20 e 29 semanas, respectivamente. Essa metodologia resultou na diminuição dos níveis de GAGs acumulados no cérebro dos animais afetados. Tendência de melhora locomotora foi detectada, através de teste de campo aberto de exposição única, mesmo nos animais tratados em fase mais avançada da neuropatologia. A queda de expressão do transgene, observada com frequência neste tipo de abordagem, pode ter sido um entrave para que a correção cruzada (*cross correction*) fosse mais

duradoura. Baseando-se nessa experiência, nós desenvolvemos um novo vetor retroviral (MSCV IDUA) para ser avaliado *in vitro* com ferramenta de transferência gênica para células-tronco mesenquimais. Esse vetor promoveu expressão sustentável do transgene e rapidez de seleção das células geneticamente modificadas, via resistência à blasticidina. A administração *in vivo* desse vetor, através de injeção intraventricular bilateral no cérebro de camundongos MPSI jovens, levou à produção local de níveis detectáveis de IDUA ao término do experimento (30 dias). Esse estudo *in vivo* de terapia gênica (TG) para mucopolissacaridose do tipo I murina também avaliou a potencialidade de outros dois vetores virais (HIV e MLV), nessas mesmas condições experimentais. O vetor baseado em HIV também foi capaz de promover a expressão *in situ* da enzima, enquanto que o MLV não. Redução no conteúdo total de GAGs extraídos do cérebro dos animais foi detectada apenas para os grupos tratados com os vetores MSCV e HIV. Intensa redução na proporção de dermatan/heparan sulfato nessas amostras também foi detectada. Concluindo, o vetor MSCV mostrou-se uma boa ferramenta de transferência gênica *in vitro* e *in vivo*, favorecendo a produção *in situ* da enzima de forma sustentável, o que é altamente desejável na realização de TG *in vivo*. Devido à possibilidade de seleção rápida das células geneticamente modificadas após a transdução com esse vetor, a realização de um novo protocolo pré clínico de TG *ex vivo* baseada em células-tronco mesenquimais oriundas desse modelo murino de MPSI também poderá ser beneficiada.

Abstract

Mucopolysaccharidosis type I (MPSI) is an autosomal recessive disease due to mutations in the alpha-L-iduronidase gene (*IDUA*). MPSI is a multisystemic disorder, in which all vital functions are compromised, including neurocognition. Currently, the only two options of treatment available are bone marrow/hematopoetic stem cell transplantation and enzyme replacement therapy (ERT). Transplantations depend on availability of compatible donors and the age at diagnosis. ER is a weekly and life-time procedure, invasive and a high cost treatment. It ameliorates visceral and joint/bone diseases, however, the same effect on neuropathology is not achieved, mainly in most advanced stages of the disease. Mesenchymal stem cells transduced with MLV vector containing the IDUA transgene were injected intraventricularly in the brain of MPSI adult mice (12 and 25 weeks). The study was concluded when animals were 20 and 29 weeks old, respectively. This protocol resulted in a reduction of GAGs accumulated in the brain. Locomotion improvement was observed through open field analysis, even in animals treated at advanced stages of neuropathology. The decrease of transgene expression, which is frequently observed in this type of approach, may have restrained the mechanism of cross-correction. Based on that, we developed a new retroviral vector (MSCV IDUA) to be analyzed *in vitro* as a gene transfer tool to mesenchymal stem cells. This vector demonstrated a sustainable expression

of the transgene and enabled a fast selection of genetically modified cells through blasticidin resistance. *In vivo* administration of the vector, through bilateral intraventricular injection in the brain of young MPSI mice, led to the production of detectable local levels of IDUA 30 days after the procedure. This *in vivo* study of gene therapy for murine MPSI also evaluated the potentiality of two other viral vectors (HIV and MLV), at the same experimental conditions. HIV based vector was also able to induce *in situ* production of the enzyme, whilst MLV was not. A reduction of total GAGs extracted from mice brains was detected only in the groups treated with MSCV and HIV, as well as an intense reduction of dermatan/heparan sulfate proportion. Therefore, MSCV vector was shown to be an efficient gene transfer tool *in vitro* and *in vivo*, enabling *in situ* sustainable production of the enzyme, which is highly desirable for *in vivo* gene therapy. Due to the possibility of fast selection of genetically modified cells after transduction with this vector, the accomplishment of a new *ex vivo* pre clinical gene therapy protocol based on mesenchymal stem cells from the murine model of MPSI may also be benefited.

Introdução

"Certainly, an important theme that recurred throughout the meeting was analysis of the chromosomal integration sites of gene transfer vectors. The question of how random is retroviral integration has been addressed numerous times in prior years, but we are learning that the answer depends on what the meaning of "how random is" is. New techniques for integration site recovery and genomic analysis are producing a wealth of information on integration patterns of retroviral, lentiviral and AAV-based vectors, as well as transposons. We are learning about the relative frequency of vector integration, the category of preferred integration sites and the interactions between genetic elements of the vector and juxtaposed cellular sequences. Understanding these properties of each specific gene transfer vector should allow more informed estimations of their relative risks and benefits in clinical applications."

Don B. Kohn (*Gene Therapy:Breadth and vigor*, 2003).

Introdução

A mucopolissacaridose do tipo I

A perda da função lisossomal para o metabolismo celular ocasiona o acúmulo de glicosaminoglicanos (GAGs), o que caracteriza as doenças de acúmulo lisossomal (DAL). O tipo de GAGs acumulado em decorrência da perda parcial ou total da função de uma enzima lisossômica específica determina um tipo específico de DAL. Assim, a mucopolissacaridose do tipo I (MPSI) é uma DAL decorrente da perda de função da enzima alfa-L-iduronidase (IDUA), levando ao acúmulo de dermatan e heparan sulfato. É uma doença monogênica recessiva, cuja freqüência estimada é de 1:100.000 nascidos vivos (Neufeld and Muenzer, 2001). Devido à sua variabilidade clínica, à progressão e à idade da manifestação de alguns sintomas, a MPSI é atualmente classificada em duas formas: a forma grave (Síndrome de Hurler) e a forma atenuada (Síndrome de Hurler-Scheie e Scheie) (Muenzer *et al.*, 2009).

Os pacientes MPSI, de uma forma geral, apresentam esplenomegalia, disostoses, mal funcionamento cardiorrespiratório, entre outros sintomas (Neufeld and Muenzer, 2001). A forma atenuada de MPSI apresenta imensa variabilidade em termos de perda de função neurológica, morbidade e outros sintomas. A forma grave (Síndrome de Hurler) é assim classificada devido ao seu intenso comprometimento neurológico, que ocorre de forma progressiva e leva à morte ainda na infância. Dois tipos de tratamento estão disponíveis para os pacientes MPSI. Os transplantes (de medula óssea ou de

células tronco hematopoiéticas) são limitados pelos poucos doadores compatíveis, pela alta mortalidade e pelo alto custo do procedimento. A segunda alternativa é a reposição enzimática (RE), bastante efetiva para as doenças visceral e articular/óssea, o que contribui para a qualidade de vida do paciente. Nem o transplante nem a reposição enzimática, contudo, evitam o processo neurodegenerativo (Ponder and Haskins, 2007). Se a terapia convencional é iniciada cedo, portanto, ela pode retardar a manifestação dos sintomas da patologia, mas não da neuropatologia. Infelizmente, o diagnóstico para MPSI através de triagem neonatal ainda não é rotina e a maioria das crianças afetadas é diagnosticada tardiamente (Ponder and Haskins, 2007). É importante lembrar que aproximadamente 50-80% desses pacientes apresentam a forma grave da doença (Muenzer *et al.*, 2009); conseqüentemente, há necessidade urgente de busca por novas alternativas terapêuticas para essa patologia, voltadas especialmente para o cérebro. Um estudo de caso foi publicado recentemente sobre RE via injeção intratecal por punção lumbar (Munoz-Rojas *et al.*, 2008); contudo, a RE seguiria sendo um procedimento semanal de alto custo (Pastores, 2008), o que reforça ainda mais essa necessidade. Baseando-se na introdução feita acima para a fisiopatologia da MPSI, fica evidente que o tratamento do sistema nervoso central precisa ser uma prioridade. A terapia gênica (TG), que se baseia na transferência de genes terapêuticos para obtenção de um fim terapêutico, se enquadra bem nesse contexto.

Terapia gênica para MPSI: buscando alternativas para o tratamento em nível pré-clínico

A expressão *in situ* do gene *IDUA* através da transferência gênica *in vivo* ou *ex vivo* poderá trazer um grande benefício clínico, mesmo que apenas uma pequena população de células locais seja modificada geneticamente. Isso porque a enzima IDUA secretada pelas células modificadas geneticamente é captada por células vizinhas, através de um processo conhecido por correção cruzada – mesmo mecanismo explorado na reposição enzimática ou nos transplantes. A captação da enzima é realizada através de um processo mediado por receptores de manose-6-fosfato. É importante ressaltar que a atividade da IDUA ocorre apenas em pH ácido, o que também contribui para que a atividade da enzima esteja restrita ao lisossomo. Esse é o destino final da translocação da IDUA após a captação via receptor (Neufeld and Muenzer, 2001).

Os estudos de TG para MPSI têm sido realizados com diferentes modelos animais (cão, camundongo ou gato), transgenes selecionados (gene repórter ou terapêutico), vetor empregado (viral ou não viral) e desenho molecular do cassete de expressão (Ponder and Haskins, 2007) (Tabela 1).

Tabela 1: Compilação de protocolos *in vivo* de terapia gênica para MPSI

Referência	Vetor	Transgene	Modelo animal	Especificação	Tempo de análise	Via de administração	Benefício clínico
Shull & Lu, 1996	retro MLV	cDNA canino	canino	adulto		infusão de céls transduzidas	não (RI)
Lutzko *et al* ., 1999	retro MLV	cDNA canino	canino	adulto		infusão de céls transduzidas	não
Lutzko *et al* ., 1999	retro MLV	cDNA canino	canino	prenatal - intrauterina		injeção intravenosa de céls transduzidas	não
Hartung *et al.* , 2004	AAV	cDNA humano	murino	neonatal	5 meses depois	injeção intravenosa (veia temporal)	sim (total)
Camassola *et al* ., 2005	plasmidial	cDNA humano	murino	adulto		injeção hidrodinâmica	sim (cérebro também)
Di Domenico *et al* ., 2005	lenti HIV	cDNA humano	murino	adulto	1 e 6 meses depois	injeção intravenosa (veia caudal)	sim (fígado, baço; RI tardia)
Kobayashi *et al* ., 2005	lenti HIV	cDNA humano	murino	neonatal e adulto	20 semanas de idade	injeção intravenosa (veia temporal e caudal)	sim (parcial)
Liu *et al* ., 2005	retro MLV	cDNA canino	murino	neonatal	8 meses depois	injeção intravenosa (veia temporal)	sim (total)
Ciron *et al* ., 2006	AAV	cDNA canino	canino	adulto	3, 5, 7 meses depois	injeção intratecal	não (RI)
Di Domenico *et al* ., 2006	lenti HIV	cDNA humano	murino	adulto	6 meses depois	injeção intravenosa (veia caudal)	sim (fígado, baço; RI tardia)
Ponder *et al* ., 2006	retro MLV	cDNA canino	felino	neonatal	3 meses depois	injeção intravenosa (veia temporal)	sim (parcial)
Watson *et al* , 2006	AAV	cDNA humano	murino	adulto	6-10 semanas depois	injeção intratecal	sim (parcial)
Aranovich *et al* ., 2007	transposon SB	cDNA humano	murino	adulto		injeção hidrodinâmica	não (RI)
Chung *et al* ., 2007	retro MLV	cDNA canino	murino	neonatal	8 meses depois	injeção intravenosa (veia temporal)	sim (na alta dose do vetor)
Ma *et al.* , 2007	retro MLV	cDNA canino	murino	adulto	8 meses depois	injeção intravenosa	sim (imunomodulação)
Traas et al., 2007	retro MLV	cDNA canino	canino	neonatal	1 ano, aprox.	injeção intravenosa	sim (total)
Herati *et al.* , 2008	retro MLV	cDNA canino	murino	adulto	6.5 meses depois	injeção intravenosa	sim (menos aorta)

As referências assinaladas referem-se aos protocolos *ex vivo* ou *in vivo* envolvendo vetores não-virais. Legenda: retro – retrovetor; MLV – *Murine Leukemia Virus*; AAV – *Adenoassociated Virus*; lenti – lentivetor; HIV – *Human Immunodeficiency Virus*; SB – *Sleeping Beauty*; RI – resposta immune.

A maioria desses trabalhos focou na administração intravenosa, representando a mesma rota de administração atualmente empregada na terapia de resposição enzimática. Protocolos de transferência gênica intravenosa em fase neonatal resultaram em produção de níveis de enzima detectáveis no cérebro de camundongos MPSI, com correção da maioria dos sintomas da doença (Liu *et al.*, 2005; Chung *et al.*, 2007). Injeções sistêmicas de vetores plasmidiais, lentivirais ou retrovirais com imunossupressores em animais adultos também produziram níveis detectáveis de enzima no cérebro, com diferentes taxas de redução de GAGs totais (Camassola *et al.*, 2005; Di Domenico *et al.*, 2005; Di Domenico *et al.*, 2006; Ma *et al.*, 2007; Herati *et al.*, 2008). Um estudo comparativo para fase de administração do vetor mostrou que a correção metabólica da doença foi atingida apenas para o grupo de camundongos neonatos tratados (Kobayashi *et al.*, 2005). Correção metabólica, craniofacial e neurológica foi reportada em um protocolo de administração intravenosa de vetor AAV em fase neonatal (Hartung *et al.*, 2004).

Terapia gênica *in situ* voltada ao sistema nervoso central em modelo animal de MPSI foi pouco explorada. Para cães MPSI as injeções intracerebrais foram combinadas a um regime de imunossupressão – ineficiente para evitar a encefalite subaguda (Ciron *et al.*, 2006). Em modelo murino adulto, a administração intratecal levou a redução da patologia vacuolar quando altas doses de vetor AAV foram empregadas – o que levou a uma superprodução de enzima no local (Watson *et al.*, 2006).

Como o acúmulo de GAGs é a consequência primária da doença, sua dosagem traz informação relevante após a intervenção terapêutica. Por isso os trabalhos acima citados buscaram quantificar de alguma forma esses GAGs totais. Entretanto, é muito importante lembrar que os GAGs de um tecido podem ser compostos por vários tipos, e no caso da MPSI ocorre acúmulo específico de dermatan e heparan sulfato nos lisossomos. Historicamente, essa redução de GAGs totais tem sido quantificada bioquímica ou histologicamente, por método colorimétrico ou por contagem de vacúolos oriundos dos depósitos (Tabela 2). Nenhuma dessas técnicas, contudo, discrimina especificamente o teor de dermatan e heparan sulfato acumulados dos demais GAGs característicos do tecido em análise. A contagem de vacúolos é uma medida relacionada aos depósitos lisossomais de GAGs e, portanto, uma medida relacionada aos GAGs acumulados em cada tipo de MPS.

Tabela 2: Método empregado para avaliação de GAGs em protocolos *in vivo* de terapia gênica para MPSI

Referência	**Vetor**	**Modelo animal**	**Fase**	**Avaliação de GAG**	
				colorimetria	**histologia**
Hartung *et al* ., 2004	AAV	murino	neonatal		X
Camassola *et al* ., 2005	plasmidial	murino	adulto	X	
Di Domenico *et al* ., 2005	lenti HIV	murino	adulto	X	
Kobayashi *et al* ., 2005	lenti HIV	murino	neonatal e adulto		X
Liu *et al.* , 2005	retro MLV	murino	neonatal		X
Chung *et al* ., 2007	retro MLV	murino	neonatal	X	X
Ciron *et al* ., 2006	AAV	canino	adulto	X	
Di Domenico *et al* ., 2006	lenti HIV	murino	adulto	X	
Watson *et al* ., 2006	AAV	murino	adulto		X
Ma *et al* ., 2007	retro MLV	murino	adulto	X	X
Traas *et al* ., 2007	retro MLV	canino	neonatal	X	X
Herati *et al* ., 2008	retro MLV	murino	adulto	X	

A resposta imune parece ser um outro obstáculo na busca por tratamentos alternativos para MPSI. Para o modelo canino de MPSI, a resposta imune contra a proteína exógena e/ou as células geneticamente modificadas bloqueou a correção cruzada (Shull *et al.,* 1996; Lutzko *et al.*, 1999a). Quando um protocolo similar baseado no vetor Sleeping Beauty foi realizado, a produção e manutenção da enzima durante 6 meses foi atingida somente com o uso de imunossupressor. Nessas condições, apenas o fígado apresentou redução de GAGs totais depositados, e os hepatócitos apresentaram sinais claros de apoptose 10-14 dias após a administração do vetor, como conseqüência de resposta inflamatória (Aronovich *et al.*, 2007). Foi proposto que o modelo felino é capaz de montar uma potente resposta imune contra o vetor MLV empregado no protocolo de terapia gênica em fase neonatal (Ponder *et al.*, 2006). É importante ressaltar que o transgene introduzido no vetor referia-se ao IDUA canino. Finalmente, a imunomodulação favoreceu a terapia gênica via vetor MLV em modelo murino adulto de MPSI (Ma *et al.*, 2007).

Coletivamente, essas informações nos mostram que modificações no *design* do estudo, alterando variáveis como tipo e quantidade do vetor administrado ou idade do animal selecionado para realização do protocolo, podem contribuir para a eficácia do tratamento. Procurando avaliar a eficiência de um protocolo de terapia gênica baseado em vetor viral, camundongos MPSI foram tratados em fase neonatal e adulta. Níveis superiores de enzima circulante e expressão sustentável do transgene foram observados apenas quando a intervenção foi feita precocemente (Kobayashi

et al., 2005). Isso está de acordo com a tendência atual de antecipar o diagnóstico para favorecer a intervenção precoce (Karolewski and Wolfe, 2006); contudo, a maioria dos diagnósticos, ainda é feita tardiamente (Ponder and Haskins, 2007).

Uma vez que as terapias atuais não são capazes de evitar a neuropatologia, seria importante avaliar a potencialidade de um protocolo *in situ* de terapia gênica visando redução de GAGs, restauração da atividade da IDUA e melhora locomotora e cognitiva. Estudos anteriores são mostram que nem todos esses aspectos são avaliados após TG em nível pré-clínico. Injeção neonatal de vetores adeno-associados em modelo de MPSVII resultou em níveis detectáveis de enzima no cérebro (Passini and Wolfe, 2001). Camundongos adultos MPSI tratados, também em fase neonatal com doses menores desse mesmo tipo de vetor, apresentaram redução parcial dos depósitos de GAGs e níveis detectáveis de enzima no cérebro (Watson *et al.*, 2006). Em um modelo canino de MSPI, essa mesma abordagem resultou em redução substancial de GAGs no cérebro dos animais, mas mesmo com a administração de um imunossupressor a resposta imune gerada contra o vetor e contra a proteína IDUA desencadearam encefalite subaguda nos animais (Ciron *et al.*, 2006). Nenhum desses estudos, entretanto, avaliou a função locomotora/cognitiva dos animais após a TG.

Esse cenário mostra que o protocolo de TG voltado ao cérebro precisa atingir níveis locais de enzima suficientes para reduzir GAGs depositados especificamente na MPSI (dermatan e heparan sulfato), através de uma

intervenção não muito agressiva, de preferência única e sem ocasionar efeitos colaterais indesejáveis. Além disso, é de fundamental importância que a avaliação da função locomotora/cognitiva faça parte das análises posteriores à intervenção terapêutica voltada ao cérebro – em nível clínico e pré-clínico. Já existe relato de aplicação clínica de TG viral voltada ao cérebro de dez crianças com lipofuscinose com melhora de função neurológica; infelizmente, algumas reações adversas foram relatadas (Worgall *et al.*, 2008).

Para os pacientes MPSI de qualquer tipo, a função neurocognitiva pode variar enormemente, e esse parâmetro avaliativo é incluído no algoritmo de escolha de tratamento proposto, em conjunto com a idade (Muenzer *et al.*, 2009). O objetivo desse algoritmo é auxiliar na seleção de uma abordagem terapêutica que contribua de forma mais consistente para a atenuação da neuropatologia, além da doença sistêmica. Em nível pré-clínico, poucos trabalhos avaliando a função cognitiva após a execução de um protocolo *in vivo* de TG para DAL foram realizados, a maioria deles voltados para o cérebro de camundongos adultos em diferentes faixas etárias de outros tipos de DAL (Cressant *et al.*, 2004; Liu *et al.*, 2005; Fu *et al.*, 2007). Um estudo comportamental pós terapia gênica voltado para MPSI foi realizado com camundongos tratados em fase neonatal e com redução dos GAGs (por contagem de vacúolos) (Hartung *et al.*, 2004). Os autores relataram melhora significativa da função cognitiva. Interessantemente, já existem relatos na literatura comprovando que esses camundongos MPSI de fato possuem problemas cognitivos progressivos

(Reolon *et al.*, 2006; Pan *et al.*, 2008). Essas janelas de déficit, no modelo murino, são de extrema importância, uma vez que elas poderiam mimetizar os diferentes níveis de comprometimento mental evidenciados nos pacientes MPSI, simulando algumas situações descritas no algoritmo de tratamento. Em conclusão, a TG para MPSI em modelo animais tem sido explorada através do emprego de vetores virais e não-virais e muitas questões relacionadas ao design do vetor, a sua via de administração, a produção da enzima exógena nas células alvo e as conseqüências dessa reposição permanecem em aberto (para uma revisão sobre os mesmos consultar Ponder and Haskins, 2007).

A TG *in vivo* e *ex vivo* para MPSI

A TG para MPSI busca a restauração da atividade de IDUA, mesmo que parcialmente. Foi proposto que um nível residual (1-5% em relação ao normal) poderia fazer uma transição de fenótipo: de forma grave para branda – ou da forma neuropática para a não-neuropática. Esse processo seria dependente de uma nova tecnologia voltada especificamente para o sistema nervoso central (Ioannou 2000; Ioannou *et al.*, 2003). Tratamentos sistêmicos para MPSI não conseguem atingir o cérebro, conforme mencionado anteriormente. A ruptura de barreira hematoencefálica não é recomendável a cada RE (Bosch *et al.*, 2000). Assim sendo, de fato uma nova alternativa terapêutica faz-se necessária para a clínica.

A TG tem sido proposta como uma terapia alternativa para a MPSI (Ponder and Haskins, 2007). A TG *ex vivo* em nível clínico voltada a outro tipo de MPS já foi iniciada, baseado na transdução de células tronco

hematopoiéticas humanas e posterior reinfusão das mesmas ao paciente (Cheng and Smith, 2003). Esse estudo mostra que a aplicação da TG *ex vivo* para DAL pode ser uma alternativa clínica factível, considerando-se a *expertise* acumulada com os protocolos para imunodeficiência combinada (SCID) e genotoxicidade de transferência gênica em células progenitoras (Havenga *et al.*, 1997; Bushman, 2007). Outra proposta seria a combinação de protocolos – sistêmico e *in situ* – para obtenção de correção global ou parcial da patologia. É possível que essa nova terapia possa ser somada às convencionais, promovendo uma atenuação da doença neurológica em concomitância à doença sistêmica. Tem-se discutido de forma pouco intensa, ainda, os benefícios que poderiam ser obtidos através de uma proposta desse tipo. Para tanto, é necessário que novas ferramentas de transferência gênica eficientes para as células do sistema nervoso central sejam desenvolvidas e bem caracterizadas. Mais uma vez a pesquisa pré-clínica tem um papel de destaque no desenho futuro de terapias avançadas.

Objetivos

O presente projeto de pesquisa teve por objetivo geral o estabelecimento de protocolos de terapia gênica *ex vivo* e *in vivo* em modelo murino de Mucopolissacaridose do tipo I (MPSI) empregando vetores retrovirais. Os objetivos específicos compreenderam:

Terapia gênica *ex vivo*:

- Transdução de células-tronco mesenquimais oriundas de camundongos MPSI com vetor baseado em MLV (Vírus da Leucemia Murina – *Moloney Leukemia Virus*) contendo o transgene *IDUA* e avaliação dos níveis enzimáticos obtidos após a transformação genética.
- Injeção das células transduzidas através da via intraventricular em camundongos MPSI de duas faixas etárias (12 e 25 semanas), com o intuito de avaliar a eficiência do protocolo para fases distintas da neuropatologia já estabelecida, através da correlação de parâmetros bioquímicos e comportamentais.

Terapia gênica *in vivo*

- Construção de um novo vetor retroviral baseado no MSCV (Vírus da Célula Tronco Murina – *Murine Stem Cell Virus*) como ferramenta de transferência gênica mais adequada às células progenitoras e/ou com baixa atividade mitótica.

- Introdução de um gene de resistência à blasticidina como meio de seleção rápida para enriquecimento da fração de células geneticamente modificadas.
- Caracterização *in vitro* do vetor em células-tronco mesenquimais.
- Avaliação comparativa de três vetores retrovirais distintos (baseados em MLV, HIV e MSCV) sob as mesmas condições experimentais como vetores de transferência gênica para o sistema nervoso central de camundongos MPSI, através de injeção intraventricular bilateral.
- Avaliação de sustentabilidade da expressão do transgene e da redução dos depósitos de GAGs no cérebro dos animais tratados ao término do experimento.

Injection of mesenchymal stem cells modified with IDUA in adult MPSI mice brains decreases GAGs deposits and improves exploratory behavior

Artigo a ser submetido a "*Molecular Genetics and Metabolism*"

"(...) We have seen the maturation of gene transfer technology, the design of excellent clinical research studies, and, of course, the therapeutic successes in the X-SCID and ADA-SCID studies, promising clinical results in a broad set of cancer gene therapy studies, exciting effects of genetic correction in animal models of blindness and imminent human clinical studies of genetic forms of blindness, and even some hopeful findings in neurodegenerative, orthopaedic, and cardiovascular disease, among others. (...)"

T. Friedmann, "Happy Anniversary" *Mol Ther* 2007, 15(6).

Injection of mesenchymal stem cells modified with IDUA in adult MPSI mice brains decreases GAGs deposits and improves exploratory behavior

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Abstract

Mucopolysaccharidosis type I (MPSI) is caused by the deficiency of alpha-L iduronidase (IDUA), which leads to lysosomal accumulation of glycosaminoglycans (GAGs) dermatan and heparan sulfate. Unfortunately, currently available therapies are not able to prevent neuropathology. In this study we hypothesized that mesenchymal stem cells (MSC) transduced with MLV-IDUA vector and injected in KO adult mice brain could reduce brain GAG deposits and improve mice exploratory activity. After 1 or 2 months of follow-up, the presence of transgene in the mice brain tissues was confirmed by PCR in almost all treated mice, in addition to an intense reduction of total GAGs. Despite that, IDUA activity was undetectable in these samples. These results indicate that the initial level of IDUA was not sustainable for a month, but they were enough to reduce GAGs content in the treated mice. An important consequence of this treatment was seen in the behavioral tests, which showed a tendency of exploratory behavior improvement. These results indicate a significant improvement in motility of adult KO animals, at least in a part, due to the brain GAGs reduction. By this study we suggest that the IDUA gene therapy associated with MSC and injected directly in the brain could be an efficient way to ameliorate neuropathology.

Keywords: MPSI, gene therapy, retrovectors, lysosomal storage disorder, mesenchymal stem cell

Abbreviations: GAGs - glycosaminoglycans; LSD - lysosomal storage disorders; MPSI - mucopolysaccharidosis type I; ERT - enzyme replacement therapy; CNS - central nervous system; MSC - mesenchymal stem cells; MOI - multiplicity of infection; IDUA - alpha-L-iduronidase; CS - chondroitin sulfate; DS - dermatan sulfate; HS - heparan sulfate; WT - wild type; KO – knockout; KO/GFP - KO mice injected with MSC transduced with the

MLV-GFP construct; KO/IDUA - KO mice injected with MSC transduced with the MLV-IDUA construct

1. Introduction

The disruption of lysosomal competence in cellular metabolism results in accumulation of glycosaminoglycans (GAGs), which leads to a group of inherited diseases called lysosomal storage disorders (LSD). Mucopolysaccharidosis I (MPSI) is a type of LSD in which the GAGs dermatan and heparan sulfate accumulate due to deficiency of alpha-L iduronidase (IDUA - EC 3.2.1.76). The frequency of MPS I is 1:100,000 living births and it presents itself as a syndrome with three phenotypes: Hurler (OMIM #607014), Hurler-Scheie (OMIM #607015) and Scheie (OMIM # 607016). Recently it has been suggested that MPSI patients should be classified into two forms: attenuated (Hurler-Scheie and Scheie) and severe (Hurler) [1]. MPS I patients develop splenomegaly, bone/articular diseases and cardiorespiratory malfunctioning, among other problems. Hurler syndrome also results in progressive and irreversible neurodegeneration, which leads to death in early childhood [2].

Two types of treatment are currently available for MPSI. Bone marrow or stem cell transplantation is efficient, but limited by the scarcity of compatible donors, high mortality due to the procedure and high financial cost. The second alternative is enzyme replacement therapy (ERT), which is effective for visceral disease, diminishing hepatosplenomegaly and also improving articular movements. However, neither transplantation nor ERT can avoid the neurodegenerative process [3]. Although preclinical studies have shown that infusion of high levels of enzyme may reach the central nervous system (CNS) with therapeutic benefits [4], these levels are not attainable at the clinical level, since more frequent infusions and higher amount of enzyme would be necessary. Therefore, even initiating ERT in early childhood, it will not bring a significant benefit to the CNS despite improving visceral disease. In addition, MPS I syndrome is not routinely diagnosed in newborns and most of the affected

children are diagnosed much later in life [3]. These observations force to search for alternative procedures aiming specifically to treat CNS.

Intravenous gene transfer of viral vectors to reach the brain of LSD animals is feasible at neonatal stage with AAV, lentivector or retrovetor [5-8]. In adult MPSI mice, systemic injection of AAV and MLV vectors have been shown to produce significant level of enzyme in blood, but much less activity was detectable in brains [6, 8-11]. It is important to note that the correction of neurological abnormalities, seen by behavioral tests, was evaluated only after neonatal AAV viral gene transfer in this murine MPSI model [5]. Brain gene therapy for MPSI models with viral vectors results in local IDUA production and GAGs reduction for mice and dogs [9, 12], but unfortunately these works have not employed behavior tests.

In the literature, there are no gene therapy studies comparing adult mice in different stages of advanced illness, which is a more interesting model if we consider the future implementation of clinical trials. In MPSI mice, a progressive and temporal evolution of behavioral malfunction is detected according to age onset [13], which should be the consequence of accumulation of GAGs in the brain. How GAGs deposits affect neurocognition, even in patients, is still under investigation [1]. Thus, it will be of great importance to include behavioral tests and correlate such results with GAGs quantification, especially in brain gene therapy studies for MPSI. Behavioral tests have already been performed in other types of MPS [7, 14-17].

Mesenchymal stem cells (MSC) present the potential to differentiate into various cell types of mesenchymal origin such as osteoblasts, chondrocytes and adipocytes [18, 19]. These cells have shown the ability to respond to guidance cues in the brain, and may be used as vehicles for the treatment of neurologic disorders [20]. This strategy has been exploited in

the preclinical study of different pathologies [21, 22], and has also shown success in terms of cognitive gain of function in MPS VII mice [23].

In this paper, based on the characteristics of MSC and the necessity to provide additional neurotrophic factors in situ, we hypothesized that MSC modified with IDUA delivered directly into the ventricular brains of MPSI mice can bring a synergistic therapeutic effect, especially in adult MPSI animals. To validate our hypothesis, adult MPSI mice were used evaluating locomotor and exploratory behavior functions besides molecular analyses.

2. **Material and methods**

2.1. *IDUA* -/- mice

IDUA -/- (knockout – KO) mice were produced by targeted disruption of the IDUA gene [24]. The colony (kindly provided by Dr Elizabeth Neufeld, UCLA, Los Angeles, CA, USA) was maintained by breeding heterozygous animals. Maintenance conditions and experimental protocols were approved by the Research Ethics Committee of the Federal University of São Paulo (CEP 1201/07). In our experiments, 12-week old (12w) and 25-week old (25w) KO mice were used. At the end of experiments, these mice were 20 (20w) and 29-week old (29w), respectively.

2.2. MLV vectors and mesenchymal stem cell culture and transduction

Two PT67 PCLs cell lines were used in our experiments: one containing the human IDUA cDNA (NM_000203.3) (MLV-IDUA) and another *gfp* reporter gene (MLV-GFP) in the same backbone, described by M Camassola *et al*. (unpublished results). Vectors were collected as described elsewhere [25, 26] and were concentrated in a Sorvall centrifuge (rotor SS34) at 16000 rpm for 2 hours. For each 20 mL of viral vector, 4 mL of 20% sucrose in water were added. After centrifugation, the supernatant was drained off and the pellet was ressuspended in the desired volume of serum-free DMEM medium without antibiotics and glutamine and incubated overnight at 4^{o}C. Vectors were titrated using NIH 3T3 cells with 8 μg/ml of polybrene [25, 26]. G418 sulfate and cell culture reagents were purchased from Gibco/Invitrogen Canada Inc (Burlington, ON, Canada); protamine sulfate and polybrene from SIGMA (St Louis, MO, USA).

Mesenchymal stem cell (MSC) cultures were established from the bone marrow of 8 week-old IDUA -/- mice as previously described [27]. MSC were transduced in two different MOIs

(multiplicity of infection) as indicated in the Results section, always using 5 x 10^4 cells plated on 25 cm^2 dishes. The transduction proceeded for 24 hours in the presence of 10 μg/mL of protamine sulfate. The medium was then replaced with a fresh one and the cells were cultivated for 7 days at 37°C in 5% CO_2, when they were screened for IDUA activity. MSC cultures positive for IDUA activity were then split and allowed to expand for additional 3 days in 75 cm^2 dishes for the in vivo experiments. Cells with the passage 3/4 were used for in vivo experiments.

For transduction of NIH3T3 mouse fibroblast cell line, the same procedure from MSC transduction was used. After transduction, the cells were selected with G418 (1 mg/ml final concentration) for at least 15 days. To determine transduction efficiency of MSC with *gfp* reporter gene, GFP fluorescence positive cells were counted using fluorescence microscope.

2.3. Alpha-L-iduronidase activity

Cells were collected after tripsinization and the pellet was ressuspended in homogenization buffer (10 mM $NaPO_4$ pH 5.8, 0.1 mM DTT, 0.1% Triton X-100). IDUA dosage protocol is described in M Camassola *et al*. (submitted manuscript). Briefly, in a microplate, 10 μL of the samples were mixed with 10 μL of 2.85 mM 4-methylumbelliferyl-alpha-L-iduronide (4MU-I, Toronto Research Chemicals Inc., North York, ON, Canada) and 40 μL of formate buffer 0.2 M pH 2.8. The plate was incubated at 37°C for 1 h and the reaction was stopped by the addition of 175 μL of stop buffer (0.5 M glycine-NaOH, pH 10.3). Fluorescence of the 4MU product was determined using a Spectramax M2 fluorimeter (Molecular Devices Inc., Sunnyvale, CA, USA) using 365 and 455 nm of wavelength for excitation and emission, respectively. Protein concentration was determined with the Bio Rad protein dosage kit (Bio

Rad Laboratories, Hercules, CA, USA). IDUA activity is expressed in units (U), representing nmols/h/mg ptn.

2.4. GAGs measurement

One third of mice brain tissue was cut and grounded in 10 volumes of acetone. After standing overnight at room temperature, acetone was changed during five days. Afterwards, acetone was discarded and the sample was completely dried at 50^{o}C. The dry weight was taken, and the sample incubated twice with maxatase 4 mg/mL (Tris HCl 0.05M pH8.0 NaCl 0.15M) for 24 h at 60^{o}C. Trichloroacetic acid was added (10% of final volume) and allowed to stand for 20 minutes at 4^{o}C. The precipitate formed was removed by centrifugation at 5000 g for 20 minutes at room temperature. The GAGs from supernatant were precipitated with two volumes of ethanol. After overnight incubation at -20^{o}C, GAGs were collected by centrifugation, vacuum dried, and submitted to deoxyribonuclease I treatment (Sigma, Saint Louis, EUA). The GAGs were analyzed by agarose gel electrophoresis [28] and quantified by densitometry with Quick Scan 2000 Software and using standard known concentration of chondroitin sulfate (CS), dermatan sulfate and heparan sulfate (DS-HS). Each sample for electrophoresis represents the minimal of one third of all GAGs extracted from brain tissue destined to this analysis. We have not used less brain tissue to extract GAGs, neither less sample to load the gel. GAGs are expressed in µg/g of dry tissue (total GAG) or percentual of GAGs composition depicted in eletrophoretic profiles (% DS-HS and CS).

2.5. In vivo injection of transduced MSC

This protocol is a modification of [29]. IDUA KO mice were anesthetized as specified in the mentioned reference and treated with 10^6 MLV-transduced MSCs, previously screened for IDUA activity, ressuspended in 5 µL PBS. The cells were injected directly in the left

ventricule of the brain with a 30-gauge needle. Five minutes after injection, the needle was removed and the incision was sutured. Mice were allowed to recover on a heating pad before returned to microisolators. At different times after treatment, mice were sacrificed and the brain was collected. Fractions (1/3) of the tissue were kept at -80^{o}C for genomic DNA extraction and GAGS determination. One third of the brain was directly processed in 1mL of homogenization buffer with the help of a Potter device, for determination of IDUA activity.

2.6. Behavioral tests

All behavioral tests were conducted in the same room, at controlled temperature and luminosity. Mice were separated into four cohort groups, based on treatment: wild type - control mice (*IDUA* +/+, WT), knockout MPSI control mice (*IDUA* -/-, KO), KO mice injected with MSC transduced with the MLV-GFP construct (KO/GFP) and KO mice injected with MSC transduced with the MLV-IDUA construct (KO/IDUA). Animals were tested before and after treatment, to evaluate the effect of treatment with transduced MSC on motor function and anxiety in each group, by two behavioral tests. The elevated plus maze test was conducted as previously described [17]. The number of entries in closed/open arms, fecal pellets and urine foci were counted. The time spent in closed/open arms was registered, in seconds. For the open field test, we used a circular arena, and the one trial exposition was conducted similarly as previously described [30], except for the second exposition. The time of freezing and grooming, and numbers of rearings, crossings (internal, external and total), fecal pellets and urine foci were counted. Data are presented as mean and standard deviation values for the pre trial test, when KO mice were compared to WT ones through the nonparametric Mann-Whitney. The threshold for statistical significance was established at $P< 0.05$. Results obtained after MSC treatment are presented as individual scores for each parameter.

2.7. Molecular analyses

Genomic DNA (gDNA) was extracted from the brain with the QIAamp DNA extraction kit (QIAGEN GmbH, Hilden, Germany). PCR reactions for IDUA and endogenous control were performed as previously described [31, 32]. PCR conditions for IDUA were also employed for *gfp* primers in *gfp* PCR (5'gag cct ggg gac ttt cca cac cc 3' and 5' gaa aat tgt gat gct att gc 3'). Reagents were purchased from Fermentas Inc. (Glen Burnie, MD, USA).

3. Results

3.1. Transduction of NIH 3T3 and MPSI MSCs cultures

To evaluate the efficiency of the concentrated viral vectors, NIH 3T3 cells were transduced with 5.2 MOI and IDUA activity was evaluated (Table 1). These cells were used as internal control for IDUA activity even if these fibroblasts were not isolated from MPSI mice, because it was possible to submit these cells to selection with G418. The endogenous activity of IDUA in non-transduced fibroblasts was at least 245 times lower than G418-selected cells (23.8 ± 5.8 versus 5849.1 ± 851.6 U) (Table 1).

MPSI MSC were transduced with 5.2 and 77 MOI to estimate the transduction rate. After one week of transduction, cells transduced with 77 MOI presented a similar level of IDUA activity in relation to NIH3T3 MLV-IDUA transduced cells. After 4 weeks, enzyme production in these cells was decreased to basal level. On the other hand, MPSI MSC transduced with 5.2 MOI had 40 % of activity in the 1st week, but in the 4th week enzyme production increased to 51%, reaching 3016.5 U. However, this group also lost IDUA activity at 6th week (around 15.0 U) (Table 1). MSC transduced with MLV-GFP vector, which express only the reporter *gfp* gene useful to determine transfection rates, presented some level of activity (Table 1). This basal and non-specific activity can be explained by the interference of gfp fluorescence, which emits weak signal of light at 455 nm.

Transfection rate determined by *gfp* expression showed a similar profile from cells transduced with MLV-IDUA vector (Table 1). G418-selected NIH3T3-GFP cells were 100% positive for GFP, whereas MPSI MSC transduced with 5.2 and 77 MOI had 8.9 and 21.3% of GFP-positive cells, respectively, in the 1st week of transduction. After 4 weeks, cells

transduced with 77 MOI had no signal of GFP, while cells transduced with 5.2 MOI maintained the same percentage of GFP-positive cells detected on week one. The 5.2 MOI, however, resulted in undetectable levels of GFP on week 6, indicating that the extension of *gfp* expression time is also followed by some kind of transgene silencing.

3.2. Pre trial behavioral evaluation

Since our research focused on already established illness, wild type (WT) and knockout (KO) mice were submitted to open field test to evaluate exploratory behavior. Thus, this pre trial test was performed in order to determine the degree of basal exploratory capacity (Tables 2 and 3). Results of open field test show that KO mice were less active than WT ones. Elevated zero maze did not detect differences among phenotypes.

3.3. Pos trial behavioral evaluation

Thirty to 60 days after *ex vivo* gene therapy, a second round of behavioral test was performed, after which brains were collected for enzymatic dosage, PCR analysis and GAGs quantification. Mice treated at 12 weeks of age were analyzed when 20-week old (20w). Mice treated at 25 weeks were analyzed when 29-week old (29w).

Open field test showed a tendency of exploratory behavior improvement after ex vivo gene therapy for both age groups (Table 4). In the 20w group, treated mice seemed to rear more than non treated ones. Vertical movement (internal, external and total squares crossed in open field test) showed the same tendency, being KO/IDUA more alike to normal mice. For the 29w group, rearings were greatly improved in KO/IDUA mice only, and vertical movement in this group seemed to be similar to normal mice. For KO/GFP mice belonging to 20w group, the injection of new and low passage MSCs may explain the possible improvement in these two parameters – which was not detected for KO/GFP mice from 29w group.

3.4. Quantification of IDUA and PCR analysis

Enzyme activity was undetectable in brain tissues from both study groups. PCR analyses showed that almost all treated mice were positive for IDUA sequence, except in 3 samples (Figure 1).

3.5. GAGs analysis

GAGs content was reduced in treated mice, as seen in Tables 4 and 5. Results, however, point to a better outcome in 25 week-old age treated mice. Representative eletrophoretic profiles allowed us to depict the proportion of chondroitin and dermatan-heparan sulfate in each sample (Figure 2).

4. Discussion

In gene therapy studies, the characterization of a vector, in terms of level and time of gene expression, is the first and fundamental step of research, because those terms depend on the therapeutic gene, target cell and administration route. In the case of human MSC, it is known that the rate of transduction with MLV is low [33], consequently high MOI is used to use to increase transduction efficiency. To estimate a number of MOI necessary to obtain high level of IDUA gene expression, two MOI values were tested in MSC and compared with the NIH 3T3 cells transduced with the same vector and selected with G418 to have 100% of genetic modified cells. Use of 77 MOI reached 21% of transduction efficiency, seen by GFP positive cells, but, curiously, this expected efficiency with MLV-IDUA was sufficient to reach the level of IDUA provided by the NIH3T3 selected cells (Table 1). Additionally, the use of only 5.2 MOI resulted in the transduction of about 10% of MSC and produced about 50% of IDUA activity seen with 77 MOI, in the same cells. These results indicate that the concentration of virus required to increase transduction rate is not linearly proportional, and the level of IDUA gene expression per cell is highly variable according to the cell type. It has been said that in cells lacking IDUA, this heterologous expression may be adaptative [34].

As in our study the long-term gene expression is desired to correct permanently IDUA deficiency, the MLV transduced MSC were followed measuring IDUA activity and also counting GFP-positive cells. IDUA activity in 77 MOI-transduced MSC decreased to the basal level in 4 weeks, and this result was confirmed by the absence of GFP-positive cells detectable in fluorescence microscopy at the same time. However, the 5.2 MOI transduced MSC lasted 2 weeks longer than 77 MOI transduced cells, as seen by IDUA activity and GFP positive cells. Silencing of gene expression after MLV transduction is a known

phenomenon, especially in the progenitor context [35-37]. Our results indicate that this silencing phenomenon is accentuated by the augmentation of virus concentration. Even though the 77 MOI transduced MSC produced double level of gene expression in comparison to the 5.2 MOI transduced MSC, we decided to use low MOI in the next experiments because of the biosafety [38, 39] besides just the longer term gene expression.

The main goal of our gene therapy study was to reduce brain GAGs deposits after injection of MSC modified with MLV-IDUA in the lateral ventricles, expecting exploratory improvement as a consequence. In our study, we used adult mice because MPSI pathology is already established in this phase. To validate our study, behavior characteristics were initially analyzed. As neurodegeneration is a progressive and slow process that affects animal behavior, there is not a specific age at which the animal may be diagnosed as fully affected. Therefore, it is very important to determine the behavioral state of KO animals before starting gene therapy experiments. Two behavioral tests were applied in our study: open field and elevated plus maze. In this pre-evaluation, the difference between normal and KO mice in terms of vertical and horizontal movements (rearing and internal crossings) and fecal pellets parameters, which are indicative of exploratory activity and anxiety, respectively, was statistically significant (Tables 1 and 2). These results indicate that the movement of KO mice was clearly affected. A previous study using the open field test with habituation found statistical significance only for the number of rearings [30]. In terms of the elevated plus maze analysis, which is an important indicator of anxiety, we have not detected any evident distinction among phenotypes, which is in accordance with the literature [13].

To evaluate the therapeutic effect of ex vivo gene transfer, we established 30 and 60 days of observation for mice treated at 25 and 12 weeks of age, respectively, after gene therapy because the life expectancy of MPSI mice [13]. A second round of behavioral tests

was applied after the observation periods, and then the animals were sacrificed to collect brain tissue for enzymatic dosage, PCR analysis and GAGs quantification. Enzyme activity was undetectable in both study groups. As shown by the in vitro study, the enzyme activity of MSC transduced with MLV-IDUA is lost 6 weeks after transduction (Table 1), therefore, these in vivo results are not unexpected. A similar decline of IDUA levels after in vivo MLV gene transfer has been observed [8].

Quantitative and qualitative analyses of GAGs and monitoring of animal behavioral may be related to the state of MPSI neuropathological evolution. In our experiments, IDUA activity was not detected in treated mice brain collected at the end of the study. However, the enzyme supplied by MLV-IDUA-transduced MSC was sufficient to trigger GAGs degradation and improve exploratory function. As seen in Tables 4 and 5, total GAGs content was diminished in a great number of treated mice, despite the maintenance of CS, DS-HS proportion in brain tissue. We have used the dermatan-heparan sulfate denomination for GAGs accumulated in the brain (besides chondroitin), because they are the specific GAGs that accumulate in MPSI [2]. It is known that the heterogeneity of GAGs fragment sizes may decrease the sensitivity of GAGs dosage in routine assays [40]. The method employed in our work to quantify GAGs may offer an advantage in sensitivity for brain tissue, but still is not as refined as mass spectrometry [41].

In conclusion, in some IDUA-treated mice (#8-10 and 19), GAGs reduction was more prominent, which resulted in more exploratory activity. Interestingly, the results point to a better outcome in mice belonging to the 29w group (#8-10). Since mice of the 20w group were analyzed 2 months after ex vivo gene transfer, and in vitro follow-up of IDUA activity from duplicates of injected cells indicated an intense decline in transgene expression in 6 weeks, it is possible that in this group GAGs accumulation restarted. Such phenomenon is

seen in urine GAGs from MPSI patients treated with enzyme replacement therapy [42]. Despite that, these results evidence a possible relationship of accumulated GAGs and exploratory activity, which importantly occurs regardless of mice age.

Since we have used MSC in ex vivo gene therapy with *IDUA*, injection of MSC transduced with MLV-GFP vector in some animals was carried out to investigate the effect of MSC cells alone. In 25-weeks treated mice, the use of MSC alone did not bring any improvement of exploratory activity. However, in 12-weeks animals (#17 and #18) GAGs deposit were diminished and exploratory activities augmented. As the MSC injected may secret factors to modulate the metabolism of neighboring cells, these results suggest that they had a role in the reduction of total GAGs biosynthesis. This observation could be corroborated by the maintenance of the relationship of DS-HS versus CS in brains, despite total GAGs reduction (Tables 4 and 5).

The treatment of KO mice with MSC transduced with MLV-IDUA vector diminished significantly total brain GAGs, which resulted in the improvement of exploratory activity. In mice treated at advanced stages of pathology (25 weeks old), this resulted in higher scores of vertical and horizontal movements. But it is also important to note that those animals were analyzed after a month of therapy versus two months in 12 week-old treated animals. Even though this may be similar to the already known two-phase phenomenon of urine GAGs decline in patients under enzyme replacement therapy [42], the improved exploratory behavior was considered a very surprising outcome. Last, as MSC transduced with MLV vectors presented a transitory transgene expression (around 6 weeks), it is likely that GAGs deposit accentuated in brain after the first month of treatment, as seen in mice from 20w group. In these animals, MSC alone brought some improvement in exploratory behavior,

despite the absence of a therapeutic gene. Additional experiments are needed, as discussed above, to further explore these results.

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Table 1. Transduction efficiency and gene expression analysis of MPSI MSC and NIH 3T3 cells with MLV-IDUA and MLV-GFP vectors

	MPSI-MSC				NIH3T3	
	MOI 5.2		MOI 77		G418 selected	
	IDUA (U)	GFP (%)	IDUA (U)	GFP (%)	IDUA (U)	GFP (%)
Control	ND		ND		23.8 ± 5.8	0
MLV-GFP			20.7 ± 21.6		29.5 ± 29.5	100
1w	2278 ± 249	8.9±1.3	5340.4 ± 137.0	21.3 ± 6.65		
3-4w	3016.5 ± 780	9.3±2.2	59.7 ± 16.8	ND	58491.1 ± 851.6	100
6w	15 ± 1.41	ND	NE	ND		

MPSI-MSC were transduced with 5.2 and 77 MOI and evaluated for transgene expression during 6 weeks. NIH 3T3 cells transduced with the same vectors and selected with G418 were used as a positive control cells. All experiments were performed at least in duplicates. IDUA is expressed in units of activity (U) and GFP-positive cells are expressed in frequency. Cells injected in KO mice are indicated in italic numbers. ND= non detectable; NE= non-evaluated.

Table 2. Pre trial behavioral evaluation of wild type (WT) and knockout (KO) mice in open field.

	Open field							
Group (n)	**Freezing**	**Grooming**	**Rearing**	**IS**	**ES**	**TS**	**FP**	**Urine**
12w								
WT (5)				79.0	136.6	215.6		
	9.4 ± 0.5	10.4 ± 6.6	46.4 ±10.7	±21.9	±25.2	±44.8	5.0 ±1.58	0.8 ±0.4
KO (10)				42.5	114.7	157.2		
	15.1 ± 9.5	12.1 ±6.7	35.7 ±8.8	±14.3	±38.4	±42.6	4.0 ±2.1	0.9 ±1.2
p	> 0.05	> 0.05	> 0.05	= 0.00	> 0.05	= 0.02	> 0.05	> 0.05
25w								
WT (3)				49.0	74.0	123.0		
	25.3 ±15.8	10.0 ±4.0	37.3 ±24.0	±34.6	±15.8	±50.3	4.6 ±1.1	0.6 ±0.5
KO (7)				33.8	70.8	104.7		
	24.4 ±10.0	9.1 ±6.0	23.7 ±12.9	±13.5	±37.9	±46.4	0.8 ±1.2	0.0 ±0.0
p	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	= 0.01	> 0.05

Results (mean ± SD) show freezing and grooming in seconds; rearings and square crossings (internal, external and total) in absolute number of events. Fecal pellets and urine foci were counted too. The non parametric Mann-Whitney test was used to compare the two phenotypes. Statistically significant p values are presented. 12w = 12 week-old treated mice; 25w = 25 week-old treated mice; IS, ES, TS = internal, external and total squares, respectively; FP = fecal pellets.

Table 3. Pre trial behavioral evaluation of wild type (WT) and knockout (KO) mice in elevated zero maze.

	Elevated zero maze					
Group (n)	**OAE**	**OAS**	**CAE**	**CAS**	**FP**	**Urine**
20w						
WT (5)	2.8±1.4	29.0±24.8	9.2±3.2	223.2±23.4	0.6±0.8	0.0±0.0
KO (10)	2.8±2.7	42.9±47.3	6.4±4.2	194.5±60.2	0.7±0.6	0.4±0.9
p	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05
29w						
WT (3)	2.3±0.5	55.3±64.6	8.3±5.6	182.0±113.5	3.0±2.0	0.6±0.5
KO (7)	2.5±3.1	25.8±25.9	4.5±3.0	187.7±46.6	0.5±0.5	0.1±0.3
p	> 0.05	> 0.05	> 0.05	> 0.05	= 0.06	> 0.05

Results (mean ± SD) show time spent in each arm in seconds (S) and the number of entries in each arm (E). The non parametric Mann-Whitney test was used to compare the two phenotypes. Fecal pellets and urine foci were counted too. Statistically significant p values are presented. 12w = 12 week-old treated mice; 25w = 25 week-old treated mice; OAE, OAS = open arm entries and open arm time spent in seconds, respectively; FP = fecal pellets.

Table 4. Post trial behavioral evaluation of wild type (WT), knockout (KO), knockout mice injected with MLV-GFP-transduced MPSI MSC (KO/GFP) and knockout mice injected with MLV-IDUA-transduced MPSI MSC (KO/IDUA).

		Open field									
#	**Group**	**Freezing**	**Grooming**	**Rearing**	**FP**	**Urine**	**IS**	**ES**	**TS**	**Total GAG**	**% DS-HS**
1	WT 29w	6	8	4	4	0	21	69	90	*NE*	23.8
2	WT 29w	5	2	5	5	0	11	15	26	2057.0	*NE*
3	WT 29w	15	18	11	6	1	19	52	71	1757.3	9.8
4	KO 29w	6	0	0	0	0	2	7	9	4345.0	49.8
5	KO 29w	5	9	0	4	0	10	28	38	3235.5	47.7
6	KO/GFP 29w	1	2	0	0	0	7	37	44	3819.0	41.8
7	KO/GFP 29w	3	0	0	1	0	10	22	32	*NE*	*NE*
8	KO/IDUA 29w	5	3	0	3	0	7	26	33	1656.0	42.6
9	KO/IDUA 29w	8	3	6	2	0	10	34	44	1778.9	39.8
10	KO/IDUA 29w	4	6	27	4	0	31	108	139	1094.2	55.6
11	WT 20w	5	0	11	3	1	34	79	113	1140.4	6.2
12	WT 20w	0	10	6	4	0	16	78	94	1281.2	9.9
13	WT 20w	12	5	12	3	0	29	68	97	1997.3	32.1
14	WT 20w	18	17	20	4	1	35	121	156	1849.6	29.0
15	KO 20w	5	15	25	1	0	22	46	68	2741.0	40.0
16	KO 20w	9	2	2	3	0	7	31	38	2889.3	48.8
17	KO/GFP 20w	11	4	22	4	1	29	110	139	2224.2	66.5
18	KO/GFP 20w	5	0	14	2	0	9	86	95	1756.3	36.8
19	KO/IDUA 20w	9	14	20	2	0	36	119	155	1220.2	37.3
20	KO/IDUA 20w	2	8	24	0	0	29	49	78	2323.0	42.1

21	KO/IDUA 20w	4	8	9	0	0	28	96	124	2404.4	46.1
22	KO/IDUA 20w	25	7	10	2	0	29	60	89	2296.0	49.2
23	KO/IDUA 20w	3	9	18	1	0	25	65	90	2083.0	49.4

Results show freezing and grooming in seconds; rearings, fecal pellets, urine foci and square crossings (internal, external and total) in absolute number of events. IS, ES, TS = internal, external and total squares, respectively; FP = fecal pellets. Total GAG is presented in µg GAG/dry tissue g. Dermatan-heparan sulfate content in each sample is presented in percentual (% DS-HS). 20w = 12 week-old treated mice analyzed after treatment; 29w = 25 week-old treated mice analyzed after treatment; NE = non evaluated.

Table 5. Post trial behavioral evaluation of wild type (WT), knockout (KO), knockout mice injected with MLV-GFP-transduced MPSI MSC (KO/GFP) and knockout mice injected with MLV-IDUA-transduced MPSI MSC (KO/IDUA).

		Elevated zero maze							
#	**Group**	**AOE**	**OAS**	**CAE**	**CAS**	**FP**	**Urine**	**Total GAG**	**% DS-HS**
1	WT 29w	0	0	1	290	3	0	*NE*	23.8
2	WT 29w	1	5	3	284	2	0	2057.0	*NE*
3	WT 29w	0	0	4	284	1	0	1757.3	9.8
4	KO 29w	0	0	0	0	1	0	4345.0	49.8
5	KO 29w	3	82	3	95	1	0	3235.5	47.7
6	KO/GFP 29w	2	44	4	237	1	0	3819.0	41.8
7	KO/GFP 29w	0	0	0	0	0	0	*NE*	*NE*
8	KO/IDUA 29w	0	0	1	294	3	0	1656.0	42.6
9	KO/IDUA 29w	0	0	**4**	288	1	0	1778.9	39.8
10	KO/IDUA 29w	2	12	5	267	1	0	1094.2	55.6
11	WT 20w	2	6	4	265	1	0	1140.4	6.2
12	WT 20w	0	0	2	293	2	0	1281.2	9.9
13	WT 20w	4	147	7	123	0	0	1997.3	32.1
14	WT 20w	0	0	4	0	0	0	1849.6	29.0
15	KO 20w	1	4	4	187	4	0	2741.0	40.0
16	KO 20w	0	0	2	267	1	1	2889.3	48.8
17	KO/GFP 20w	0	0	3	288	4	1	2224.2	66.5
18	KO/GFP 20w	1	5	4	278	3	0	1756.3	36.8
19	KO/IDUA 20w	2	21	13	238	3	0	1220.2	37.3
20	KO/IDUA 20w	0	0	2	285	2	0	2323.0	42.1

21	KO/IDUA 20w	1	4	3	285	2	0	2404.4	46.1
22	KO/IDUA 20w	1	16	5	266	1	0	2296.0	49.2
23	KO/IDUA 20w	1	15	3	280	0	0	2083.0	49.4

Results show time spent in each arm in seconds (S) and the number of entries in each arm (E). Fecal pellets and urine foci were counted too. OAE, OAS = open arm entries and open arm time spent in seconds, respectively; FP = fecal pellets. Total GAG is presented in µg GAG/dry tissue g. Dermatan-heparan sulfate content in each sample is presented in percentual (% DS-HS). 20w = 12 week-old treated mice analyzed after treatment; 29w = 25 week-old treated mice analyzed after treatment; NE = non evaluated.

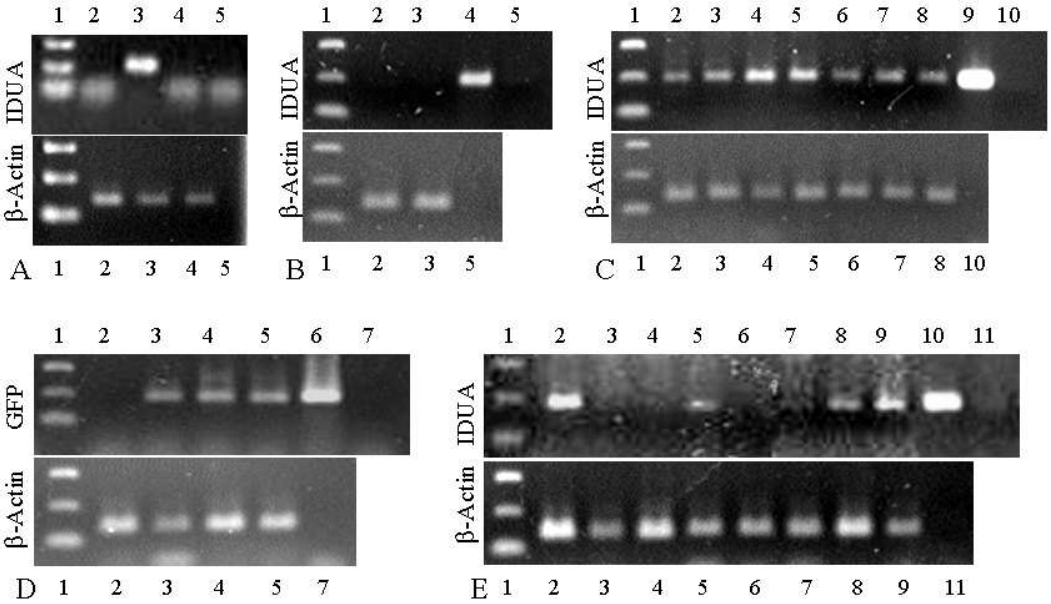
1 2 3 4 5
IDUA
β-Actin
A 1 2 3 4 5
1 2 3 4 5
IDUA
β-Actin
B 1 2 3 5
1 2 3 4 5 6 7 8 9 10
IDUA
β-Actin
C 1 2 3 4 5 6 7 8 10
1 2 3 4 5 6 7
GFP
β-Actin
D 1 2 3 4 5 7
1 2 3 4 5 6 7 8 9 10 11
IDUA
β-Actin
E 1 2 3 4 5 6 7 8 9 11

Fig. 1 - Molecular analysis of transduced MSC and treated mice. Molecular markers have 300, 200 and 100-bp bands. Negative control, blank. Positive control, plasmid viral vector pertinent for each analysis.

(A) PCR amplification of IDUA and β-actin in transduced MSC from KO mice. 1, molecular marker; 2, negative control; 3, MSC transduced with MLV-IDUA; 4, MSC transduced with MLV-GFP; 5, non-tansduced MSC.

(B) PCR amplification of IDUA and β-actin in brain samples from non-treated KO mice. 1, molecular marker; 2, young KO mouse (#15); 3, old KO mouse (#16); 4, positive control; 5, negative control.

(C) PCR amplification of IDUA and β-actin in brain samples from normal mice. 1, molecular marker; 2-5, young normal mice (#11-14, respectively); 6-8, old normal mice (#1-3, respectively); 9, positive control; 10, negative control.

(D) PCR amplification of GFP and β-actin in brain samples from KO mice treated with MLV-GFP. 1, molecular marker; 2-3, young mice (#17 and 18, respectively); 4-5, old mice (#6 and 7, respectively); 6, positive control; 7, negative control.

(E) PCR amplification of IDUA and β-actin in brain samples from KO mice treated with MLV-IDUA. 1, molecular marker; 2-6, young mice (#19-23, respectively); 7-9, old mice (#8-10, respectively); 10, positive control; 11, negative control.

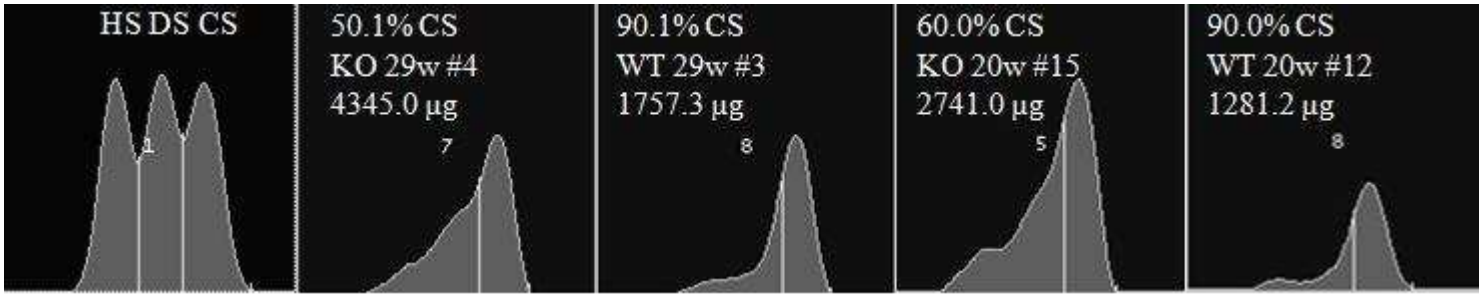
HS DS CS
1
50.1% CS
KO 29w #4
4345.0 µg
7
90.1% CS
WT 29w #3
1757.3 µg
8
60.0% CS
KO 20w #15
2741.0 µg
5
90.0% CS
WT 20w #12
1281.2 µg
8

Figure 2: Eletrophoretic profiles obtained after densitometry of GAGs samples analyzed through eletrophoresis. Representative profiles of mice from both age groups are used to show how the proportion of chondroitin (CS) and dermatan (DS)-heparan (HS) sulfate is determined. Total GAG is expressed in µg/g dry tissue. Phenotype, age group and sample code (#) are presented below the percentual of chondroitin sulfate (CS). On the left, profile from a purified know concentration of HS, DS and CS mixture of known concentration used in the process of GAGs quantification.

Retroviral vectors trilogy: evaluating transgene expression and its effects on GAG deposits after *in vivo* gene therapy with HIV, MLV and MSCV vectors to treat mucopolysaccharidosis I in adult mice

A ser submetido a "*Molecular Therapy*"

"(...)but to some extent, perception and expectation in parts of the scientific and public media have swung far in the direction away from an acceptance of those results. Although most ASGT members are increasingly convinced of the validity of our current direction, the perception of gene therapy in important parts of the public media and even the general scientific community continues to be of expected failure and clinical ineffectiveness. Some remain unconvinced of the progress and disappointed by the time required to take concept to the clinic despite the knowledge that other areas of indispensable modern medical technology such as tissue transplantation, cancer chemotherapy and applications of monoclonal antibodies struggled for several decades in the limbo of setbacks, disbelief, and criticism. Remember that gene therapy has been in the clinic for only 17 years. "

T. Friedmann, "Happy Anniversary" *Mol Ther* 2007, 15(6).

Retroviral vectors trilogy: evaluating transgene expression and its effects on GAG deposits after i*n vivo* gene therapy with HIV, MLV and MSCV vectors to treat mucopolysaccharidosis I in adult mice

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Short title: MPSI *in vivo in situ* gene therapy

Abstract

Mucopolysaccharidosis type I (MPSI) is a type of lysosomal storage disease (LSD) in which undegraded glycosaminoglycans (GAG) dermatan and heparan sulfate are accumulated, compromising the normal function of cells. Two therapeutic alternatives are currently available, bone marrow/ hematopoietic stem cell transplantation and enzyme replacement, but both do not avoid neuropathology. Brain *in situ* MPSI gene therapy is a good option, but the therapeutic efficacy is primarily dependent on viral transduction efficiency. In this study, we compared MLV, HIV and MSCV as gene transfer tools for *in situ* gene therapy in adult MPSI mice, through evaluation of GAGs quantification and IDUA dosage. *In vitro* assays in NIH 3T3 and mesenchymal stem cells (MSC) obtained from wild type and MPSI mice were performed with MSCV to evaluate transgene expression levels and time required to select target cells based on *blasticidin S deaminase* gene. In conclusion, MSCV was very functional *in vitro* and *in vivo*, leading to intense IDUA production and GAGs reduction. Percentual of dermatan and heparan sulfate accumulated in brain was reduced in MSCV and HIV treated mice. Additional experiments will clarify the benefits we could obtain from our MSCV construct for *in vivo* and *ex vivo* gene therapy.

Introduction

Mucopolysaccharidosis type I (MPSI) is a type of lysosomal storage disease (LSD) in which undegraded glycosaminoglycans (GAG) are accumulated, compromising the normal cell function. Consequently, bone, visceral and cardiac diseases are present in all forms of MPSI. The severe form (Hurler phenotype) presents neuropathology, while mild forms (Hurler/Scheie and Scheie phenotype) do not. Dermatan and heparan sulfate are the GAG deposited as consequence of partial or total loss of function of the lysosomal enzyme alfa-L-iduronidase [1].

Two therapeutic alternatives are now available for MPSI. Bone marrow or hematopoietic stem cell transplantation both rely on availability of compatible donors. Yet, they both also have high mortality due to the procedure and high financial cost [2]. Enzyme replacement therapy (ERT) is very effective in diminishing hepatosplenomegaly and improving articular movements, which has major impact in the quality of life of patients. The neurodegenerative process cannot be avoided by these processes, unfortunately [2]. Preclinical studies have shown that high levels of enzyme may reach the central nervous system (CNS) and be therapeutically efficient [3], but how to make this procedure compatible with the weekly enzyme replacement regimen remains unclear. Besides that, the ERT is already a high cost procedure in its conventional form [4]. Interestingly, important contribution from preclinical studies can improve ERT [4, 5], but still CNS remain a challenge.

In resume, since this LSD causes mental retardation and early death, the ultimate clinical benefit will be obtained when early diagnosis and treatment can be performed before the onset of pathology [6]. However, newborn screening for MPSI diagnosis is not routinely performed and affected children are still diagnosed much later in life [2]. While

preclinical neonatal/fetal gene therapy protocols are designed to avoid the establishment of the disease [6-8], later phase intervention is still required for late diagnosis cases. In this context, brain gene therapy may contribute to delay the neurodegenerative process through *in situ* expression of the transgene. The use of adult mouse models may represent an important research tool to depict questions related to blood brain barrier, efficacy of *in situ* vector administration and immune responses [9-12]. Questions to still be addressed in gene therapy concern low level and transient proviral gene expression - that would reinforce the need for a better molecular design of expression cassette, markers of selection also transferred to target cells, vector silencing, among others [13]. In addition, a proper direct comparison among vectors may contribute to identify pro and cons of each system and trigger subsequent investigation including all mentioned aspects, in special long term benefit coming from a sustained transgene expression. This should include GAGs reduction and improvement in cognitive functions, since MPSI knockout mice (KO) present cognitive deficits [14, 15].

In this study we investigated the effect of a brain gene transfer protocol in adult MPSI mice, using three different viral vectors: MLV, HIV and MSCV. The use of MSCV resulted in higher IDUA levels and more intense GAGs reduction than other vectors, including the proportion of dermatan and heparan sulfate. *In vitro*, it allowed rapid selection of transduced cells. Additional experiments will clarify the benefits that could be obtained with this MSCV construct for *in vivo* gene therapy, as well as with *ex vivo* gene therapy protocols.

Results

Production of viral vectors: concentration and validation of batches

HIV vectors and MLV vectors

The final titers of HIV and MLV batches are presented in Table 1. Transduction with bicistronic vectors (containing IRES sequence) resulted in less GFP positive cells than the PLL3.7 construct. Despite that, IDUA levels were around eight fold the endogenous level for HEK 293T cells, seven days after transduction. Concentration of MLV vectors rendered similar titers to HIV vectors, after selection with G418. IDUA levels were at least ten fold above the endogenous ones.

MSCV vectors

Four independent harvestings of MSCV vectors were performed, derived from four different PCLs, identified as presented in Table 2. Despite titers variation among concentrated batches, final titers were considered adequate for our experimental design of *in situ* injection of viral vectors, being similar to HIV and MLV ones. Blasticidin-based selection was fast and in five days BS resistant colonies could be identified in transduced NIH 3T3 cells, as demonstrated in our microplate transduction protocol (Figure 1). Each batch presented a different efficiency of transduction, resulting in different time of culture until reaching confluence. These observations agree with titers obtained with CFU assay. Consecutive experiments were performed also in NIH 3T3 in order to validate the batches through IDUA dosage on transduced and selected cells, as performed for MLV vectors.

For IDUA screening, a protocol similar to that used for titration was employed. We kept a sample of unselected cells to evaluate IDUA expression prior to blasticidin selection (Figure 2). Here we present results relative to endogenous values, to better see the increase in IDUA production. IDUA activity in seven days was around 20 fold the endogenous values for the higher titer batch (33). For batch 37, on the other hand, it was only eight fold the endogenous value. However, 15 days after transduction, selected cultures were proliferating normally, including those transduced with batch 37. These results were maintened during the course of the study (30 days follow up). Selection pressure was omitted after five days of selection.

MSCV functionality in MSC from normal mice (*IDUA* +/+)

Next, we started to evaluate transduction efficiency and blasticidin selection of MSCV vector in MSC from normal mice (*IDUA* +/+). After one day of recovery, 1:5 transduced cells were submitted to blasticidine selection (8 µg/mL final concentration). We used a 24-well microplate in order to visualize the results in a comparative fashion for one and 10 µL of concentrated batches. Individual colonies were visualized for all transduction experiments (Figure 3, left), after five days of selection. The area occupied by cells seems to be in correspondence with the volume of viral vector used. Non-transduced cells exposed to selection pressure were eliminated after five days of selection. In conclusion, it was possible to transduce and select MSC in five days with blasticidin, in these experimental conditions. Another interesting aspect regarding selection of transduced cells was the relationship between maintenance of the selecting agent in the culture medium and transgene expression, after the selection period was completed. Duplicates of selected cells were kept in culture with the continuous supply of blasticidin. Figure 4 (upper panel)

shows that no strong decline in IDUA expression was observed when the selecting agent was removed.

MSCV functionality in MSC from MPSI mice (*IDUA* -/-)

Since MSCV vectors were capable of transducing MSC cells from normal mice and blasticidin selection was not harmful to cell cultures, the next step involved *in vitro* transduction of MSC from MPSI mice (*IDUA* -/-). Repeting the microplate transduction protocol (Figure 3, right) we observed that indeed transduction efficiency in these cells was reduced, when compared to MSC from *IDUA* +/+ mice. In fact, if we used a split ratio of 1:10 (as employed for NIH 3T3), we obtained few individual clones, and only for high titer batches. In conclusion, the protocol showed efficiency in transducing and selecting MSC from MPSI mice, but the efficiency was lower than that for *IDUA* +/+ cells.

Despite these results, we performed a new set of transductions in order to screen IDUA activity. As seen in Figure 4 (lower panel), despite difficulty in transducing MPSI MSC (*IDUA* -/-), IDUA levels were higher than those obtained in NIH 3T3 or MSC (*IDUA* +/+). In consequence to lower levels of transduction, cultures took longer to achieve confluence, so that IDUA levels were only evaluated 30 days after transduction. In view of our previous results with MSC (*IDUA* +/+), we decided to exclude blasticidin after the selection period was concluded. In conclusion, MPSI MSC (*IDUA* -/-) were more difficult to transduce and took longer periods of time to reach confluence after selection than MSC (*IDUA* +/+) transduced cultures, in these experimental conditions. Yet, blasticidin selection showed no obvious deleterious effects on transduced MPSI MSC (*IDUA* -/-). Higher levels of IDUA were obtained after cells were normally proliferating even without the continous supply of a selecting agent.

***In vivo* injection of concentrated batches**

Next, we evaluated if IDUA would be detectable in the brain from treated mice, 30 days after *in situ* injection. As seen in Figure 5A, KO IDUA levels are extremely low in comparison to NL and MSCV treated mice (p=0.00 and p=0.01, respectively). Interestingly, no differences were observed between NL and MSCV-treated mice (p=0.06). HIV-treated mice presented intermediary IDUA levels in comparison to NL and MSCV-treated animals, differing only from NL ones (p= 0.00). The mock group presented IDUA levels similar to that of KO mice, but different from NL (p=0.00) and MSCV-treated animals (p=0.01). In summary, the MSCV vector was more efficient in transducing brain cells, leading to stable expression of *IDUA*. The HIV vector presented intermediary efficiency, and the MLV vector did not induce the production of detectable IDUA levels, with results similar to those of mock and KO experimental groups.

Finally, we dosed GAG from these brains samples to evaluate if there was a reduction of GAG deposits whithin 30 days. As seen in Figure 5B, the total GAG content presented a tendency of reduction (p=0.07), more evident for MSCV-treated mice. When analyzing specifically dermatan and heparan sulfate however (Fig. 5C), it becomes clear that MSCV-treated mice presented an intense reduction of deposits, which reached levels similar to NL mice (p=1.00). KO mice were different only from NL and MSCV groups (both p=0.00). Mock and MLV groups rendered similar results. HIV-treated mice were still different from NL (p=0.00) and MSCV animals (p=0.01). Finally, results for chondroitin sulfate reflected the results for dermatan and heparan sulfate, as expected since this is the characteristical GAG in brain tissue and any increase in the proportion of dermatan and heparan sulfate would lead to reduction of chondroitin proportion (Figure 5D).

In conclusion, after 30 days of treatment with MSCV vectors, the proportion of dermatan and heparan sulfate accumulated in the brain tissue was reduced more markedly than treatment with the HIV vector. The MLV vector did no present any benefit in these experimental conditions.

Discussion

HIV and MLV vectors

These vectors were characterized previously, by M Camassola *et al*. (manuscript submitted). Here we focused on evaluating the efficiency of concentrated batches developed for our experimental protocol. GFP levels obtained with HIV GFP and HIV IDUA were very distinct from PLL 3.7, which may be explained by molecular features of each construct with reflection on transduction efficiency (Table 1). Different retroviral constructs have been shown to present different transduction efficiency and transgene expression levels despite being tested on the same cell background [13]. IDUA levels detected in HIV and MLV-treated cells (eight fold the endogenous levels) were compatible with reports from the literature, even for different viral vectors [16-18]. Higher IDUA levels obtained with HIV under the CMV promoter were reported [19]. Another work with a bicistronic HIV containing IRES *gfp* detected a huge variation in *gfp* expression levels in each cell lineage tested, achieving 10-45% of transduced cells with MOI of 20 [12]. Since we fixed our experiments in 10 µL of concentrated batch obtained with our experimental conditions, we have not employed such high MOI *in vitro* neither *in vivo*.

The MSCV vector containing *blasticidin S deamnase* and *IDUA* genes was first tested in NIH 3T3 (Figure 1). Blasticidin was proposed to be a versatile selection agent for mammalian cells [20]. In fact, in transduced NIH 3T3 cultures the blasticidin based selection was efficient and fast. High number of colonies was detected in each transduction experiment. When transducing NIH 3T3 to analyze IDUA levels, we detected low enzymatic activity when cells were unselected, but after selection these cells reached a plateau (Figure 2). It has already been reported that IDUA expression could increase under selection pressure, as seen for MTX selection in transduced lymphocytes and

hematopoietic stem cells and that a steady state could be achieved [13]. We believe that this was our case and further experiments were analyzed always at 7 and 30 days post transduction (unselected and selected cells, respectively).

Progenitor cell transduction depends on different factors. Important reviews are available for hematopoietic stem cells, discussing vector design, MOI, adjuvants employed, genotoxicity and stability of expression [21, 22]. Many aspects discussed there can be used for MSC transduction. Lentivectors pseudotyped with RD114 env GP presented better results at higher MOI (4) when transducing human MSC [23]. MOI of 140 was required to achieve transduction efficiency bigger than 30% (these values changed according to the HIV construct evaluated) also in human MSC [24]. We have worked with a fixed amount of vector, so we were not considering the MOI component directly. Because of these considerations, we decided to use a split ratio of 1:5 rather than 1:10 in our first evaluation of MSC transduction with MSCV vector (Figure 3, left). Good transduction efficiency was detected in those experimental conditions, but efficiency seemed to be lower than with NIH – even with experimental differences attributed to microplate transduction protocol. We had better results with batches with higher titer, probably a reflexion of MOI variation [12]. Analyzing IDUA expression (Figure 4, upper panel), we once again detected an increase in transgene expression after blasticidin based selection. This agrees with previous findings about MTX selection being beneficial to improve IDUA levels in a dose-dependent fashion [25]. Next, we decided to evaluate if the continuous supply of blasticidin would result in higher IDUA levels. No obvious differences were detected in cultures that were kept with blasticidin. This indicates that the rapid selection of transduced cells is not followed by an obvious silencing mechanism related to selection pressure. This does not agree with a suggestion that an intense decrease

in transgene expression is seen when the selection agent is removed [26]. We have not tested if blasticidin in higher concentrations could select cells with higher IDUA activity [13], but at PCL level we employed 16 µg/mL (final concentration) of selection agent. If this was helpful to select better virus-producing PCL we cannot assure. In conclusion, 30 days after selection, we still detected high IDUA activity in MSC (*IDUA* +/+) transduced with MSCV vector. For murine hematopoietic progenitors, comparisons among HIV and MLV showed that in progenitors, MLV presented an intense fall of transgene expression [27]. So, our MSCV construct presented stable expression in a progenitor context, which was interesting for the next set of experiments with MSC.

Finally, we transduced MSC from MPSI mice (*IDUA* -/-). Large differences were already detected in the microplate assay (Figure 3, right) in comparison to MSC (*IDUA* +/+). With the same amount of vector, fewer colonies were detected after the 1:5 split. It was impossible to obtain visible colonies with a split of 1:10 - as seen for NIH 3T3 cells. We speculate that intracellular GAG may be partially responsible for this, since they could interfere with the endocytic pathway of internalized vectors. For lentivectors and airway epithelial cells, it has already been shown that GAG interfere with transduction [28]. *In vitro*, different IDUA and GAG levels were detected in two lineages of fibroblasts [29], but the authors have not proposed a connection among GAG content and transduction efficiency. Another factor that may be relevant is calcium imbalance in these cells. Calcium is required for the proper function of the endocytic pathway and autophagy in LSD [30]. Collectively, these processes definitely interfere with basic cell functions and internalization/ transport of viral vector in these conditions are probably more difficult to occur. Even enzyme traffic / exocytose may be affected.

Last, IDUA levels in these cultures were higher than those detected for NIH 3T3 and MSC (*IDUA* +/+) (Figure 4, lower panel). These increases are compatible with those seen in MPSI fibroblasts in relation to normal ones after transduction with retroviral vector [29]. Since we kept these cultures for 30 days and we have not quantified IDUA every week, we cannot say that splits may be interfering with IDUA production, as proposed previously for human MPSI MSC transduced with retroviral vector [31]. In that paper, authors emphasize that the growing rate of cultures established from different patients varied. Interesting, batch 37 did not transduce cells as efficiently as other batches, but after the culture achieved confluence, culture 37b was producing high IDUA levels, in contrast to culture 37a. Culture 37a took longer to achieve confluence. As mentioned before, it is possible that transduction efficiency and enzyme production are not totally linked. Thus, the best vector for transduction may not be the best one for inducing enzyme production, meaning that using a drug selection agent to assess transduction efficiency is different than quantifying enzyme expression [13]. Location of provirus and promoter interferences may account for such differences too [32]. We have not investigated these aspects in greater detail.

In conclusion, transduced MPSI MSC (*IDUA* -/-) presented high IDUA levels right after selection. We speculate that the high IDUA levels detected already at seven days post transduction are a consequence of selective advantage of IDUA-producing cells, as seen previously [18], rather than pseudotransduction of target cells [25]. Thirty days after the experiment, no apparent transgene silencing was detected.

***In vivo* protocol**

Considering the background obtained in our *in vitro* experiments, MSCV would be an efficient gene transfer tool for *in situ* gene therapy in MPSI mice. Neonatal gene therapy

rendered detectable levels of IDUA in mice brain [7, 8, 33, 34]. Systemic administration of plasmidial, lentiviral or retroviral vectors in adult MPSI mice resulted in IDUA activity in the brain too, leading to different total GAG reduction values as a consequence [11, 12, 19, 35, 36]. Our MSCV vector *in situ* injected virtually normalizes IDUA levels in brain of adult treated mice (Figure 5A). The intermediary levels obtained with HIV may be also be consequence of concentrated titer batch, which could be tested by improving initial titers at transient transfection or testing another concentration protocol [37].

Accumulated GAG is consequence of IDUA malfunction or absence and its dosage is extremely important after testing a gene transfer protocol. It is important to remember that each tissue may present component GAG besides those deposited in MPSI. Therefore, total GAG dosage includes tissue GAG. Historically, GAG have been quantified based on colorimetric methods or histology (counting of vacuoles, for example) [12, 33, 35]. These methods, however, do not discriminate accumulated dermatan and heparan sulfate from GAG tissues. Since there is considerable structural heterogeneity in the specific GAG accumulated in MPSI and considering their different sizes resulting in a low molecular weight GAG fragments, we used another method to quantify GAG from brain tissue (Figure 5B). As seen for IDUA results, GAG reduction was more evident in MSCV-treated mice. *In vivo* use of MSCV vectors for other diseases had positive results in the biochemical parameters analyzed for each pathology, with different target cells: hematopoietic progenitor cells for murine galactosialidosis [38], murine bone marrow for X-linked chronic granulomatous disease [39] and GM1-gangliosidosis [40] and MPSI dog stem cells [41]. We have performed an initial biochemical evaluation, and further experiments are required to better understand how this gene transfer tool interacts with brain cells. Interestingly, a HIV/MSCV hybrid vector was described as efficient in

improving transgene expression [42], which demonstrates how important vectorology is for gene therapy by the combination of molecular features of each viral vector. Finally, blasticidin may be an important tool to work with this specific MPSI murine model because it is impossible to employ G418 as a selection system. In addition, it is important to evaluate new drug resistance genes for progenitor cells coupled to fast selection [43], preserving their stemness and/or basic functions.

Material and methods

IDUA -/- mice

IDUA -/- mice (KO) were produced by targeted disruption of the murine IDUA gene [44]. The colony, derived from animals kindly provided by Dr Elizabeth Neufeld (UCLA, Los Angeles, CA, USA), is maintained by breeding heterozygous animals. Maintenance conditions and experimental protocols were approved by the Research Ethics Committee of the Federal University of São Paulo (CEP 1201/07).

Experimental groups

Mice of 12-14 week-old were divided randomly into 7 experimental groups. Two untreated groups were included in the study: wild type mice (*IDUA* +/+) (WL) (n = 11) and MPS I mice (*IDUA* -/-) (KO) (n = 4). Treated animals were divided in 5 groups, based on viral vectors injected: HIV GFP (MOCK) (n = 3), HIV IDUA (HIV) (n = 6), MLV IDUA (MLV) (n = 4), MSCV blasticidin (MOCK) (n = 1) and MSCV IDUA blasticidin (MSCV) (n = 3). MOCK group was represented by the sum of HIV GFP and MSCV blasticidin injected mice. The study was followed-up for 30 days after the *in situ* bilateral injection of viral vectors.

Cell culture

All cell culture reagents were purchased from Gibco/Invitrogen (Burlington, ON, Canada). Mesenchymal stem cells (MSC) were established from the bone marrow of *IDUA* -/- (KO) and wild type *IDUA* +/+ (WT) mice and maintained as previously described [45]. The same cell culture condition was applied to NIH 3T3 and HEK 293T. G418 sulfate was purchased from Gibco/Invitrogen Canada Inc (Burlington, ON, Canada); protamine sulfate and polybrene from SIGMA (St Louis, MO, USA); Blasticidin S HCl from ICN Biomedicals Ins (Aurora, Ohio, USA).

MLV vectors

The MLV vectors used to establish packaging cell lines (PCL) were described previously (M Camassola *et al*., manuscript submitted). Two different PCLs were produced: one containing the human IDUA cDNA and the other one the *gfp* reporter gene, in the same vector backbone. Transfection procedures and PCL establishment were performed as described elsewhere [46]. To collect viral vector, 2.6x10E6 cells from each PCL were plated in 75 cm2 culture dish (final volume 10 mL). Harvesting was performed as described previously [46]. To concentrate these viral vectors, 4 mL of 20% sucrose (in water) were added to each 20 mL of harvested vector. Batches were concentrated in a Sorvall centrifuge (rotor SS34), at 16000 rpm for 2 hours. The supernatant was drained off and the pellet was ressuspended in the desired volume of serum-free DMEM medium, after a spin 5000 rpm for 10 minutes. After 6 hours, the tubes were vortexed and once again centrifuged 5000 rpm for 10 minutes. Aliquots were immediately frozen at -80^{o}C. This protocol is slightly different from our previous method (FH Silva *et al*., manuscript submitted). Vectors were titrated using NIH 3T3 cells and final titers were expressed as CFU/mL or CFU/µl, based on G418 resistant cell colonies (1 mg/mL, final concentration) [46].

HIV vectors

The HIV vectors and the protocol of transient transfection of HEK 293T cells were described previously (M Camassola *et al*., manuscript submitted). They will be referred here as HIV IDUA, for the construct containing the therapeutic gene and HIV GFP for the mock construct. Both constructs present the *gfp* gene downstream to the IRES element, so the HIV IDUA is a bicistronic vector expressing *IDUA* and *gfp* from its heterologous

promoter, but independently translated due to IRES sequence. Vector PLL3.7 was a kind gift from Dr. Didier Trono (EPFL SV-DO, Lausanne, Switzerland).

Concentration of viral batches were performed as described above. Titration was performed as previously described [47], applying 5 or 10 µL of batch. We counted GFP positive cells in 10 random fields photographed in fluorescence microscopy seven days after transduction. This was possible due to the bicistronic nature of the vectors. Vector PLL3.7 was used as control of transfection and concentration procedures.

MSCV vectors

The *blasticidin S deaminase* (BS) gene was cloned into vector MSCVpac (Clontech, Palo Alto, EUA) with Hind III and Sac II sites, under promoter activity of PGK. The resulting MSCV BS vector was digested with EcoRI enzyme (Fermentas Inc, Glen Burnie, MD, USA), the same enzyme applied to obtain IDUA cDNA from a vector from Dr. Melissa Camassola. After phenol-chloroform: isoamilic alcohol purification/ sodium acetate precipitation (all from SIGMA, St Louis, MO, USA) and proper procedures related to CIAP treatment (Fermentas Inc, Glen Burnie, MD, USA), the IDUA fragment was cloned into MSCV BS vector with T4 ligase (Invitrogen, Carlsbd, CA, USA), under promoter activity of its modified 5' LTR. After restriction analysis and sequencing on Abi Prisma 3100 (Applied Biosystems, Foster City, CA, USA), the vector was purified using a QIAgen midi prep kit (QIAGEN GmbH, Hilden, Germany) and used to establish PCLs. Transient transfection of ecotropic virus packaging cell line BOSC23 was performed as described elsewhere [48], with a minor modification: we allowed transfected cells to recover one more day than previously described before viral harvesting. The ecotropic viral supernatant was then employed in transduction of amphotropic PT67 cells [46], allowing transduced cells to recover for 24 hs before seeding 1:10 of this culture in 6 cm

diameter plate dish with cell culture medium supplemented with blasticidin (16 µg/mL final concentration). When single cell colonies were individualized, we pooled them together, for each plate in selection. The best polyclonal PCLs in titers (CFU/mL or CFU/µL based on blasticidin resistant cell colonies) and IDUA activity in transduced cells were expanded for use in concentration protocols. Viral vectors were collected in the same way as MLV ones. Titer of concentrated vectors was determined in the same fashion as MLV, always allowing transduced cells to recover one day before selecting cells with blasticidin (8 µg/mL final concentration). Thus, our MSCV IDUA construct is a bicistronic vector, with two independent transcription units.

Validation of concentrated batches

After titration of the concentrated batch, we performed a transduction assay with 10 µL of the viral vector, in order to screen for IDUA activity, to assay the IDUA activity obtained with the same amount of virus that would be injected in mice brain. We employed our standard transduction protocols for each batch, matching each transduction condition with the type of vector. Thus, for MLV vectors we employed NIH 3T3 cells, for HIV vectors we employed HEK 293T cells and for MSCV we used NIH 3T3 and MSC from wild type and MPSI knockout mice (referred also as *IDUA* +/+ and MPSI MSC or *IDUA* -/-). This was decided based on the molecular features presented by MSCV vector backbone – designed to achieve stable, high-level gene expression in hematopoietic and embryonic stem cells and to enhance transcriptional activation, preventing molecular silencing in progenitors cells [49]. Validation of MSCV batches became our set of results to evaluate its *in vitro* efficiency.

MLV and NIH 3T3 transduction

1x10E5 cells were plated on 6 cm diameter dishes, with 4 mL of cell culture medium, one day before transduction. Ten µL of vector were used to transduce the cells, recently fed with fresh medium. Polybrene (8 µg/mL final concentration) was added and after 20 hours the transduction was stopped by changing the cell culture medium. 1:10 of cell culture was seeded to perform G418 selection (1 mg/mL final concentration), until enough colonies could be pooled together – at least 15 days – and expanded to measure IDUA activity.

HIV and HEK 293T transduction

We employed the same protocol used in titration and IDUA activity was quantified after proper fluorescence microscopy analyses – seven days after transduction.

MSCV and NIH3T3, wild type and MPSI MSC transduction

As this was the first time we were using MSCV vectors containing a *blasticidin S deaminase* resistance gene in MPSI studies, we decided to quantify IDUA at timepoints other than just at the end of selection. Cells in selection took no more than 5 days to present individualized colonies in enough numbers to be pooled together and expanded. NIH 3T3 was split 1:10 and MSC 1:5. Experiments were performed in duplicates.

NIH 3T3 transduction

In general, the same protocol of MLV was applied for NIH 3T3 cells, but allowing cells to recover one day before selection with blasticidin (8 µg/mL final concentration).

Wild type and MPSI MSC transduction

5x10E4 cells were plated on 6 cm diameter dishes one day before transduction, with 4 mL of cell culture medium. Ten µL of vector were applied to the culture, recently fed with fresh medium. Protamine sulfate (10 µg/mL final concentration) was added and

after 20 hours the transduction was stopped by changing the cell culture medium. 1:5 of cell culture was seeded to perform blasticidin selection, until enough colonies could be pooled together – as fast as five days – and expanded to measure IDUA activity. MPSI MSC took longer to be pooled together than normal MSC – a consequence of differences in transduction efficiency. In order to evaluate if the removal of selection agent in MSC would interfere in transgene expression, we allowed duplicates of transduced wild type MSC to be cultured always in the presence of blasticidin (8 µg/mL). Since we detected no interference on that matter, for MPSI MSC we have only evaluated IDUA activity at the end of study (30 days), removing BS after five days of selection.

Coomassie blue staining of NIH 3T3, wild type and MPSI MSC transduced in microplate

We applied the same transduction protocols described above (in terms of viral vector volume and cell density) to transduce cells in a microplate condition. For NIH 3T3 we used 12-well plates and for MSC 24-well plates, in order to obtain visible colonies after the desired 5 days of selection. The individual clones obtained were stained with Coomassie Blue solution. Briefly, after 5 days of selection, we fixed cells with glutaraldehyde 2.5% (in water), for 1 minute. Then we washed the plate two times with PBS and Coomassie brilliant G solution (0.5% w/v prepared in ethanol 35%, glacial acetic acid 10%) (all from SIGMA, St Louis, MO, USA) was added. After 2 minutes, cells were washed with water and allowed to air dry.

Alpha-L-iduronidase activity

Cells were collected after tripsinization and the pellet was ressuspended in homogenization buffer (10 mM $NaPO_4$ pH 5.8, 0.1 mM DTT, 0.1% Triton X-100). IDUA dosage protocol is described in M Camassola *et al*. (manuscript submitted). Briefly, in a

microplate, 10 μL of the samples were mixed with 10 μL of 2.85 mM 4-methylumbelliferyl-alpha-L-iduronide (4MU-I, Toronto Research Chemicals Inc., North York, ON, Canada) and 40 μL of formate buffer 0.2 M pH 2.8. The plate was incubated at 37°C for 1 hour and the reaction was stopped by the addition of 175 μL of stop buffer (0.5 M glycine-NaOH, pH 10.3). Fluorescence of the 4MU product was determined using a Spectramax M2 fluorimeter (Molecular Devices Inc., Sunnyvale, CA, USA) using 365 and 455 nm of wavelength for excitation and emission, respectively. Protein concentration was determined with the Bio Rad protein dosage kit (Bio Rad Laboratories, Hercules, CA, USA). IDUA is expressed in nmol/ptn mg/h or relative values to endogenous enzyme concentrations.

GAGs measurement

One third of mice brain tissue was cut and grounded in 10 volumes of acetone. After standing overnight at room temperature, acetone was changed everyday during five days. Afterwards, acetone was discarded and the sample was completely dried at 50°C. The dry weight was taken, and the sample incubated with 8 mg of maxatase (4 mg/mL in Tris HCl 0.05M pH8.0 NaCl 0.15M) for 24 hours at 60°C. Trichloroacetic acid was added (10% of final volume) and allowed to stand for 20 minutes at 4°C. The precipitate formed was removed by centrifugation at 5,000 g for 20 minutes at room temperature. The GAGs from supernatant were precipitated with two volumes of ethanol. After overnight incubation at -20°C, GAGs were collected by centrifugation, vacuum dried, and submitted to deoxyribonuclease I treatment (Sigma, Saint Louis, EUA). The GAGs were analyzed by agarose gel electrophoresis [50] and quantified by densitometry with Quick Scan 2000 Software and using standard known concentration of chondroitin, dermatan sulfate and heparan sulfate. Each sample for electrophoresis represented the minimal of one third of all

GAG extracted from brain tissue destined to this analysis. We have not used less brain tissue to extract GAG, neither less sample to load the gel. GAGs are expressed as µg / g dry tissue.

***In vivo* injection of viral batches**

This protocol is a modification of [51]. Mice were anesthetized as specified in the mentioned reference and injected with 10 µl total of viral vector (5 µL per injection) [52], bilaterally with a 30-gauge needle (5 minutes interval between each injection). 200 ng of protamine sulfate were mixed to each viral vector suspension. Five minutes after the last injection, the needle was removed and the incision was sutured. Mice were allowed to recover on a heating pad before returning to micro isolators. At 30 days after treatment, mice were sacrificed and the brain was collected. Fractions of the tissue were kept at -80^{o}C for GAG quantification. One third of the brain was directly processed in 1mL of homogenization buffer with the help of a Potter device, for determination of IDUA activity.

Statistical analyses

Data are presented as mean and standard deviation values. One way ANOVA plus Bonferroni test was employed to compare data for IDUA activity in brain tissue and GAG scores, with the Statistica 6.0 software. The threshold for statistical significance was established at $P< 0.05$.

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Table 1: Titers and efficiency of transduction of concentrated HIV and MLV vectors.

CONCENTRATION OF RETROVECTORS				
	HIV VECTORS			
	HIV GFP	HIV IDUA	PLL 3.7	HEK 293T
Start volume (mL)	52	152	20	
Ressuspension volume (µL)	80	378	40	
Titer (UI /10 µL/Hek)	4x10E5	4x10E5	2x10E6	
	IDUA (GFP)			
experiment 1	4.31 (40%)	32.02(40%)	4.81(70%)	4.77
experiment 2	2.87 (35%)	30.03(42%)	4.04(80%)	5.30
	MLV VECTORS			
	MLV GFP	MLV IDUA	3T3	
Titer (CFU/mL/3T3)	4x10E6	3x10E5		
Start volume (mL)	40	80		
Ressuspension volume (µL)	40	100		
Titer (CFU /10 µL/3T3)	4x10E5	5x10E5		
	IDUA			
Experiment 1	0.34	10.25	1.18	
Experiment 2	0.41	16.56	2.38	
Experiment 3	1.72			

HIV vectors were tested in HEK 293T cells (two repeated experiments). The proportion (%) of GFP positive cells was analyzed through fluorescence microscopy 7seven days after transduction. IDUA activity was also evaluated in the same period. MLV vectors were tested in NIH 3T3 cells (three experiments). Only genetically modified cells were evaluated, since they were selected with G418 antibiotic. IDUA is expressed as nmol/mg protein/h.

Table 2: Titers obtained for four concentrated batches of MSCV vectors in NIH 3T3 cells after selection with blasticidin.

	MSCV VECTORS			
Titer (CFU/mL/3T3)	2x10E5	3x10E5	5x10E5	3x10E5
Start volume (mL)	80	80	80	80
Ressuspension volume (µL)	100	100	100	100
Titer (CFU /10 µL/3T3)	10E6	2x10E5	10E6	6x10E4
	STOCK IDENTIFICATION			
Id number	32	33	36	37
Transgene	BS	IDUA BS	BS	IDUA BS

Legend: BS – blasticidin

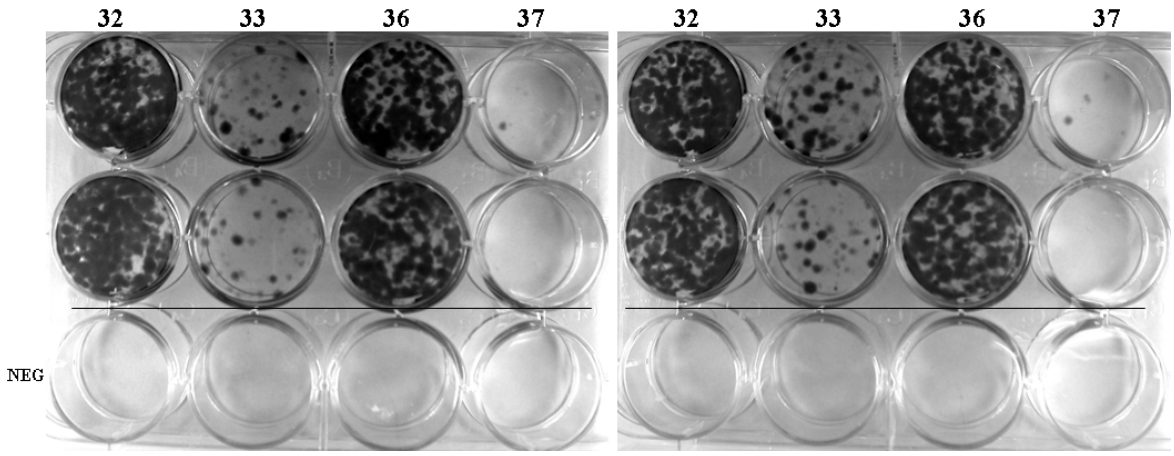

32
33
36
37
32
33
36
37
NEG

Figure 1: Efficiency of transduction of concentrated MSCV vectors in NIH 3T3 cells. After transducing NIH 3T3 cells with 1 µL of vector with the same titration protocol, cells were split 1:10 and submitted to blasticidin selection. Five days after the antibiotic was added, non-transduced cells were eliminated. Some batches presented more efficiency of transduction, as shown by the higher number of individual colonies. This agrees with titration results. Experiments were performed in duplicates, twice**.** Legend: 32 and 36 – MSCV BS; 33 and 37 – MSCV IDUA BS; BS - blasticidin. NEG refers to non-transduced cells submitted to selection pressure.

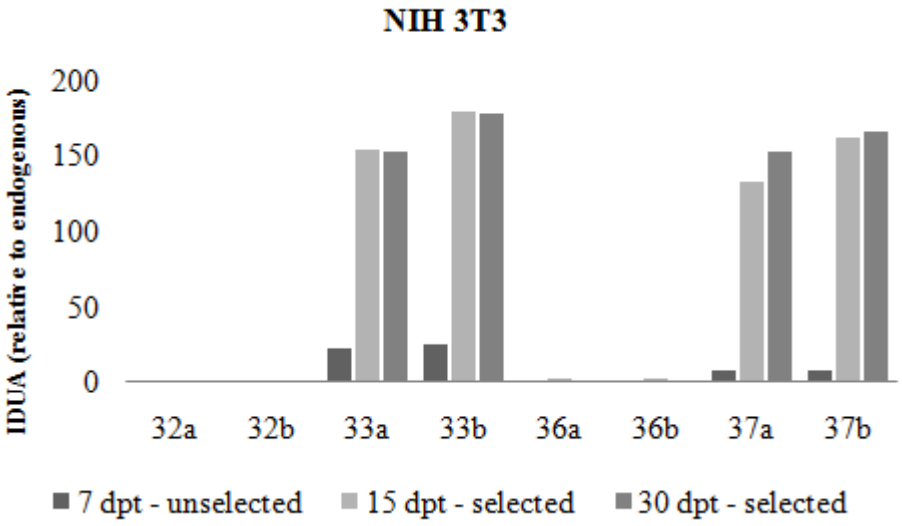
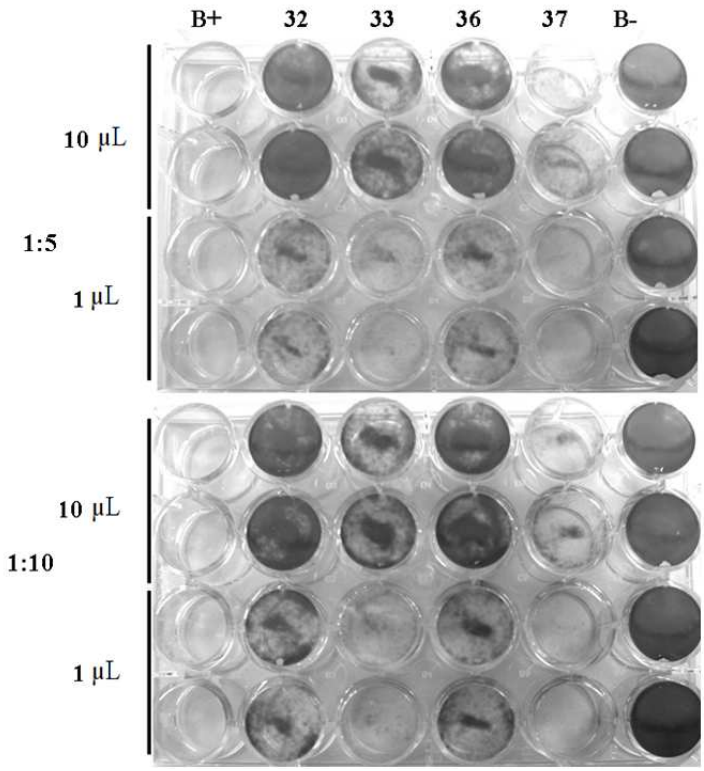

NIH 3T3
IDUA (relative to endogenous)
200
150
100
50
0
32a
32b
33a
33b
36a
36b
37a
37b
7 dpt - unselected
15 dpt - selected
30 dpt - selected

Fig. 2: Validation of concentrated MSCV batches in NIH 3T3 cells (IDUA activity). Ten µL of each viral batch was used to transduce 1X10E5 NIH 3T3 cells. 1:10 of transduced cells were cultured in the presence of blasticidin (8 µg/mL final concentration) and 1:10 in blasticidin free medium (unselected cells). Unselected cells were kept in culture for seven days in order to evaluate initially IDUA levels. Selected cells were evaluated for IDUA levels 15 and 30 days after transduction. Experiments were performed in duplicates for each batch (a and b). IDUA activity is expressed relative to endogenous levels (2.67 ± 1.38; n=6). Legend: dpt – days post transduction; 32 and 36 – MSCV BS; 33 and 37 – MSCV IDUA BS; BS - blasticidin


B+
32
33
36
37
B-
10 µL
1:5
1 µL
10 µL
1:10
1 µL

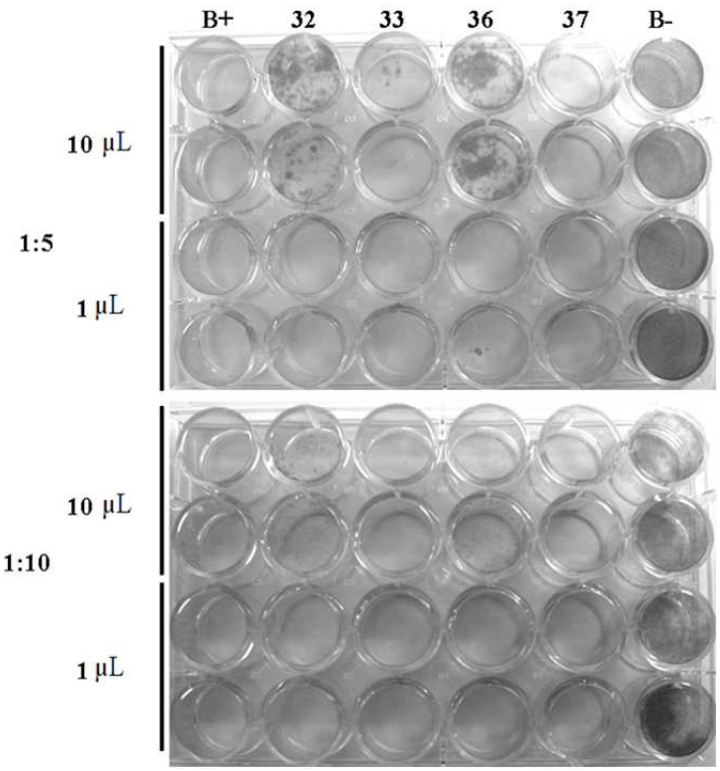
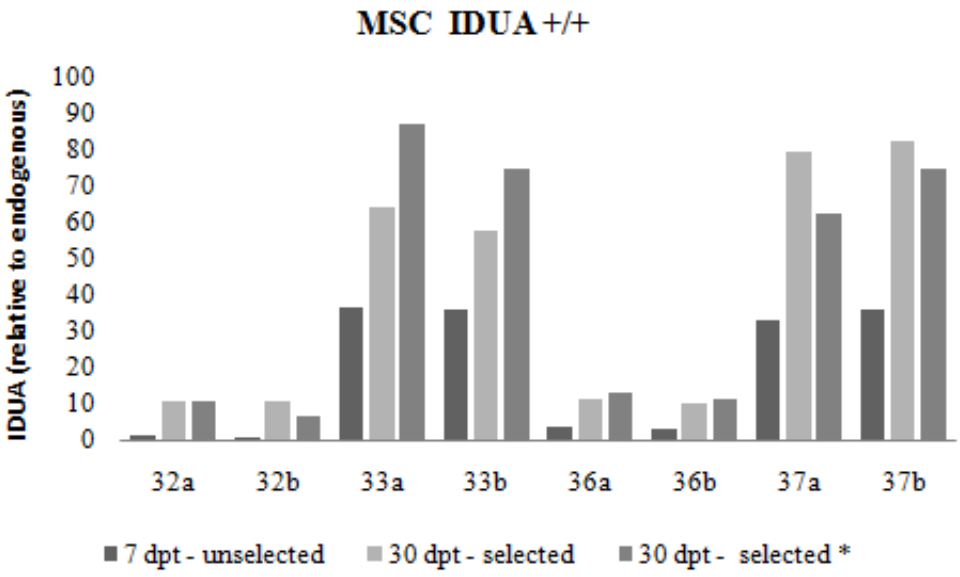

B+
32
33
36
37
B-
10 µL
1:5
1 µL
10 µL
1:10
1 µL

Figure 3: Efficiency of transduction of concentrated MSCV vectors in MSC from wild type mice (IDUA +/+) (left) and MPSI MSC (IDUA -/-) (right). 48 hs after transducing MSC cells with 1 and 10 µL of vector, cells were splited 1:5 and submitted to blasticidin selection (8 µg/mL final concentration). Five days after the antibiotic was added, non-transduced cells were eliminated. Experiments were performed in duplicates, twice. Legend: 32 and 36 – MSCV BS; 33 and 37 – MSCV IDUA BS; BS – blasticidin; B+ - non-transduced cells exposed to selection pressure; B- non-transduced cells not exposed to selection culture medium.


MSC IDUA +/+
IDUA (relative to endogenous)
100
90
80
70
60
50
40
30
20
10
0
32a
32b
33a
33b
36a
36b
37a
37b
7 dpt - unselected
30 dpt - selected
30 dpt - selected *

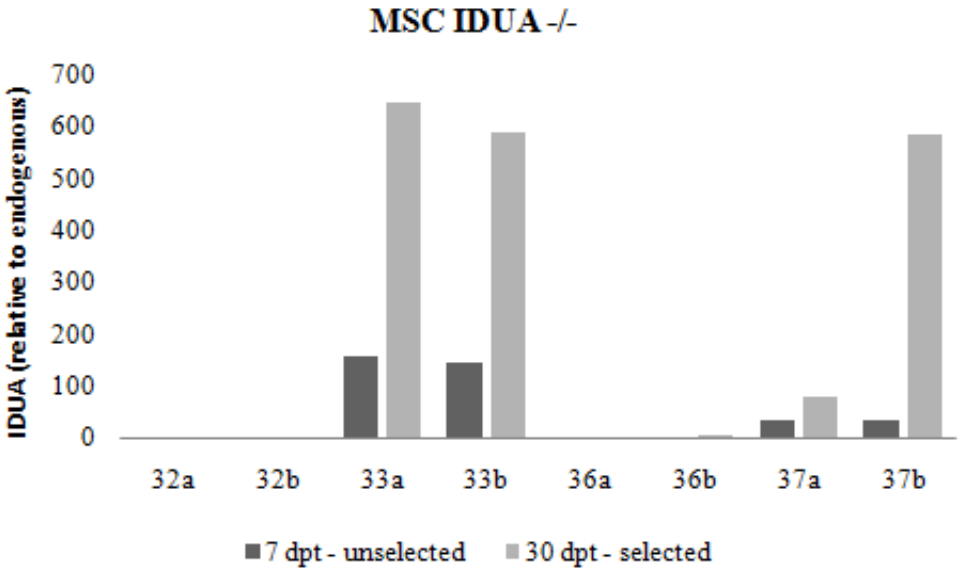

MSC IDUA -/-
IDUA (relative to endogenous)
700
600
500
400
300
200
100
0
32a
32b
33a
33b
36a
36b
37a
37b
7 dpt - unselected
30 dpt - selected

Figure 4: Validation of concentrated MSCV batches in MSC cells (IDUA activity). Upper panel: Effect of maintenance of blasticidine selection agent in transduced MSC from wild type mice (IDUA +/+). Ten µL of each viral batch was employed to transduce 5X10E4 MSC MPSI cells. 1:5 of transduced cells were cultured in the presence of blasticidin (8 µg/mL final concentration) and 1:5 in blasticidin free medium (unselected cells). Unselected cells were kept in culture for seven days in order to evaluate initially IDUA levels. Duplicates of selected cultures (1:5) were cultured in continuos supply of selection agent and dosed at the end of the study. Experiments were performed in duplicate (as indicated by a and b). IDUA activity is expressed relative to endogenous enzyme levels (1.46±0.67; n=7). Lower panel: IDUA levels in transduced and selected cultures of MPSI MSC (IDUA -/-). The same transduction protocol described above was applied to these cells. Unselected cells were evaluated in seven days post transduction and selected cultures 30 days post transduction. Experiments were performed in duplicate (as indicated by a and b). IDUA activity is expressed relative to endogenous levels (0.25±0.27, n=8). Legend: 32 and 36 – MSCV BS; 33 and 37 – MSCV IDUA BS; * signs selected cultures that were kept with the continous supply of blasticidine; dpt – days post transduction.

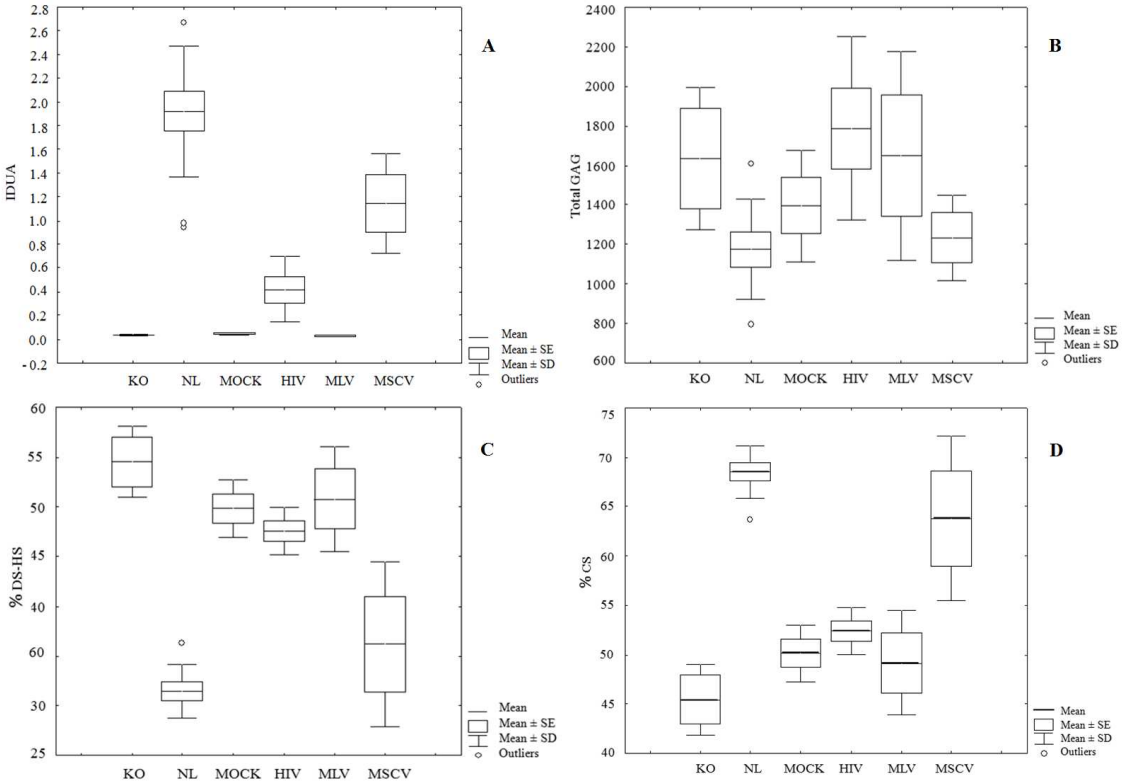

A
IDUA
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
KO
NL
MOCK
HIV
MLV
MSCV
Mean
Mean ± SE
Mean ± SD
Outliers
B
Total GAG
2400
2200
2000
1800
1600
1400
1200
1000
800
600
KO
NL
MOCK
HIV
MLV
MSCV
Mean
Mean ± SE
Mean ± SD
Outliers
C
% DS-HS
60
55
50
45
40
60
30
25
KO
NL
MOCK
HIV
MLV
MSCV
Mean
Mean ± SE
Mean ± SD
Outliers
D
% CS
75
70
65
60
55
50
45
40
KO
NL
MOCK
HIV
MLV
MSCV
Mean
Mean ± SE
Mean ± SD
Outliers

Figure 5: IDUA and GAG evaluation in brain tissue from *in situ* injected mice. A) Ten µL of viral vectors were injected bilaterally in mice brain, with 200 ng of protamine sulfate. Thirty days after, samples were evaluated for IDUA activity (nmol/ptn mg/h). Groups were distributed: KO (n= 4), NL (n=11), mock (n=4), HIV(n=6), MLV (n=4) and MSCV (n=3). HIV, MLV and MSCV carry IDUA transgene. B-D) Total GAG and percentual of dermatan, heparan and chondroitin sulfate in brain tissue from in situ injected mice. 30 days after experiment, brain homogenates were evaluated for total GAG (µg/g dry tissue) or percentage of specific GAG (% of DS-HS and CS). Groups were distributed: KO (n= 2), NL (n=8), mock (n=4), HIV (n=5), MLV (n=3) and MSCV (n=3). HIV, MLV and MSCV carry IDUA transgene. The graphic in D shows the results for chondroitin sulfate. Its percentual tends to increase if dermatan and heparan sulfate deposits are diminished. Legend: DS;HS – dermatan and heparan sulfate; CS – chondroitin sulfate.

Discussão

"(...) both reports present cautiously optimistic interpretation of the data and propose that the studies provide the basis for additional clinical studies in these and other retinal degenerative diseases. Indeed, the expectation is that younger patients who may have a higher number of preserved retinal cells may possess greater chance for benefit from the procedure. (...) These studies highlight the continued and important diversification of gene transfer technology from the original focus on hematopoetic stem cells to other cell targets and pathologic conditions.(...) Given the trial details, noted above, the modest results and the understandable lack of controls (e.g., placebo injections in the contralateral eye), a cautious outlook is warranted, but the trial clearly represents another step forward for the field"

D. A. Williams "Progress reported in two studies of clinical gene transfer into the retina" *Mol Ther* 16(7) 2008.

Discussão

1.Terapia gênica para MPSI

A TG *in vivo* caracteriza-se pela administração dos vetores diretamente ao organismo alvo, enquanto que a TG *ex vivo* caracteriza-se pela administração de células-alvo previamente transduzidas ao organismo-alvo. Ambas abordagens foram exploradas para MPSI no passado (Tabela 1, p. 24) (Ponder and Haskins, 2007). A vetorologia viral parece predominar nos estudos, muito provavelmente devido ao fato de a MPSI ser uma doença monogênica recessiva, o que leva a uma necessidade contínua da produção da enzima exógena.

1.1 Expressão transiente *versus* expressão permanente

Para atender a essa necessidade constante do produto terapêutico, é importante que o vetor de transferência gênica apresente expressão sustentável do transgene, evitando a necessidade de intervenções repetidas. Para que os sistemas plasmidiais possam fornecer tal sustentabilidade, eles precisam ser modificados de forma a se tornarem integrativos (Aronovich *et al.,* 2007). Os vetores virais integrativos são considerados ferramentas de transferência gênica permanentes, o que não significa necessariamente que a expressão do transgene será duradoura. Uma breve revisão dos vetores virais integrativos é apresentada na Tabela I (p.158). Mecanismos descritos com participação ativa na sustentabilidade de expressão do gene exógeno incluem fatores epigenéticos como cromatina repressiva levando a silenciamento, efeito de posição após integração com subseqüente variegação e/ou silenciamento de expressão gênica, metilação de sequências promotoras levando a

redução e/ou perda de expressão do transgene, entre outros (Chen and Townes, 2000; Emery *et al.*, 2000; Zentilin *et al.*, 2000; Li and Emery, 2008). Tais problemas devem ser investigados para qualquer tipo de vetor viral integrativo, portanto. Existe, contudo, uma tendência evidente de valorização de certos sistemas virais em detrimento de outros, apesar da riqueza dos estudos moleculares já disponibilizados mostrando que a eficiência do vetor em termos de expressão gênica depende também da célula-alvo.

Expressão não-sustentável em contexto de progenitores hematopoiéticos através de vetor baseado em MLV devido à metilação de sequências promotoras já foi descrita (Mostoslavsky *et al.*, 2005). Anteriormente, vetores lentivirais eram classificados como não-sujeitos ao silenciamento em células progenitoras, inclusive em células-tronco embrionárias (Case *et al.*, 1999; Barrette *et al.*, 2000; Pfeifer *et al.*, 2002; Ma *et al.*, 2003). Recentemente, entretanto, foram detectados casos de silenciamento pós transcricional e também de metilação de sequência proviral de lentivetores em células-tronco hematopoiéticas (revisado em Chang and Sadelain, 2007), o que questiona a imposição de estabilidade sempre associada aos mesmos. Inclusive os vetores adenoassociados (AAV), ultimamente propostos como alternativa não-retroviral de transferência gênica permanente, podem permanecer episssomais quando empregados *in vivo* em modelo primata (Penaud-Budloo *et al.*, 2008). Isso mostra como cada elemento participante do *design* do vetor viral selecionado deve ser analisado dentro do contexto proposto para seu emprego, seja *in vivo versus in vitro* ou célula progenitora *versus* não-progenitora.

1.2 Vetores baseados em HIV, MLV e MSCV

Os protocolos *in vivo* de TG para MPSI murina compilados na Tabela II (p. 159) apresentam maior ocorrência de uso de vetor baseado em MLV, seguida pelos vetores

baseados em HIV. OS vetores MSCV não foram testados ainda nessa patologia específica – apenas para outros tipos de DALs (Tabela III, p. 160). Existe uma imensa variação em termos de fase de intervenção e dose viral administrada nesses protocolos, o que dificulta enormemente a discussão dos resultados de forma comparativa. Faltam estudos mais homogêneos nesse sentido, analisando vetores sob as mesmas condições experimentais, e quantificados dentro dos mesmos sistemas válidos para seu grupo de origem. Nosso projeto de pesquisa desenvolveu dois protocolos pré-clínicos para o modelo murino de MPSI. No protocolo *ex vivo*, foram empregadas células-tronco mesenquimais transduzidas com o vetor MLV em camundongos MPSI adulto de 12 e 25 semanas, simulando experimentalmente os déficits cognitivos detectados nos pacientes – e incluídos no algoritmo que auxilia na seleção de tratamento para a patologia (Muenzer *et al.*, 2009). No protocolo *in vivo* avaliou-se comparativamente o emprego de vetores virais baseados no MLV, HIV e MSCV sob as mesmas condições experimentais, através da injeção bilateral intraventricular no cérebro dos mesmos. A discussão referente a cada protocolo é apresentada na sequência.

2. TG *ex vivo* para MPSI

2.1 Transdução *in vitro* de células-tronco mesenquimais

Para inicialmente validar um protocolo de transdução de célula tronco mesenquimal com vetor MLV contendo o gene *IDUA*, fibroblastos NIH 3T3 foram empregados como controle de MOI e de nível de expressão obtida após seleção. Os níveis de IDUA detectados nesse experimento (com baixa MOI) eram altamente desejáveis para a execução do protocolo *ex vivo* com células oriundas do próprio animal MPSI. Como não seria possível selecionar essas culturas de MSC do camundongo MPSI transduzidas com o vetor

MLV, nós decidimos trabalhar com uma alta MOI (Tabela 1, p. 60) Esse racional experimental procurava garantir um maior número de células transduzidas, diminuindo um pouco a razão de células não-transformadas/células geneticamente modificadas. Dados da literatura mostram que para transdução de células-tronco mesenquimais humanas isoladas de medula óssea altas MOI são desejáveis (Van Damme *et al.*, 2006).

Apesar dos baixos níveis de transdução obtidos com o vetor MLV GFP (Tabela 1, p. 60), altos níveis de IDUA foram detectados nas culturas transduzidas com MLV IDUA, inicialmente. A expressão do transgene, contudo, caiu drasticamente, para os dois vetores. No caso do MLV GFP, não era mais possível detectar células GFP positivas na microscopia de fluorescência 3-4 semanas após a transdução, enquanto que para o MLV IDUA a atividade enzimática detectada foi muito inferior à inicial. Dados da literatura mostram que quando o MLV é empregado na transdução de progenitores hematopoiéticos, uma queda similar na expressão do GFP éi detectada (Mostoslavsky *et al.*, 2005). Isso representou uma redução de 40 a 0% de células GFP positivas, em 56 dias, quando o gene estava sob atividade promotora do CMV. Em células humanas, MOI de 140 resultaram em 5,2% de células GFP positivas (Van Damme *et al.*, 2006). A administração sistêmica de um construto também baseado em MLV em modelo murino de MPSI apresentou uma similar queda de expressão (Herati *et al.*, 2008).

Ainda que esses níveis iniciais de IDUA fossem tão intensos a ponto de disparar a cascata de degradação de GAG, questões remanescentes em relação à genotoxicidade desse processo baseado em alta MOI levaram ao desenho de um segundo protocolo experimental. Considerando que a manutenção das características das células progenitoras pudesse depender de transdução com baixas MOI (Yoshimitsu *et al.*, 2007), um protocolo alternativo de transdução foi proposto, combinando baixa densidade celular, sulfato de

protamina como adjuvante e baixa MOI. Altos níveis de IDUA por um período maior de tempo antes da queda de expressão do transgene foram detectados. Foram essas células, transduzidas com baixa MOI e produtoras de altos níveis de IDUA sete dias após transdução que nós injetamos nos camundongos MPSI (Tabela 1, p. 60). Os experimentos com MLV GFP refletiram diretamente essa queda de MOI, uma vez que o percentual de células GFP positivas foi inferior ao observado com altas MOI. Conforme dito anteriormente, os níveis de IDUA não foram tão prejudicados – eram inferiores, mas ainda dentro da faixa desejável de trabalho. Interessantemente, talvez questões adaptativas relacionadas à produção da enzima nessas células oriundas do modelo murino de MPSI tenham contribuído para essa intensidade de expressão de IDUA apesar dos níveis baixos de transdução sugeridos pelos experimentos com MLV GFP ou da ausência de seleção das células geneticamente modificadas (Di Natale *et al.,* 2002).

2.1.1 Sulfato de protamina

O uso de sulfato de protamina para transdução de células-tronco mesenquimais baseou-se em dois aspectos: o uso de polybrene havia sido deletério para a expansão de células progenitoras, ao contrário da protamina (Flasshove *et al.,* 1995) e boas taxas de transdução em células progenitoras hematopoiéticas humanas de pacientes MPSI haviam sido detectadas na presença desse adjuvante, apesar de não terem sido feitas comparações diretas com o polybrene (Pan *et al.,* 2004). Nós acreditamos que essa escolha favoreceu também a execução do protocolo *in vivo* (próxima seção) e esse adjuvante foi mantido como condição padrão para nossas transduções de células progenitoras. É importante ressaltar que o vetor baseado em MLV depende de mitoses celulares para a efetiva

transdução de células-alvo, o que reforça a observação feita acima em relação à expansão das células (Biffi and Naldini, 2005; Baum *et al.*, 2006).

2.2 Protocolo *ex vivo*

A seleção da faixa de idade dos animais MPSI empregados no trabalho baseou-se na comprovação de déficit já estabelecido e progressivo, conforme mostrado na literatura (Reolon *et al.*, 2006; Pan *et al.*, 2008). Nosso foco experimental eram os animais adultos com pelo menos dois níveis claros de neuropatologia – por isso 12 *versus* 25 semanas de idade. A injeção intraventricular de CTM MPSI transduzidas com MLV IDUA nesses animais levou a uma tendência de melhora locomotora e de redução de GAGs totais (artigo 1, p. 34). A redução intensa dos GAGs acumulados no cérebro corroborou nossa idéia de que os altos níveis de IDUA produzidos pelas células injetadas foram suficientes para o disparo da cascata de degradação dos mesmos. Assim como no caso das CTM MPSI transduzidas, é melhora de função locomotora foi avaliada através de teste comportamental de campo aberto de exposição única. Nos animais tratados, os deslocamentos vertical e horizontal (avaliados através do número de *rearings* e de *crossings* internos e externos) estavam aumentados, aproximando-se mais do fenótipo normal do que do animal MPSI.

O protocolo pré-clínico proposto, portanto, apresentou resultados positivos em termos de redução de GAGs e de melhora da neuropatologia, que foi mais evidente nos animais em fase mais avançada da doença. Essa observação nos levou a considerar a hipótese de que talvez em fases mais iniciais da patologia um protocolo *in vivo* fosse mais adequado, em vista da maior facilidade de transduzir as células-alvo e da menor sinalização para retenção de células-tronco. À medida em que a doença progride, as condições de deterioração tecidual favoreceriam a retenção de células-tronco no ambiente,

concomitante a uma maior dificuldade de transdução das células-alvo. Nessas condições, o fornecimento de IDUA é altamente adaptativo, podendo desencadear a degradação dos GAGs. Um esquema representativo dessa hipótese é apresentado na Figura I (p. 163). Interessante, foi proposto que a inflamação aumenta os níveis de fusão das células progenitoras de medula óssea injetadas *in situ* com as células de Purkinje no cérebro de camundongos com dermatite ulcerativa e encefalite (Johansson *et al.*, 2008; Singec and Snyder, 2008). Talvez um processo semelhante pudesse ocorrer nos camundongos MPSI, em função da ativação de microglia e inflamação já descritas (Ohmi *et al.*, 2003). Apesar de experimentos específicos relacionados a esse tema não terem sido feitos, sugere-se que nos animais em fase avançada da neuropatologia essa inflamação poderia estar acentuada – favorecendo o procedimento *ex vivo* baseado em CTM. Uma ferramenta de transferência gênica que fornecesse expressão mais sustentável nas CTM e que permitisse enriquecer a fração de células geneticamente modificadas se tornou altamente desejável para que esses aspectos pudessem ser investigados mais detalhadamente no futuro.

3. TG *in vivo* para MPSI

Nosso protocolo *in vivo* de TG para MPSI objetivava a comparação direta entre 3 tipos de vetores (baseados em MLV, HIV e MSCV), sob as mesmas condições experimentais. Os vetores baseados em HIV e MLV já haviam sido caracterizados previamente (Camassola M *et al.*, manuscrito submetido); a discussão aqui apresentada refere-se, portanto, a aspectos relacionados ao protocolo de concentração dos estoques virais e sua validação para uso nos animais (seção 3.1 para HIV e MLV e 3.2 para MSCV). A discussão referente ao vetor MSCV aborda também sua caracterização de funcionalidade *in vitro*, em vista do seu emprego inédito nesse modelo.

3.1 A concentração dos vetores baseados em HIV e MLV e sua validação

Os lentivetores foram concentrados conforme um protocolo padrão recomendado pelo Dr. Didier Trono (revisado em Tiscornia *et al.,* 2006). A maior dificuldade em adaptar o referido protocolo ocorreu no momento da ressuspensão do estoque viral, uma vez que pequenos volumes deveriam ser empregados devido à injeção *in situ* dos mesmos no ventrículo dos camundongos, posteriormente. Isso pode ter afetado a qualidade de recuperação do sobrenadante viral. De qualquer maneira, os títulos obtidos estavam dentro do desejável para nossas condições laboratoriais. Como critérios de validação dos estoques foram aplicados microscopia de fluorescência (para detecção e contagem de células GFP positivas) e produção de IDUA (Tabela IV, p. 161; Figura II, p. 164).

A maior diferença notada entre os estoques PLL 3.7 e HIV IDUA provavelmente deve-se a um conjunto de características moleculares relacionadas à construção de cada um dos vetores, com reflexo direto na eficiência de transdução e na intensidade de GFP detectada. Os níveis de GFP obtidos com o construto PLL 3.7 pareceram ser maiores do que para os construtos HIV IDUA e HIV GFP. Uma figura representativa mostra a comparação entre células HEK 293T transduzidas com o vetor PLL 3.7 e com o HIV IDUA (Figura II, p. 164). A natureza bicistrônica desses vetores poderia justificar tais variações, uma vez que o gene *gfp* está localizado a jusante da sequência IRES. A posição dos genes em relação à sequência IRES pode ter reflexo direto na sua intensidade de expressão (Kaufman *et al.,* 2008). O PLL 3.7 não é um vetor bicistrônico com sequência IRES; ele apresenta unidades de transcrição independentes (cada uma com um promotor heterólogo). Ainda assim, os estoques concentrados para HIV GFP e HIV IDUA

mostraram uma boa eficiência de transdução e bom título após a concentração, de uma forma geral.

O emprego desse mesmo protocolo para vetores baseados em MLV mostrou resultados similares, o que indicou uma boa reprodutibilidade da técnica. Os títulos foram coletados pelo menos 15 dias após a seleção, para visualização das colônias individuais e contagem das mesmas. A dosagem de IDUA deu-se 30 dias após transdução para que essas colônias pudessem confluir, levando à obtenção de uma monocamada celular a partir de células geneticamente modificadas. Os níveis de IDUA obtidos com ambos vetores foram considerados compatíveis com dados descritos na literatura, considerando-se os títulos e a MOI estimada empregada naquelas condições experimentais (Fairbairn *et al.,* 1996; Hartung *et al.,* 1999; Di Natale *et al.,* 2002). Valores mais altos do que os reportados pelo nosso grupo de pesquisa foram descritos também para um vetor baseado em HIV contendo promotor CMV (Di Domenico *et al.,* 2005). Um construto bicistrônico contendo uma sequência IRES à montante do gene *gfp* apresentou variação intensa nos níveis detectáveis dessa proteína nas linhagens celulares testadas, oscilando de 10 a 45% nas células transduzidas com MOI de 20 (Kobayashi *et al.,* 2005). Nossos experimentos foram realizados com um volume fixo de vetor, em vista da limitação do volume de injeção permitido no cérebro dos animais, o que nos impediu de analisar variações de MOI.

3.2 A concentração dos vetores baseados em MSCV

A primeira alteração realizada na produção desses vetores foi em relação ao protocolo de concentração – descrito no manuscrito. A introdução de etapas adicionais no protocolo deu-se devido à dificuldade de ressuspensão evidenciada na etapa do trabalho com os vetores HIV e MLV. Aqui também foi reduzido o tempo de incubação dos estoques

a 4^{o}C, com a finalidade de otimizar a ressuspensão dos estoques, conforme sugerido especificamente para a concentração de MSCV (Kanbe and Zhang, 2004). Nós acreditamos que essas modificações levaram a uma melhor recuperação de partículas que poderiam estar presas às paredes dos tubos SS34 empregados. Assim sendo, o volume final de ressuspensão deveria incluir o volume de líquido recuperado após esses *spins*. Os títulos finais obtidos foram considerados satisfatórios e o novo protocolo foi definido como o padrão para concentração de estoques retrovirais, de uma forma geral (inclusive o MLV).

Conforme mencionado anteriormente, protocolos específicos para concentração de cada tipo de vetor viral estão disponíveis na literatura - basicamente dependentes de centrifugações, podendo utilizar filtros específicos (Kanbe and Zhang, 2004; Tiscornia *et al*., 2006). Otimizações da concentração dos vetores já foram propostas. Para HIV, por exemplo, é possível uma metodologia específica para o emprego *in vivo* desses estoques (Coleman *et al.*, 2003). Quando pseudotipados com envelope ecotrópico, contudo, o processamento sugerido é outro (Schambach *et al*., 2006). Para vetores baseados em MLV havia sido sugerido o emprego de polímeros aniônicos e catiônicos como otimizadores de transdução (Le Doux *et al.*, 2001) e posteriormente o emprego dos mesmos na concentração desses vetores (via floculação) (Landazuri *et al.*, 2006). Isso mostra que várias técnicas permitem a concentração dos estoques; cada uma, contudo, com sua específica taxa de rendimento – o que deve ser considerado em função da intenção de aplicação dos mesmos.

3.3 Linhagens empacotadoras dos vetores virais empregados no protocolo *in vivo*

Uma otimização da transfecção transiente para obtenção de vetores lentivirais não foi buscada durante a execução desse projeto. Isso poderia ter contribuído para os títulos obtidos após a concentração serem maiores devido a um maior *input* de partículas virais no estoque não-processado. Para HIV essas otimizações também podem se relacionar às características moleculares dos construtos empregados na transfecção transiente (Morita *et al.*, 2000; Hughes *et al.*, 2001; Kohno *et al.*, 2002).

Para os retrovetores (MLV e MSCV) as linhagens empacotadoras eram estáveis, o que significa que alguns critérios foram aplicados na seleção das mesmas. A relação entre os elementos necessários à produção da partícula viral e sua liberação para o meio extracelular depende de uma estequiometria rigorosa e do *background* das células selecionadas para o processo (Yap *et al.*, 2000). Para a obtenção de estoques pseudotipados com envelopes ecotrópico e anfotrópico existem linhagens diferentes disponíveis (Miller and Rosman, 1989; Miller 1990; Pear *et al.*, 1993; Miller, 1996; Miller and Chen, 1996). As linhagens produtoras de MLV foram obtidas através de uma combinação de linhagem eco e anfotrópica diferente das de MSCV, o que também pode ter contribuído para diferenças observadas entre esses estoques (em termos de título).

A relação observada entre o título obtido para os retrovetores (MLV e MSCV) e a expressão do transgene também deve ser analisada a partir da perspectiva de *design* molecular. O promotor heterólogo empregado na expressão do transgene terapêutico e do gene de resistência desejado pode refletir no título e na infectividade dos estoques, dificultando o empacotamento do vetor (Logan *et al.*, 2004). Essas interferências podem ser oriundas da competição entre esses promotores, da disponibilidade das proteínas empacotadoras e dos transcritos a serem encapsidados (estequiometria) e da

autotransdução da própria linhagem empacotadora – deletéria para a produção dos vetores (Schambach *et al.*, 2006; Brandtner *et al.*, 2007; Carrondo *et al.*, 2008). Finalmente, nem sempre a expressão do transgene nas linhagens empacotadoras é correlacionada diretamente ao maior título e nem sempre o maior título (medida em geral dependente do gene de resistência e das unidades formadoras de colônias) indica maior expressão do transgene nas células alvo (Huang *et al.*, 1997; Pan *et al.*, 2000). No caso do nosso transgene IDUA, a enzima precisa ser adequadamente processada após tradução e a glicosilação final da mesma é indispensável a sua atividade (Neufeld and Muenzer, 2001). Esse processo parece ser mais complexo do que conferir resistência a um antibiótico. Nossas avaliações referentes às linhagens empacotadoras dos retrovetores não incluíram metodologias mais refinadas relacionas à estequiometria ou aos rearranjos. Nenhuma delas, contudo, apresentou problemas relacionados à contaminação dos estoques com vetor replicação competente quando testes específicos foram executados (Miller and Chen, 1996) (dados não mostrados). Conclui-se, portanto, que a etapa de produção de vetores virais concentrados para uso *in vivo* em modelo murino de MPSI foi bem sucedida e adequada às nossas instalações laboratoriais e necessidades experimentais.

3.4 A caracterização *in vitro* dos estoques virais baseados em MSCV

O teste inicial do vetor MSCV nos fibroblastos NIH 3T3 analisou a eficiência da seleção baseada na blasticidina (Kimura *et al.*, 1994). Já havia sido chamada atenção para a necessidade de utilização de novos genes de resistência que não fossem o da neomicina em vetores de transferência gênica– pela dificuldade de sua utilização *in vivo* (Huang *et al.*, 1997). Para a seleção de células progenitoras geneticamente modificadas, foi proposta uma seleção rápida e livre de interferências nas características tronco das mesmas, evitando

longos períodos de cultura (Treschow *et al.*, 2007; Yoshimitsu *et al.*, 2007). Por fim, é importante enriquecer a fração de células geneticamente modificadas para aumentar a possibilidade de sucesso da terapia, o que reforça esses dois conceitos apresentados anteriormente (Ohashi *et al.*, 1992). As células NIH 3T3 foram facilmente transduzidas com uma quantidade mínima de vetor, evidenciado através do ensaio de transdução em microplaca corado com solução de Coomassie (Figura 1, p. 105).

A transdução de células progenitoras envolve uma séria de aspectos relacionados à vetorologia viral: *design* do vetor, MOI, adjuvantes selecionados, genotoxicidade e expressão sustentável (Nienhuis *et al.*, 2006; Chang and Sadelain, 2007). Alguns dados da literatura apontavam para o emprego de altas MOI na transdução de CTM humanas (Zhang *et al.*, 2004; Van Damme *et al.*, 2006), indicando uma maior dificuldade para a transformação genética desse tipo celular. Como nosso interesse estava voltado para CTM murinas e não havia possibilidade de testar diferentes MOI, optamos por inferir que uma menor eficiência de transdução seria obtida nesse tipo celular. De fato, a seleção das CTM transduzidas com o vetor MSCV mostrou que essas células representavam um desafio maior à transdução do que os fibroblastos NIH 3T3 (Figura 3, p. 109). Os melhores resultados foram obtidos com o estoque de maior título, indicando que uma maior MOI de fato renderia uma melhor taxa de transdução (Kobayashi *et al.*, 2005). Isso, contudo, é uma medida de avaliação atrelada ao gene de seleção e ao emprego de blasticidina como agente seletivo.

Os níveis de IDUA nas culturas não-selecionadas foram menores do que após a adição de blasticidina, indicando que de alguma forma a pressão seletiva estava associada ao aumento da expressão do transgene (Figura 4, p. 111). No caso do protocolo clínico para ADA-SCID, inferiu-se que os linfócitos transduzidos possuidores de inserções

permissivas à expressão sustentável do transgene tenham apresentado uma vantagem seletiva em termos de expansão (Aiuti and Cassani, 2007). No caso da MPSI, foi reportado que a pressão de seleção via agente MTX em linfócitos e células-tronco hematopoiéticas selecionava os clones com mais produção de enzima, como se os padrões de expressão do gene terapêutico e de resistência estivessem correlacionados, nesse vetor (Pan *et al.*, 2000).

Nos nossos experimentos, a expressão do IDUA aumentou consideravelmente após a seleção, corroborando dados prévios da literatura (Pan, 2004). A dúvida remanescente referia-se à manutenção desses níveis de expressão na completa ausência do agente seletivo, ou seja, após a seleção ser concluída. Já havia sido demonstrado na literatura que a remoção do agente seletivo levava à variação nos níveis de expressão do transgene, alcançando inclusive o silenciamento (Kauffman, 2008). Nós não realizamos uma análise dose-dependente para a concentração de blasticidina empregada na seleção e para os níveis de expressão do transgene obtidos, o que poderia nos levar a inferir que concentrações mais altas desse agente seletivo isolariam os clones mais produtores de enzima (Pan *et al.*, 2000). Interessante, no momento da seleção das linhagens empacotadoras, nós empregamos concentrações bastante altas desse antibiótico, o que poderia ter influenciado na coleta de estoques virais com mais potencialidade para a produção de IDUA. Essa hipótese não foi testada diretamente, entretanto.

As conclusões finais relacionadas à transdução das CTM indicaram que uma menor eficiência de transdução foi obtida nessas células do que em NIH 3T3; contudo, após a seleção, as células geneticamente modificadas apresentaram sustentabilidade de expressão até ao término do estudo (30 dias) não se detectou nenhuma queda intensa de produção de IDUA. O construto baseado em MSCV, portanto, apresentou as características necessárias

para que se pudesse elaborar um protocolo de transdução de CTM MPSI com seleção rápida e permissiva ao enriquecimento da fração de células geneticamente modificadas em cultura.

As diferenças percebidas entre as linhagens de CTM normal e MPSI foram marcantes. Mesmo com mais vetor (e por consequência, MOI maior), o número de clones isolados no ensaio de transdução em microplaca é menor do que para as CTM normais transduzidas nas mesmas condições (Figura 3, p. 109). Fatores que estariam dificultando a transdução dessas células poderiam incluir componentes de matriz extracelular/GAG (Le Doux *et al.,* 1996; Copreni *et al.,* 2008). Adicionalmente, GAG intracelulares também poderiam contribuir nesse sentido, dificultando a internalização do vetor e seu acesso até o núcleo da célula. Não foi testada diretamente uma correlação entre os níveis intracelulares de GAG e a eficiência de transdução em fibroblastos MPSI, apesar de variações na expressão do IDUA terem sido detectadas nessas condições (Anson *et al.,* 1992). Fatores adicionais como funcionalidade das proteínas de citoesqueleto, desequilíbrio nos níveis de cálcio e de autofagia poderiam atuar sinergisticamente nesse contexto (Settembre *et al.,* 2008). Assim, processos dependentes de tráfego intracelular deveriam ficar comprometidos: transdução, produção e secreção de enzima, etc. As vantagens adaptativas relacionadas à produção da enzima nas células deficientes ficam mais evidentes nesse contexto.

Devido aos resultados anteriores com CTM normais nos terem mostrado uma estabilidade de expressão, as CTM MPSI transduzidas foram avaliadas apenas ao término do experimento (Figura 4, p. 111) – o que nos impediu de avaliar se os repiques nessas culturas poderiam ter interferido nos níveis de IDUA conforme proposto para CTM humanas transduzidas com MLV (Baxter *et al.,* 2002). Trinta dias após transdução, as

CTM MPSI produziam níveis bastante altos de enzima, mesmo após a seleção, o que era altamente promissor para a aplicação desses vetores *in vivo*. Isso excluiu totalmente a possibilidade de pseudotransdução nessas culturas (Pan *et al.*, 2004).

3.5 Aplicação *in situ* dos estoques

A produção de IDUA no cérebro de animais neonatos tratados via TG foi relatada para uma série de vetores de transferência gênica (Hartung *et al.*, 2004; Liu *et al.*, 2005; Chung *et al.*, 2007; Traas *et al.*, 2007). A administração sistêmica de vetores também levou à detecção de IDUA no cérebro de animais adultos, através de sistemas plasmidiais, lentivirais ou retrovirais, com diferentes taxas de redução de GAG como consequência (Camassola *et al.*, 2005; Di Domenico *et al.*, 2005; Kobayashi *et al.*, 2005; Di Domenico *et al.*, 2006; Ma *et al.*, 2007). O vetor baseado em MSCV praticamente normalizou os níveis de enzima nos animais tratados, enquanto que o vetor baseado em HIV apresentou níveis intermediários. O vetor baseado em MLV não levou a produção de IDUA – o que era esperado uma vez que ele é dependente de atividade mitótica para eficiente transdução e esse não é o caso da maioria das células do sistema nervoso central (Figura 5, p. 113).

3.6 Análise quantitativa dos GAGs

Os GAGs acumulados no tecido cerebral apresentaram uma tendência à diminuição, mesmo para o grupo tratado com o vetor MSCV (Figura 5, p. 113). Quando analisamos o percentual de dermatan e heparan sulfato depositado, contudo, as taxas de redução foram extremamente significantes (Figura 5, p. 113). A dosagem de dermatan/heparan e condroitin sulfato no tecido cerebral é complementar, de forma que a redução do teor de dermatan/heparan sulfato nos animais tratadas é acompanhada de um

aumento no percentual de condroitin sulfato (Figura 5, p. 113). Nós optamos por um sistema de quantificação de sque pudesse fornecer uma informação referente aos GAGs totais – o que incluiria também os GAGs característicos do tecido em análise – e também aos tipos de GAGs possíveis de serem discriminados via eletroforese – o que permitiria quantificar o percentual de dermatan e heparan sulfato nesses tecidos. Essas informações são de extrema relevância para a avaliação final da funcionalidade do protocolo de transferência gênica. Os níveis de enzima produzidos via TG deveriam permitir a degradação de material já acumulado no interior dos lisossomos e ao mesmo tempo evitar novos depósitos. A intensidade com que esses dois preocessos ocorrem definiria a correção parcial ou total da patologia, ou a correção *versus* a atenuação de fenótipo. Interessante, a atenuação de fenótipo no contexto do comprometimento nuerológico da MPSI envolveria a transição da forma grave (neuropática) para a forma branda (não–neuropática), o que já conferiria mais qualidade de vida ao paciente.

Os diferentes métodos empregados na detecção de GAGs apresentam suas limitações técnicas e essas distinções poderiam explicar alguns resultados conflitantes detectados na literatura. Conforme explicitado na Tabela V (p. 162), nem todos os trabalhos conseguiram detectar diferença no teor de GAGs total do tecido cerebral de animais MPSI quando comparados aos normais (Di Domenico *et al.*, 2005; Herati *et al.*, 2008). Interessante, em alguns desses trabalhos as análises histológicas mostraram diferenças significantes na patologia vacuolar, apesar disso (Chung *et al*., 2007; Ma *et al*., 2007). Parece inconsistente, portanto, que uma patologia com um forte componente de deterioração cerebral progressiva não apresente diferenças no teor de GAGs total acumulado nos cérebros dos animais normais e afetados. De alguma forma os limites de detecção ou as questões metodológicas relacionadas à extração e análise dos GAGs estão

contribuindo para isso. Por fim, recentemente um novo modelo murino de MPSI foi descrito (Garcia-Rivera *et al.,* 2007). Nesse artigo os autores reforçam a questão de o método de dosagem de GAGs selecionado por eles ter sido capaz de demonstrar o depósito diferencial nos cérebros dos animais afetados. Os autores declararam que a modificação de técnica proposta é menos sujeita à interferência por contaminantes como ácidos nucleicos e à perda dos oligosacarídeos de cadeias mais curtas nas etapas de precipitação. O protocolo selecionado no nosso trabalho também elimina contaminantes de ácidos nucleicos e captura oligosacarídeos de cadeias curtas, devido à etapa de eletroforese (Nader *et al.,* 1984; de Sousa Junior *et al.,* 1990). Um refinamento no sistema de dosagem seria o emprego de espectometria de massa, capaz de quantificar precisamente oligos de tamanho bem diminutos (Fuller *et al.,* 2004). Esse método extremamente preciso, contudo, é trabalhoso e custoso demais para as técnicas de rotina no diagnóstico e na pesquisa préclínica, infelizmente. Em conclusão, o vetor MSCV levou à produção sustentável da enzima e à intensa redução dos níveis de dermatan e heparan acumulados *in vivo* – o que só foi possível de ser evidenciado através de uma técnica alternativa de dosagem de GAGs. *In vitro,* esse vetor mostrou-se uma excelente ferramenta de transferência gênica para fibroblastos e CTM murinas, permitindo a rápida seleção dessas células-alvo. Essa foi apenas uma caracterização inicial da potencialidade desse vetor na TG para MPSI. Estudos futuros são necessários para o melhor entendimento dessa ferramenta em nível molecular. Interessante, híbridos de HIV e MSCV já foram descritos (Choi *et al.,* 2001).

4. Conclusões finais e perspectivas

A TG para MPSI começa a refletir o tipo de abordagem científica já evidenciada para outros tipos de DAL: a transferência gênica *in situ* voltada ao sistema nervoso central

em modelo animal de grande e pequeno porte (Ciron *et al.,* 2006; Watson, Bastacky *et al.,* 2006). A comparação da eficiência desses protocolos se torna complicada nesse contexto, uma vez que os demais trabalhos de injeção intracerebral de vetores referem-se a outros tipos de DAL. A produção *in situ* de enzima recombinante foi obtida através de injeções intracerebrais de vetores virais realizadas em fase neonatal ou intrauterina para modelo murino de MPS VII (Daly *et al.,* 1999; Passini and Wolfe, 2001; Passini *et al.,* 2003; Karolewski and Wolfe, 2006). Em fase adulta, essa mesma abordagem permitiu a restauração de níveis enzimáticos para MPS VII (Stein *et al.,* 1999; Cearley and Wolfe, 2007) e melhora de função comportamental foi detectada nesses animais adultos tratados (Liu *et al.,* 2005; Liu *et al.,* 2007). No caso de MPSIIIB, a administração intraparenquimal ou intracisternal de vetores AAV também resultou em melhora de função cognitiva (Cressant *et al.,* 2004; Fu *et al.,* 2007). Para MPSI, contudo, à exceção dos trabalhos relacionados à avaliação comportamental do modelo murino (Reolon *et al.,* 2006; Pan *et al.,* 2008), a função cognitiva após TG somente foi avaliada para o protocolo aplicado em fase neonatal (Hartung *et al.,* 2004).

Em relação à TG *ex vivo*, a introdução de células-tronco de medula óssea em modelo murino de Niemann Pick levou a produção sustentável de enzima e normalização de função cerebral transitoriamente (Jin and Schuchman, 2003; Shihabuddin *et al.,* 2004). A expressão transiente do transgene também foi detectada em modelo canino de MPSI com células progenitoras hematopoiéticas, devido à resposta imune (Shull *et al.,* 1996; Lutzko *et al.,* 1999a; Lutzko *et al.,* 1999b) e para MPS VII (Sakurai *et al.,* 2004). No caso do modelo murino da doença de Fabry, a injeção de progenitores hematopoiéticos modificados via lentivetor restaurou os níveis enzimáticos sistêmicos (Yoshimitsu *et al.,* 2007). Atualmente, esse mesmo racional experimental foi aplicado em um protocolo

clínico para MPSIIIB (Cheng and Smith, 2003), mostrando que a translação da pesquisa pré clínica para a clínica é possível. Recentemente, um estudo pré clínico sobre reposição enzimática em modelo canino de MPSI propôs um novo regime de imunossupressão que reduziu enormemente o título de anticorpos neutralizantes contra IDUA – uma dificuldade enfrentada rotineiramente na clínica, durante a reposição enzimática (Dickson *et al.*, 2008). Já foi sugerida a adoção desse protocolo como medida potencializadora da terapia (Ponder, 2008), o que reforça a importância da pesquisa básica para a medicina translacional.

Nos últimos anos, relatos de incidentes relacionados aos protocolos de TG têm recrutado a atenção da mídia, mais do que os sucessos. A ciência tem discutido esses resultados adversos e proposto soluções para uma série de dilemas éticos. Entre os maiores tópicos controversos relacionados à TG estão a biosegurança, a transferência gênica *in utero*, a regulação da pesquisa envolvendo linhagem germinativa e a transferência gênica em modelo animal (Wilson, 2007; Kimmelman, 2008). Nos últimos anos temos vivenciado a questão das leucemias induzidas envolvendo retrovetores, dos protocolos bem sucedidos para imunodeficiência severa, amaurose congênita de Leber (entre outros), da aprovação do primeiro produto clínico de TG (Gendicine), dos dois casos fatais envolvendo vetores Ad e AAV, da genotoxicidade dos vetores integrativos, entre outros (Wilson, 2007; Bainbridge *et al.*, 2008; Friedmann, 2008; Kimmelman, 2008; Maguire *et al.*, 2008). Como resposta prática, tem-se estruturado centros para a produção de vetores virais AAV com certificação de qualidade para o uso clínico (Moullier and Snyder, 2008). Para retro e lentivetores, testes de biosegurança padronizados estão sendo desenvolvidos, com o intuito de facilitar a qualificação dos estoques para emprego em estudos *in vivo* (Manilla *et al.*, 2005; Schambach *et al.*, 2006; Schambach *et al.*, 2007; Bauer *et al.*, 2008). Essas importantes contribuições auxiliarão na execução de protocolos clínicos futuros, talvez

avaliando novos fármacos baseados nessa terapia avançada. Atualmente, vários protocolos clínicos em andamento para uma ampla gama de patologias e de vetores já avaliam segurança e eficácia da TG na clínica, inclusive para doenças pediátricas (Aiuti and Bachoud-Levi, 2007; Alexander *et al.*, 2007; Worgall *et al.*, 2008).

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"... fechou o livro (sinal de identificação de uma irmandade secreta) e ela teve vontade de saber o que ele estava lendo".

Milan Kundera (*A insustentável leveza do ser*)

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Anexos

"(...) In assessing the state of in vivo gene therapy with AAV vectors we have entered into the face of translational research referred to as "bench to bedside and back". The clinical phenomena described above have stimulated novel hypotheses and additional basic research. However, substantial gaps remain in our understanding of vector biology and host-vector interactions. (...) Potential considerations include increasing vector potency, decreasing immunogenicity of the capsid and vector preparations"

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Tabela I: Resumo das características principais dos vetores virais empregados na terapia gênica

Sigla do vetor viral	**Vírus de origem**	**É integrativo?**	**É dependente do ciclo celular para transdução?**
MLV	*Murine Leukemia Virus*	sim	sim
MSCV	*Murine Stem Cell Virus*	sim	não
HIV	*Human Immunodeficiency Virus*	sim	não
FIV	*Feline Immunodeficency Virus*	sim	não
AAV	*Adenoassociated Virus*	sim	não
Ad	*Adenovirus*	não	não

Tabela II: Compilação de protocolos de terapia gênica para MPSI

Terapia gênica *in vivo* para MPSI					
Referência	**Vetor**	**Transgene**	**Modelo animal**	**Especificação**	**Carga viral**
Shull & Lu, 1996	retro MLV	cDNA canino	canino	adulto	
Lutzko *et al*., 1999	retro MLV	cDNA canino	canino	adulto	
Lutzko *et al*., 1999	retro MLV	cDNA canino	canino	prenatal - intrauterina	
Hartung *et al.*, 2004	AAV	cDNA humano	murino	neonatal	1X10E10
Camassola *et al*., 2005	plasmidial	cDNA humano	murino	adulto	
Di Domenico *et al*., 2005	lenti HIV	cDNA humano	murino	adulto	5, 10, 15 ug de p24
Kobayashi *et al*., 2005	lenti HIV	cDNA humano	murino	neonatal e adulto	1.65X10E11 TU/Kg
Liu *et al*., 2005	retro MLV	cDNA canino	murino	neonatal	10E8 e 10E9 TU/Kg
Ciron *et al*., 2006	AAV	cDNA canino	canino	adulto	4.8X10E10 VG
Di Domenico *et al*., 2006	lenti HIV	cDNA humano	murino	adulto	0.6X10E9 TU/Kg
Ponder *et al*., 2006	retro MLV	cDNA canino	felino	neonatal	5X10E8 e 1X10E10 TU/Kg
Watson *et al*, 2006	AAV	cDNA humano	murino	adulto	2X10E9 e 4X10E10 TU/Kg
Aranovich *et al*., 2007	transposon SB	cDNA humano	murino	adulto	
Chung *et al*., 2007	retro MLV	cDNA canino	murino	neonatal	10E8 e 10E9 TU/Kg
Ma *et al*., 2007	retro MLV	cDNA canino	murino	adulto	5X10E9 TU/Kg
Traas *et al*., 2007	retro MLV	cDNA canino	canino	neonatal	4.3 ± 3.4X10E9 TU/Kg
Herati *et al*., 2008	retro MLV	cDNA canino	murino	adulto	0.5-1.7X10E10 TU/kg

Os protocolos assinalados referem-se à TG *ex vivo* ou ao emprego de sistemas não-virais, como é possível de se observar na coluna “Vetor”. “Especificação” refere-se à fase da intervenção. Legenda: TG – terapia gênica; MPSI – mucopolissacaridose do tipo I; retro – retrovetor; lenti – lentivetor; TU – *transducing unit* (unidade transdutora).

Tabela III: Compilação de protocolos de TG em modelo murino para DAL envolvendo o vetor MSCV

Emprego de vetores MSCV em TG em modelo murino de DAL					
Referência	**Patologia**	**Transgene**	**TG - tipo**	**Células-alvo**	**Resultado obtido**
Dinauer *et al* ., 1999	Doença granulomatosa crônica ligada ao X	NADPH oxidase	*ex vivo*	células de medula óssea	atividade enzimática detectada em células-alvo 20-25% (em relação ao normal)
Leimig *et al* ., 2002	Galactosialidose	PPCA	*ex vivo*	célula tronco hematopoiética murina	correção completa da patologia sistêmica correção cruzada de células não-transduzidas correção parcial no cérebro - área perivascular
Sano *et al* ., 2005	GM1-gangliosidase	Beta galactosidase	*ex vivo*	células de medula óssea	correção parcial no cérebro

Apenas protocolos *ex vivo* foram descritos empregando o vetor MSCV em modelo murino de DAL. Legenda: TG – terapia gênica; DAL – doenças de acúmulo lisossomal.

Tabela IV: Dados representativos referentes à validação dos estoques concentrados (baseados em HIV)

VETORES BASEADOS EM HIV				
	HIV GFP	HIV IDUA	PLL 3.7	HEK 293T
Volume inicial (mL)	52	152	20	
Volume de ressuspensão (µL)	80	378	40	
Título (UI /10 µL/Hek)	4x10E5	4x10E5	2x10E6	
IDUA (GFP)	4.31 (40%)	32.02(40%)	4.81(70%)	4.77
	2.87 (35%)	30.03(42%)	4.04(80%)	5.30
n	2	2	2	2

Volume inicial refere-se ao sobrenadante viral coletado após a transfecção transiente e submetido ao protocolo de concentração. Volume final refere-se ao volume de ressuspensão dos estoques. O título foi medido baseado no número de células GFP positivas (unidades infectantes – UI) nos volumes e linhagem celular especificados. IDUA foi medida em nmol/mg ptn/h e o valor entre parênteses refere-se ao percentual de células GFP positivas contadas nas fotomicroscopias de fluorescência. Dados coletados após sete dias de transdução.

Tabela V: Compilação de protocolos de dosagem de GAGs em modelo animal de MPSI, agrupados por tipo de metodologia e capacidade de distinção entre o fenótipo normal e afetado

Referência	Vetor	Modelo animal	Fase	Avaliação de GAG		
				colorimetria	histologia	Distinção entre fenótipos?
Hartung *et al* ., 2004	AAV	murino	neonatal		X	sim
Camassola *et al* ., 2005	plasmidial	murino	adulto	X		sim, cérebro inclusive
Di Domenico *et al* ., 2005	lenti HIV	murino	adulto	X		sim, exceto cérebro
Kobayashi *et al* ., 2005	lenti HIV	murino	ıeonatal e adulto		X	sim
Liu *et al.* , 2005	retro MLV	murino	neonatal		X	sim
Chung *et al* ., 2007	retro MLV	murino	neonatal	X	X	sim; cérebro apenas na histologia
Ciron *et al* ., 2006	AAV	canino	adulto	X		sim
Di Domenico *et al* ., 2006	lenti HIV	murino	adulto	X		sim, não avaliou cérebro
Watson *et al* ., 2006	AAV	murino	adulto		X	sim, focado no cérebro
Ma *et al* ., 2007	retro MLV	murino	adulto	X	X	sim; cérebro apenas na histologia
Traas *et al* ., 2007	retro MLV	canino	neonatal	X	X	sim, cérebro inclusive
Herati *et al* ., 2008	retro MLV	murino	adulto	X		sim, exceto cérebro

Os protocolos assinalados não relataram diferenças de teor de GAGs total no tecido cerebral entre animais normais e afetados ou detectaram diferença apenas no número de vacúolos presentes nas amostras de tecidos analisadas histologicamente.

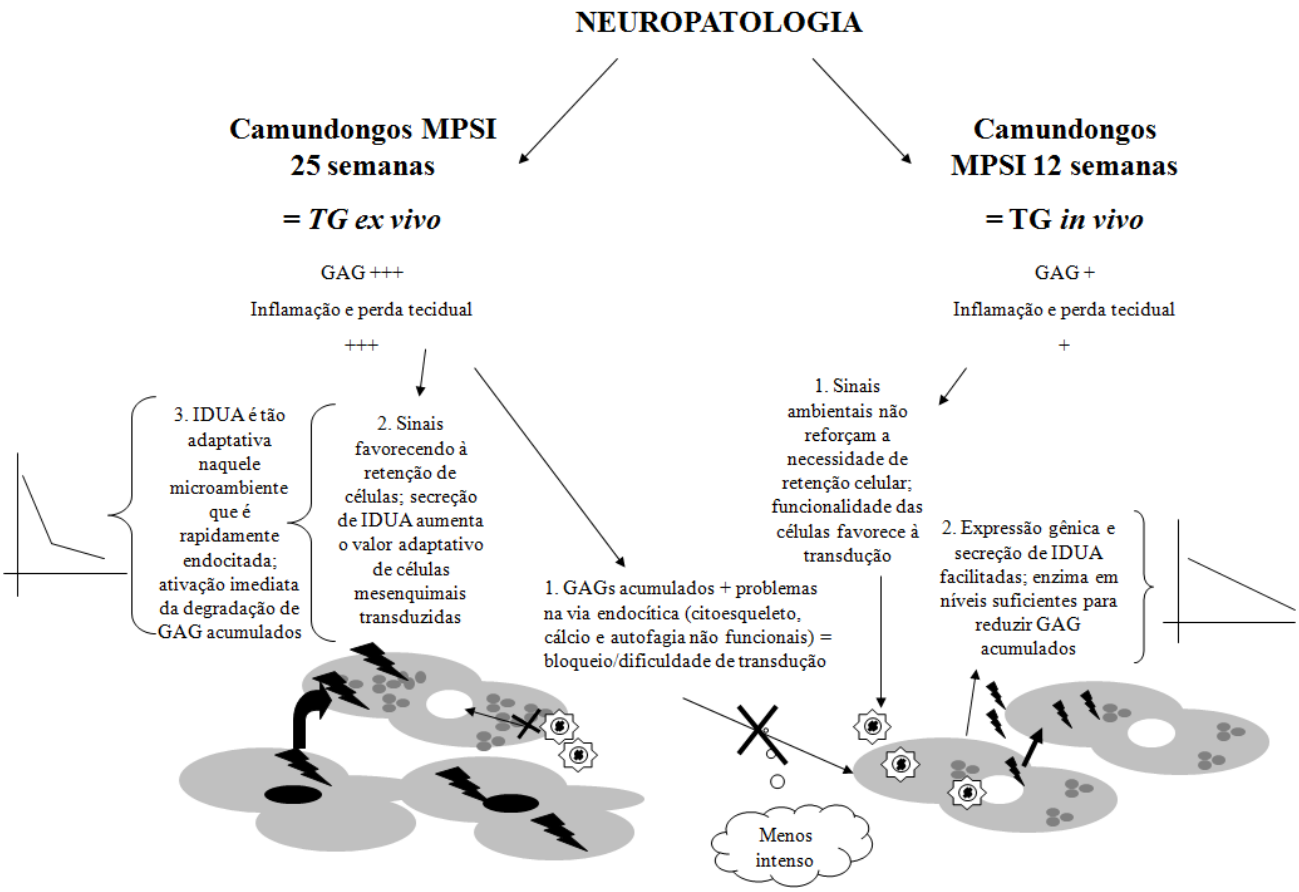

Figura I: Diagrama esquemático da hipótese de adequação do tipo de terapia gênica à fase da patologia. Problemas relacionados a funcionalidade das proteínas de citoesqueleto, ao balanço de cálcio e à regulação da autofagia estão resumidos entre parênteses. Legenda: TG – terapia gênica; GAG – glicosaminoglicanos.

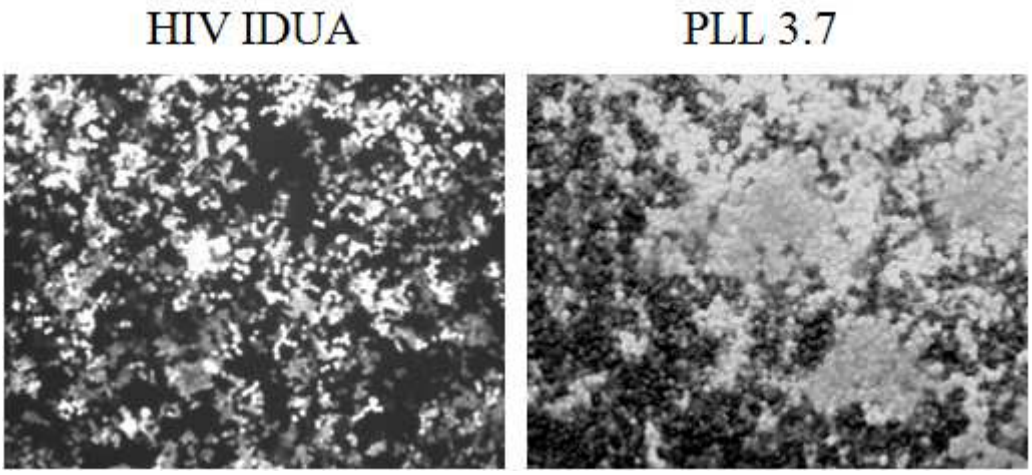

Figura II: Imagens representativas dos estoques concentrados HIV IDUA e PLL 3.7 em microscopia de fluorescência. HIV IDUA parece apresentar mais variação do que o PLL 3.7. Também é possível evidenciar um maior número de células GFP positivas no painel referente ao estoque PLL 3.7, que também apresentou maior título pós- concentração. Dados coletados 7 dias após transdução. Aumento: 100X.