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Avaliação do potencial terapêutico de células-tronco de tecido adiposo para Distrofias Musculares Progressivas

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Tese apresentada ao Instituto de Biociências da Universidade de São Paulo, para a obtenção de Título de Doutor em Ciências, na Área de BIOLOGIA/GENÉTICA.

Orientador(a): Profa. Dra. Mayana Zatz

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Orientadora

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À minha família que sempre esteve ao
meu lado independentemente do
caminho que escolhi.

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Epígrafe

“Most people say that is it is the intellect which makes a great scientist. They are wrong: it is character.”

(Albert Einstein)

Nota do Autor

A proposta deste trabalho é a avaliação do potencial terapêutico de células-tronco de tecido adiposo (*human Adipose-derived Stem Cells – hASCs*) para terapia de distrofias musculares progressivas (DMPs).

Esta tese está organizada no formato de capítulos, sendo o primeiro deles composto por uma revisão geral e objetivos deste estudo. Os capítulos seguintes consistem de artigos científicos publicados em revistas internacionais ou em processo de publicação, os quais descrevem os resultados obtidos durante o período de doutoramento. No último capítulo é feita uma discussão geral sobre todos os resultados obtidos.

Desde o início deste projeto foram delineadas metas e somente após a finalização de cada uma delas era iniciada a próxima. Propor uma terapia celular para DMPs requer ultrapassar diversas barreiras. A primeira delas é o tamanho do tecido afetado, o músculo estriado, que em humanos representa certa de 40% do corpo. Antes do início deste trabalho era sabido que hASCs são capazes de se diferenciar em músculo estriado. A fonte de onde são obtidas as células-tronco (CT) é um ponto muito crítico por que a reconstituição desse tecido requer grandes quantidades de CT, o que nos levou a escolher o tecido adiposo. As perguntas a serem respondidas eram: Qual é a melhor via de injeção: local ou sistêmico? Uma injeção é suficiente ou são necessárias múltiplas injeções? Quanto tempo as células injetadas permanecem no músculo após a última injeção? A imunosupressão é necessária? Enquanto essas perguntas foram sendo respondidas novas questões foram surgindo e algumas delas ainda permanecem em aberto.

Sumário

Resumo

As Distrofias Musculares Progressivas (DMP) constituem um grupo de doenças genéticas caracterizadas por uma degeneração progressiva e irreversível da musculatura esquelética. A Distrofia Muscular de Duchenne (DMD) é a forma mais comum e grave de DMP. Obedece a herança recessiva ligada ao X e é caracterizada pela ausência de distrofina na membrana das fibras musculares. Atualmente não existe nenhum tratamento efetivo para este grupo de doenças. Deste modo, este trabalho tem como objetivo principal avaliar o potencial terapêutico das células-tronco mesenquimais de tecido adiposo humano (*human Adipose-derived Stem Cells – hASCs)* visando à regeneração ou diminuição da degeneração muscular. Para tanto, verificamos o potencial miogênico destas células *in vitro*, utilizando células musculares de pacientes DMD e *in vivo* utilizando como modelo camundongos distróficos e cães da raça Golden Retriever portadores de distrofia muscular (GRMD – *Golden Retriever Muscular Dystrophy*). Demonstramos neste estudo que hASCs são capazes de restaurar a expressão de distrofina *in vitro*, quando co-cultivadas com células musculares de pacientes DMD. Frente a estes resultados, continuamos nossos estudos em modelos animais, *in vivo, e* demonstramos que as hASCs são capazes de chegar à musculatura de camundongos distróficos e de cães GRMD, quando injetadas por via venosa, e de restaurar a expressão da proteína muscular defeituosa. Foi possível observar uma melhora funcional nos camundongos injetados. Nos cães GRMD encontramos distrofina humana seis meses após a última injeção entretanto é difícil julgar se houve melhora clínica. Todos esses experimentos de xenotransplantes foram feitos sem imunosupressão e não observamos rejeição. Concluímos que o tecido adiposo é uma fonte de células-tronco com potencial para regeneração muscular *in vivo*. Contudo é de extrema importância repetir os experimentos em um número maior de cães GRMD e ainda investigar novas estratégias visando melhorar os resultados obtidos neste trabalho, antes de começar qualquer teste clínico.

Abstract

Progressive muscular dystrophies (PMD) are a clinically and genetically heterogeneous group of disorders caused by the deficiency or abnormal muscle proteins, resulting in progressive degeneration and loss of skeletal muscle function. As effective treatments for these diseases are still unavailable, they have been widely investigated as possible candidates for stem cell therapy. Duchenne muscular dystrophy (DMD), a lethal X-linked disorder, is the most common and severe form of muscular dystrophies, affecting 1 in 3000 male births. Mutations in the DMD gene lead to the absence of muscle dystrophin. The aim of this study is to evaluate the therapeutic potential of human Adipose-derived Stem Cells (hASCs) for muscle regeneration. First we verified the myogenic potential of these cells *in vitro* co-culturing them with muscle cells from DMD patients and verifying that hASCs are able to restore dystrophin expression *in vitro*. Subsequently we repeated this experiment *in vivo* using the dystrophic mice *SJL* and the dystrophic golden retriever dogs (GRMD - Golden Retriever Muscular Dystrophy) as animal models. We demonstrated that the hASCs are able to reach the muscles of the dystrophic mice and the GRMD dogs when injected systemically and restore expression of absent muscle protein, without any immunosupression. We observed a functional improvement in the injected mice. We found human dystrophin in injected dogs up to 6 months after the last injection. However it is difficult to evaluate if there was clinical improvement in the GRMD dogs due to their great phenotypic variability. We conclude that the adipose tissue is a source of stem cells with potential for muscle regeneration *in vivo* and that human cells are not rejected even in xenotransplants without immunosuppression. However it is important to repeat the experiments on a larger number of GRMD dogs and to investigate new strategies to improve our findings before starting any clinical trial.

Introdução Geral

Distrofias Musculares Progressivas

As Distrofias Musculares Progressivas (DMP) constituem um grupo de doenças genéticas caracterizadas por uma degeneração progressiva e irreversível da musculatura esquelética. As DMPs podem ser subdivididas com base na distribuição corporal da fraqueza muscular. Mais de 30 formas de DMPs, causadas por mutações em diferentes genes, foram descritas, entretanto algumas características encontradas no tecido muscular são comuns a todas as DMPs. Entre elas estão: variação no calibre das fibras musculares, áreas de necrose, invasão de macrófagos, reposição do tecido muscular por tecido adiposo e/ou tecido conjuntivo e aumento nos níveis séricos da proteína creatina kinase (CK)[1]. As formas mais comuns de DMPs são: a Distrofia Muscular de Duchenne (DMD), a Distrofia Muscular tipo Becker (DMB) e a Distrofias Musculares do tipo Cinturas (*Limb-Girdle Muscular Dystrophy* - LGMD)[2].

A Distrofia Muscular de Duchenne (DMD) foi descrita originalmente pelo médico inglês Edward Meryon em 1851, em uma reunião da Sociedade Real de Medicina e Cirúrgica[3]. A DMD, de herança ligada ao cromossomo X, tem incidência de 1 entre 3000 nascimentos do sexo masculino. Os sinais clínicos iniciam-se entre 3-5 anos de idade, o confinamento em cadeira de rodas ocorre aproximadamente aos 12 anos de idade e os afetados raramente sobrevivem após a terceira década de vida[4]. A DMD é causada por mutações no gene da distrofina[5], proteína que mantém a estabilidade do sarcolema[6], ancorando as proteínas do citoesqueleto às proteínas presentes na membrana plasmática[7] através do complexo de proteínas associado à distrofina[8]. Dentre as mutações que causam DMD 60% são deleções, 5-6% duplicações e 34% mutações de ponto[9,10], estas geram uma proteína truncada, ou sem função, que é rapidamente degradada pela célula[10].

A Distrofia Muscular de Becker (DMB), alélica a DMD, também é causada por mutações no gene da distrofina, no entanto, estas mutações ocorrem em fase, mantendo o quadro de leitura do mRNA, gerando uma

proteína truncada, de tamanho diminuído, mas parcialmente funcional[10]. A incidência de DMB é de 1:30000 nascimentos. Os sinais clínicos dos pacientes portadores de DMB iniciam-se, em geral, na segunda década de vida, os afetados conseguem andar após os 16 anos de idade, sendo a velocidade de progressão da doença extremamente variável[4].

Além da manutenção do quadro de leitura do RNA mensageiro a gravidade do quadro clínico pode estar associada à localização da deleção. Deleções nas regiões de ligação da distrofina com a actina (região carboxi-terminal) ou na região de ligação com o complexo de glicoproteínas (região amino-terminal) resultam em um quadro mais grave[11,12]. Foram observados casos de deleção de 50% do gene, restrita à região central (domínio em bastão ou *rod domain*), associadas a um quadro clínico leve[13].

A análise da presença de distrofina no músculo pode ser feita por *Western blot* (WB) e imunofluorescência utilizando anticorpos para região amino-terminal e carboxi-terminal. Os pacientes DMD apresentam ausência de distrofina em WB. Entretanto se a análise por imunofluorescência é feita com o anticorpo amino-terminal uma marcação positiva parcial de algumas fibras pode ser observada, geralmente sem correlação com a gravidade do quadro clínico. Além disso, algumas fibras isoladas, distrofina positiva podem ser observadas, denominadas fibras revertentes. A análise de proteínas em pacientes DMB por WB mostra bandas de distrofina com peso molecular alterado ou em quantidade diminuída. A imunoflorescência apresenta uma marcação positiva para distrofina[8], embora diferente do músculo normal.

As LGMD são caracterizadas pelo comprometimento dos músculos das cinturas pélvica e escapular, sem o comprometimento da inteligência. O início e a progressão da doença é muito variável, com um amplo espectro de variação[14,15]. Vinte e dois genes já foram identificados por gerar LGMDs, sendo 7 deles com herança autossômica dominante e 15 com herança autossômicos recessiva[15] (www.musclegenetable.org). O fenótipo mais comum é caracterizado por fraqueza proximal das cinturas pélvica e escapular, sem comprometimento dos músculos faciais ou da inteligência[16].

Apesar da grande heterogeneidade genética as LGMD apresentam muitas características em comum que são importantes para o diagnóstico. Os afetados têm em geral um aumento da enzima sérica creatinoquinase (CK),

sendo esta a mais utilizada para um diagnóstico inicial. Na biopsia muscular observa-se um padrão distrófico típico, mas com marcação positiva para distrofina[17]. Anticorpos específicos para outras proteínas musculares devem ser utilizados para verificar qual proteína está ausente/deficiente.

A maioria das proteínas codificadas pelos genes que causam LGMD pertencem ao complexo glicoproteico associado à distrofina (*Dystrophin Glycoproteins Complex,* DGC). Mutações em qualquer um dos genes do complexo sarcoglicano pode levar a perda parcial ou total do DGC ou a deficiência secundária de uma proteína sarcoglicana[18]. Portanto espera-se que alterações em qualquer proteína do DGC possam causar um quadro de deficiência muscular.

Distrofina e o complexo glicoproteico associado à distrofina

Em 1985 o gene responsável pela DMD foi localizado no braço curto do cromossomo X (Xp21)[5]. Com 2,3 megabases (Mb), o gene DMD é o maior gene humano já caracterizado. É constituído por 79 exons, sendo transcrito em um mRNA de 14 kilobases (Kb). O produto protéico deste gene, denominado distrofina, possui 3685 aminoácidos e uma massa molecular de 427 kilodaltons (kDa)[19].

A distrofina, proteína mutada em DMD e DMB, está localizada na face citoplasmática da membrana celular[20]. Acredita-se que o papel do DGC é estrutural, realizando a ligação entre o citoesqueleto de actina à matriz extracelular, e responsável pela estabilização do sarcolema durante os ciclos de contração e relaxamento da célula muscular, transmitindo a força gerada nos sarcômeros à matriz extracelular[21]. Há também evidências de que o DGC esteja envolvido na sinalização celular[22]. Vários membros da DGC, como proteínas sarcoglicanas, foram ligados a uma série de doenças musculares, o que ilustra o papel vital que este complexo desempenha na manutenção da integridade do músculo.

Modelos animais para DMPs

Mesmo com os grandes avanços na elucidação dos mecanismos

moleculares das DMPs, nenhum tratamento efetivo está disponível. O progresso nesta área depende de modelos animais fiéis à patologia clínica humana para realização de testes pré-clinicos a fim de verificar a eficácia e segurança das terapias.

A descoberta do gene responsável pela DMD e de seu produto protéico, a distrofina, permitiu a identificação de modelos animais homólogos à DMD humana em cachorros, gatos, camundongos e peixes[23-26]. Nos humanos e nos cães o processo de degeneração e fibrose predominam, levando a uma perda progressiva da estrutura e função muscular. Já nos modelos felino e murino, observa-se pouca fibrose e o processo de regeneração supera a necrose do músculo, gerando um fenótipo brando ou ausente. Dessa forma, a utilização do modelo murino e felino tornam-se limitados, servindo apenas como modelo genético e bioquímico, mas não como modelo clínico-patológico da DMD humana[27]. O *Sapje* é o modelo *zebrafish* para DMD. Surgiu recentemente como um modelo animal promissor para o estudo de distrofias musculares e outras doenças humanas devido ao seu pequeno tamanho, grande número de descendentes (50-350 por semana), rápido desenvolvimento da musculatura esquelética e transparência nas fases embrionária / juvenil. O *Sapje* apresenta musculatura normal até o dia 2 pós-fertilização, porém seu músculo vai perdendo a organização gradativamente levando a perda da mobilidade por volta do dia 5 pós-fertilização[28].

O camundongo *mdx*, modelo murino para DMD, apresenta deficiência da proteína distrofina causada por uma mutação no exon 23 do gene da distrofina, resultando em um *stop codon* prematuro. Entretanto, diferentemente da DMD humana, o camundongo *mdx* não apresenta fraqueza muscular evidente. Portanto é um bom modelo molecular para DMD, porém não funcional[26].

O modelo animal que apresenta um fenótipo semelhante aos pacientes portadores de DMD são os cães da raça Golden Retriever (*Golden Retriever Muscular Dystrophy* – GRMD), suas alterações musculares assemelham-se a patogênese humana mais fielmente que o modelo murino, além de possuírem uma massa muscular comparável a dos pacientes distróficos. A deficiência de distrofina nos cães GRMD é causada por uma

mutação de ponto (A>G) no sitio 3' de splicing no íntron 6 do gene da distrofina causando o *skipping* do exon 7 no RNA mensageiro. Esta mutação causa um erro no quadro de leitura gerando um *stop codon* prematuro que leva a síntese de um peptídeo com 5% do tamanho normal da distrofina[23].

Alguns modelos murinos de DMPs apresentam características fenotípicas semelhantes às encontradas em seres humanos. O camundongo *SJL*, modelo da LGMD2B (disferlinopatia), apresenta uma DMP que afeta primeiramente os músculos proximais. Estes animais apresentam uma mutação em um sítio de *splicing* do gene que codifica a disferlina, levando a perda do exon 45 (171 pb) no RNA mensageiro. As alterações histopatológicas podem ser observadas nas primeiras três semanas de vida, enquanto as alterações clínicas são evidentes somente quando o animal é erguido pela cauda e após os 9 meses de idade[29]. Os camundongos *dy/dy* e dy^{2J}/dy^{2J} são modelos naturais da distrofia muscular congênita 1A, de herança autossômica recessiva. Os camundongos *dy/dy* e dy^{2J} apresentam degeneração muscular e desmielinização do sistema nervoso periférico causada por uma ausência completa ou parcial da proteína LAMA2, respectivamente[30,31].

Estratégias terapêuticas para DMPs

Diferentes estratégias estão sendo testadas na tentativa de criar uma terapia para DMD: terapia celular; administração de drogas sistêmicas, como o antibiótico aminoglicosídeo[32]; corticóides como a prednisona[33]; utilização de um RNA antisenso para causar o *skipping* do exon mutado[34,35], bem como terapias gênicas usando vetores virais e plasmídios, vem mostrando resultados bastante promissores[7].

O transplante de mioblastos, apesar de restaurar a expressão de distrofina em camundongos *mdx* e pacientes com DMD, ainda apresenta limitações como baixa distribuição das células após injeção, rejeição imune e baixa sobrevivência celular[36-38]. Outra estratégia bastante testada é a terapia gênica. Durante muitos anos, o grande tamanho do mRNA da distrofina (14kb) foi o grande empecilho nesta área, pois dificultava a sua manipulação em vetores virais. Entretanto, a criação de miniaturas deste gene (micro e

minidistrofinas) tornou possível sua introdução em vetores virais adenoassociados (AVV) e a realização de ensaios pré-clínicos em camundongos *mdx*[39-41]. Atualmente, os grandes obstáculos a serem ultrapassados envolvem: a dose necessária para transdução viral em todo o corpo humano sem causar toxicidade; a baixa permanência do vetor e necessidade de re-injeção sem causar rejeição; e o teste da efetividade da terapia gênica em modelos animais de grande porte, como os cães GRMD[42,43]. Concomitante aos estudos em modelos animais, já se encontra em andamento estudos clínicos avaliando a segurança da administração de AVV em pacientes com DMD, administração de oligonucleotídeos antisenso para indução de exon *skipping* e avaliação de drogas que ignoram o códon de parada em pacientes com mutações nonsense[35].

Todas essas abordagens ainda são experimentais e ainda não existe nenhum tratamento efetivo para esse grupo de doenças.

Terapia celular para DMPs

O músculo esquelético adulto regenera novas fibras musculares ativando uma população de precursores musculares[44]. No entanto, a degeneração muscular contínua encontrada nas DMPs leva a uma depleção de células satélites e, conseqüentemente, a capacidade de restaurar o músculo esquelético é perdida[45,46]. Uma abordagem terapêutica promissora para tratar as DMPs é a utilização de células-tronco, afim de que estas células atuem como precursores na regeneração das fibras musculares, restaurando a expressão da proteína defeituosa.

Células-tronco são definidas pela sua capacidade de auto-renovação e diferenciação em múltiplos tipos celulares[47]. Estas células possuem origem tanto adulta como embrionária. Dentre as fontes adultas destacam-se o cordão umbilical, a medula óssea, polpa dentária, tecido adiposo, dentre outros[48-51]. As células-tronco embrionárias podem ser obtidas da massa celular interna de blastocistos, ou até de um único blastômero sem interferir no potencial de desenvolvimento do embrião ou a partir da reprogramação de células diferenciadas como os fibroblastos da pele, também conhecidas com células iPS - *induced pluripotent stem cells*[52-55].

Duas populações principais de células-tronco adultas se destacam: células-tronco hematopoéticas (CTHs) e células-tronco mesenquimais (CTMs). Tradicionalmente as CTHs são isoladas da medula óssea, porém, mais recentemente, o mesmo protocolo de separação de CTHs está sendo realizado no sangue do cordão umbilical. As CTHs por definição são capazes de originar toda a linhagem de células sanguíneas e podem ser identificadas por uma série de marcadores de superfície celular como: CD34, CD38, CD45, CD133, dentre outros. Ao contrário das CTHs que crescem em suspensão *in vitro*, as CTMs são cultivadas aderidas às placas de cultura. CTMs compreendem uma população de células progenitoras multipotentes capazes de diferenciar em vários tipos celulares de origem mesodérmica incluindo condrócitos, osteócitos e adipócitos[56,57] e células musculares. Além disso, estas células podem ser caracterizadas com os marcadores de superfície celular CD29, CD90, SH2, SH3, SH4, dentre outros. Entretanto as CTMs são populações heterogêneas de células[58], existindo uma diferença entre doadores[59] ou até mesmo do local de coleta[60].

Uma fonte muito utilizada para terapia celular é o sangue do cordão umbilical, que é geralmente descartado após o parto. Sua coleta é obtida por um método simples, seguro e não doloroso; não causando nenhum dano para a mãe ou para a saúde do recém-nascido. Nosso grupo comparou a eficiência do isolamento e a origem das CTMs do sangue e do tecido do cordão umbilical (*human umbilical cord tissue mesenchymal stem cells* – hUCT MSC) do mesmo doador e observamos que não o sangue, mas sim o próprio cordão, o qual é rotineiramente descartados durante a coleta de células-tronco, é a fonte mais rica em CTMs[61] e que estas células, apesar de mesenquimais, apresentam um perfil de expressão diferente [62].

O tecido adiposo também é uma fonte de CTMs. Contém uma população de células que pode ser facilmente isolada, apresenta crescimento estável, alta taxa de proliferação *in vitro* (mais de 200 PD com uma passagem a cada 4-5 dias). Estas células, chamadas de hASCs (*human Adipose-derived Stem Cells*), têm a capacidade de se diferenciar em células musculares, adiposas, de osso e de cartilagem quando submetidas a fatores de diferenciação específicos[57,63].

Entretanto antes de falar-se em terapia celular para tratamento para

DMPs, vários problemas ainda devem ser enfrentados. Dentre eles deve-se encontrar uma fonte de fácil obtenção, pouco invasiva, que apresente grande quantidade de células-tronco, que possam ser expandidas mantendo suas características originais e que apresente potencial de se infiltrar nos tecidos, multiplicar-se e manter-se após o transplante. Porém os resultados de experimentos pré-clínicos no transplante de CTMs visando a terapia de DMPs ainda são muito controversos.

Benchaouir et al. (2007)[64] demonstrou a restauração da expressão de distrofina após a injeção de células-tronco CD133 positivas geneticamente modificadas retiradas de pacientes DMD, em camundongos *mdx* imunodeficientes. Estes resultados mostram que o transplante autólogo de células-tronco geneticamente modificadas pode representar uma terapia promissora para as DMP. Entretanto é importante salientar que o uso de vetores virais, modificações genéticas pode levar a alguns problemas, especialmente no que se diz respeito à segurança uma vez que estes integram seu DNA no DNA genômico da célula.

CTMs de medula óssea foram utilizadas com sucesso em transplantes para reparo muscular em *mdx*[65]. Entretanto a quantidade de CTMs obtidas da punção de medula óssea é pequena (aproximadamente 1 célula-tronco por 10^5 células aderentes) necessitando de uma grande expansão celular *in vitro* para obter um número suficiente de células para um transplante. Ao contrário dos resultados anteriores, mais recentemente foi relatada a injeção de CTMs de medula óssea, transduzidas com vetores virais, expressando PAX3, em camundongos *mdx*. Neste trabalho os autores observaram que, apesar da restauração da expressão da distrofina, não ouve melhora funcional da musculatura[66]. O mesmo grupo havia demonstrado anteriormente a eficiência do transplante de células-tronco embrionárias murinas, transduzidas com um retrovírus contendo o gene Pax3, em camundongos *mdx*. Este transplante levou à restauração da expressão de distrofina e melhora clínica dos animais injetados[67].

O resultado aparentemente mais promissor envolvendo CTMs para terapia em DMPs veio do transplante de células-tronco caninas originadas de vasos, chamadas mesoangioblastos, em cães GRMD. Neste trabalho foi observada a restauração da expressão de distrofina em 70% das fibras

musculares[68], acompanhado de uma melhora funcional dos animais injetados. Entretanto apesar do grande impacto do trabalho, este foi criticado internacionalmente devido ao uso de cyclosporina – que poderia ser responsável por si só pela melhora clínica – e pelo método de avaliação física dos animais[37].

Sabe-se que o grau de degeneração muscular pouco influencia no *engraftment* e regeneração de células satélites. Este fato é de grande importância visto que, no futuro, pacientes com um grau avançado de degeneração muscular poderão se beneficiar da terapia celular[69]. Entretanto é importante verificar quais fatores estão presentes no músculo distrófico em diferentes estágios da doença e como estes podem ser modulados a fim de melhorar o potencial da terapia. Os resultados discrepantes envolvendo o transplante de CTMs, mostram que é muito importante a elucidação da ação das células transplantadas no músculo distrófico e de seus efeitos parácrinos. Uma vez que estas células podem propiciar uma efeito benéfico mesmo sem se diferenciarem em células musculares[70].

As abordagens terapêuticas para a DMPs baseiam seu sucesso na analise da restauração da expressão da proteína ausente no músculo distrófico. No entanto, recentemente, diversos estudos têm mostrado que o resgate da expressão de distrofina pode não resultar em uma melhora clínica[41,71]. Além disso, é sabido que os camundongos *mdx* são quase assintomáticos, apesar da ausência de distrofina no músculo[26]. Mais recentemente, observamos que isso também pode ocorrer em cães GRMD[72], sendo este um modelo animal de grande porte com uma sobrevida muito maior. Isso demonstra que o sucesso dos ensaios terapêuticos não devem basear-se unicamente na análise molecular para avaliar o resgate da expressão defeituosa de proteínas musculares.

Na tentativa de estabelecer uma terapia celular para DMPs, este trabalho visa avaliar o potencial das hASCs de diferenciar-se em células musculares, de restaurar a expressão protéica e a morfologia do músculo distrófico atenuando os sinais clínicos associados à degeneração muscular.

I. OBJETIVOS

1. Padronização da separação e congelamento das células ASCs humanas e caninas
2. Caracterização das ASCs
3. Avaliação do potencial miogênico e interação com células musculares das hASCs *in vitro*
4. Avaliação do potencial miogênico das hASCs *in vivo* em modelos animais (murino e canino)

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Capítulo 2

Células-tronco mesenquimais de tecido adiposo são capazes de restaurar expressão de distrofina de células musculares de Duchenne *in vitro*

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Human multipotent adipose derived stem cells restore dystrophin expression of Duchenne skeletal muscle cells in vitro

Natássia Moreira Vieira, Vanessa Brandalise, Eder Zucconi, Tatiana Jazedje, Mariane Secco, Viviane Abreu Nunes, Bryan E Strauss, Mariz Vainzof e Mayana Zatz

Abstract

BACKGROUND INFORMATION: Duchenne muscular dystrophy (DMD) is a devastating X-linked disorder characterized by progressive muscle degeneration and weakness. The possibility of replacing the defective muscle through cell therapy is being pursued as a future treatment for DMD. Mesenchymal stem cells have the potential to differentiate to a myogenic phenotype in vitro. Since human liposuctioned fat is available in large quantities, it may be an ideal source of stem cells for therapeutic applications. Adipose derived stem cells (ASCs) are able to restore the dystrophin expression in *mdx* mice muscle. However, the ability of these cells to interact with human dystrophic muscle is still unknown. RESULTS: We explored the potential of ASCs to participate in myotube formation when cultured together with differentiating human DMD myoblasts, and their potential to interact with DMD myotubes. ASCs restore dystrophin expression in both situations. Experiments with GFP-positive ASCs and DAPI stained DMD myoblasts indicated that ASCs participate inhuman myogenesis through cellular fusion. CONCLUSIONS: These results show that ASCs have the potential to interact with dystrophic muscle cells restoring dystrophin expression of DMD cells in vitro. The possibility to use adipose tissue as a source for stem cell therapies for muscular diseases is extremely exciting.

Resumo

INFORMAÇÕES GERAIS: A Distrofia Muscular de Duchenne (DMD) é uma doença de herança recessiva ligada ao cromossomo X, caracterizada por uma degeneração e fraqueza progressiva dos músculos esqueléticos. A possibilidade de reparo do tecido muscular através da terapia celular é foco de estudos para o futuro tratamento de DMD. As células-tronco mesenquimais têm potencial de se diferenciar em células musculares *in vitro*. Visto que a gordura lipoaspirada está disponível em grandes quantidades, esta pode ser uma fonte ideal de células-tronco para aplicações terapêuticas. Células-tronco derivadas de tecido adiposo (*Adipose-derived stem cells* - ASC) foram capazes de restaurar a expressão de distrofina no músculo de camundongos *mdx*. No entanto, a capacidade dessas células de interagir com músculo distrófico humano ainda é desconhecida. RESULTADOS: Neste trabalho demonstramos o potencial da ASC de participar na formação de miotubos quando co-cultivadas com mioblastos DMD humanos e seu potencial de interagir com miotubos DMD. As ASC foram capazes de restaurar a expressão da distrofina em ambas as situações. Os co-cultivos foram realizados com ASC GFP-positivas e mioblastos DMD corados com DAPI indicaram que ASCs participam na miogênese através de fusão celular. CONCLUSÕES: Estes resultados mostram que a ASC têm o potencial de interagir com as células musculares distróficas e restaurar a expressão de distrofina de células DMD *in vitro*.

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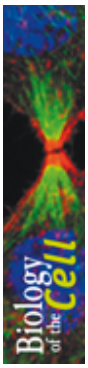
Research article



Human multipotent adipose-derived stem cells restore dystrophin expression of Duchenne skeletal-muscle cells *in vitro*

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Background information. DMD (Duchenne muscular dystrophy) is a devastating X-linked disorder characterized by progressive muscle degeneration and weakness. The use of cell therapy for the repair of defective muscle is being pursued as a possible treatment for DMD. Mesenchymal stem cells have the potential to differentiate and display a myogenic phenotype *in vitro*. Since liposuctioned human fat is available in large quantities, it may be an ideal source of stem cells for therapeutic applications. ASCs (adipose-derived stem cells) are able to restore dystrophin expression in the muscles of *mdx* (X-linked muscular dystrophy) mice. However, the outcome when these cells interact with human dystrophic muscle is still unknown.

Results. We show here that ASCs participate in myotube formation when cultured together with differentiating human DMD myoblasts, resulting in the restoration of dystrophin expression. Similarly, dystrophin was induced when ASCs were co-cultivated with DMD myotubes. Experiments with GFP (green fluorescent protein)-positive ASCs and DAPI (4′,6-diamidino-2-phenylindole)-stained DMD myoblasts indicated that ASCs participate in human myogenesis through cellular fusion.

Conclusions. These results show that ASCs have the potential to interact with dystrophic muscle cells, restoring dystrophin expression of DMD cells *in vitro*. The possibility of using adipose tissue as a source of stem cell therapies for muscular diseases is extremely exciting.

Introduction

Muscular dystrophies are a clinically and genetically heterogeneous group of disorders characterized by progressive degeneration and loss of skeletal muscle (reviewed by Zatz et al., 2003). Adult skeletal muscle has the potential to regenerate new muscle fibres by activating a population of mononucleated precursors, which otherwise remain in a quiescent and non-proliferative state (Schultz and McCormick, 1994). However, the continuous and gradual muscle degeneration in progressive muscular dystrophies leads to a depletion of satellite cells and, consequently, the capacity to restore the skeletal muscle is lost (Laguens, 1963; Heslop et al., 2000).

DMD (Duchenne muscular dystrophy), an X-linked lethal disorder that affects 1 in 3–4000 male births, is the most prevalent form of muscular dystrophy (Leturcq and Kaplan, 2005). DMD is caused by genetic mutations in the dystrophin gene at Xp21, resulting in the absence of this protein in muscle. Dystrophin is a component of the dystrophin-associated glycoprotein complex and

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Key words: adipose, Duchenne muscular dystrophy (DMD), DMD therapy, *in vitro* study, skeletal-muscle cell, stem cell.
Abbreviations used: ASC, adipose-derived stem cell; DAPI, 4′,6-diamidino-2-phenylindole; DMD, Duchenne muscular dystrophy; DMEM-HG, Dulbecco's modified Eagle's medium with high glucose; FBS, fetal bovine serum; FM, fusion medium; GFP, green fluorescent protein; GM, growth medium; hASC, human ASC; HLA-ABC, human leucocyte antigens, MHC class I; HLA-DR, human leucocyte antigens, MHC class II; hMSC, human mesenchymal stem cells; HS, horse serum; *mdx*, X-linked muscular dystrophy; PLA, processed lipoaspirate; RT, reverse transcription; SH3 domain, Src homology 3 domain; SVF, stromal-vascular fraction.

links the muscle fibre cytoskeleton to the extracellular matrix (Burghes et al., 1987).

A promising approach to the treatment of DMD is to restore dystrophin expression by repairing the defective muscle through cell therapy. Previous studies have suggested that haematopoietic stem cells can contribute to skeletal-muscle regeneration (Gussoni et al., 1999; McKinney-Freeman et al., 2002; Camargo et al., 2003; Corbel et al., 2003; Bachrach et al., 2004). In normal and *mdx* (X-linked muscular dystrophy) mice, BM (bone marrow)-derived stem cells were shown to participate in skeletal-muscle repair after induced damage (Ferrari et al., 1998; Fukada et al., 2002; LaBarge and Blau, 2002). However, the clinical usefulness of haematopoietic cell transplantation in muscular dystrophies, including DMD, has been a subject of great controversy (Lakshmipathy and Verfaillie, 2005; Sampaolesi et al., 2006).

An abundant and accessible source of stem cells is adipose tissue. Several groups have demonstrated that mesenchymal cells within the SVF (stromal-vascular fraction) of subcutaneous adipose tissue [PLA (processed lipoaspirate) cells] are capable of differentiation along multiple lineages, including myocytes, in the presence of lineage-specific inductive medium (Halvorsen et al., 2001a, 2001b; Zuk et al., 2001, 2002; Erickson et al., 2002; Mizuno et al., 2002; Safford et al., 2002, 2004; Justesen et al., 2004; Miranville et al., 2004; Planat-Benard et al., 2004; Rehman et al., 2004; Brzoska et al., 2005; Seo et al., 2005; Rodriguez et al., 2006).

Recently, the ability of hASCs [human ASCs (adipose-derived stem cells)] to differentiate into muscle was demonstrated *in vitro* (Lee and Kemp, 2006; Rogriguez et al., 2006). *In vivo* studies showed that implantation of ASCs in *mdx* mice restored dystrophin expression in the dystrophic mouse cells (Rodriguez et al., 2005).

In the present study, we explore the concept of using ASCs to restore dystrophin expression, but in the context of human DMD muscle cells. We also demonstrate that the participation of ASCs in human myogenesis occurs through cellular fusion.

Results

Characterization of ASCs

ASCs from six unrelated donors were characterized by flow cytometry for the expression of 12 cell surface proteins [HLA-DR (human leucocyte antigens, MHC class II), HLA-ABC (human leucocyte antigens, MHC class I), CD13, CD29, CD31, CD34, CD44, CD45, CD73, CD90, CD105 and CD117]. Cell viability was above 96% by Guava ViaCount reagent (Guava Technologies).

At passage 4, hASCs did not express either endothelial markers (CD31-PECAM1) or haematopoietic markers (CD34, CD45 and CD117-c-kit). Most of the ASCs expressed high levels of CD13, CD44, adhesion markers (CD29-integrin β1 and CD90-Thy-1) and mesenchymal stem cell marker CD73 [SH3 domain (Src homology 3 domain)]. Expression of some markers, such as CD105 (SH2), was variable among the donors. ASCs were negative for HLA-class II (HLA-DR), but positive for HLA-class I (HLA-ABC) (Figure 1).

The plasticity of ASCs was assessed 3 weeks after lineage induction. Myogenic, adipogenic, chondrogenic and osteogenic differentiations were demonstrated by the expressions of myogenic markers (myosin and desmin), lipid vacuoles, mucopolysaccharide-rich extracellular matrix and calcium deposits respectively (Figure 2). These results confirmed the mesenchymal nature of the isolated cells as well as their multipotent potential.

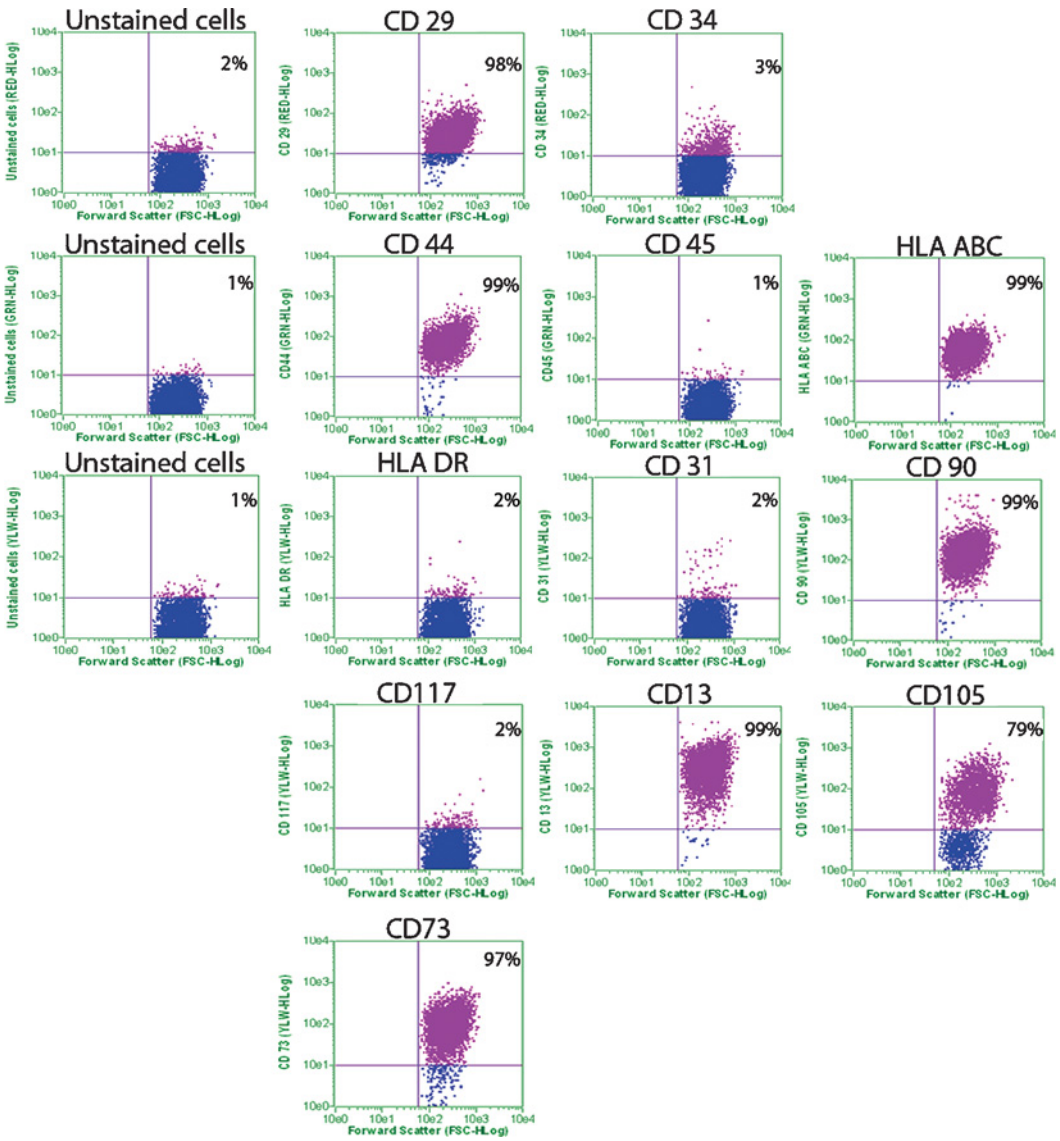
Spontaneous myogenic differentiation of ASCs

The undifferentiated control ASC cultures were maintained in GM (growth medium) to preserve their multipotent capacity (Zuk et al., 2001). Surprisingly, we observed spontaneous fusion between ASCs and the formation of myotube-like cells (Figures 3B and 3E) in the control culture plates from two of the donors (fresh or at passages 1 and 2). However, differentiation was not observed in cultures maintained in a low cell density (Figure 3A).

Immunofluorescence confirmed the expression of myosin in the differentiated ASC culture (Figure 3D). RT (reverse transcription)–PCR revealed the expression of MyoD, telethonin and dystrophin in first passage/high density culture but not at first passage/low density nor in passage 4/high density cultures (Figure 3F). Western blot confirmed the presence of dystrophin in the differentiated cells (Figure 3G). Since uncultured ASCs do not show expression of these markers (Figures 3F and 3G), these results suggest that ASCs when submitted to culture

Figure 1 | Immunophenotyping of ASCs at passage 4

Values represent the mean percentage of positively stained cells as analysed by flow cytometry. Graphs of forward scatter against fluorescence intensity of the indicated antigen are shown.



conditions in earlier passages and in high density show autonomous myogenic potential and can spontaneously differentiate into skeletal muscle. On the other hand, since we observed such a spontaneous differentiation only in the initial passages and not in later stages, even at a high cell density (Figure 3C), we decided to use ASCs at passages 4 or later in the co-culture experiments.

ASCs co-cultured with primary DMD myoblasts differentiate into dystrophin-positive myotubes

To test whether the ASCs were able to interact with DMD myoblasts and acquire a myogenic phenotype, we co-cultured equal amounts of ASCs expressing GFP (green fluorescent protein) along with primary, GFP-negative DMD myoblasts previously stained with DAPI (4′,6-diamidino-2-phenylindole;

Figure 2 | Differentiation potential of ASCs at passage 4

(**A**) The adipogenic differentiation was detected by the formation of intracytoplasmic lipid droplets stained with Oil Red O. Scale bar, 200 μm. (**A′**) Undifferentiated ASCs stained with Oil Red O. Scale bar, 200 μm. (**B**) Osteogenic differentiation was demonstrated by calcium deposition, which was shown by von Kossa stain. Scale bar, 200 μm. (**B′**) Undifferentiated ASCs stained with von Kossa. Scale bar, 200 μm. (**C**) Chondrogenic differentiation in monolayer culture was demonstrated by staining with Toluidine Blue. Scale bar, 200 μm. (**C′**) Undifferentiated ASCs stained with Toluidine Blue. Scale bar, 200 μm. (**E**, **F**) Myogenic differentiation was assessed by immunofluorescence. Induced cells were labelled with anti-human myosin monoclonal antibody (**E**) or with anti-human; desmin monoclonal antibody (**F**). (**E′**) Undifferentiated ASCs labelled with anti-human myosin monoclonal antibody. (**F′**) Undifferentiated ASCs labelled with anti-human desmin monoclonal antibody. Scale bar, 50 μm.

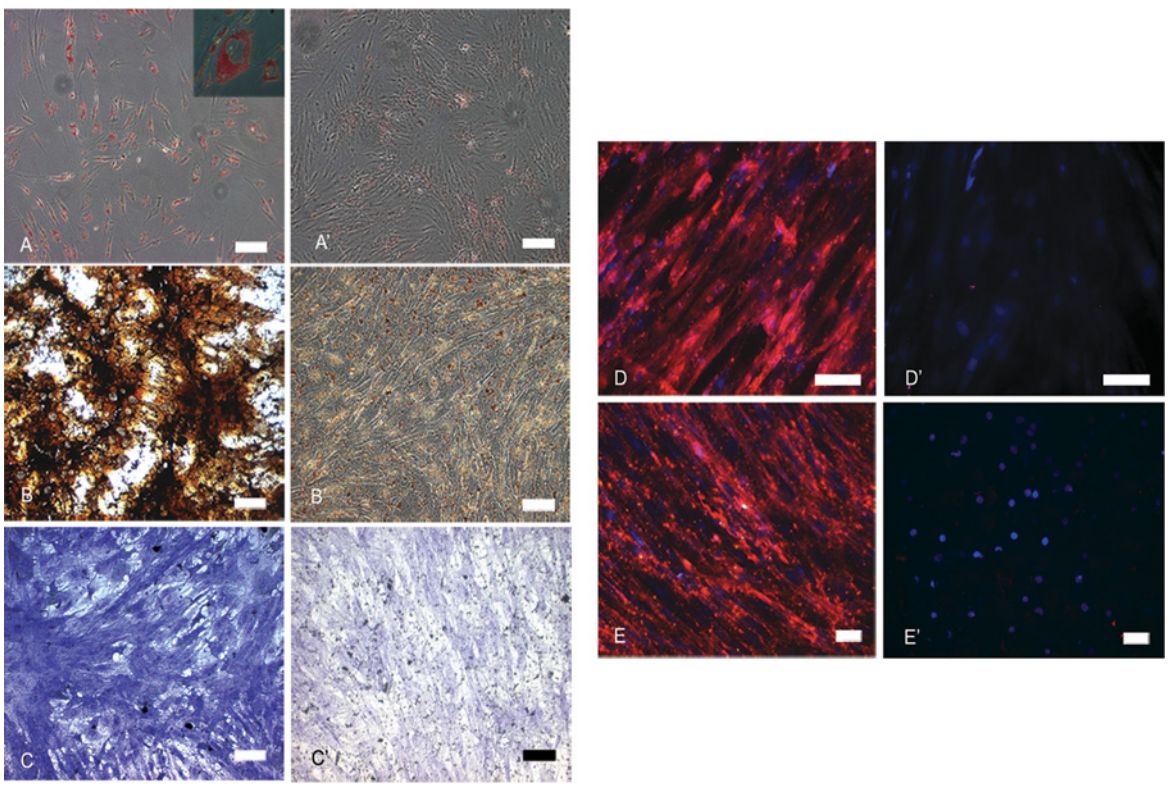
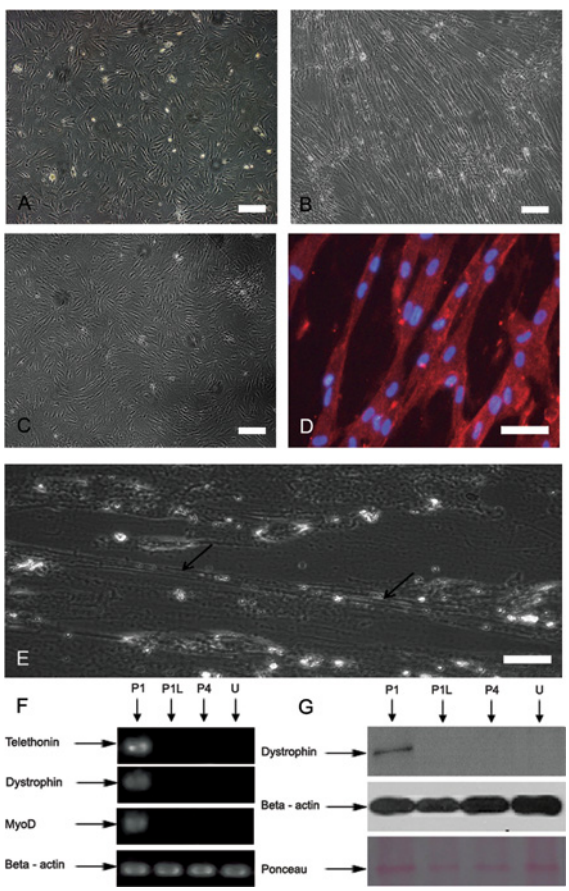


Figure 4A). The ASCs (passage 4) were stably transfected with GFP in order to distinguish them from DMD myoblasts in the co-cultures. After establishment of the mixed cultures, the cells were exposed to FM (fusion medium) that induces myoblasts to coalesce and form multinucleated structures. After 10 days, multinucleated myotubes were observed with areas of GFP-positive syncytia in the resulting cultures (Figure 4B). Controls containing exclusively GFP-positive ASCs subjected to the same experimental conditions did not contain any multinucleated structures, whereas syncytium formation in cultures of DMD myoblasts occurred to the same extent as in the mixed cultures.

To evaluate whether ASCs contributed to the pool of human myotubes by differentiation, fusion, or both, the co-culture was kept under myogenic differentiation conditions and visualized through direct fluorescence microscopy analysis (Figure 4C). We concluded that ASCs participate in the generation of human myotubes through cellular fusion, because all GFP-positive syncytia presented at least one DAPI-stained nucleus.

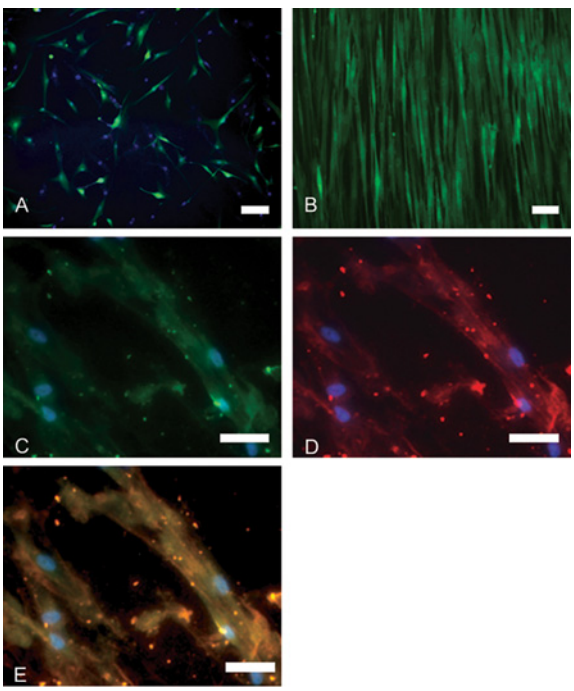
To confirm that the fluorescently labelled multinucleated structures were expressing the heretofore absent dystrophin, an immunofluorescence assay was performed for this protein after 45 days (Figure 4D). The specificity of this assay was corroborated by the

Figure 3 | Spontaneous differentiation
(**A**) Typical morphology of ASCs at passage 1/low-density culture. Scale bar, 200 μm. (**B**) ASCs at passage 1/high-density culture show the characteristic pattern of muscle fibres. Scale bar, 200 μm. (**C**) Spontaneous differentiation was not observed in passage 4/high-density culture. Scale bar, 200 μm. (**D**) Expression of myosin in passage 1/high-density culture. Scale bar, 50 μm. (**E**) Phase-contrast microscopy image showing that at passage 1/high-density culture, ASCs fuse, forming myotube-like structures (arrows). Scale bar, 200 μm. (**F**) Gene expression of skeletal-muscle markers at passage 1/high-density (P1), passage 1/low-density (P1L), passage 4 (P4) and uncultured ASCs (U) determined by RT–PCR using β-actin as a control gene. (**G**) Dystrophin expression confirmed by Western-blot analysis.



absence of staining ASCs and DMD myotubes. No revertant fibres that spontaneously expressed dystrophin were found in the DMD myotube cultures. Merging the DAPI, GFP and Dys1-specific signals revealed that the GFP-positive myotubes, containing DAPI-stained nuclei, were expressing dystrophin

Figure 4 | ASCs co-cultured with primary DMD myoblasts
(**A**) DMD myoblasts stained with DAPI and GFP-positive ASCs on the first day in culture. Scale bar, 200 μm. (**B**) GFP-positive fibres observed after 10 days in culture. Scale bar, 50 μm. (**C**) GFP-positive myotubes. Scale bar, 50 μm. (**D**) Expression of dystrophin in myotubes containing blue DMD nuclei by immunofluorescence. Scale bar, 50 μm. (**E**) Merged image confirming the fusion between the two cell types. Scale bar, 50 μm.



(Figure 4E). These results demonstrated that ASCs can contribute to the generation of human myotubes in the presence of differentiating myoblasts.

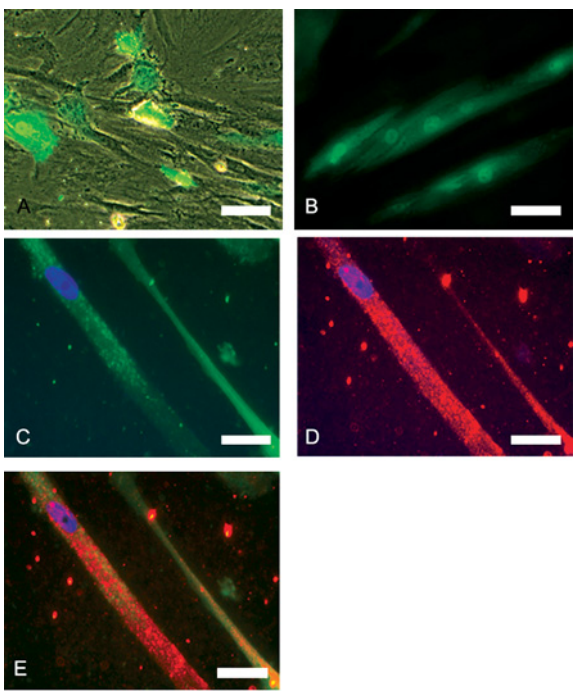
ASCs plated on DMD myotubes restore dystrophin expression

In order to investigate the behaviour of ASCs in the presence of DMD myotubes, we differentiated DAPI-stained DMD myoblasts into myotubes and added undifferentiated GFP-positive ASCs to the myotube cultures. The co-cultures were maintained only with GM to exclude the possibility of myogenic induction by the medium.

After 1 h of co-culture, the ASCs adhered to the DMD myotubes (Figure 5A). GFP-positive myotubes were found after 10 days (Figure 5B). Immunofluorescence showed GFP-positive myotubes containing DAPI-stained nuclei and expressed dystrophin on

Figure 5 | ASCs plated on DMD myotubes

(**A**) GFP-positive ASCs adhered to the DMD myotubes after 1 h. Scale bar, 50 μm. (**B**) GFP-positive fibres observed within 10 days in culture. Scale bar, 50 μm. (**C**) GFP-positive myotubes. Scale bar, 50 μm. (**D**) Expression of dystrophin in myotubes containing blue DMD nuclei by immunofluorescence. Scale bar, 50 μm. (**E**). Merged image confirming fusion between the two cell types. Scale bar, 50 μm.



day 45 (Figure 5C), which was not limited to the proximity of the normal nuclei (Figure 5D). This observation demonstrates that ASCs are able to fuse to DMD myotubes and restore dystrophin expression.

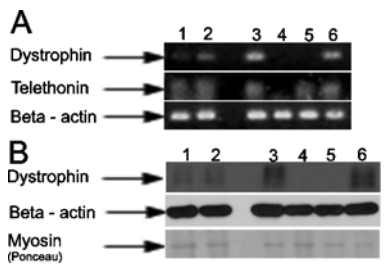
Dystrophin expression through RT–PCR and Western-blot analyses

Dystrophin expression was evaluated by RT–PCR. In order to assess whether the expression of dystrophin was proportional to the amount of ASCs present in the co-culture, we plated equal proportions of DMD myoblasts and ASCs. In this co-culture assay, we observed a greater dystrophin expression than that found when the ratio of ASCs to plated myoblasts was 1:3 (Figure 6A).

In addition, the co-culture of ASCs with normal myoblasts showed a dystrophin expression similar to that found in normal myotubes (Figure 6A). This indicates that ASCs, when differentiated into muscle

Figure 6 | Gene expression in the co-culture experiments

Samples shown are the following: (1) co-culture of DMD myoblasts/GFP-positive ASCs at a ratio of 1:1. (2) Co-culture of DMD myotubes/GFP-positive ASCs at a ratio of 1:3. (3) Co-culture of normal myoblasts/GFP-positive ASCs at a ratio of 1:1. (4) GFP-positive ASCs. (5) DMD myotubes. (6) Normal myotubes. (**A**) Dystrophin and telethonin gene expressions determined by RT–PCR by using β-actin as a control gene. (**B**) Dystrophin expression confirmed by Western-blot analysis.



cells, can express dystrophin at the same level of differentiated myoblasts.

To determine whether ASCs express skeletal-muscle proteins, we analysed the expression of telethonin, which is expressed exclusively in skeletal-muscle fibres (Moreira et al., 2000). We observed that the telethonin expression in the co-cultures was comparable with that found in normal controls or DMD myoblasts cultures. This result indicates that these cells can differentiate into skeletal-muscle cells (Figure 6A).

Finally, we analysed the dystrophin protein levels in the two different co-cultivation assays. As seen in Figure 6(B), the level of dystrophin protein was quite similar as assessed by Western-blot analysis between the co-cultivation assays and as compared with normal myotubes. This indicates that the vast majority of co-cultivated cells, including ASCs, were expressing this protein.

Discussion

In response to muscle injury, muscle satellite cells are activated to become myogenic precursor cells. These cells divide and fuse to repair the damaged muscle. However, the mature muscle satellite cells represent only 1–5% of the total muscle cells and their potential for self-renewal decreases with age (Schultz and Lipton, 1982). In DMD patients, the intense degeneration that occurs in muscle fibres exhaust the ability

of satellite cells to proliferate and replace damaged fibres (Heslop et al., 2000).

The possibility of stem cell-mediated myogenesis is very exciting. It has been previously suggested that hMSC (human mesenchymal stem cells) from bone marrow and human circulating AC133$^+$ stem cells, when co-cultured with mouse skeletal myoblasts, form myotubes by fusion and commit functionally in the myogenic environment (Torrente et al., 2004; Schulze et al., 2005; Goncalves et al., 2006). However, obtaining cells from bone marrow is a painful procedure and yields a low number of hMSC.

Zuk et al. (2001) have shown that human stem cells derived from adipose tissue were able to differentiate along the myogenic lineage when exposed to inductive media. The cells expressed muscle-specific proteins and fused to form multinucleated myotubes when plated under promyogenic conditions (Mizuno et al., 2002). More recently, the potential of human PLA to differentiate into smooth-muscle cells in addition to a trilineage differentiation capacity was reported (Rodriguez et al., 2006).

It was also shown that mouse adipose tissue stem cells were able to differentiate into skeletal muscle and restore dystrophin expression in *mdx* mice (Di Rocco et al., 2006). On the other hand, ASCs when injected into *mdx* mice revealed dystrophin expression just at the site of injection (Rodriguez et al., 2005). The authors suggest two mechanisms to account for the contribution of ASCs to muscle regeneration: *de novo* generation of muscle-specific cells from ASCs or modification in gene expression after direct fusion of ASCs with host cells. The relative contribution of transdifferentiation compared with nuclear fusion in the long-term engraftment of ASCs into the host muscle was not investigated by these authors.

However, to the best of our knowledge, the interaction between human adipose stem cells and human skeletal-muscle cells *in vitro* has not yet been reported. In the present study, we first co-cultured human ASCs with human DMD myoblasts or DMD myotubes and assessed whether dystrophin expression was restored *in vitro*. We observed that ASCs were able to fuse into myoblasts as well as myotubes and express dystrophin in both situations. Whether ASCs directly fuse to DMD muscle cells or first acquire muscle progenitor phenotype remains to be investigated.

We found that human ASCs show spontaneous skeletal-muscle differentiation in early passages when cultured in high cell density without any induction, as observed for mouse ASCs (Di Rocco et al., 2006). Therefore we decided to use ASCs in passage 4 or later for co-culture experiments. Apparently, sequential passages favour hMSC expansion since we did not find any spontaneous differentiation after passage 4.

Assessment of dystrophin and telethonin expression in co-cultures of DMD myoblast/ASCs as compared with normal myoblast/ASCs suggests that most of the ASCs, under the co-culture conditions used in the present study, were involved in myotube formation.

Previous studies reported that ASCs are composed of heterogeneous cell populations including blood-derived cells, endothelial, pericytes and other progenitors (Yoshimura et al., 2006). According to Sampaolesi et al. (2006), transplantation of mesoangioblasts, a class of vessel-associated stem cells, into dystrophic dogs can achieve an extensive reconstitution of the muscle with up to 70% of total fibres expressing dystrophin. ASCs present similarities to mesoangioblasts. Both types of cells show fibroblast-like morphology, proliferate efficiently and display a similar surface protein profile. However, while mesoangioblasts were isolated from the outgrowth of small, vessel-containing, tissue fragments from muscle biopsies, which are not easily obtainable, human liposuctioned fat is available in large quantities. If the *in vivo* potential of ASCs is similar to that found with mesoangioblasts, it might be an ideal source of stem cells for therapeutic applications.

Our *in vitro* results reinforce the observation that ASCs are able to restore dystrophin expression in *mdx* mice (Rodriguez et al., 2005) and support the idea that ASCs could be an important source of muscle cell therapy. In short, we demonstrated here that ASCs, when co-cultured with primary DMD myoblasts, fuse and generate dystrophin and skeletal myotubes. We also show that ASCs plated on DMD myotubes fuse into muscle cells and re-establish dystrophin expression. This suggests that cellular fusion of ASCs with muscle cells takes place during the process of syncytium formation.

The *in vitro* expression of dystrophin supports the hypothesis that ASC cells have the potential to be used for DMD therapy. Adipose tissue is abundant and liposuction procedures are relatively safe. Therefore the possibility of using adipose tissue as a source of stem cell therapies not only for DMD but also

for different muscular diseases is extremely exciting. However, further *in vivo* studies, which are currently under way, will be essential for identifying the factors determining their definitive myogenic differentiation and 'homing' as well as potential clinical effects in animal models before any therapeutic trial in DMD patients.

Materials and methods

All experiments were approved by the research ethics committee of the Biosciences Institute, University of São Paulo. All the present studies were performed in the Human Genome Research Center, at the Biosciences Institute, University of São Paulo.

Myoblasts cell culture and differentiation

The human DMD skeletal-muscle cells were obtained from primary cultures of muscle biopsies taken for diagnostic purposes, following informed consent. Normal skeletal muscle was obtained from healthy DMD fathers. The biopsies were processed according to protocol 11.4 described in Freshney (2000).

The myoblasts were cultured in DMEM-HG (Dulbecco's modified Eagle's medium with high glucose; Gibco) supplemented with 20% (v/v) FBS (fetal bovine serum; Gibco), 100 units/ml of penicillin and 100 g/ml of streptomycin (Gibco). Cells were maintained at 37°C and 5% CO_2. To differentiate human myoblasts into myotubes, the cells were rinsed twice in PBS and cultured in FM [DMEM-HG containing 3% (v/v) HS (horse serum; Gibco)] for 1 week.

ASC isolation and expansion

Human adipose tissue was obtained from elective liposuction procedures. Cells were isolated using methods previously described (Gimble and Guilak, 2003). Briefly, the unprocessed lipoaspirate was washed extensively with equal volumes of PBS containing antibiotics (100 units/ml of penicillin and 100 g/ml of streptomycin) and then dissociated with 0.075% collagenase (Sigma). Enzyme activity was neutralized with DMEM-HG containing 10% FBS. The infranatant was centrifuged at 1200 ***g*** for 5 min to pellet the cells. The cells from the pellet SVF were filtered to remove debris and seeded on to tissue culture plates (Nunc) at 1000–3500 cells/cm^2 in DMEM-HG containing 10% FBS. Cultures were washed with PBS 24–48 h after plating to remove unattached cells and fed with fresh medium.

The cultures were maintained at 37°C with 5% CO_2, in GM (DMEM-HG containing 10% FBS). When they achieved approx. 70% confluence, the cells were trypsinized (0.025%; Invitrogen) and plated at a density of 5000/cm^2. Cultures were passaged repeatedly after achieving a density of 70–80% until passage 4. The remaining cells were cryopreserved in cryopreservation medium (10% DMSO, 10% DMEM-HG and 80% FBS), frozen at −80°C in a cryo 1°C freezing container (Nalgene) and stored in liquid nitrogen the next day.

Multilineage differentiation

Cells were analysed for their capacity to differentiate towards adipogenic, osteogenic, chondrogenic and myogenic lineages as described by Zuk et al. (2001).

Adipogenic differentiation

Subconfluent cells were cultured in GM supplemented with 1 μM dexamethasone (Sigma), 500 μM IBMX (isobutylmethylxanthine; Sigma), 60 μM indomethacin (Sigma) and 5 μg/ml insulin (Sigma). Adipogenic differentiation was confirmed on day 21 by intracellular accumulation of lipid-rich vacuoles stainable with Oil Red O (Sigma). For the Oil Red O stain, cells were fixed with 4% (w/v) paraformaldehyde for 30 min, washed and stained with a working solution of 0.16% Oil Red O for 20 min.

Chondrogenic differentiation

Subconfluent cells were cultured in chondrogenic differentiation medium consisting of DMEM with low glucose supplemented with 100 nM dexamethasone, 50 μM ascorbic acid-2 phosphate (Sigma), 1 mM sodium pyruvate (Gibco), 10 ng/ml TGF-β1 (transforming growth factor-β1; R&D Systems) and 1% ITS-Premix (Becton Dickinson). Medium was changed every 3–4 days, and cells were fixed on day 21 with 4% paraformaldehyde. Chondrogenesis was demonstrated by staining with Toluidine Blue.

Osteogenic differentiation

To promote osteogenic differentiation, subconfluent cells were treated with GM supplemented with 50 μM ascorbate-2 phosphate, 10 mM β-glycerophosphate (Sigma) and 0.1 μM dexamethasone for 21 days. Osteogenesis was demonstrated by the accumulation of mineralized calcium phosphate, which was shown by von Kossa stain. Briefly, cells were stained with 1% silver nitrate (Sigma) for 45 min under UV light, followed by 3% sodium thiosulfate (Sigma) for 5 min, and then counterstained with van Gieson.

Myogenic differentiation

For myogenic differentiation, ASC cells passage 4 were cultured in GM supplemented with 0.1 μM dexamethasone (Sigma), 50 μM cortisol (Sigma) and 5% HS (Gibco) for 45 days.

Flow cytometry

Early passages from six different donors (aged 19–40 years) were evaluated for cell surface protein expression using flow cytometry. The flow cytometry was performed on a Guava EasyCyte system (Guava Technologies) using a blue laser (488 nm). Cells were pelleted, resuspended in PBS at a concentration of 1×10^5 cells/ml and stained with saturating concentration of antibodies. Cells were incubated in the dark for 45 min at room temperature (20°C). After incubation, cells were washed three times with PBS and resuspended in 0.25 ml of cold PBS. Cell viability was assessed with Guava ViaCount reagent (Guava Technologies).

ASCs were incubated with the following primary antibodies: HLA-DR-PE, HLA-ABC-FITC, CD13-PE, CD29-PECy5, CD31-PE, CD34-PerCP, CD44-FITC, CD45-FITC, CD73, CD90-PE, CD105 and CD117-PE (Becton Dickinson) for 45 min. Unconjugated markers were reacted with anti-mouse PE secondary antibody (Guava Technologies) for 15 min.

Flow cytometer gates were set using unstained cells. Cells were gated by forward scatter to eliminate debris. To eliminate the possible autofluorescence of ASCs, we removed the contribution of unstained cells in the measurement channel. A minimum of 10 000 events was counted for each analysis.

ASCs transduction with lentivirus vector

Supernatant containing the FUGW lentiviral vector (Lois et al., 2002) was produced as described previously (Strauss et al., 2006) and concentrated by ultracentrifugation. Undifferentiated ASCs at passage 2 were incubated at 37°C, in a 6-well plate (Nunc), using a minimal volume of GM in the presence of vector particles [20 pfu (plaque-forming units) per cell] and 8 μg/ml Polybrene (Sigma). After 4 h, 2 ml of GM was added and the medium was changed on the next day. At 72 h post-transduction, transgene expression was examined by flow cytometry. Approx. 80% of the cells were GFP-positive, and GFP expression did not decline during culture passages. To evaluate whether GFP interfered with the multipotent capacity of ASCs, both GFP-positive and -negative cells at successive passages were analysed by flow cytometry and multilineage differentiation.

Co-cultures

Two different types of co-culture were tested. In the first, we mixed equal amounts of GFP-negative myoblasts stained with DAPI and GFP-positive ASCs plated at a density of 5000 cell/cm^2 on culture dishes in GM. After 90% confluence was reached, the cultures were washed with PBS and fed with FM to promote myotube differentiation. Fresh medium was added every 2–3 days for 6 weeks.

In the second type of co-culture, DMD myotubes stained with DAPI were cultured and the GFP-positive ASCs were added to the myotube cultures at a ratio of 3:1 of plated myoblasts and ASCs. The co-cultures were maintained in GM, and fresh medium was added every 2–3 days for 6 weeks.

For co-culture experiments, we used DMD muscle cells from patients with known deletions, which allowed the use of primers containing the mutation and quantify the mRNA of the dystrophin gene by RT–PCR.

Co-culture control experiments were: GFP-positive ASCs maintained undifferentiated; normal control myoblasts co-cultured with GFP-positive ASCs; normal control myoblasts; and DMD myoblasts. The myoblast controls were exposed to FM as the co-culture experiments.

The visualization of ASC cell differentiation and their identification in the myoblasts co-cultures were performed with GFP transduction since it has been shown that GFP did not influence the multilineage potential of human ASCs (Lin et al., 2006) although it had been reported that GFP might impair actin–myosin interaction in muscle cells (Agbulut et al., 2006).

RNA isolation and RT–PCR

Total RNA was harvested from cultured cells using TRIzol® (Invitrogen) following the manufacturer's instructions. The RNA was treated with DNase (Invitrogen). A total of 5 μg of total RNA was reverse-transcribed with a SuperScript™ III First-strand Synthesis System (Invitrogen). All amplifications were performed in an MJ Research PTC-200 thermocycler (MJ Research) for 24 cycles after the initial 2 min denaturation at 94°C. The PCR primers are listed in Table 1. The PCR products were analysed by electrophoresis of 5 μl aliquots in 2% agarose gel, and the amplicons were visualized by ethidium bromide staining.

Immunofluorescence

Cells grown in a one-chamber slide (Nalgene; Nunc) were fixed in 4% paraformaldehyde in PBS for 20 min at 4°C and permeabilized in 0.05% Triton X-100 in PBS for 5 min. Non-specific binding was blocked with 10% FBS in PBS for 1 h at room temperature. Cells were incubated with primary antibody overnight at 4°C and with secondary antibody for 1 h at room temperature. The following primary antibodies were used: anti-desmin (1:100; Sigma), anti-myosin, skeletal (1:100; Sigma), NCL-Dys1 (1:20; Novocastra), NCL-Dys2 (1:20; Novocastra) combined with a rabbit anti-mouse IgG secondary antibody, Cy3-conjugated (1:100; Chemicon). The fluorescence signal was examined in Axiovert 200 (Carl Zeiss) and in an ApoTome imaging system (Carl Zeiss).

Table 1 | Oligonucleotide primer sequences and expected PCR product sizes

Gene	Oligonucleotide primers	Product size (bp)
Dystrophin		196
Sense	5′-ttgtcggtctcctgctggtcagtg-3′	
Antisense	5′-caaagccctcactcaaacatcaagc-3′	
MyoD		430
Sense	5′-cgatataccaggtgctctgaggg-3′	
Antisense	5′-gggtgggttacggttacacctgc-3′	
Telethonin		150
Sense	5′-ggctgctccctgcatga-3′	
Antisense	5′-ctggtagggcagctggta-3′	
β-Actin		242
Sense	5′-gtggggcgccccaggcacca-3′	
Antisense	5′-ccccccctgaaccccaaggccaa-3′	

Western-blot analysis

Cells were harvested from culture samples and proteins were extracted by treatment with a buffer containing 10 mM Tris/HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100 and 60 mM octyl glucoside. Samples were centrifuged at 13 000 *g* for 10 min to remove insoluble debris. Soluble proteins were resolved by SDS/PAGE (6 or 13% gel) and transferred on to nitrocellulose membranes (Hybond; Amersham). All membranes were stained with Ponceau (Sigma) to confirm equal loading and transfer of protein. Blots were blocked for 1 h in TBST (Tris-buffered saline+Tween) containing 5% (w/v) non-fat dried skimmed milk powder and reacted overnight with the following primary antibodies against dystrophin: NCL-Dys1 (1:100; Novocastra), NCL-Dys2 (1:25; Novocastra) and anti-β-actin (1:10000; Sigma) kindly provided by Dr Patrícia Gama (Instituto Ciências Biomédicas, São Paulo, SP, Brazil). Blots were incubated for 1 h at room temperature with secondary antibodies and immunoreactive bands were detected with an ECL® chemiluminescence detection system (GE Healthcare).

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Capítulo 3

Expressão de proteínas musculares humanas após transplante de células estromais de tecido adiposo, sem imunossupressão, no camundongo distrófico *SJL*

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SJL dystrophic mice express a significant amount of human muscle proteins following systemic delivery of human adipose-derived stromal cells without immunosupression

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Abstract

Limb-girdle muscular dystrophies (LGMD) are a heterogeneous group of disorders characterized by progressive degeneration of skeletal muscle caused by the absence or defective muscular proteins. The murine model for Limb-Girdle Muscular Dystrophy 2B (LGMD2B), the *SJL* mice, carry a deletion in the dysferlin gene that causes a reduction in the protein levels to 15% of normal. The mice show muscle weakness that begins at 4–6 weeks and is nearly complete by 8 months of age. The possibility to restore the defective muscle protein and improve muscular performance by cell therapy is a promising approach for the treatment of LGMD or other forms of Progressive Muscular Dystrophies (PMD). Here we have injected human adipose stromal cells (hASCs) in the *SJL* mice, without immunosupression, aiming to assess their ability to: engraft into recipient dystrophic muscle after systemic delivery; form chimeric human/mouse muscle fibers; express human muscle proteins in the dystrophic host and improve muscular performance. We show for the first time that hASCs are not rejected after systemic injection even without immunosupression, are able to fuse with the host muscle, express a significant amount of human muscle proteins and improve motor ability of injected animals. These results may have important applications for future therapy in patients with different forms of muscular dystrophies.

Resumo

As distrofias musculares do tipo cinturas (*Limb-girdle muscular dystrophies*, LGMD) são um grupo heterogêneo de doenças caracterizadas por uma degeneração progressiva do músculo esquelético causada pela ausência ou defeito de proteínas musculares. O modelo murino da LGMD2B é o camundongo *SJL*. Estes carregam uma deleção no gene disferlina que provoca uma redução dos níveis de proteína a 15% do normal. Os *SJL* apresentam fraqueza muscular que começa a partir de 04-06 semanas de vida. A possibilidade de restaurar a expressão da proteína muscular deficiente e melhorar o desempenho muscular através de terapia celular é uma abordagem promissora para o tratamento de DMC ou outras formas de Distrofias Musculares Progressivas (DMP). Neste trabalho injetamos células estromais do tecido adiposo (*human Adipose-derived Stromal Cells* - hASCs) em camundongos *SJL*, sem imunossupressão, com o objetivo de avaliar a sua capacidade de: atingir o músculo distrófico após injeção sistêmica; formar fibras musculares quiméricas (humano/camundongo); expressar proteínas musculares humanas no músculo dos camundongos e melhorar o desempenho muscular. Nós mostramos, pela primeira vez, que hASCs não são rejeitados após a injeção sistêmica, mesmo sem imunossupressão, são capazes de fundir-se ao músculo do camundongo, expressar uma quantidade significativa de proteínas musculares humanas e melhorar a habilidade motora dos animais injetados. Esses resultados podem ter aplicações importantes para o tratamento futuro em pacientes com diferentes formas de distrofias musculares.

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SJL Dystrophic Mice Express a Significant Amount of Human Muscle Proteins Following Systemic Delivery of Human Adipose-Derived Stromal Cells Without Immunosuppression

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Key Words. Human adipose stromal cells • Xenotransplantation • Muscular dystrophy • Therapy

ABSTRACT

Limb-girdle muscular dystrophies (LGMDs) are a heterogeneous group of disorders characterized by progressive degeneration of skeletal muscle caused by the absence of or defective muscular proteins. The murine model for limb-girdle muscular dystrophy 2B (LGMD2B), the *SJL* mice, carries a deletion in the *dysferlin* gene that causes a reduction in the protein levels to 15% of normal. The mice show muscle weakness that begins at 4–6 weeks and is nearly complete by 8 months of age. The possibility of restoring the defective muscle protein and improving muscular performance by cell therapy is a promising approach for the treatment of LGMDs or other forms of progressive muscular dystrophies. Here we have injected human adipose stromal cells (hASCs) into the *SJL* mice, without immunosuppression, aiming to assess their ability to engraft into recipient dystrophic muscle after systemic delivery; form chimeric human/mouse muscle fibers; express human muscle proteins in the dystrophic host and improve muscular performance. We show for the first time that hASCs are not rejected after systemic injection even without immunosuppression, are able to fuse with the host muscle, express a significant amount of human muscle proteins, and improve motor ability of injected animals. These results may have important applications for future therapy in patients with different forms of muscular dystrophies. STEM CELLS *2008; 26:2391–2398*

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

INTRODUCTION

Progressive muscular dystrophies (PMDs) are a clinically and genetically heterogeneous group of disorders caused by deficiency or abnormal muscular proteins and characterized by progressive degeneration and loss of skeletal muscle. The limb-girdle muscular dystrophies (LGMDs) constitute a subgroup characterized by the involvement of the pelvic and shoulder girdle musculature. To date, 20 different forms have been identified: 7 with autosomal dominant LGMD1A to LGMD1G and 13 with autosomal recessive inheritance [1]. Among the autosomal recessive forms, one of the most prevalent is caused by mutations in the dysferlin gene resulting in two phenotypes: Miyoshi myopathy, which affects distal muscles at onset, with preferential early involvement of the gastrocnemius, and LGMD2B, with a more pronounced limb-girdle involvement [2]. Dysferlin expression is reduced or absent in these patients [3, 4].

A 171-bp deletion in the murine dysferlin gene was detected in a mouse model, the *SJL* mice, with a corresponding reduction in dysferlin levels to 15% of normal. The spontaneous myopathy of *SJL* mice begins at 4–6 weeks of age and is nearly complete by 8 months of age, showing a progressive inflammatory change in muscle [5]. The mutation in *SJL* mice is in-frame and is probably one of the reasons why its phenotype is milder than that of patients with dysferlinopathy, as in the case of Becker type muscular dystrophy compared with Duchenne type [6]. Adult skeletal muscle has the potential to regenerate new muscle fibers by activating a population of mononucleated precursors, which otherwise remain in a quiescent and nonproliferative state [7]. However, the continuous and gradual muscle degeneration in progressive muscular dystrophies leads to a depletion of satellite cells and, consequently, the capacity to restore the

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skeletal muscle is lost [8, 9]. The possibility of repairing the defective muscle through cell therapy is a promising approach for the potential treatment of PMDs.

Different sources of stem/progenitor cells (SCs) that show extended proliferation in vitro and also have the ability to generate normal muscle fibers in vitro and in vivo were recently described [10–17]. Recently reported were two different strategies attempting cell therapy for PMDs in a dystrophic dog: transplantation of autologous genetically corrected cells or transplantation of normal donor cells. Apparently, the second approach showed better results [13].

An abundant and accessible source of stem cells is adipose tissue. The ability of human adipose-derived stromal cells (hASCs) to differentiate into skeletal muscle when in contact with dystrophic muscle cells was demonstrated by us in vitro [18] and in vivo [19]. This last study showed that implantation of hASCs directly into the muscle of mdx mice restored dystrophin expression in the area nearby the injected place. However, the *mdx* mice have no evident muscular weakness and therefore are not a good model to assess potential functional effects of SC therapy.

Here we have injected hASCs, without any immunosuppression, into the *SJL* mouse model to assess whether hASCs cells (a) can reach and engraft into recipient dystrophic muscle cells after systemic delivery, without immunosuppression; (b) form chimeric human/mouse muscle fibers; (c) express human muscle proteins in the dystrophic host; and (d) improve muscular function. In addition, since it had been suggested that cells already committed to the myogenic phenotype, and therefore are able to autonomously differentiate into skeletal muscle in vitro, possess the highest regenerating potential in vivo when injected locally [20] we also analyzed the engraftment potential of hASCs previously exposed to myogenic induction media through systemic transplantation. We showed for the first time the successful engraftment and myogenic differentiation of undifferentiated hASCs through systemic delivery without immunosuppression, which resulted in a significant improvement in the muscular function of the injected mice.

MATERIALS AND METHODS

All experiments were approved by the research ethics committee of the Biosciences Institute, University of São Paulo. All human samples were obtained after written informed consent from the donors. All research was carried out in the Human Genome Research Center, at the Biosciences Institute, University of São Paulo.

ASC Isolation and Expansion

Human adipose tissue was obtained from elective liposuction procedures. Cells were isolated using methods previously described [18]. Briefly, the unprocessed lipoaspirate was washed extensively with equal volumes of phosphate-buffered saline (PBS) containing antibiotics (100 U/ml of penicillin and 100 g/ml of streptomycin; Gibco, Grand Island, NY, http://www.invitrogen.com) and then dissociated with 0.075% collagenase (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com). Enzyme activity was neutralized with Dulbecco's modified Eagle's media–high glucose (DMEM-HG–HG; Gibco) containing 10% fetal bovine serum (FBS; Gibco). The infranatant was centrifuged at 1,200*g* for 5 minutes to pellet the cells. The cells from the pellet were filtered to remove debris and seeded in tissue culture plates (Nunc, Rochester, NY, http://www.nuncbrand.com) at 1,000–3,500 cells per cm^2 in DMEM-HG 10% FBS. Cultures were washed with PBS 24–48 hours after plating to remove unattached cells and fed with fresh media.

The cultures were maintained at 37°C with 5% CO_2, in growth media (GM; DMEM-HG 10% FBS). When they achieved approximately 70% confluence, the cells were trypsinized (0.025%, TrypLE Express; Gibco) and plated at a density of 5,000 cells per cm^2. Cultures were passaged repeatedly after achieving a density of 70%–80% until passage 4. The remaining cells were cryopreserved in cryopreservation media (10% dimethylsulfoxide, 10% DMEM-HG, 80% FBS), frozen at −80°C in isopropanol-jacked closed container, and stored in liquid nitrogen the next day.

Flow Cytometry

The flow cytometry was performed on Guava EasyCyte System (Guava Technologies, Hayward, CA, http://www.guavatechnologies.com) using a blue laser (488 nm). Cells were pelleted, resuspended in PBS at concentration of 1×10^5 cells per milliliter, and stained with saturating concentration of antibodies. Cells were incubated in the dark for 45 minutes at room temperature. After incubation, cells were washed three times with PBS and resuspended in 0.25 ml of cold PBS. Cell viability was accessed with Guava ViaCount reagent (Guava Technologies).

ASCs were incubated with the following primary antibodies: HLA-DR-PE, HLA-ABC-FITC, CD13-PE, CD29-PECy5, CD31-PE, CD34-PerCP, CD44-FITC, CD45-FITC, CD73, CD90-PE, CD105-PE, and CD117-PE (Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com). Unconjugated markers were reacted with anti-mouse PE secondary antibody (Guava Technologies).

Flow cytometer gates were set using unstained cells. Cells were gated by forward scatter to eliminate debris. To eliminate the possible autofluorescence of ASCs, we removed the contribution of unstained cells in the measurement channel. A minimum of 10,000 events was counted for each analysis.

Multilineage Differentiation

Cells were analyzed for their capacity to differentiate toward adipogenic, osteogenic, chondrogenic, and myogenic lineages as described in Zuk et al. (2001) [11].

Myogenic Differentiation Induction

hASCs were cultured in GM supplemented with 0.1 μM dexamethasone (Sigma-Aldrich), 50 μM hydrocortisone (Sigma-Aldrich), and 5% horse serum (Gibco) for 10 days before transplantation to the *SJL* mice. At this stage, hASCs cells express *MyoD* [21].

Transplantation

SJL mice were purchased from the Jackson Laboratory (Bar Harbor, ME, http://www.jax.org). Animal care and experiments were performed in accordance with animal research ethics committee of the Biosciences Institute, University of São Paulo.

Two-month-old *SJL* mice were divided into three groups ($n = 7$) for the transplantation experiments (groups A, B, and C). Each animal was injected in the tail vein with 1×10^6 of either hASCs (A group) or myogenic-induced hASCs (B group). The animals were injected for 6 months, weekly in the first month and then monthly. Animals were analyzed blindly in these three groups (A, B, and C); the code for each of the mouse groups was disclosed only after completion of all the studies. Two months after the last cell transplantation, the animals were euthanized using a CO_2 chamber.

Human DNA Analysis

The DNA was obtained using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany, http://www1.qiagen.com). The presence of human DNA in the host samples were evaluated as described in Pelz et al. (2005) [22]. Centromeric region of human chromosome 7 and mouse chromosome 8 was amplified by polymerase chain reaction (PCR; 35 cycles, annealing at 59°C). The PCR products were separated by electrophoresis on 2% agarose gels and stained with ethidium bromide. Nonsaturated digital images were obtained using an ImageQuant imaging system (GE Healthcare, Little Chalfont, U.K., http://www.gehealthcare.com).

Immunofluorescence

Proximal and distal muscles of the foreleg and hind leg of *SJL* mice were removed and frozen in liquid N^2. Serial muscle cryosections

were fixed in 4% paraformaldehyde in PBS for 20 minutes at 4°C and permeabilized in 0.05% Triton X-100 in PBS for 5 minutes. Nonspecific binding was blocked with 10% FBS in PBS for 1 hour at room temperature. Muscle sections were incubated with primary antibody overnight at 4°C and with secondary antibody for 1 hour at room temperature. The following primary antibodies were used: anti-dysferlin NCL-hamlet (1:300; Novocastra Ltd., Newcastle upon Tyne, U.K., http://www.novocastra.co.uk) and anti-human dystrophin MANEX 12/16E2 G10 (1:100) [23], kindly provided by Dr. Glenn E. Morris at Center for Inherited Neuromuscular Diseases (Oswestry, Shropshire, U.K.), combined with Cy3-conjugated (1:200) or FITC-conjugated (1:100) rabbit anti-mouse IgG secondary antibody (Chemicon, Temecula, CA, http://www.chemicon.com). We visualized nuclei with 4′,6′-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). The fluorescence signal was examined in Axiovert 200 (Carl Zeiss, Jena, Germany, http://www.zeiss.com) and in AxioImager Z1 (Carl Zeiss).

Western Blot Analysis

Muscle sample proteins were extracted through treatment with a buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and 60 mM octyl glucoside. Samples were centrifuged at 13,000*g* for 10 minutes to remove insoluble debris. Soluble proteins were resolved by 6% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membranes (Hybond; Amersham Biosciences, Piscataway, NJ, http://www.amersham.com). All membranes were stained with Ponceau (Sigma-Aldrich) to evaluate the amount of loaded proteins. Blots were blocked for 1 hour in Tris-buffered saline Tween (TBST) containing 5% powdered skim milk and reacted overnight with the following primary antibodies: anti-mouse CD3 (1:1000; Becton, Dickinson and Company), anti-human dystrophin MANEX 12/16E2 G10 (1:100), and anti-dysferlin NCL-hamlet (1:25). Blots were incubated for 1 hour with secondary antibodies and immunoreactive bands were detected with enhanced chemiluminescence detection system (GE Healthcare).

Reverse Transcription Reactions and Quantitative Real-Time PCR

To test the presence of human *Dysferlin* in the host muscle, total RNA from skeletal gastrocnemius of all uninjected and injected with undifferentiated hASCs animals and controls (five normal Swiss mice and human muscle) was extracted. Total RNA (0.2 μg) was reverse transcribed into cDNA using Superscript III reverse transcription kit (Invitrogen, Carlsbad, CA, http://www.invitrogen.com). Quantitative real-time PCR was performed using 50 ng of cDNA and SYBR Green PCR master mix in an ABI Prism 7100 system (Applied BioSystems, Foster City, CA, http://www.appliedbiosystems.com). The PCR conditions were 94°C for 15 seconds, 58°C for 30 seconds, and 72°C for 30 seconds, for 40 cycles.

Since the host lacks a 171-bp region in the cDNA, primers were designed to amplify cDNA fragment from inside the *SJL* deleted region, amplifying the normal mice and the human samples. The endogenous control (β-actin) primers were designed to amplify *SJL*, mouse, and human cDNAs. The authenticity of the PCR products was verified by melt-curve analysis and agarose gel electrophoresis. Primers sequences were as follows: *Dysferlin*: sense: GCCTCTGGAGAAGGACCTAAAG and antisense: ACAGCGAGCCCCAAACTTG (accession no. NM_003494, GenBank); *Beta-actin*: sense: GGCTGTATTCCCCTCCATCG and antisense: CCAGTTGGTAACAATGCCATGT (accession no. NM_007393, GenBank).

Samples were run in triplicates, and the threshold suggested by the instrument software was used to calculate Ct. To normalize the readings we used Ct values from the β-actin as internal standard in each run, obtaining a delta Ct value for *dysferlin*.

Skin Transplantation

Allogeneic or syngeneic tail skin was grafted on the back of *SJL* or BALB/c recipients. Skin was removed from the donor tail, cut into 1-cm^2 pieces, and kept in PBS at room temperature until use. Grafts were placed on a bed prepared by removing an area on the back dermis of the recipient, sutured, and covered with plaster. Donor skin was considered rejected when 90% of the area was destructed.

Functional Assessment

To verify whether injected hASCs would improve motor ability in *SJL* injected mice, we performed motor ability tests before and after 6 months of SC injection period. Mice were examined, weighed, and submitted to the following tests: (a) the inclined plane test measured the maximal angle of a wood board on which the animal was placed until it slipped; (b) the wire hanging test determined the ability of the mouse, suspended on a horizontal thread by its forelegs, to reach it with its hind legs and the length of time the mouse was able to stay hanging; (c) the ambulation test determined the mean length of a step measured in hind foot ink prints while mice freely ran in a corridor (length, 50 cm; width, 8 cm; height of lateral walls, 20 cm) [24].

Statistical Analysis

Observations were quantified blindly by two authors (N.M.V. and C.R.B.) independently from one another. Numerical data are the mean ± SD. The statistical analysis of the equivalence between the injected and uninjected mice was achieved by the one-tailed Student's *t* test, at the significance level of $p = .05$, and the results were expressed by the percentage variation between their performance before and after SC transfer period.

RESULTS

Characterization of ASCs

ASCs were previously characterized [18] by flow cytometry for the expression of 12 cell surface proteins (HLA-DR, HLA-ABC, CD13, CD29, CD31, CD34, CD44, CD45, CD73, CD90, CD105, and CD117). Cell viability was above 96% by Guava ViaCount reagent.

At passage 4, hASCs did not express endothelial markers (CD31-PECAM1) or hematopoietic markers (CD34, CD45, and CD117-c-kit). The majority of ASCs expressed high levels of CD13, CD44, adhesion markers (CD29-integrin β1, CD90-Thy-1), and mesenchymal stem cell marker CD73 (SH3). Expression of some markers, such as CD105 (SH2), was variable among the donors. ASCs were negative for HLA-class II (HLA-DR), but positive for HLA-class I (HLA-ABC) (data not shown).

The plasticity of hASCs was assessed 3 weeks after lineage induction [18]. Myogenic, adipogenic, chondrogenic, and osteogenic differentiation was demonstrated by the expression of myogenic markers (myosin and desmin), lipid vacuoles, mucopolysaccharide-rich extracellular matrix, and calcium deposits, respectively. These results confirmed the mesenchymal nature of the isolated cells as well as their multipotent potential (data not shown).

hASC Capacity to Reach and Engraft at the Host Muscle

To assess the potential of hASCs to reach and colonize the host muscle we injected undifferentiated hASCs, previously characterized by flow cytometry and differentiation potential [17], into the caudal vein of *SJL* mice ($n = 7$). The PCR method as previously reported by Pelz et al. (2005) [22] was used to evaluate the presence of human cells in different tissues of the injected *SJL* mice. Human DNA was found in the foreleg and hind leg muscles of all seven injected mice (Fig. 1A). One among the seven animals showed human DNA only in the proximal muscles, whereas in the remaining six the cells were found in both distal and proximal muscles. Among other analyzed tissues, human DNA was found in the liver, lung, and kidney of the injected animals (Fig. 1B).

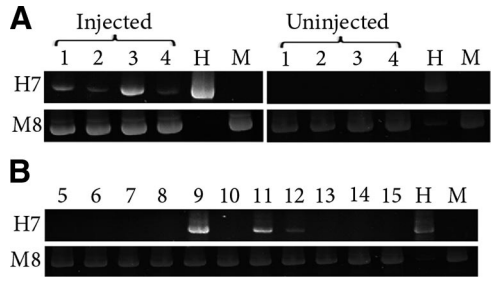

Figure 1. Polymerase chain reaction analysis for human chromosome 7 α-satellite sequences (H7) and mouse chromosome 8 centromeric repeat sequence (M8) of *SJL* mice. **(A):** Muscles of the injected and uninjected *SJL* mice. **(B):** Various tissues of the injected animal. Tissues were harvested at day 60 after injection. Samples shown are the following: (1) distal foreleg muscle; (2) proximal foreleg muscle; (3) distal hind leg muscle; (4) proximal hind leg muscle; (5) intestine; (6) urinary bladder; (7) heart; (8) brain; (9) liver; (10) spleen; (11) lung; (12) kidney; (13) inguinal fat; (14) tail; (15) diaphragm; (H) human DNA; and (M) mouse DNA.

Muscle Differentiation in the Host Muscle

To explore the myogenic differentiation followed by the engraftment of hASCs we analyzed the expression of dysferlin and human dystrophin in the host muscle.

The NCL-hamlet antibody against dysferlin showed poor labeling on human and mouse muscle sections through immunofluorescence (IF) analysis (Fig. 2A) and it also recognizes the *SJL* mutant dysferlin in IF (Fig. 2A) and Western blot (WB) analysis (Fig. 2C). Therefore, we evaluated dysferlin expression through real-time PCR. The expression of exogen dysferlin was variable among the seven injected animals, ranging from 14%–26% (Fig. 3).

To evaluate the presence of human muscle proteins in the host muscle, we assessed the presence of human dystrophin, using human dystrophin antibody [23]. Through WB analysis, human dystrophin bands were found in the proximal and distal muscles of foreleg and hind leg of the injected animals (Fig. 2B). IF analysis revealed that in the hASC-injected mice, approximately 50% $\pm$ 2% ($p = 3.623\ \mathrm{E}{-13}$, Student's t test, $n = 7$) of the fibers showed a positive labeling, whereas no positive labeling of fibers was observed in the muscles of noninjected animals (Fig. 2A and Fig. 4A). These labeled fibers were seen both in clusters and spread out through the muscle tissue, with some totally labeled and others partially positive for human dystrophin (Fig. 4B, 4C).

Capacity of hASCs to Reach and Engraft at the Host Muscle After Myogenic Commitment

To assess whether cells already committed to the myogenic phenotype possess a higher or lower regenerating potential in vivo, we evaluated the effect of in vitro differentiation in the migration ability of hASCs in a second group of animals. In seven mice hASCs were transplanted following in vitro exposure to myogenic differentiation media. Interestingly, after myogenic induction, we did not find human DNA in the muscle of any of the injected animals (supplemental online Fig. 1).

Lymphocyte T Infiltration

The evaluation of lymphocyte T infiltration in the host muscle expressing human muscle proteins was performed by WB analysis for anti-CD3. Positive bands were detected in both injected and uninjected animals with different intensities in samples from both groups. These data indicate that the presence of hASCs neither reduces nor increases the inflammatory changes typically found in the *SJL* dystrophic muscle (supplemental online Fig. 2).

Skin Graft Survival

To evaluate whether *SJL* naïve mice are immunocompetent, we performed allogeneic skin transplantation and tested whether these mice were able to reject allografts. Our data show that *SJL* mice rejected skin from C57Bl/6 donors at the same period as immunocompetent BALB/c mice. Acceptance of syngeneic skin grafts suggests that *SJL* mice are also capable of maintaining tolerance to self-antigens (supplemental online Fig. 3). Together these data indicate that these mice are capable of driving normal immune responses as well as modulating reactions toward self.

Functional Assessment

We performed standardized motor ability tests [24] and compared the performance of each *SJL* mouse (injected and uninjected) before (2 months of age) and after (9 months of age) the injection period. The treated group showed an improvement of 15.2% $\pm$ 7.0% in their performance, whereas the untreated worsened 6.12% $\pm$ 6.0% in their performance ($p = .013$, Student's t test, $n = 7$). The performance of the animals submitted to hASC transplantation after myogenic induction was very similar to the control group, with a 7.21% $\pm$ 7.0% ($p = .449$, Student's t test, $n = 7$) worsening of motor ability (Fig. 4 and supplemental online Videos 1–3).

Typical behavior of the *SJL* mice is observed when they are suspended by the tail. The first reflex of normal mice is to spread their limbs and digits while the trunk is held in an extended position. However, the *SJL* mice, in contrast, keep their limbs in an adducted and flexed position and tend to curl in on themselves, grasping at their own fur. They often fail in attempts to extend their trunk and they are not able to turn around and reach the suspending hand, indicating trunk muscle weakness [5].

We also observed a different behavior in injected mice when they were suspended by the tail. Injected animals were able to spread their limbs and digits with their trunk held in an extended position, whereas the uninjected ones showed the typical reflex found in *SJL* mice (Fig. 5 and supplemental online Videos 4–6).

DISCUSSION

Two different studies reported that ASCs are able to differentiate in vivo when injected directly into the muscle of the *mdx* mice [19, 20]. However the potential of these cells to reach the muscle through systemic transplant as well as their potential functional effect in treated mice were not evaluated. The successful use of stem cells for clinical applications in cell therapy for PMDs requires the investigation of the capacity of these cells, after systemic injections, to reach the target, engraft, and restore the defective protein in the dystrophic muscle. A significant obstacle in designing cell therapy for PMDs is the necessity to reach the entire body musculature, a problem that cannot be easily overcome unless systemic cell delivery methods are proved effective.

Here we show for the first time that systemic delivery of hASCs into the tail vein of the *SJL* mice resulted in human dystrophin and dysferlin expression in the host muscles and amelioration of functional parameters in the treated animals after 6 months of injections. In addition, we demonstrate that these positive results were obtained without the use of any immunosuppression, which has been reported by local injection of hASCs into the mdx mouse muscle [19] but is described here for the first time with systemic transplantation.

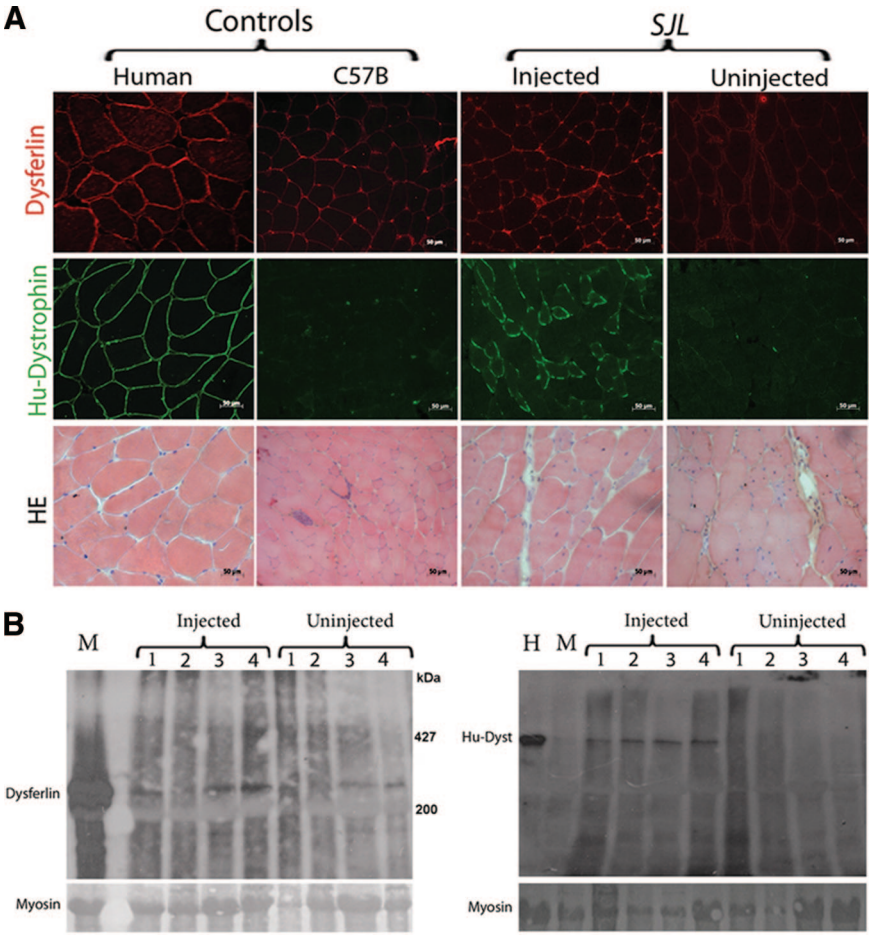

Figure 2. Expression of dysferlin and human dystrophin in the muscle from human and mouse (C57B) normal controls, in one *SJL* mouse of the injected and one of the uninjected group. **(A):** Immunofluorescence analysis of dysferlin and human dystrophin (Hu-Dystrophin) of the gastrocnemius muscle of the injected and uninjected animal. Histopathological analysis in skeletal muscles from both injected and uninjected *SJL* mice showed comparable alterations including size variation among individual muscle fibers, fiber splitting, small regenerated basophilic fibers, numerous fibers with centrally located myonuclei, and significant connective tissue replacement. **(B):** Western blot analysis for human dystrophin and dysferlin of the muscles of injected and uninjected animal. Samples shown are the following: (1) distal foreleg muscle; (2) proximal foreleg muscle; (3) distal hind leg muscle; (4) proximal hind leg muscle; (myosin) myosin band detected in the Ponceau S prestained blot, for the evaluation of loaded muscle proteins; (H) human muscle; and (M) mouse muscle.

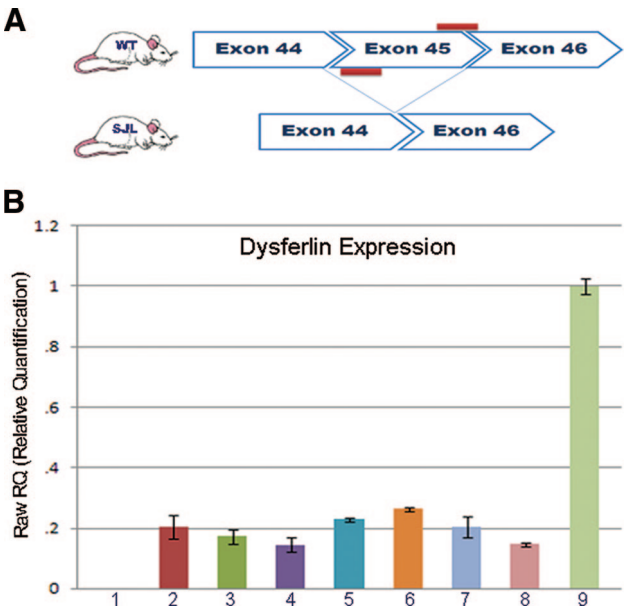

Figure 3. Quantitative real-time polymerase chain reaction analysis for exogen dysferlin expression. **(A):** Primers (in red) were designed to amplify cDNA fragment from inside the *SJL* 171-bp deleted region, amplifying the normal mouse and human samples. **(B):** Relative quantification of exogen dysferlin expression in all injected (2–8) and one uninjected animal (1) compared with normal Swiss mouse expression (9). Data are normalized to β-actin expression and expressed as means ± SEM. Abbreviation: WT, wild type.

Sampaolesi et al. (2006) [13] reported that systemic injections of normal dog mesoangioblasts to the muscle of dystrophic dogs resulted in the restoration of dystrophin expression. The mesoangioblasts show similarities with ASCs in cell surface protein analysis, proliferation, and differentiation capacity. However, all dogs that received a transplant were maintained on steroids as standard treatment and received immunosuppressant drugs, which makes it difficult to evaluate functional results, since it is known that immunosuppressive and anti-inflammatory drugs can ameliorate the phenotype in muscular dystrophy patients [35].

More recently, Benchaouir et al. (2007) [26] reported that exon-skipping-engineered Duchenne muscular dystrophy (DMD) stem cells restored human dystrophin when transplanted into dystrophic mice. In their experiment, the authors performed both intramuscular and intra-arterial delivery of genetically corrected CD133+ cells expressing myogenic progenitors isolated from the blood and muscle of DMD patients into the scid/mdx mice. According to the authors, a significant recovery of muscle morphology, function, and dystrophin expression was observed. These results show that autologous transplantation of muscle-derived CD133+ cells may represent a promising approach to treat DMD. However the authors highlighted that the use of gene therapy raises some problems, specifically in terms of biosafety, since they used lentivirus vectors to correct the genetic defect into DMD stem cells prior to in vivo transplantation. They also called the attention to the fact that hazardous integration of the provirus can theoretically disturb the control of some housekeeping genes or tumor suppressor genes, and for clinical purposes, this risk of developing a tumor after in vivo injection

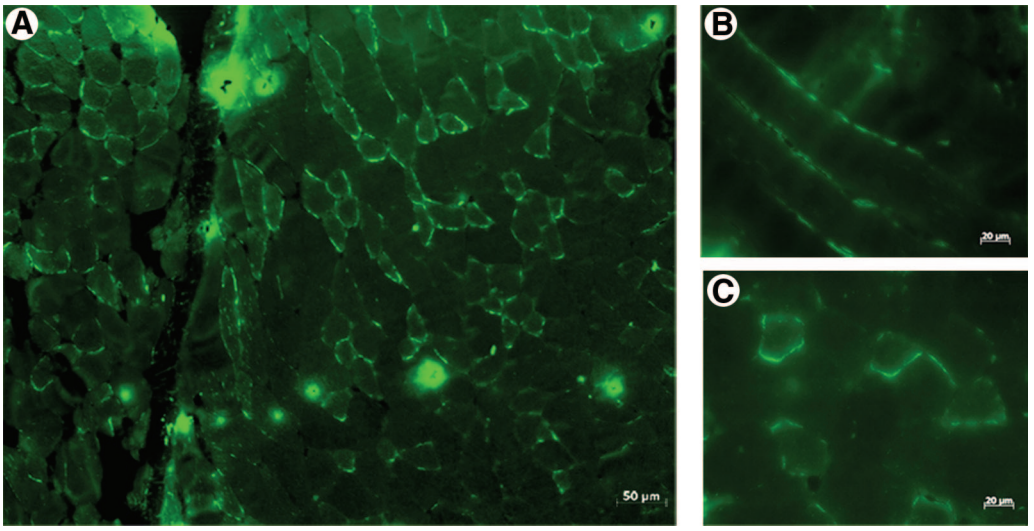

Figure 4. Human dystrophin positively labeled fibers in the gastrocnemius muscle of the injected *SJL* mice. **(A):** Global distribution of the positively labeled fibers and details of partially positive fibers in **(B)** longitudinal section and **(C)** transversal section.

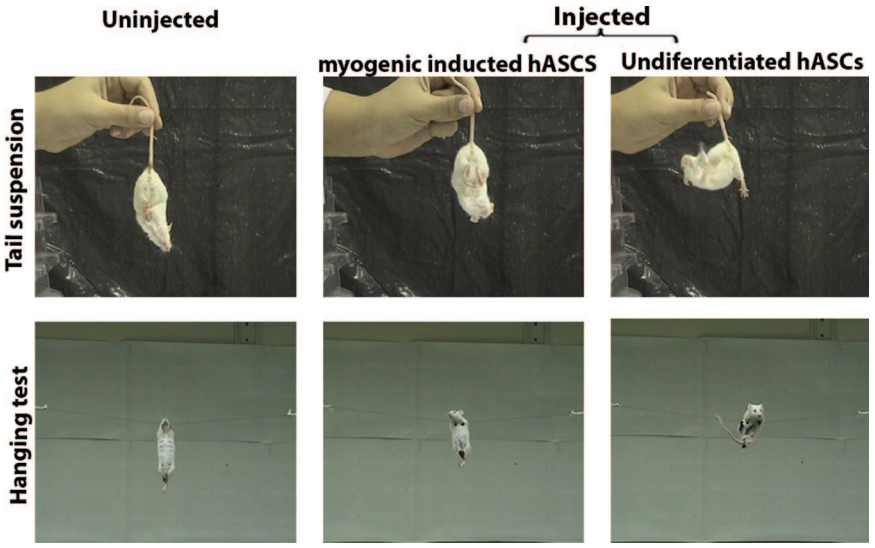

Figure 5. Functional improvement in injected animals. One animal of each group injected with undifferentiated human adipose stromal cells (hASCs), injected with myogenic inducted hASCs, and uninjected in two different situations: when suspended by the tail and in the hanging test, showing the different observed behaviors. Details are shown in the supplemental online Videos 1–6.

has to be carefully addressed. In addition, in contrast to our approach, the possibility of transforming DMD into a less severe form of Becker muscular dystrophy, through the restoration of the RNA reading frame, is not applicable to all cases since it depends on specific mutation deletions.

Here, we show through real-time PCR, WB, and IF analysis, that 6 months of systemic injections of nonengineered hASCs resulted in the expression of human muscle proteins in the host muscle. Interestingly the IF analysis revealed the presence of labeled fibers either in clusters or spread out through the muscle tissue, with some totally labeled and others partially positive for human dystrophin. This pattern of labeling might be explained by the fusion of hASC nuclei into host muscle fiber segments, demonstrating nuclear domains along the fibers [27]. On the other hand, Di Rocco et al. (2006) [20] also observed dystrophin-positive fibers organized in clusters following local transplantation of mouse ASCs. These results are in agreement with our findings and reinforce these authors' suggestion of clonal proliferation of donor cells in the host muscle.

Leriche-Guérin et al. (2002) [28] reported the myoblast transplantation into the *SJL* mouse muscle with immunosuppression. The percentage of positively labeled fibers obtained in their study was lower than the percentage of dystrophin-positive fibers usually observed following the transplantation of normal myoblasts in *mdx* mice (30%–90%) [29]. Since the immunosuppressive drug efficiently controlled the humoral and cellular immune reactions, the authors concluded that the immune rejection is not the cause of the low myoblast transplantation success in the *SJL* mice.

It has also been suggested that cells already committed to the myogenic phenotype, and therefore able to autonomously differentiate into skeletal muscle in vitro, possess the highest regenerating potential in vivo when injected locally [20]. To address this issue, which has important implications for therapeutic approaches, we assessed the effect of in vitro differentiation in the migration ability of hASCs. Surprisingly, in opposition to these authors, we did not find human DNA in the muscle of any of the injected animals after myogenic induction through systemic delivery. Our findings are in agreement with other authors [30] who observed that cultured myogenic progenitor cells injected intra-arterially into *mdx* mice resulted in only 1% of engraftment, which led the authors to suggest that these cells extravasate poorly from the circulation to the muscle locally. Alternatively, it is possible that differentiated cells

behave differently or are rejected by the recipient animals following systemic transplantation. In this respect, it might be interesting to assess the effect of local injections of myogenic committed cells in combination with systemic transfer of undifferentiated stem cells for therapeutic purposes in PMDs.

The functional ability was evaluated by standardized motor ability tests [24] that were already shown to be efficient for other rodent models [31–33]. With these selected tests we could assess the skeletal muscle function of each mouse before and after the cell treatment. Finally, our results revealed that muscle engraftment and the body-wide distribution of hASCs determined a significant (+15%) functional improvement in the injected animals. However, it is relevant that although hASC muscle engraftment was widespread, it did not reach the entire population of muscle cells. Since the functional recovery was considerable this may indicate that partial muscle colonization is enough for a significant functional amelioration.

These results are of clinical relevance in the development of appropriate stem cell approaches for human patients, suggesting that it might not be necessary to correct the entire muscle to gain a functional benefit. It is also important to highlight that it is not known whether this clinical improvement is due to the enhanced expression of the faulty muscle proteins derived from hASCs or to their known immunomodulatory effect [34]. However, the lymphocyte T infiltration data, where no difference was found between injected and uninjected animals, suggest that the presence of hASCs does not significantly reduce the inflammatory changes typically found in the *SJL* dystrophic muscle.

Our data indicating that systemic delivery of hASCs to the muscle can be done efficiently without immunosuppression may be explained because hASCs in vitro (a) do not induce proliferation of allogeneic T cells, (b) suppress the proliferation of T cells induced by either mitogens or allogeneic cells, (c) secrete a soluble factor(s) that inhibits the production of inflammatory cytokines (TNF-α, IFN-γ, and IL-12) of T cells stimulated by nonspecific mitogenic and by allogeneic stimuli, and (d) maintain these properties even after differentiation [34, 35]. This is supported by the present work since human cells were found in the host muscles for at least 2 months after the last injection.

In short, here we show for the first time that hASCs are not rejected when transferred systematically to the *SJL* mice without any immunosuppression, are able to fuse with the host muscle cell, express human skeletal muscle proteins, and ameliorate motor ability of affected animals. Although the *SJL* mice have a mild phenotype, and further in vivo studies in different animal models, which are currently under way, will be essential to corroborate the present observations, the observed functional recovery after 6 months of in vivo treatment is encouraging. These results open new avenues for preclinical research that may have important applications for future therapy, with the following advantages: (a) it may be applicable to patients affected with different forms of progressive muscular dystrophies, regardless of their specific disease-causing mutation; (b) human liposuctioned fat is available in large quantities; and (c) hASCs can be easily obtained without any in vitro genetic modification or induction [36]. Although it remains to be seen whether allogeneic transplantation can also be performed in humans without any immunosuppression, this simple approach here reported may represent a great step toward clinical application for the future therapy of different forms of progressive muscular dystrophies.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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Complementação

Injeção sistêmica de hASCs em camundongos *Swiss* normais.

A fim de observar o direcionamento de hASCs em animais normais em comparação ao observado em camundongos *SJL*, injetamos camundongos *Swiss* utilizando o mesmo protocolo e linhagem de células.

Nestes animais não foram encontradas células humanas dois meses após a última injeção em nenhum dos músculos analisados (Figura 6).

Essa observação sugere que o músculo distrófico secreta fatores que atraem (*homing) as* células-tronco provavelmente para ajudar no processo de reparo.

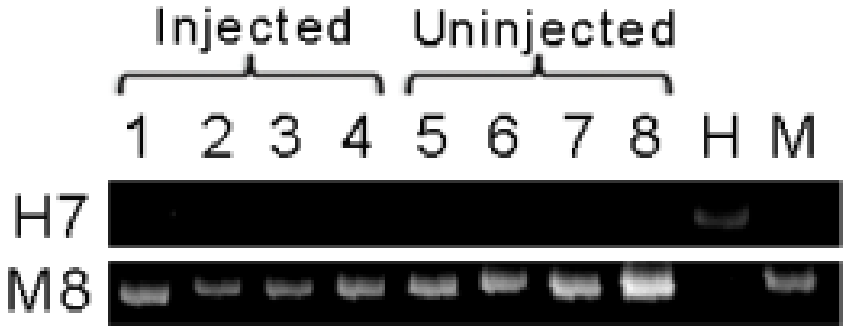

Figura 6 - Analise de PCR (Polymerase chain reaction) para o cromossomo 7 humano: H7; cromossomo 8 murino: M8; do camundongo SJL. Amostras: 1 e 5) músculo distal posterior; 2 e 6) músculo proximal posterior; 3 e 7) músculo distal anterior; 4 e 8) músculo proximal anterior; dos animais injetados (*Injected*) e não injetados (*Uninjected*). H) DNA Humano; M) DNA murino.

Capítulo 4

Células mesenquimais estromais humanas de diferentes fontes apresentam diferente potencial de diferenciação miogênica *in vivo* quando injetadas em camundongos distróficos

Artigo publicado em 2010 na revista Stem Cells Reviews and Reports

Human mesenchymal stem-cells from distinct sources show different in vivo potential to differentiate into muscle cells when injected in dystrophic mice

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Abstract

Limb-girdle muscular dystrophies are a heterogeneous group of disorders characterized by progressive degeneration of skeletal muscle caused by the absence or deficiency of muscle proteins. The murine model of Limb-Girdle Muscular Dystrophy 2B, the *SJL* mice, carries a deletion in the dysferlin gene. Functionally, this mouse model shows discrete muscle weakness, starting at the age of 4–6 weeks. The possibility to restore the expression of the defective protein and improve muscular performance by cell therapy is a promising approach for the future treatment of progressive muscular dystrophies (PMD). We and others have recently shown that human adipose multipotent mesenchymal stromal cells (hASCs) can differentiate into skeletal muscle when in contact with dystrophic muscle cells in vitro and in vivo. Umbilical cord tissue and adipose tissue are known rich sources of multipotent mesenchymal stromal cells (MSCs), widely used for cell-based therapy studies. The main objective of the present study is to evaluate if MSCs from these two different sources have the same potential to reach and differentiate in muscle cells in vivo or if this capability is influenced by the niche from where they were obtained. In order to address this question we injected human derived umbilical cord tissue MSCs (hUCT MSCs) into the caudal vein of *SJL* mice with the same protocol previously used for hASCs; we evaluated the ability of these cells to engraft into recipient dystrophic muscle after systemic delivery, to express human muscle proteins in the dystrophic host and their effect in functional performance.

Resumo

As distrofias musculares do tipo cinturas (*Limb-Girdle Muscular Dystrophy*, LGMD) são um grupo heterogêneo de doenças caracterizadas por uma degeneração progressiva do músculo esquelético causada pela ausência ou defeito de proteínas musculares. O modelo murino da distrofia muscular do tipo cinturas 2B (LGMD2B) é o camundongo *SJL*. Este é portador de uma deleção no gene disferlina que provoca uma redução dos níveis da proteína a 15% do normal. Os *SJL* apresentam fraqueza muscular que começa a partir de 04-06 semanas de vida. A possibilidade de restaurar a expressão da proteína muscular deficiente e melhorar o desempenho muscular através de terapia celular é uma abordagem promissora para o tratamento de LGMD e de outras formas de Distrofias Musculares Progressivas (DMP). Recentemente nosso grupo e outros demonstraram que as células mesenquimais estromais do tecido adiposo humano (*human adipose-derived stromal cells* - hASCs) podem se diferenciar em músculo esquelético quando em contato com células musculares distróficas *in vitro* e *in vivo*. O tecido do cordão umbilical e o tecido adiposo são fontes ricas em células-tronco mesenquimais (CTM), amplamente utilizadas para estudos de terapia celular. O principal objetivo do presente estudo é avaliar se as CTMs destas duas fontes diferentes têm o mesmo potencial para atingir e se diferenciar em células musculares *in vivo* ou se essa capacidade é influenciada pelo nicho de onde foram obtidas. A fim de abordar esta questão foram injetadas CTMs humanas, derivados de tecido do cordão umbilical (*human Umbilical Cord Mesenchymal Stem Cells* - hUCT MSCs) na veia caudal dos camundongos *SJL* utilizando o mesmo protocolo utilizado anteriormente para injeção de hASCs. Avaliamos a capacidade dessas células de atingir o músculo

dos camundongos após injeção sistêmica, de expressar proteínas musculares humanas e seu efeito no desempenho funcional dos animais.

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Human Multipotent Mesenchymal Stromal Cells from Distinct Sources Show Different In Vivo Potential to Differentiate into Muscle Cells When Injected in Dystrophic Mice

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Abstract Limb-girdle muscular dystrophies are a heterogeneous group of disorders characterized by progressive degeneration of skeletal muscle caused by the absence or deficiency of muscle proteins. The murine model of Limb-Girdle Muscular Dystrophy 2B, the *SJL* mice, carries a deletion in the dysferlin gene. Functionally, this mouse model shows discrete muscle weakness, starting at the age of 4–6 weeks. The possibility to restore the expression of the defective protein and improve muscular performance by cell therapy is a promising approach for the future treatment of progressive muscular dystrophies (PMD). We and others have recently shown that human adipose multipotent mesenchymal stromal cells (hASCs) can differentiate into skeletal muscle when in contact with dystrophic muscle cells *in vitro* and *in vivo.* Umbilical cord tissue and adipose tissue are known rich sources of multipotent mesenchymal stromal cells (MSCs), widely used for cell-based therapy studies. The main objective of the present study is to evaluate if MSCs from these two different sources have the same potential to reach and differentiate in muscle cells *in vivo* or if this capability is influenced by the niche from where they were obtained. In order to address this question we injected human derived umbilical cord tissue MSCs (hUCT MSCs) into the caudal vein of *SJL* mice with the same protocol previously used for hASCs; we evaluated the ability of these cells to engraft into recipient dystrophic muscle after systemic delivery, to express human muscle proteins in the dystrophic host and their effect in functional performance. These results are of great interest for future therapeutic application.

Keywords Human multipotent mesenchymal stromal cells · Xenotransplantation · Muscular dystrophy · Therapy

Introduction

Multipotent mesenchymal stromal cells (MSCs) are potentially useful for therapeutic approaches as well as models for developmental biology studies. MSC can be isolated from different tissues, such as adipose tissue, dental pulp, placenta, umbilical cord and fallopian tube [1–6]. However an important question is whether MSCs from different sources are comparable in their differentiation potential *in vivo* or whether this potential is influenced by the niche from where they were obtained.

Progressive muscular dystrophies (PMD) are a clinically and genetically heterogeneous group of disorders caused by the deficiency or abnormal muscle proteins, resulting in progressive degeneration and loss of skeletal muscle function. As effective treatments for these diseases are still unavailable, they have been widely investigated as possible candidates for stem cell therapy.

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Among the different forms, the Limb Girdle Muscular Dystrophies (LGMDs) constitute a sub-group characterized by the involvement of the pelvic and shoulder girdle musculature. A 171-bp in-frame deletion in the murine dysferlin cDNA was identified in a mouse model, the *SJL* mice, with a corresponding reduction in dysferlin levels to 15% of normal [7]. The *SJL* mice deletion is in-frame, and therefore does not cause a total absence of the protein.

The continuous and gradual muscle degeneration in PMDs leads to a depletion of satellite cells and, consequently, the capability to restore the skeletal muscle is lost [8, 9]. Different sources of stem/progenitor cells that show extended proliferation *in vitro* and also have the ability to generate normal muscle fibers *in vitro* and *in vivo* have been described in several publications [1, 2, 10–14].

We recently showed that human adipose-derived stromal cells (hASCs) can differentiate into skeletal muscle when in contact with dystrophic muscle cells *in vitro* [15] and *in vivo* [16]. In addition we also observed that the human umbilical cord tissue (hUCT) is a much richer source of MSC than umbilical cord blood [1, 17] and that they have different expression profiles [18]. However it is not known if all MSCs show the same capacity *in vivo.* Do MSCs from adipose and umbilical cord tissue have the same potential to reach and differentiate into muscle cells *in vivo*? Or, this capability is influenced by the niche from where they were obtained?

In order to address this question we have injected hUCT MSCs intravenously into the *SJL* mice, aiming to compare their potential to differentiate into skeletal muscle with our previous data with hASCs [20]. Differently from hASCs, hUCT MSCs reached the muscle but did not differentiate into muscle cells. These results suggest that according to the source from which MSCs were obtained they may show a greater potential to differentiate into determined cell lineages. This may have important implications depending on the intended therapeutic use.

Results

hUCT MSCs Capacity to Reach and Engraft at the Host Muscle

In order to assess the potential of hUCT MSCs to reach and colonize the host muscle we injected undifferentiated, previously characterized, hUCT MSCs, into the caudal vein of *SJL* mice (n=7). PCR analysis detected human DNA in the foreleg and hindleg muscles of all seven injected mice (Fig. 1a).

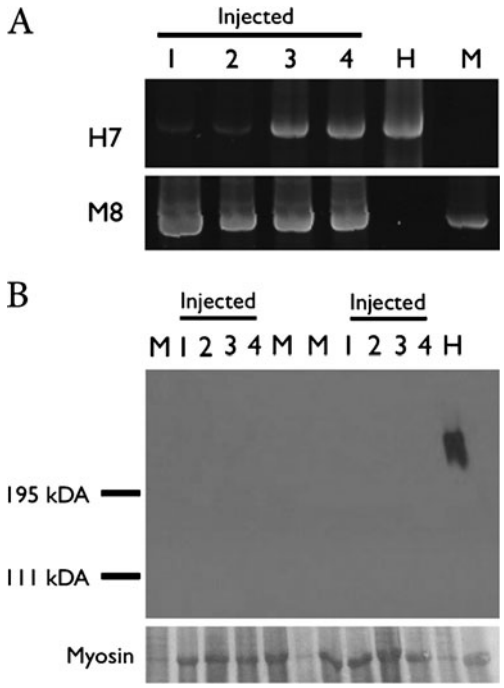

Fig. 1 **a** Polymerase chain reaction analysis for human chromosome 7 α-satellite sequences (H7) and mouse chromosome 8 centromeric repeat sequence (M8) of *SJL* mice. Muscles of the injected *SJL* mice, samples shown are the following: 1–Distal foreleg muscle; 2–Proximal foreleg muscle; 3–Distal hindleg muscle; 4–Proximal hindleg muscle; H–Human DNA; M–Mouse DNA. **b** Western blot analysis for human-dystrophin of the muscles of two injected animals. Samples shown are the following: 1–Distal foreleg muscle; 2–Proximal foreleg muscle; 3–Distal hindleg muscle; 4–Proximal hindleg muscle; H–Human muscle protein; M–Mouse muscle protein. Myosin = myosin band detected in the Ponceau S pre-stained blot, for the evaluation of loaded muscle proteins

Muscle Differentiation in the Host Muscle

To explore the myogenic differentiation followed by the engraftment of hUCT MSCs we analyzed the expression of dysferlin and human-dystrophin in the host muscle.

The analysis of dysferlin is not sufficient to infer if the injected muscles are expressing human or mouse proteins [16]. Therefore, we assessed the presence of human-dystrophin, using a specific anti-human-dystrophin antibody [19]. Through western blot (WB) analysis, no human dystrophin was found in the muscles of the injected animals (Fig. 1b).

Functional Assessment

We performed three standardized motor ability tests [20] and compared the performance of each *SJL* mouse (injected and uninjected) before (2-months of age) and after (9-months of age) the injections period, in blind test (Table 1). We observed that for the tests that required trunk strength (inclined plane and wire hanging tests) the

Table 1 Results of 3 motor ability tests in injected (n=7) and uninjected mice (n=7) before and after 6 months of injection

Test/Animal	Uninjected						Injected					
	Inclined plane (degrees)		Wire hanging		Ambulation (cm)		Inclined plane (degrees)		Wire hanging		Ambulation (cm)	
			Time hanging (seconds)						Time hanging (seconds)			
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
1	76.67	75.33	120.00	40.50	5.56	0.23	72.00	72.00	103.33	64.00	6.04	4.70
2	72.33	75.00	120.00	10.50	5.42	5.97	78.67	76.80	120.00	108.20	4.65	4.00
3	79.00	73.67	120.00	41.00	6.30	5.10	74.33	72.00	120.00	111.50	6.06	4.89
4	76.00	70.67	116.33	28.50	5.84	5.67	77.67	74.00	104.33	113.33	5.64	5.46
5	78.33	74.33	120.00	120.00	5.49	5.24	85.67	82.50	95.50	106.00	4.51	5.68
6	76.33	73.00	91.33	59.00	6.25	5.70	68.67	68.67	116.50	61.00	5.74	5.69
7	76.24	73.10	92.01	48.90	5.20	4.90	73.07	75.80	97.00	90.00	5.50	5.38
Average	76.42	73.59	111.38	49.77	5.72	4.69	75.72	74.54	108.10	93.43	5.45	5.11

At the *inclined plane test* the uninjected animals worsened their performance (p=0.008, *t-Student test,* n=7) while in the injected animals it did not differ (p=0.33, *t-Student test,* n=7)

For the *wire hanging test* the uninjected animals worsened their performance (p=0.0012, *t-Student test,* n=7) while the injected animals showed no significant difference (p=0.07, *t-Student test,* n=7)

At the *ambulation test* there was no difference in the performance of uninjected animals (p=0.11, *t-Student test,* n=7) and *injected animals* (p=0.16, *t-Student test,* n=7) after the injection period

uninjected animals showed a significantly worse performance while in the injected animals there were no statistically significant changes (Table 1). The deambulation test did not show a significant difference before and after the injections period in both groups.

Discussion

The successful use of stem cells for clinical applications in therapy for PMD requires the finding of a rich and easily obtainable source of cells, which must have the ability to reach the entire body musculature, engraft and restore the defective protein in the dystrophic muscle.

Sampaolesi *et al* (2006) [12] reported that systemic injections of normal dog mesoangioblasts to the muscle of dystrophic dogs resulted in the restoration of dystrophin expression. However all transplanted dogs were maintained on steroids and received immunosuppressant drugs, which makes difficult to evaluate functional results, since it is known that immunosuppressive and anti-inflammatory drugs can ameliorate the phenotype in muscular dystrophy patients [21].

Leriche-Guérin et al. (2002) [22] investigated the effect of myoblast transplantation into the *SJL* mice muscle with immunosupression. The percentage of dysferlin positive labeled fibers obtained in their study was lower than the percentage of dystrophin-positive fibers usually observed following the transplantation of normal myoblasts in *mdx* mice (30–90%) [23]. Since the immunosupressive drug efficiently controlled the humoral and cellular immune reactions, the authors concluded that the immune rejection is not the cause of the low myoblast transplantation success in the *SJL* mice.

We have previously shown that systemic delivery of hASCs into the *SJL* mice, without immunosuppression, resulted in human muscle proteins expression in the host muscles and functional amelioration [16].

MSCs may be found in different tissues which are routinely discarded [1, 6, 15–17, 24]. However an important question to be addressed is whether stem-cells obtained from different sources have the same potential to differentiate into different cell lineages or if there is already a pre-commitment depending on the niche from which they were obtained.

Since umbilical cord is a rich source of MSCs, we investigated their ability to originate muscle proteins and ameliorate functional parameters using the same animal model and methodology proved to be successful in our previous experiment with hASCs [16].

DNA analysis showed that the hUCT MSCs were able to reach the host muscle through systemic delivery. However we did not find human muscle proteins in the same muscle samples where the human DNA was present.

The functional ability in the previous and current study was evaluated by standardized motor ability tests [16, 25–27]. However, for the *SJL* model, the most affected muscles are the ones that are responsible for trunk strength [7].

In opposition to our previous study with hASCs [16] the injected animals with hUCT MSCs did not show clinical improvement, but, surprisingly, the performance of non-injected animals was significantly worse than in the "treated" animals. The mice from the latter group maintained their performance at the end of the injection period, in particular for the wire hanging test, which requires most trunk strength, suggesting an apparent stabilization of the dystrophic process. That is, even without differentiating in muscle cells, the injected hUCT MSCs may have a positive effect when interacting with the host muscle. Indeed there are growing evidences in the literature describing the immunosuppressive properties of MSCS [28]. Inflammatory infiltration is observed in the dystrophic muscle but little is known about the mechanisms involved in mesenchymal immunomodulation. It is possible that secreted known cytokines factors (TNF-α, IFN-γ and IL-12) could act, by protecting the dystrophic muscle. Several authors showed that mesenchymal stem cells suppress proliferation of activated lymphocytes *in vitro* in a dose-dependent, non-HLA-restricted manner [29–31]. Antibody-mediated depletion of CD4+ and CD8+ T cells in *mdx* mice has been found to result in a reduction in muscle pathology [32]. MSCs are also being tested in clinical trials aiming to ameliorate graft-versus-host disease after haemopoietic-stem-cell transplantation in humans [33]. Therefore, the immunomodulation effect of MSCs in patients affected by progressive muscular dystrophies could be a promising additional benefit to cell therapy.

Although MSCs from different sources show similar ability to differentiate into muscle cells *in vitro* [1, 15, 34] preclinical studies are of utmost importance to verify it this also happens *in vivo*. The apparent greater potential of adipose tissue than umbilical cord derived MSCs to differentiate into muscle cells here observed could be explained by a recently described population of mesenchymal progenitors, distinct from satellite cells, in the skeletal muscle [35, 36]. These progenitors have many similarities with hASCs and according to the authors they may have the same origin. These cells do not generate myofibers but enhance the rate of differentiation of primary myogenic progenitors, and have adipogenic differentiation potential both *in vitro* and *in vivo*. The interaction between muscle cells and these mesenchymal progenitors has a considerable impact on muscle homeostasis since adipogenesis is strongly inhibited by the presence of satellite cell-derived myofibres [35, 36]. It remains unclear however which cell population participates in the regeneration process by fusing to the degenerated myotubes or forming new myofibers. The identification of this subpopulation will be extremely important for the establishment of clinical trial protocols. Interestingly, it has been recently shown that there is an epigenetic memory in induced pluripotent stem-cells according to the tissue of origin [37] which might occur also with adult MSCS derived cells.

In short, here we compared, for the first time, the ability of MSCs obtained from human umbilical cord tissue and adipose tissue to engraft into recipient dystrophic muscle after systemic delivery; express human muscle proteins in the dystrophic host and their effect in functional performance using the same animal model and protocol. Our results showed that although umbilical cord MSCs apparently do not have the same potential to differentiate in human muscle proteins *in vivo* as hASCs they were able to reach the muscle and showed an apparent therapeutic benefit in injected animals as compared to the control group, probably due to their immunomodulatory effect.

The present investigation suggests that although MSC from different sources show apparently similar properties *in vitro* they may be more or less efficient to differentiate into specific cell lineages *in vivo* according to the niche from where they were obtained. Preclinical studies in different animal models, which are currently underway, will be essential to corroborate the present observations, which will have important implications aiming future cell therapy replacement.

Materials and Methods

Ethics Statement

This study was approved by the human research ethics committee (*Comitê de ética em pesquisa—seres humanos*—CEP) and by the animal research ethics committee (*Comissão de ética no uso de animais em experimentação*—CEUA) of Institute of Bioscience and University Hospital of University of São Paulo. hUCT MSCs were collected from donated umbilical cord units (UC), after all mothers signed the writhen informed consent, in accordance with the ethical committee of Institute of Bioscience and University Hospital of University of São Paulo (CEP), permit number 040/2005. *SJL* mice were purchased from the Jackson Laboratory. Animal care and experiments were performed in accordance with the animal research ethics committee (CEUA) of the Biosciences Institute, University of São Paulo, permit number 034/2005.

Harvesting of hUCT MSCs

UCs were filled with 0.1% collagenase (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com) in PBS and incubated at 37°C for 20 min. Each UC was washed with proliferation medium (DMEM low glucose, 10% fetal bovine serum), and the detached cells were harvested after gentle massage of the UC. Cells were centrifuged at 300 g for 10 min, resuspended in proliferation medium, and seeded in 25-cm2 flasks at a density of 5×10^7 cells per ml. After 24 h of incubation, non-adherent cells were removed and cultivated.

Immunophenotyping

To analyze cell-surface expression of specific markers, adherent cells were incubated with the following anti-human primary antibodies: CD29-PECy5, CD34-PerCP, CD31-phycoerythrin (PE), CD45-fluorescein isothiocyanate (FITC), CD90-R-PE, CD73-PE, CD13-PE, CD44-PE, CD117-PE, human leukocyte antigen (HLA)-ABC-FITC, HLA-DR-R-PE (Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com). A total of 10,000 labeled cells were analyzed using a Guava EasyCyte flow cytometer running Guava ExpressPlus software (Guava Technologies Hayward, CA, http://www.guavatechnologies.com).

Characterization of hUCT MSCs

The evaluation of MSCs properties included immunophenotyping by flow cytometric analysis, using a panel of surface markers. hUCT MSCs were negative for CD31 (endothelial cell marker), CD34, CD45, CD117 (hematopoietic cell markers), and HLA-DR (human leukocyte differentiation antigen class II), whereas they were positive for CD29, CD44 (adhesion markers), CD90, CD73, CD13 (mesenchymal markers), and HLA-ABC (human leukocyte differentiation antigen class I) [1] (data not shown).

The plasticity of hUCT MSCs was assessed by *in vitro* differentiation capacity, after three weeks of lineage induction [1]. Myogenic, adipogenic, chondrogenic and osteogenic differentiation was demonstrated by the expression of myogenic markers (myosin and desmin), lipid vacuoles, mucopolysaccharide-rich extracellular matrix and calcium deposits, respectively. These results confirmed the mesenchymal nature of the isolated cells as well as their multipotent potential (data not shown).

Cell Differentiation Procedures

To evaluate MSCs properties, hUCT MSCs (third passage, at 80%–90% confluence) were subjected to adipogenic, chondrogenic, myogenic, and osteogenic differentiation in vitro, according to established protocols [1]. Normal human dermal fibroblasts were used as a negative control in the differentiation studies.

Adipogenic Differentiation

Subconfluent cells were cultured in proliferation medium supplemented with 1 μM dexamethasone (Sigma-Aldrich), 500 μM 3-isobutyl-1-methylxanthine (Sigma-Aldrich), 60 μM indomethacin (Sigma-Aldrich), and 5 μg/ml insulin (Sigma-Aldrich). Adipogenic differentiation was confirmed on day 21 by intracellular accumulation of lipid-rich vacuoles stainable with oil red O (Sigma-Aldrich). For the oil red O stain, cells were fixed with 4% paraformaldehyde for 30 min, washed, and stained with a working solution of 0.16% oil red O for 20 min.

Chondrogenic Differentiation

A pellet culture system was used for chondrogenesis. Cells ($2{,}5\times10^5$) were centrifuged in a 15-ml polypropylene tube at 500 g for 5 min, and the pellet was resuspended in 10 ml of basal medium consisting of DMEM-LG supplemented with 100 nM dexamethasone, 50 μM ascorbic acid-2 phosphate (Sigma-Aldrich), 1 mM sodium pyruvate (Invitrogen-Gibco), and 1% ITS-Premix (Becton Dickinson). Without disturbing the pellet, cells were resuspended in 0.5 ml of chondrogenic differentiation medium consisting of basal medium supplemented with 10 ng/ml transforming growth factor-B1 (R&D Systems Inc., Minneapolis, http://www.rndsystems.com). On day 1, tubes were flipped gently to acquire a single floating cell sphere. Medium was changed every 3-4 days, and cells were fixed on day 21 with 4% paraformaldehyde. Cryosections (10 um thick) were stained with toluidine blue to demonstrate extracellular matrix mucopolysaccharides.

Osteogenic Differentiation

To promote osteogenic differentiation, subconfluent cells were treated with proliferation medium supplemented with 50 μM ascorbate-2 phosphate, 10 mM B-glycerophosphate (Sigma-Aldrich) and 0.1 μM dexamethasone, for 21 days. Osteogenesis was demonstrated by accumulation of mineralized calcium phosphate assessed by von Kossa stain. Briefly, cells were stained with 1% silver nitrate (Sigma-Aldrich) for 45 min under ultraviolet light, followed by 3% sodium thiosulfate (Sigma-Aldrich) for 5 min, and then counterstained with van Gieson stain.

Transplantation

Fourteen two-months *SJL* mice were divided into two groups of 7: transplanted animals (group A) and control group B (uninjected animals). Each animal from group A was injected in the tail vein with 1×10^6 of hUCT MSC in 0.1 ml of Hank's Buffered Salt Solution (HBSS). The animals were injected for 6 months, weekly in the first month and then monthly. All results were analyzed blindly. The code for each of the mice groups was disclosed only after completion of all the studies. Two months after the last cell transplantation, the animals were euthanized using a CO_2 chamber.

Human DNA Analysis

The DNA was obtained using DNeasy Blood & Tissue Kit (Qiagen). The presence of human DNA in the host samples

were evaluated as described in Pelz et al (2005) [38]. Centromeric region of human chromosome 7 and mice chromosome 8 was amplified by PCR (35 cycles, annealing at 59°C). The PCR products were separated by electrophoresis on 2% agarose gels and stained with ethidium bromide. Non-saturated digital images were obtained using an ImageQuant imaging system (GE HealthCare).

Western Blot Analysis

Muscle sample proteins were extracted through treatment with a buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100 and 60 mM octylglucoside. Samples were centrifuged at 13,000 × *g* for 10 min to remove insoluble debris. Soluble proteins were resolved by 6% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membranes (Hybond; Amersham). All membranes were stained with Ponceau (Sigma) to evaluate the amount of loaded proteins. Blots were blocked for 1 h in Tris-buffered saline Tween (TBST) containing 5% powdered skim milk and reacted overnight with the following primary antibody: anti human-dystrophin MANEX 12/16E2 G10 (1:100) kindly provided by Dr. Glenn E. Morris at Center for Inherited Neuromuscular Diseases, Oswestry, Shropshire, UK. Blots were incubated one hour with secondary antibodies. Immunoreactive bands were detected with ECL chemiluminescence detection system (GE Healthcare).

Functional Assessment

In order to verify whether injected hUCT MSCs would improve motor ability in *SJL* injected mice, we performed motor ability tests before and after 6 months of SC injection period. Mice were examined, weighed, and submitted to the following tests: (a) the *inclined plane test* evaluated by measuring the maximal angle of a wood board on which the animal was placed until it slipped; (b) *the wire hanging test* to determine the ability of the mouse suspended on a horizontal thread by its forelegs, to reach it with its hindlegs and the length of time they were able to stay hanging; (c) *the ambulation test* which was performed to determine the mean length of a step measured in hindfoot ink prints while mice freely run in a corridor (length, 50 cm; width, 8 cm; height of lateral walls, 20 cm) [20].

Statistical Analysis

Observations were quantified blindly. Numerical data are the mean sd (standard deviation). The statistical analysis of the equivalence between the injected and uninjected mice was achieved by the one-tailed t-student test, at the significance level of $p=0.05$ and the results were expressed by the percentage variation between their performance before and after hUCT MSCs transfer period.

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Capítulo 5

Isolamento, caracterização e potencial de diferenciação de células-tronco de tecido adiposo canino

Artigo publicado em 2010 na revista Stem Cells Reviews and Reports

Isolation, characterization and differentiation potential of canine adipose-derived stem cells

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Abstract

Adipose tissue may represent a potential source of adult stem cells for tissue engineering applications in veterinary medicine. It can be obtained in large quantities, under local anesthesia and with minimal discomfort. In this study, canine adipose tissue was obtained by biopsy from subcutaneous adipose tissue or by suction-assisted lipectomy (i.e., liposuction). Adipose tissue was processed to obtain a fibroblast-like population of cells similar to human Adipose-derived Stem (hASC) cells. These canine Adipose-derived Stem Cells (cASCs) can be maintained in vitro for extended periods with stable population doubling and low levels of senescence. Immunofluorescence and flow cytometry show that the majority of cASCs are of mesodermal or mesenchymal origin. cASCs are able to differentiate in vitro into adipogenic, chondrogenic, myogenic, and osteogenic cells in the presence of lineage-specific induction factors. In conclusion, like human lipoaspirate, canine adipose tissue may also contain multipotent cells and represent an important stem cell source both for veterinary cell therapy as well as pre-clinical studies.

Resumo

O tecido adiposo representa uma fonte potencial de células-tronco adultas para aplicações na medicina veterinária. Pode ser obtido em grandes quantidades, sob anestesia local e com o mínimo de desconforto. Neste estudo, o tecido adiposo canino foi obtido através de biópsia de tecido adiposo subcutâneo ou por lipoaspiração. O tecido adiposo foi processado a fim de se obter uma população de células fibroblastóides, semelhantes às células-tronco de tecido adiposo humano (*human Adipose-derived Stem Cells* - hASC). Estas células derivadas de tecido adiposo canino (*canine Adipose-derived Stem Cells* - cASCs) podem ser mantidas *in vitro* por longo período de tempo, com crescimento estável e baixos níveis de senescência. Dados de imunofluorescência e citometria de fluxo mostram que a maioria das cASCs é de origem mesodérmica ou mesenquimais. cASCs são capazes de diferenciar *in vitro* em células adipogênicas, condrogênicas, miogênicas, e osteogênicas quando expostas a fatores de indução linhagem específicos. Em conclusão, assim como o tecido adiposo lipoaspirado humano, o tecido adiposo de cães também contém células multipotentes que representam uma importante fonte de células-tronco, tanto para a terapia celular como estudos pré-clínicos na medicina veterinária.

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Isolation, Characterization, and Differentiation Potential of Canine Adipose-Derived Stem Cells

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Adipose tissue may represent a potential source of adult stem cells for tissue engineering applications in veterinary medicine. It can be obtained in large quantities, under local anesthesia, and with minimal discomfort. In this study, canine adipose tissue was obtained by biopsy from subcutaneous adipose tissue or by suction-assisted lipectomy (i.e., liposuction). Adipose tissue was processed to obtain a fibroblast-like population of cells similar to human adipose-derived stem cells (hASCs). These canine adipose-derived stem cells (cASCs) can be maintained in vitro for extended periods with stable population doubling and low levels of senescence. Immunofluorescence and flow cytometry show that the majority of cASCs are of mesodermal or mesenchymal origin. cASCs are able to differentiate in vitro into adipogenic, chondrogenic, myogenic, and osteogenic cells in the presence of lineage-specific induction factors. In conclusion, like human lipoaspirate, canine adipose tissue may also contain multipotent cells and represent an important stem cell source both for veterinary cell therapy as well as preclinical studies.

Key words: Canine adipose-derived stem cells; Tissue engineering; Veterinary cell therapy

INTRODUCTION

A promising application in the emerging field of veterinary regenerative medicine and surgery is cell therapy, rendering the isolation and characterization of stem cells from a variety of sources areas of great interest.

An abundant and accessible source of stem cells is adipose tissue. These cells, called adipose-derived stromal cells (ASCs), are fibroblast-like cells capable of multipotential differentiation, which have been found in different species (4,27,29, 35). Several groups have demonstrated that human mesenchymal cells within the stromal-vascular fraction (SVF) of subcutaneous adipose tissue [processed lipoaspirate (PLA) cells] are capable of differentiation in multiple lineages, including myocytes, in the presence of lineage-specific inductive media (2,5,8–10,15,16,19,20,22,23,25,34,35).

In humans, ASCs for autologous transplantation are isolated relatively quickly from adipose tissue by collagenase digestion (6). We have recently shown that ASCs from human subcutaneous fat were able to differentiate in adipogenic, osteogenic, chondrogenic, and myogenic lineages and produce human muscle proteins in vitro and in vivo (30,31).

Successful transplantation of canine adipose-derived stem cells (cACSs) in dogs was reported by Li et al. (12) and Black et al. (1). However, these manuscripts lacked the full characterization of the administered cell population. Here we report the isolation, characterization, and multilineage differentiation potential of cASCs from subcutaneous adipose tissue by liposuction and biopsy procedures.

MATERIALS AND METHODS

All experimental protocols were approved by the ethics committee on animal use from the Institute of Biosciences, University of São Paulo. For this study, adipose tissue was collected from normal golden retriever dogs from the Brazilian Colony of Golden Retriever Muscular Dystrophy, Faculty of Veterinary Medicine and Zootecny, University of São Paulo. Subcutaneous adipose tissue was collected from the area over the dorsal gluteal muscles of 10 dogs (aged 4 months to 4 years).

Adipose Tissue Harvesting

Dogs were sedated upon intramuscular (IM) injection with meperidine (2 mg/kg) and acetylpromazine (0.05 mg/kg). The area over the dorsal gluteal muscles was

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asceptically prepared, and skin and subcutaneous tissues were desensitized by local infiltration of 2% lidocaine (Fig. 1A). A 0.5–1.0-cm incision was made parallel to the vertebral column. The liposuction procedure was performed by injecting infiltrate containing the vasoconstrictor epinephrine. Then adipose tissue was removed from the subcutaneous space by means of blunt-tip hollow cannula attached to a syringe at negative pressure (Fig. 1B). About 15 ml of adipose tissue was harvested over the superficial gluteal fascia for immediate cASC isolation and the skin incision apposed with nylon sutures (Fig. 1C). Adipose tissue biopsies were performed under local anesthesia. A 1–2-cm incision was made and the subcutaneous adipose tissue was collected (Fig. 1D) and the incision was closed with nylon sutures (Fig .1E).

cASC Isolation and Expansion

Cells were isolated using modified methods previously described (7). Briefly, the adipose tissue was washed extensively with equal volumes of PBS containing antibiotics (100 U/ml of penicillin and 100 g/ml of streptomycin). The infranatant containing hemopoietic cells suspended in PBS was removed. Then the tissue was dissociated with 0.075% collagenase (Sigma) for 15 min. Enzyme activity was neutralized with Dulbecco's modified Eagle's media- high glucose (DMEM-HG; Gibco) containing 10% FBS (Gibco). The infranatant was centrifuged at $1200 \times g$ for 5 min to pellet the cells. The cells from the pellet SVF were filtered to remove debris and seeded in tissue culture plates (NUNC) at 1,000–3,500 cells/cm^2 in DMEM-HG 10% FBS. Cultures were washed with PBS 24–48 h after plating to remove unattached cells and fed with fresh media.

The cultures were maintained at 37°C with 5% CO_2 in growth media (GM-DMEM-HG 10% FBS). When they achieved about 70% confluence, the cells were trypsinised (0.025%, Invitrogen) and plated at a density of 5,000/cm^2. Cultures were passaged repeatedly after achieving a density of 70–80%. The remaining cells were cryopreserved in cryopreservation media (10% dimethylsulfoxide, 10% DMEM-HG, 80% FBS), frozen at −80°C in an isopropanol-jacketed closed container, and stored in liquid nitrogen the next day.

Multilineage Differentiation

Cells were analyzed for their capacity to differentiate into adipogenic, chondrogenic, osteogenic, and myogenic lineages as described in Zuk et al. (35).

Adipogenic Differentiation. Subconfluent cells were cultured in GM supplemented with 1 μM dexamethasone (Sigma), 500 μM 3-isobutyl-1-methyl-xanthine (IBMX, Sigma), 60 μM indomethacin (Sigma), and 5 μg/ml insulin (Sigma). Adipogenic differentiation was confirmed on day 21 by intracellular accumulation of lipid-rich vacuoles stainable with Oil Red O (Sigma). For the Oil Red O stain, cells were fixed with 4% paraformaldehyde for 30 min, washed, and stained with a working solution of 0.16% Oil Red O for 20 min.

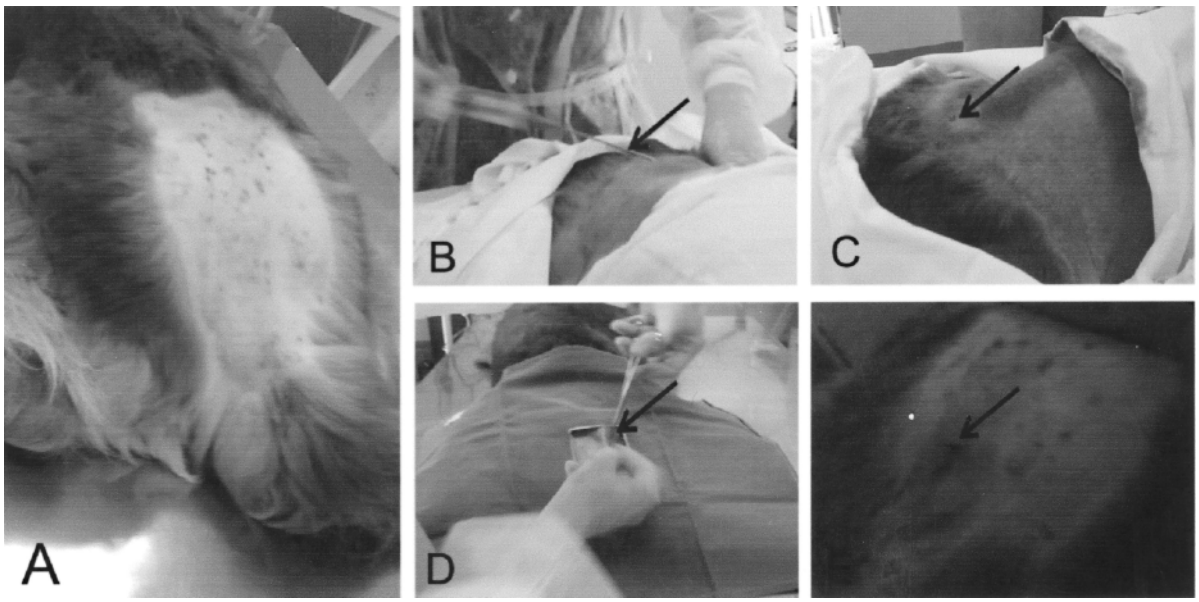

Figure 1. Adipose tissue harvest. (A) The area over the dorsal gluteal muscles prepared for the procedure. (B) Liposuction procedure. (C) Skin incision after the liposuction. (D) Subcutaneous adipose tissue biopsy. (E) Skin incision after the biopsy.

Chondrogenic Differentiation. Subconfluent cells were cultured in chondrogenic differentiation medium consisting of DMEM-low glucose supplemented with 100 nM dexamethasone, 50 μM ascorbic acid-2 phosphate (Sigma), 1 mM sodium pyruvate (Gibco), 10 ng/ml TGF-β1 (R&D Systems), and 1% ITS-Premix (Becton Dickinson). Medium was changed every 3–4 days, and cells were fixed on day 21 with 4% paraformaldehyde (PFA). Chondrogenesis was demonstrated by staining with toluidine blue and immunofluorescence using anti-collagen type II antibody (1:100, Abcam).

Osteogenic Differentiation. To promote osteogenic differentiation, subconfluent cells were treated with GM supplemented with 50 μM ascorbate-2 phosphate, 10 mM β-glycerophosphate (Sigma), and 0.1 μM dexamethasone for 21 days. Osteogenesis was demonstrated by accumulation of mineralized calcium phosphate assessed by von Kossa stain. Briefly, cells were stained with 1% silver nitrate (Sigma) for 45 min under ultraviolet light, followed by 3% sodium thiosulphate (Sigma) for 5 min, and then counterstained with van Gieson.

Myogenic Differentiation. For myogenic differentiation, cASCs cells were cultured in GM supplemented with 0.1 μM dexamethasone (Sigma), 50 μM hydrocortisone (Sigma), and 5% horse serum (Gibco) for 45 days. After that cells were labeled with anti-myosin (1:100, Sigma).

Immunofluorescence

Cells were fixed in 4% paraformaldehyde in PBS for 20 min at 4°C, permeabilized in 0.05% Triton X-100 in PBS for 5 min. Nonspecific binding was blocked with 10% FBS in PBS for 1 h at room temperature. Cells were incubated with primary antibody (1:100) overnight at 4°C. After several washes, cells were incubated with secondary (1:100, Sigma) antibodies against mouse IgG tagged with Cyanine 3 (Cy3; red) for 2 h at room temperature. Slides were counterstained with DAPI (4′-6-diamidino-2-phenylindole, Sigma). All images in the same set (samples and controls) were obtained using the same photographic parameters of exposition and speed. Images were captured using the Axiovision 3.0 image analysis system (Carl Zeiss).

Flow Cytometry

Cells were evaluated for cell surface protein expression using flow cytometry. The flow cytometry was performed on Guava EasyCyte System (Guava Technologies) using a blue laser (488 nm). Cells were pelleted, resuspended in PBS at a concentration of 1×10^5 cells/μl, and stained with saturating concentration of antibodies. Cells were incubated in the dark for 45 min at room temperature. After incubation, cells were washed three times with PBS and resuspended in 0.25 ml of cold PBS. Cell viability was accessed with Guava ViaCount reagent (Guava Technologies).

cASCs were incubated with the following primary antibodies: CD13-PE, CD29-PECy5, CD31-PE, CD34-PE, CD44-FITC, CD45, CD73, CD90-PE, CD105 e CD117-PECy5 (Becton Dickinson). The following antibodies have been raised against human cells: CD13, CD71, and CD105. Unconjugated markers were treated with anti-mouse PE secondary antibody (Guava Technologies).

Flow cytometer settings were established using unstained cells. Cells were gated by forward scatter to eliminate debris. To eliminate the possible autofluorescence of cASCs, we removed the contribution of unstained cells in the measurement channel. A minimum of 10,000 events was counted for each analysis.

RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was harvested from cultured cells using Tryzol (Invitrogen) following manufacturer's instructions. The RNA was treated with DNase (Invitrogen). A total of 1 μg of total RNA was reverse-transcribed with SuperScript™ III First-Strand Synthesis System (Invitrogen). All amplifications were performed in an MJ Research PTC-200 thermocycler (MJ Research) for 24 cycles after the initial 2-min denaturation at 94°C. The PCR primers are listed in Table 1. The PCR products were separated on 6% polyacrylamide gel by electrophoresis, stained with ethidium bromide, and visualized under UV light. Digital images were captured with ImageQuant (GE Healthcare).

cASCs Transduction With Lentivirus Vector

The visualization of cASCs cells for in vitro and in vivo studies can be improved if done with GFP-positive cells. For this purpose we transducted cASCs with GFP lentivirus.

Supernatant containing the FUGW lentivirus (13) was produced as described previously by Strauss et al. (26) and concentrated by ultracentrifugation. Undifferentiated cASCs at passage 2 were incubated at 37°C, in a six-well plate (Nunc), using a minimal volume of GM in the presence of vector particles (20 PFU/cell) and 8 μg/ml Polybrene (Sigma). After 4 h, 2 ml of GM was added and the media was changed the next day.

Karyotype Analysis

For evaluation of any chromosomal abnormality at latter passages, chromosome preparations were performed in cASC cultures. Briefly, metaphase cells were

Table 1. PCR Primers

Markers/Gene	Primer Sequence (5′–3′)	Amplicon Size	Ann. Temp. (°C)	Reference
Myogenic				
MyoD	Forward GACGGCATGATGGACTACAG	118	60	
	Reverse ACACCGCAGCACTCTTCC			
Dystrophin	Forward AAACACAGTGGTAGCCCACAAGAT	116	60	
	Reverse TGGTGACAGCCTGTGAAATC			
Myogenin	Forward GACGGCATGATGGACTACAG	102	60	
	Reverse ACACCGCAGCACTCTTCC			
Adipocytes				
FABP4	Forward ATCAGTGTAAACGGGGATGTG	117	60	17
	Reverse GACTTTTCTGTCATCCGCAGTA			
Leptin	Forward CTATCTGTCCTGTGTTGAAGCTG	102	60	17
	Reverse GTGTGTGAAATGTCATTGATCCTG			
LPL	Forward ACACATTCACAAGAGGGTCAC	132	60	17
	Reverse CTCTGCAATCACACGGATG			
Chondrocytes				
COL2A	Forward GAAACTCTGCCACCCTGAATG	156	64	17
	Reverse GCTCCACCAGTTCTTCTTGG			
SOX9	Forward GCTCGCAGTACGACTACACTGAC	101	60	17
	Reverse GTTCATGTAGGTGAAGGTGGAG			
Aggrecan	Forward ATCAACAGTGCTTACCAAGACA	122	58	17
	Reverse ATAACCTCACAGCGATAGATCC			
Osteocytes				
Osteopontin	Forward CATATGATGGCCGAGGTGATAG	114	60	
	Reverse CAAGTGATGTGAAGTCCTCCTC			
COL1A1	Forward GTAGACACCACCCTCAAGAGC	118	62	
	Reverse CCAGTCGGAGTGGCACAT			
BSP	Forward TTGCTCAGCATTTTGGGAAT	295	60	
	Reverse AACGTGGCCGATACTTAAAGAC			
Housekeeping				
GAPDH	Forward CCATCTTCCAGGAGCGAGAT	97	60	
	Reverse TTCTCCATGGTGGTGAAGAC			

arrested with 0.1 μg/ml colchicine (Sigma) for 20 min. Then, cASCs were detached from cultures flasks using TrypLE (Gibco), resuspended in a hypotonic solution (0.075M KCl), and incubated for 20 min at 37°C. Cells were pelleted at 1000 rpm for 10 min and fixed by washing three times in methanol/glacial acetic acid (3:1). Chromosome spreads were obtained by pipetting suspension drops onto clean glass slides and air dried. The best metaphases were captured with Axioplan 2 microscope (Zeiss) and analyzed with Ikaros 3 software (Zeiss).

RESULTS

Characterization of cASCs

cASC cultures were maintained in DMEM supplemented with 10% FBS. Supplementation with FBS has been shown to be important for human ASC attachment and proliferation in vitro (35). We observed that cASCs are easy to expand in vitro and show a fibroblast-like morphology, consistent with that of human ASCs (Fig. 2A–D). At both early and late passages, cells maintained a diploid karyotype of 78 chromosomes (Fig. 2E).

cASCs from four unrelated dogs were characterized by flow cytometry for the expression of 10 cell surface proteins (CD13, CD29, CD31, CD34, CD44, CD45, CD73, CD90, CD105, and CD117). Cell viability was above 96% by Guava ViaCount reagent (Guava Technologies).

At passage 4, the majority of cASCs expressed CD44, CD29 (β1 integrin) and CD90 (Thy1) adhesion molecules. Other markers, including CD14, CD34, CD45, and CD117, were consistently absent or expressed in few cells (Fig. 3). Interestingly, CD13, CD105, and CD73, known to be positively expressed in human ASCs, were negative in the canine ASC population, which might be explained by the nonspecific stain-

ing of human antibodies in canine cells. As surface markers are not sufficient for the identification or definition of mesenchymal stem cell (MSC), cASCs were subjected to differentiation studies for further confirmation of their MSC property.

The plasticity of cASCs was assessed after lineage induction. Myogenic, adipogenic, chondrogenic, and osteogenic differentiation was demonstrated by the expression of myogenic markers (myosin), lipid vacuoles, mucopolysaccharide-rich extracellular matrix, and calcium deposits, respectively (Fig. 4) and by the expression of tissue-specific mRNAs (Fig. 5). These results confirmed the mesenchymal nature of the isolated cells and their multipotent potential.

Adipogenesis. cASCs showed a rounder shape after 7 days in adipogenic medium. Two weeks after initial induction, the adipogenic differentiation was confirmed by Oil Red O staining of lipid droplets present throughout the cytoplasm (Fig. 4A). Expression of FABP4 and LPL was seen only in adipo-induced cells (Fig. 5B). On the other hand, basal level of leptin mRNA was observed in noninduced control cells, and the expression level was increased following adipogenic induction.

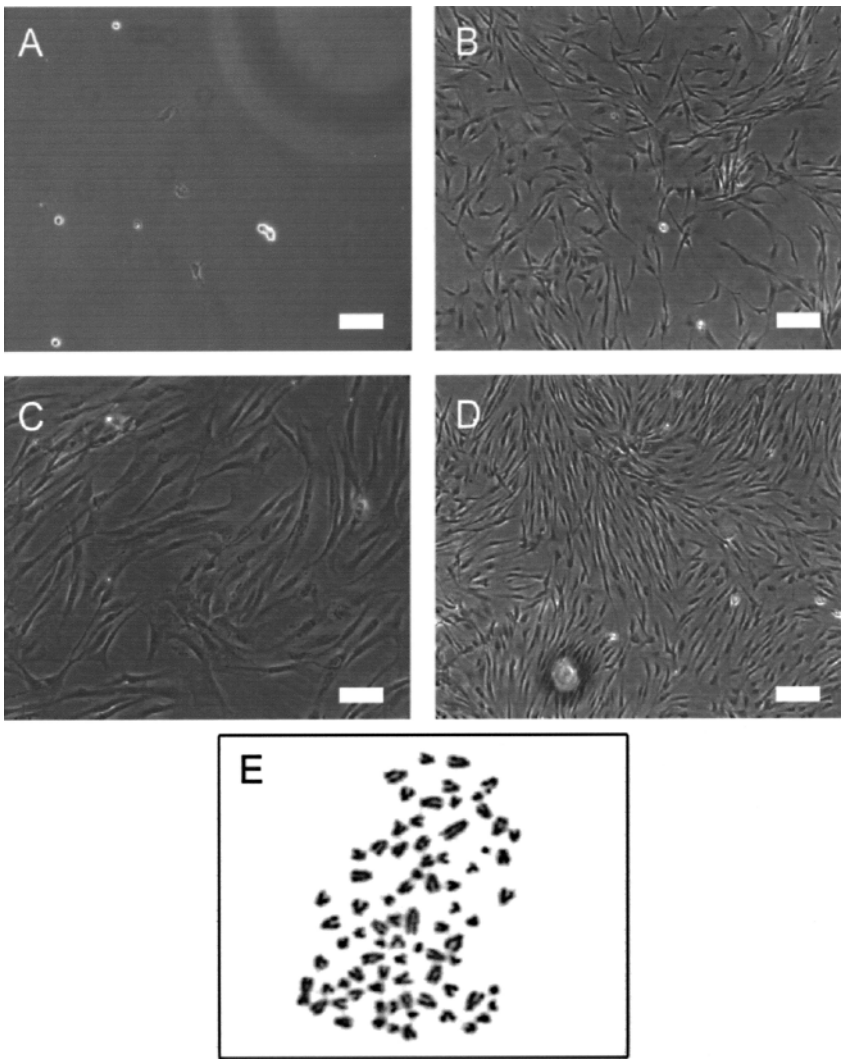

Figure 2. Typical morphology of cASCs. (A) Forty-five minutes after the establishment of the culture. Some cells remain in the supernatant. Scale bar: 200 µm. (B) cASCs at passage 3. Scale bar: 200 µm. (C) cASCs at passage 4: cASCs morphology is similar to that found in human ASCs. Scale bar: 100 µm. (D) High-density ASCs culture at passage 4. Scale bar: 200 µm. (E) Karyotype of cASC cell lineage after 10 passages, showing an euploid number of chromosomes.

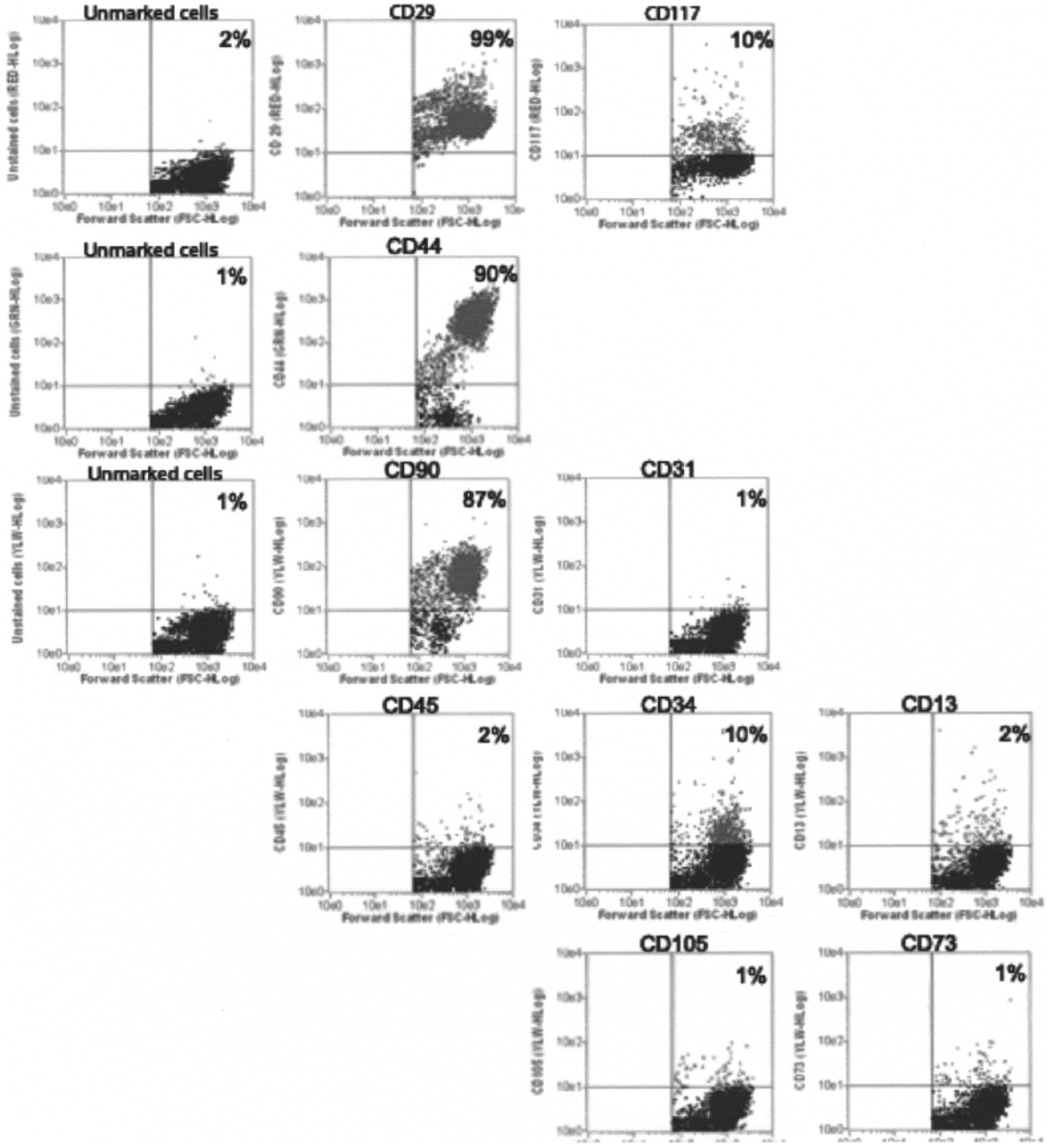

Figure 3. Immunophenotyping of ASCs at passage 4. Values represent the mean percentage of positively stained cells as analyzed by flow cytometry. Graphs show forward scatter versus fluorescence intensity of the indicated antigen.

Osteogenesis. cASCs exposed to osteogenic medium exhibited changes in cell morphology after 5 days in culture, showing a polygonal form. Mineralized nodular structures appeared in 1 or 2 weeks and were assessed by von Kossa Stain, which localized the calcium deposits (Fig. 4C). Expression of osteopontin, COL1A1, and BSP was observed only in induced cells, with no basal expression in control cells (Fig. 5C).

Chondrogenic Differentiation. After 21 days cultured in chondrogenic medium, cASCs cells were stained with toluidine blue, showing the typical metachromasia of cartilage. Chondrogenic differentiation was demonstrated by the mucopolyssaccharide-rich extracellular matrix (Fig. 4E, G). Chondrogenic treatment resulted in specific expression of COL2A, SOX9, and aggrecan, all of which were undetected or had basal expression in noninduced cells (Fig. 5D).

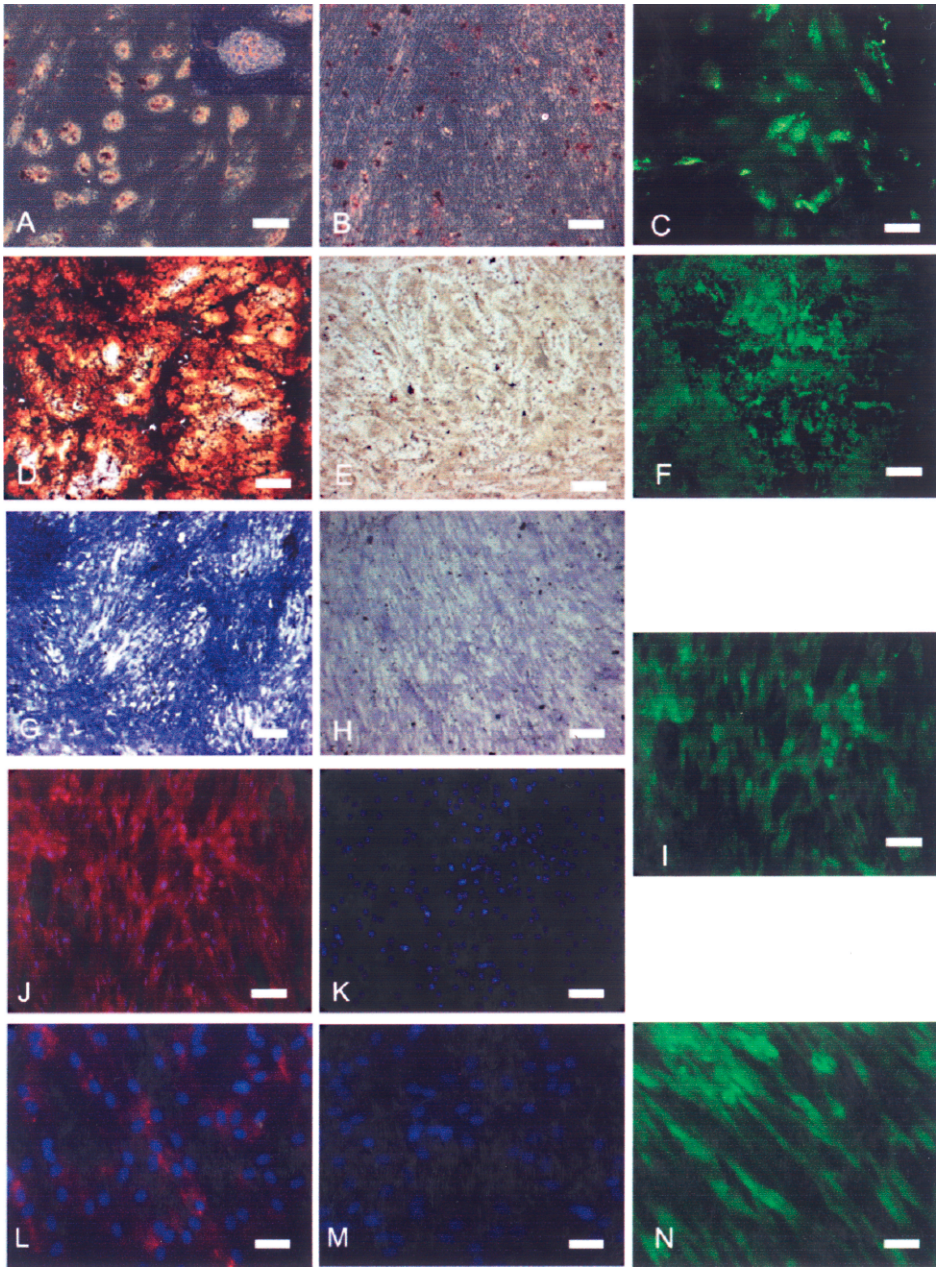

Figure 4. Differentiation potential of cASCs at passage 4. (A) The adipogenic differentiation was detected by the formation of intracytoplasmic lipid droplets stained with Oil Red O. Scale bar: 200 μm. (B) Undifferentiated ASCs stained with Oil Red O. Scale bar: 200 μm. (C) cASCs after induction with adipogenic media still show GFP expression. Scale bar: 200 μm. (D) Osteogenic differentiation was demonstrated by calcium deposition shown by von Kossa stain. Scale bar: 200 μm. (E) Undifferentiated ASCs stained with Von Kossa. Scale bar: 200 μm. (F) cASCs after induction with osteogenic media still show GFP expression. Scale bar: 200 μm. (G) Chondrogenic differentiation in monolayer culture was demonstrated by staining with toluidine blue. Scale bar: 200 μm. (H) Undifferentiated ASCs stained with toluidine blue. Scale bar: 200 μm. (I) cASCs after induction with chondrogenic media still show GFP expression. Scale bar: 200 μm. (J) Chondrogenic differentiated cells labeled with anti-collagen type II antibody. Scale bar, 200 μm. (K) Undifferentiated ASCs labeled with anti-collagen type II antibody. Scale bar: 200 μm. (L) Myogenic differentiation was assessed by immunofluorescence. Induced cells were labeled with anti-myosin monoclonal antibody. Scale bar: 50 μm. (M) Undifferentiated ASCs labeled with anti-human myosin monoclonal antibody. Scale bar: 50 μm. (N) cASCs after induction with myogenic media still show GFP expression. Scale bar: 50 μm.

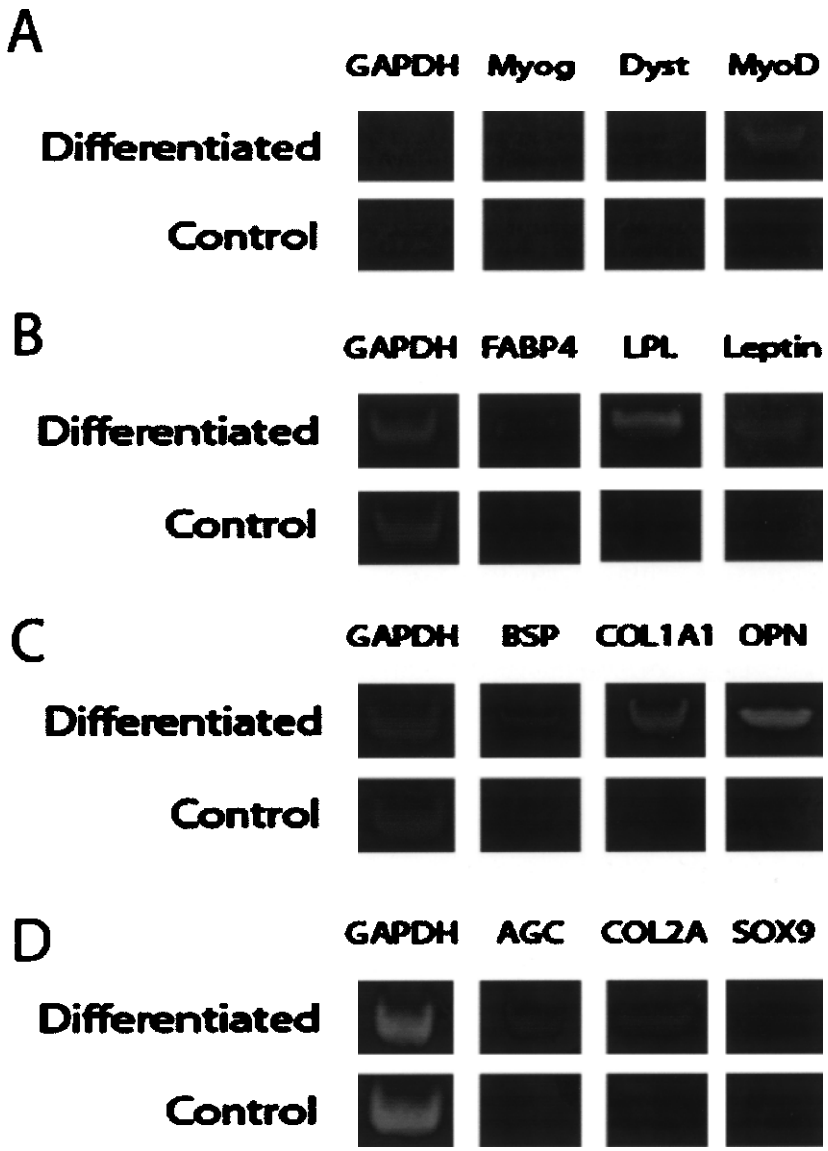

Figure 5. mRNA expression of specific differentiation markers. (A) Myogenic markers: myogenin (Myog), dystrophin (Dyst) and MyoD. (B) Adipogenic markers; FABP4, leptil, and LPL. (C) Osteogenic markers: osteopontin (OPN), COL1A1, bone sialoprotein (BSP). (D) Chondrogenic markers; COL2A, SOX9, and aggrecan (AGC). The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as reference for evaluating the quality of mRNA.

Myogenesis. After 10 days in myogenic medium, cASCs formed multinucleated structures. Controls maintained only with GM did not contain any multinucleated structures. To confirm the myogenic differentiation, the expression of myosin by immunofluorescence was assessed after 45 days (Fig. 4H). The specificity of this assay was corroborated by the absence of staining in cASCs. Expression of myogenin, dystrophin, and MyoD was observed only in induced cells, with no basal expression in control cells (Fig. 5A).

GFP Transduction of cASCs

Transgene expression was examined by flow cytometry 72 h posttransduction. About 75% of cells were GFP positive and GFP expression did not decline during culture passages (Fig. 6). To evaluate if GFP interfered with the multipotent capacity of cASCs, both GFP-positive and -negative cells at successive passages were analyzed by flow cytometry and multilineage differentiation, revealing no influ-

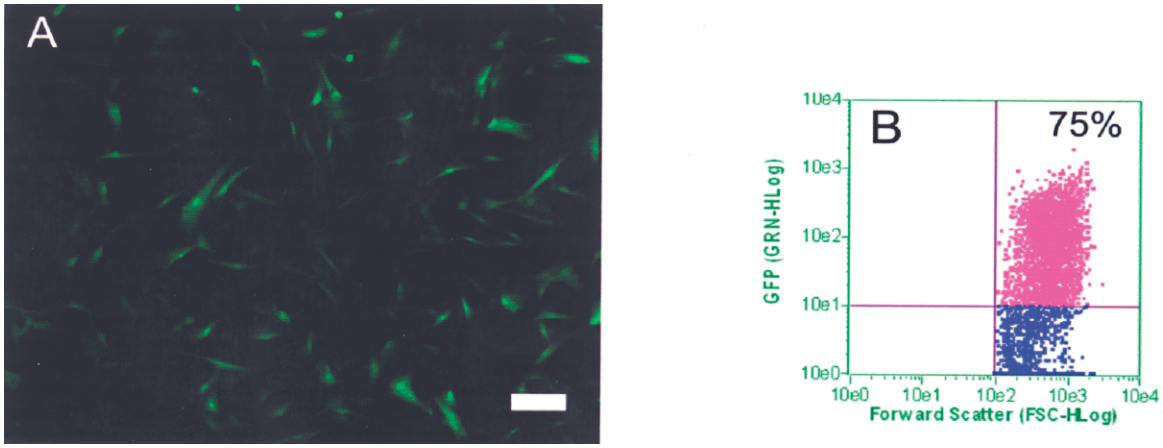

Figure 6. GFP-positive cASCs. (A) GFP-positive cASCs at passage 8. (B) Percentage of positively GFP cells as analyzed by flow cytometry.

ence of GFP on the cellular response to inductive media (Fig. 4).

DISCUSSION

Zuk et al. (35) were the first to describe the isolation and characterization of human stem cells derived from adipose tissue. These cells were able to differentiate into adipogenic, osteogenic, chondrogenic, and myogenic lineages when exposed to inductive media.

Human ASCs are usually obtained from fat tissue that is discarded after liposuction cosmetic surgery (35). Adipose tissue can be harvested in large quantities with minimal morbidity in several regions of the body and, on average, 100 ml of human adipose tissue yields about 1×10^6 stem cells (14). In dogs, the adipose tissue can be collected by a simple adapted liposuction surgery, through biopsies or in routine veterinary surgery procedures because we could isolate cASCs from just 100 µl of adipose tissue.

In the present study we show the isolation and characterization of the canine adipose-derived stromal cell (cASC) population. While this manuscript was in preparation, Neupane et al. (17) published an article reporting the isolation of canine adipose stem cells. However, their article lacked important characteristics of the isolated cell population such as the immunophenotype, the myogenic and chondrogenic potential, and karyotype analysis at late passages. These characteristics are important for veterinary cell therapy and preclinical studies.

Our results show that cASCs can be harvested by a rapid process, an important step towards preclinical studies of cell therapy. Using this methodology, we were able to harvest cells from 10 canine subcutaneous fat samples (2 from liposuction and 8 from biopsy) with a 100% rate of success. Other groups reported successful isolation of adipose stem cells from other mammals, such as rabbit, mice, horse, and pig (27,29,32,33).

We observed that the plastic adherent cells obtained after isolation can be expanded in vitro, reaching numbers that would be sufficient for a therapeutic assay, without any numeric chromosome alteration. In addition, cASCs can be stored frozen in liquid nitrogen without cell death.

During the first days in culture, endothelial cell populations were found in the plates; however, these cells were not seen after passage 4. These data are in accordance with Rodriguez et al. (21), where the isolation of hASCs by adherence properties was reported. At passage 4, cASCs show a fibroblast-like morphology commonly found in mesenchymal stem cell (MSCs). The analysis of the cell surface markers showed that the cASCs cell population expresses the known immnophenotype of MSCs (35). At passage 4 the majority of the cells are positive for CD29, CD44, and CD90. cASCs do not express the hematopoietic marker CD45, but 10% of the cells are CD34 positive. Traktuev et al. (28) described that the population of CD34-positive cells that are found in human adipose stromal-vascular fraction are reside in a periendothelial location. The authors showed that these cells are CD31 negative. This result is in accordance with our finding with cASCs, because we found CD34-positive cells but not CD31-positive cells. This adherent cASCs cell population ($CD34^+/CD31^-$) may also interact with endothelial cells at the perivascular niche. However, further studies will be essential to identify the localization of these cells at the canine adipose tissue. In order to evaluate the MSC property of cASCs, we subjected them to differentiation studies.

MSCs are defined by their ability to self-renew and

their capacity to generate committed cells in vitro and in vivo. Human ACSs can be induced to differentiate along the adipogenic, chondrogenic, and osteogenic lineages using specific culture medium (32). Even plated into scaffolds they survive long-term culture and could be terminally differentiated into adipocytes and osteoblasts (18). In all 3 of our 10 lineages obtained we demonstrated the multipotency and plasticity of cASCs by their differentiation in adipogenic, chondrogenic, myogenic, and osteogenic lineages. The differentiation was confirmed by the appearance of lipid vacuoles, mucopolysaccharide-rich extracellular matrix, myosin labeling, and calcium deposits, respectively. Although we observed a morphological change in cells submitted to adipogenic differentiation, we found poor lipid vacuoles in cASCs when compared to hASCs submitted to the same conditions.

The stable GFP expression after cASC transduction represents a great advantage in preclinical studies. GFP-positive cells could be screened though fluorescence microscopic imaging and flow cytometry, and no significant decline of GFP expression during cASC differentiation was found. This property allows their potential use for in vitro (29) and in vivo assays (3), where cell marking with GFP was used to track stem cells seeded on a scaffold, fusing to other cells and engraftment into different tissues.

Successful canine stem cell transplantation was reported by Sampaolesi et al. (24). According to these authors, the intra-arterial delivery of canine mesoangioblasts (vessel-associated stem cells) resulted in an extensive recovery of muscle morphology and function in golden retriever muscular dystrophy (GRMD) dogs. Because ASCs are much easier to be obtained and can be injected without immunosuppression (11,31), they might represent a promising alternative to canine mesoangioblasts for GRMD stem cell therapeutic trials.

In short, results from the present study demonstrate that adherent cells isolated from canine adipose tissue can be defined as multipotent MSC with the ability to differentiate into at least four mesodermal lineages. Mouse models of human diseases have been extensively used in preclinical studies. However, mouse models have several limitations when extrapolating to humans: the small size of the mouse limits the proliferative demand placed on transplanted tissue and the short life span of the mouse also prevents long-term follow-up. Large-animal models, such as the dog, more faithfully mimic human pathologies. There are many canine animal models of human genetic diseases that are well characterized, making them ideal for in vivo studies. The characterization of cASCs represents a valuable tool for in vitro and in vivo preclinical evaluation and for the screening of therapeutic drugs. These findings also have potential relevance to future canine veterinary tissue engineering and regenerative veterinary medical therapies.

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Capítulo 6

Células estromais derivadas de tecido adiposo humano são capazes de alcançar o músculo e expressar proteínas musculares humanas após injeções sistêmicas nos cães GRMD

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Human Adipose-Derived Mesenchymal Stromal cells injected systemically into GRMD dogs without immunosupression are able to reach the host muscle and express human dystrophin

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Abstract

Duchenne muscular dystrophy (DMD), a lethal X-linked disorder, is the most common and severe form of muscular dystrophies, affecting 1 in 3000 male births. Mutations in the DMD gene lead to the absence of muscle dystrophin and a progressive degeneration of skeletal muscle and wasting. The possibility to treat DMD through cell therapy has been widely investigated. We have previously shown that human adipose-derived stromal cells (hASCs) injected systemically in SJL mice are able to reach and engraft in the host muscle, express human muscle proteins and ameliorate the functional performance of injected animals without any immunosupression. However before starting clinical trials in humans many questions still need to be addressed in preclinical studies, in particular in larger animal models, when available. The best animal model to address these questions is the Golden Retriever Muscular Dystrophy (GRMD) dog that reproduces the full spectrum of human DMD. Affected animals carry a mutation that predicts a premature termination codon in exon 8 and a peptide that is 5% the size of normal dystrophin. These dogs present clinical signs within the first weeks of life involving the limbs as well as masticatory muscles. Diaphragmatic and intercostal muscles impairment leads to progressive respiratory failure. Most of them do not survive beyond age two. Here we show, for the first time, the results of local and intravenous injections of hASCs into GRMD dogs, without immunosupression. We observed that hASCs injected systemically into the dog cephalic vein are able to reach, engraft, and express human dystrophin in the host GRMD dystrophic muscle. Although there were a modest number of labeled fibers by IF we found a strong band of human dystrophin by WB up to 6 months after transplantation. Due to the great phenotypic variability seen in

GRMD dogs it is difficult to analyze the clinical impact of this procedure. But, most importantly, we demonstrated that injecting a huge quantity of human mesenchymal cells in a large animal model, without immunosupression, is a safe procedure, which may have important applications for future therapy in patients with different forms of muscular dystrophies, where heterologous transplantation is required.

Resumo

A Distrofia muscular de Duchenne (DMD), é uma doença letal ligada ao cromossomo X, é a forma mais comum e grave dentre as distrofias musculares, afetando 1 em cada 3.000 nascimentos masculinos. Mutações no gene DMD levam à ausência de distrofina no músculo e à degeneração progressiva dos músculos esqueléticos. A possibilidade de tratar a DMD através da terapia celular tem sido amplamente investigada. Recentemente nosso grupo demonstrou que células estromais humanas de tecido adiposo (*human Adipose-derived Stromal Cells* - hASCs) quando injetadas em camundongos *SJL,* sem imunossupressão, são capazes de atingir o músculo dos animais injetados, expressar proteínas musculares humanas e melhorar o desempenho funcional dos animais. Contudo, antes de começar testes clínicos em humanos, muitas perguntas ainda devem ser respondidas em estudos pré-clínicos, em particular em modelos animais de maior porte, quando disponível. O melhor modelo animal para responder a estas perguntas é o cão GRMD (*Golden Retriever Muscular Dystrophy*), que reproduz o espectro completo da DMD humana. Os animais afetados carregam uma mutação que causa um códon de parada prematuro no exon 8 e um peptídeo que é 5% do tamanho da distrofina normal. Estes cães apresentam sinais clínicos nas primeiras semanas de vida, afetando principalmente os membros e os músculos mastigatórios. A degeneração progressiva do diafragma e dos músculos intercostais leva à insuficiência respiratória. A maioria dos cães não sobrevivem além dos dois anos. Neste trabalho foi realizada a comparação de injeções locais e intravenosa de hASCs em cães GRMD, sem imunossupressão. Observamos que as hASCs injetadas na veia cefálica dos cães são capazes de atingir e expressar distrofina humana no

músculo distrófico dos cães GRMD. Embora tenhamos encontrado um pequeno número de fibras marcadas por imunoflourescência, uma banda forte de distrofina humana foi encontrada por WB até 6 meses após o transplante. Devido à grande variabilidade fenotípica observada em cães GRMD é difícil analisar o impacto clínico deste procedimento. Entretanto foi possível demonstrar que a injeção de uma enorme quantidade de células mesenquimais em um modelo animal de grande porte, sem imunossupressão, é um procedimento seguro. Este fato pode ter importantes aplicações para o tratamento futuro em pacientes com diferentes formas de distrofias musculares, onde o transplante heterólogo é necessário.

Human Adipose-Derived Mesenchymal Stromal cells injected systemically into GRMD dogs without immunosupression are able to reach the host muscle and express human dystrophin

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Running title: transplantation of human MSCs into GRMD dogs
Key words: human multipotent mesenchymal stromal cells; xenotransplantation; muscular dystrophy; therapy

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Abstract

Duchenne muscular dystrophy (DMD), a lethal X-linked disorder, is the most common and severe form of muscular dystrophies, affecting 1 in 3000-4000 male births. Mutations in the DMD gene lead to the absence of muscle dystrophin and a progressive degeneration of skeletal muscle and wasting. The possibility to treat DMD through cell therapy has been widely investigated. We have previously shown that human adipose-derived stromal cells (hASCs) injected systemically in *SJL* mice are able to reach and engraft in the host muscle, express human muscle proteins and ameliorate the functional performance of injected animals without any immunosupression. However before starting clinical trials in humans many questions still need to be addressed in preclinical studies, in particular in larger animal models, when available. The best animal model to address these questions is the Golden Retriever Muscular Dystrophy (GRMD) dog that reproduces the full spectrum of human DMD. Affected animals carry a mutation that predicts a premature termination codon in exon 8 and a peptide that is 5% the size of normal dystrophin. These dogs present clinical signs within the first weeks of life involving the limbs as well as masticatory muscles. Diaphragmatic and intercostal muscles impairment leads to progressive respiratory failure. Most of them do not survive beyond age two. Here we show, for the first time, the results of local and intravenous injections of hASCs into GRMD dogs, without immunosupression. We observed that hASCs injected systemically into the dog cephalic vein are able to reach, engraft, and express human dystrophin in the host GRMD dystrophic muscle. Although there were a modest number of labeled fibers by IF we found a strong band of human dystrophin by WB up to 6 months after transplantation. Due to the great phenotypic variability seen in GRMD dogs it is difficult to analyze the clinical impact of this procedure. But, most importantly, we demonstrated that injecting a huge quantity of human mesenchymal cells in a large animal model, without immunosupression, is a safe procedure, which may have important applications for future therapy in patients with different forms of muscular dystrophies, where heterologous transplantation is required.

Introduction

Progressive muscular dystrophies (PMD) are a clinically and genetically heterogeneous group of disorders caused by the deficiency or abnormal muscle proteins, resulting in progressive degeneration and loss of skeletal muscle function. As effective treatments for these diseases are still unavailable, they have been widely investigated as possible candidates for stem cell therapy. Among the different forms, the most frequent and severe form of muscular dystrophy is Duchenne Muscular Dystrophy (DMD), a recessive lethal X-linked disease. The onset is around 3-5 years of age and the clinical course is severe and progressive. Affected boys usually are confined to a wheelchair by 10-12 years of age and without special care and assisted ventilation death due to respiratory or cardiac failure occurs usually before the third decade[1].

The Golden Retriever Muscular Dystrophy (GRMD) dogs are the closest animal model to DMD. Affected animals carry a frameshift point mutation that causes the skipping of exon 7 and a premature stop codon, resulting in the absence of the dystrophin in their muscles. GRMD dogs and DMD patients have many phenotypic and biochemical similarities, including early progressive muscle degeneration and atrophy, fibrosis, contractures and elevated serum creatine kinase levels[2]. However, differently from the DMD patients, the GRMD dogs show difficulties in swallowing but the loss of ambulation is uncommon. They also show a great clinical variability. Death may occur within the first weeks but is most frequent around 1 or 2 years of age as a result of failure of respiratory or cardiac muscles[3].

Adult skeletal muscle regenerates new muscle fibers by activating a population of muscle cells precursors[4]. However, the continuous and gradual muscle degeneration in PMDs

leads to a depletion of satellite cells and, consequently, the capability to restore the skeletal muscle is lost[5,6]. One therapeutic approach to treat tentatively PMDs is to use stem cells as precursors to regenerate muscle fibers or compensate for the defective protein expression.

In the first pre-clinical trial involving stem cells and GRMD dogs, bone marrow hematopoietic stem cells were transplanted from normal litter mates to immunosuppressed GRMD dogs but dystrophin expression was not restored[7]. Subsequently, Sampaolesi et al (2006)[8] reported that the delivery of normal dog mesoangioblasts to the muscle of dystrophic dogs after intra-arterial injection resulted in the restoration of dystrophin expression. The mesoangioblasts show similarities with hASCs in cell surface protein analysis, proliferation and differentiation capacity. However, all transplanted dogs were maintained on steroids as standard treatment and received immunosuppressant drugs, which makes difficult to evaluate clinical results, since it is known that immunosuppressive and anti-inflammatory drugs can ameliorate the phenotype in muscular dystrophy patients[9]. Our group previously analyzed the result of early systemic delivery of human dental pulp stem cells in GRMD dogs but very few dystrophin positive labeled fibers were found in just one injected dog at the host muscle [10].

An abundant and accessible source of stem cells is adipose tissue. Human adipose-derived stromal cells (hASCs) have the ability to differentiate into skeletal muscle when in contact with dystrophic muscle cells in vitro[11] and in vivo[12-14]. Rodriguez at (2005)[12] reported that local injections of hASCs into the muscle of *mdx* mice restored dystrophin expression in the area nearby the injected place. However, the *mdx* mice have no evident muscular weakness and therefore are not a good model to assess potential functional effects of stem cell therapy. We have previously shown that hASCs injected systemically in *SJL* mice, the murine model for

LGMD2B muscular dystrophy, were able to reach the host muscle, engraft, express human muscle proteins and ameliorate their functional performance. These results led us to investigate if the hASCs have a comparable behavior in the closest animal model to human DMD, the GRMD dogs.

Here we show for the first time the results obtained with systemic transplantation of hASCs, with no immunosupression, in four affected male litter-mate GRMD dogs, with a protocol comparable to the previously used in the *SJL* mice model[15]. With this study we aimed to assess if hASCs are able to migrate, engraft, and differentiate into muscle cells expressing human dystrophin in a large animal model. We also analyzed the efficiency of local injections compared to multiple systemic injections and how long we could detect human cells after the last injection.

Results

Characterization of hASCs

hASCs were previously characterized[15] by flow cytometry for the expression of 12 cell surface proteins (HLA-DR, HLA-ABC, CD13, CD29, CD31, CD34, CD44, CD45, CD73, CD90, CD105 and CD117). Cell viability was above 96% by Guava ViaCount reagent (Guava Technologies).

At passage 4, hASCs did not express endothelial markers (CD31- PECAM1) nor hematopoietic markers (CD34, CD45 and CD117-c-kit). The majority of hASCs expressed high levels of CD13, CD44, adhesion markers (CD29-integrin ß1, CD90-Thy-1) and mesenchymal stem cell marker CD73 (SH3). Expression of some markers, such as CD105 (SH2), was variable among

the donors. hASCs were negative for HLA-class II (HLA-DR), but positive for HLA-class I (HLA-ABC) (data not shown).

The plasticity of hASCs was assessed three weeks after lineage induction[15]. Myogenic, adipogenic, chondrogenic and osteogenic differentiation was demonstrated by the expression of myogenic markers (myosin and desmin), lipid vacuoles, mucopolysaccharide-rich extracellular matrix and calcium deposits, respectively. These results confirmed the mesenchymal nature of the isolated cells as well as their multipotent potential (data not shown).

hASCs capacity to reach and engraft at the host muscle

In order to assess the potential of hASCs to reach and colonize the host muscle we injected undifferentiated hASCs, previously characterized by flow cytometry and differentiation potential[15] into the cephalic vein of four dogs. One uninjected GRMD carrier female from the same litter was analyzed as age-matched normal control. Two dogs (Peso, II-8 and Real, II-11) were euthanized due to hiatal hernia. II-8 received only six injections, the first three months of the injection protocol; II-11 received all 9 injections but was euthanized one week after the last injection. In order to compare the results of systemic injections versus local delivery we injected two homozygous affected GRMD females from another litter at the biceps femoralis.

The PCR method as previously reported by Pelz et al. (2005)[16] was used to evaluate the presence of human cells in the biceps femoralis of the systemically and locally injected animals. All systemically injected animals showed human DNA in the biceps femoralis(Fig 1a). However we did not find human DNA in any of locally injected muscles (Fig 1b).

Muscle differentiation in the host muscle

To evaluate the presence of human muscle proteins in the GRMD host muscles, we assessed the presence of human-dystrophin with specific human antibodies[17].

Through western-blot (WB) analysis, human dystrophin bands were found in the muscle of all injected animals until 6 months after the last injection (Fig 2a) but no dystrophin was found one year after the last injection (Fig 2b). By immunofluorescence (IF) analysis only a modest number of labeled fibers were found in the injected animals using the anti-human dystrophin antibody (Fig 3). Immunofluorescence analysis with anti-human nuclei antibody also showed a few positive labeled nuclei.

No human DNA was found at the injected muscles following local injections, and as expected, no positive dystrophin bands were observed by WB as well (Fig 2c).

Discussion

The role of mesenchymal stem cells (MSCs) aiming clinical applications for progressive muscular dystrophies (PMD) is still controversial. According to some authors these cells would mediate tissue repair through their multilineage differentiation potential enabling them to replace damaged cells. Others suggest that this mechanism is unlikely and believe that in response to tissue injury, MSCs home to the site of damage and enhance repair through the production of trophic factors. These include growth factors, cytokines and antioxidants, some of which providing the basis for their capacity to modulate immune responses[18] .

Two different authors reported that hASCs are able to differentiate *in vivo* when injected directly into the muscle of the *mdx* mice[12,14]. According to the first author[12] local injections of hASCs into the *mdx* mice muscle result in a huge dystrophin expression. Subsequently, we reported that hASCs are not rejected when transferred systematically to the *SJL* mice without any immunosuppression. These cells were able to fuse with the host muscle cells, express human skeletal muscle proteins and improve the motor ability of affected animals. Although the *SJL* mice have a mild phenotype we observed functional recovery after 6-months of *in vivo* treatment which was encouraging[15].

A significant obstacle in designing cell therapy for PMDs is the necessity to reach the entire body musculature, a problem that cannot be easily overcome unless systemic cell delivery methods of a large number of cells are proved to be effective. The GRMD dog, which has the weight of an 8 year-old child, is the closest animal model for DMD. They develop progressive, fatal disease strikingly similar to human condition. Therefore studies in GRMD dogs may be more likely than those in mice models to predict pathogenesis and treatment outcome in DMD[19]. Since cell therapy for DMD cannot be autologous, preclinical studies with heterologous stem-cells in larger animal model for muscular dystrophies are of utmost importance before starting clinical trials. Therefore we have repeated the same experiment done with *SJL* mice in GRMD dogs.

Here we show for the first time that hASCs delivered systemically into GRMD dogs are able to reach and express human dystrophin in the host muscles, without immunosuppression. Although two dogs died, of hiatal hernia, a frequent cause of death in GRMD dogs (Joe

Kornegay , personal communication) most likely unrelated to the stem-cell transplantation, human dystrophin was found in their necropsied muscle. In the two dogs that are alive, Yuan and Dollar, muscle biopsies were repeated two, six and twelve months after the last injection. Human dystrophin was not found after 12 months but it was still present two and six months after the last injections.

This result indicates that a huge quantity of cells can be xenotransplanted systemically, repeatedly, in non-immunosupressed animals without rejection. However, differently from previous observations in *mdx* mice[12] the hASCs injected locally in the biceps femoralis of two other GRMD dogs were not found at the host muscle after 1 month. This observation reinforces that the systemic delivery of hASCs seems much more effective than local to reach and engraft into the host muscles, in accordance to our previous results with human dental pulp stem cells[10]. On the other hand, the present results with hASCs, if confirmed, suggest that biannual systemic injections would be required for the maintenance of injected cells in the host muscles if used therapeutically.

Muscle degeneration associated with DMD is a complex process in which inflammatory events play a major role in disease progression. hASCs show enhanced immunosuppressive capacity under inflammatory conditions, without losing their differentiation capacity[20]. The fact that we found human dystrophin through WB but very little through IF is not surprising taking into account that the procedure involved a xenotransplantation in outbreed GRMD dogs. A widespread muscle expression of an AAv9 human mini-dystrophin vector following intravenous injections in neonatal dystrophin-deficient dogs was recently reported. At the end of 16 weeks, two out of three dogs showed generalized expression of mini-dystrophin in 15 to nearly 100%

of myofibers. However, although there was a widespread muscle expression of mini-dystrophin, the affected dogs had pelvic limb girdle muscle atrophy and contractures, apparently associated with an early innate immune response[19]. In another study, patients with frameshifting deletions in the DMD gene, who were injected with functional mini-dystrophin incorporated at recombinant adeno-associated virus (rAAV), showed autoreactive dystrophin-specific T cells. The authors suggest that monitoring of cellular immune responses should be a priority for any experimental therapy in PMD[21]. Differently from these two studies, here the injected stem cells carried a normal human dystrophin gene. It is possible that human dystrophin of normal length was not able to be assembled properly in the dystrophin-glycoprotein complex in the canine genetic background. The host immune response could also explain why we found a significant human dystrophin expression at the muscles of injected dogs mostly by WB since this technique allows to detect the presence of the protein even if is not well associated with the dystrophin associated protein complex or in the process of degradation.

Recent reports on the clinical impact of stem cell transplantation in PMDs animal models have shown different results. We observed an improvement in the performance of the *SJL* mice after the transplantation of hASCs[15] but not after the transplantation of human umbilical cord mesenchymal stromal cells in the same animal model[3]. Other researchers reported no functional improvement in the *mdx* mice after the transplantation of bone marrow mesenchymal stem cells, even expressing dystrophin.[22]

Yuan and Dolar, currently aged 26 months, are being followed in our kennel. One of them, Dolar, always had a milder phenotype while Yuan, who was more severely affected, showed an apparent improvement. However it is difficult to know if this occurred as a result of

the cell transplantation, a better clinical management or due to the great phenotypic variability in GRMD dogs. Therefore, it will be extremely important to repeat this experiment in a larger number of animals to be followed for longer periods.

Therapeutic approaches for DMD have analyzed their success based on dystrophin expression at the dystrophic muscle. However recently, different studies have shown that the rescue of dystrophin expression may not result in clinical improvement[19,22]. In addition, it is well known that the *mdx* mice are almost asymptomatic despite the absence of muscle dystrophin. Most important, we have reported that a milder phenotype can occur in GRMD dogs despite the complete absence of muscle dystrophin[3], which has been also described in a 7-year-old DMD boy[23] and more recently in Labrador retriever (LRMD) dogs (Diane Shelton, personal communication). The fact that is possible to find such a milder phenotype in large animal models, indicates that other still unknown factors, other than muscle dystrophin, may play a key role in muscle recover and function.

In addition, a growing body of evidence suggest that mesenchymal stromal cells have the capacity to modulate immune responses via direct and indirect interactions with a broad range of cell types[18]. These observations indicate that the success of therapeutic trials should not be based solely on the molecular analysis to assess the rescue of the defective muscle protein expression. Long term follow-up is mandatory before reaching any conclusion.

In short, here we show for the first time that hASCs are not rejected when systematically transferred intravenously to the GRMD dog without any immunosupression, are

able to fuse with the host muscle cell and express human skeletal muscle proteins at least 6 months after the last injection. Our results also showed that multiple injections, with intervals no longer than 6 months, are required to maintain the presence of exogenous dystrophin. We also observed that no human DNA or dystrophin was found in the host muscles following local injections.

These results open new avenues for pre-clinical researches which may have important applications for future therapy with the advantages that: a) it may be applicable to patients affected with different forms of progressive muscular dystrophies, regardless of their specific disease causing mutation; b) human liposuctioned fat is available in large quantities and hASCs can be easily obtained without any in vitro genetic modification or induction. Although it remains to be seen if allogenic transplantation can be also done in humans without any immunosupression, recent evidences suggest that MSCs have the property to eliminate the requirement of immunosuppressive drugs. Therefore, this simple approach here reported, if confirmed in a larger group of animals, may represent a great step toward clinical application for the future therapy of different forms of progressive muscular dystrophies.

Materials and Methods

All experiments were approved by the research ethics committee of the Biosciences Institute, University of São Paulo. All human samples were obtained after written informed consent from the donors. All researches were carried out in the Human Genome Research Center, and the GRMD Genocão (Genedog) kennel at the Biosciences Institute, University of São Paulo.

hASC isolation and expansion

Human adipose tissue was obtained from elective liposuction procedures. Cells were isolated using methods previously described[11]. Briefly, the unprocessed lipoaspirate was washed extensively with equal volumes of phosphate-buffered saline (PBS) containing antibiotics (100 U/ml of penicillin and 100g/ ml of streptomycin; Gibco) and then dissociated with 0,075% collagenase (Sigma). Enzyme activity was neutralized with Dulbecco's modified Eagle's media - high glucose (DMEM-HG – HG; Gibco) containing 10% Fetal Bovine Serum (FBS; Gibco). The infranatant was centrifuged at 1200 x g for 5 min to pellet the cells. The cells from the pellet SVF were filtered to remove debris and seeded in tissue culture plates (NUNC) at 1,000–3,500 cells/cm^2 in DMEM-LG 10% FBS. Cultures were washed with PBS 24-48 hours after plating to remove unattached cells and fed with fresh media.

The cultures were maintained at 37°C with 5% CO_2, in growth media (GM- DMEM-LG 10% FBS). When they achieved about 70% confluence, the cells were trypsinised (0,025%, TrypLE Express; Gibco) and plated at a density of 5000 per cm^2. Cultures were passaged repeatedly after achieving a density of 70%-80% until passage 4. The remaining cells were cryopreserved in cryopreservation media (10% dimethylsulfoxide, 10% DMEM-LG, 80% FBS), frozen at -80°C in isopropanol-jacked closed container and stored in liquid nitrogen the next day.

Flow Cytometry

The flow cytometry was performed on Guava EasyCyte System (Guava Technologies) using a blue laser (488 nm). Cells were pelleted, resuspended in PBS at concentration of 1 x 10^5

cells/ml and stained with saturating concentration of antibodies. Cells were incubated in the dark for 45 minutes at room temperature. After incubation, cells were washed three times with PBS and resuspended in 0.25 ml of cold PBS. Cell viability was accessed with Guava ViaCount reagent (Guava Technologies).

hASCs were incubated with the following primary antibodies: HLA-DR-PE, HLA-ABC-FITC, CD13-PE, CD29-PECy5, CD31-PE, CD34-PerCP, CD44-FITC, CD45-FITC, CD73, CD90-PE, CD105 e CD117-PE (Becton Dickinson). Unconjugated markers were reacted with anti-mouse PE secondary antibody (Guava Technologies).

Flow cytometer gates were set using unstained cells. Cells were gated by forward scatter to eliminate debris. To eliminate the possible autofluorescence of ASCs, we removed the contribution of unstained cells in the measurement channel. A minimum of 10,000 events was counted for each analysis.

Multilineage Differentiation

Cells were analyzed for their capacity to differentiate toward adipogenic, osteogenic, chondrogenic and myogenic lineages as described in Zuk et al (2001)[11].

Animals

All animals were housed and cared for in the University of São Paulo and genotyped at birth as previously described[24]. GRMD dogs were identified by microchips. Animal care and experiments were performed in accordance with animal research ethics committee of the Biosciences Institute, University of São Paulo. A total 7 dogs were used in this study.

Systemic transplantation

Four 2-month old male dogs were transplanted and one carrier female was maintained as normal control. To minimize the effects of variability previously observed in GRMD dogs we used animals from the same litter (Fig 4). Each animal was injected in the cephalic vein with 5 $.10^7$cells $.kg^{-1}$ in 0.1ml of Hank's Buffered Salt Solution (HBSS). Three animals were injected for 6 months, weekly in the first month and then monthly, receiving a total of 9 injections. The cells were injected right after their preparation. One dog (II-8) died at 4-months old, one week after the 5^{th} injection, and another (II-11) at 11-months old, one week after the 9^{th} injection. The two dogs that are still alive are currently 26-months old.

Local transplantation

Two female 2 years old affected dogs, from another litter, received a single injection of 10^7cells in 0.1ml of Hank's Buffered Salt Solution (HBSS) into the biceps femoralis.

Muscle biopsies

Biceps femoralis biopsy samples were taken from all dogs before and after hASCs transplantation.

In dogs systemically injected, DNA and muscle dystrophin were analyzed in autopsied muscle while in the two dogs that are alive, Yuan and Dolar, muscle biopsies were taken before the hASCs transplantation, 2 months, 6 months and one year after the last injection. Samples of biceps femoralis from a normal and GRMD dogs from comparable age were cryoprotected and frozen in liquid nitrogen and used as controls.

In the two female dogs that received local injections muscle biopsies were taken 1 month after being injected.

Human DNA analysis

The DNA was obtained using DNeasy Blood & Tissue Kit (Qiagen). The presence of human DNA in the host samples were evaluated as described in Pelz et al (2005)[16]. Centromeric region of human chromosome 7 and the dog dystrophin gene was amplified by PCR (35 cycles, annealing at 59ºC). The PCR products were separated by electrophoresis on 2% agarose gels and stained with ethidium bromide. Non-saturated digital images were obtained using an ImageQuant imaging system (GE HealthCare).

Immunofluorescence and histology

Muscle samples were frozen in liquid N2. 7-μm sections were used for routine histology and immunohistochemistry. Hematoxylin and eosin stain was carried out for morphology studies. Each sample was analyzed by two different and independent persons in blind test. Serial muscle crio-sections were fixed in 4% paraformaldehyde in PBS for 20 min at 4°C, permeabilized in 0,05% Triton X-100 in PBS for 5 minutes. Non-specific binding was blocked with 10% FBS in PBS for 1 hour at room temperature. Muscle sections were incubated with primary antibody overnight at 4°C and with secondary antibody for 1 hour at room temperature. The following primary antibodies were used: anti-dystrophin Dys1 (1:300; Vector); anti-human-dystrophin Mandys1062C6 (1:100), kindly provided by Dr. Glenn E. Morris at Center for Inherited Neuromuscular Diseases, Oswestry, Shropshire, UK; anti human-nuclei (1:100; Chemicon) combined with rabbit anti-mouse IgG secondary antibody Cy3-conjugated

(1:200; Chemicon) or FITC-Conjugated (1:100; Chemicon). We visualized nuclei with 4',6'-diamidino-2-phenylindole (DAPI; Sigma). The fluorescence signal was examined in Axiovert 200 (Carl Zeiss) and in AxioImager Z1 (Carl Zeiss).

Western Blot Analysis

Muscle sample proteins were extracted through treatment with a buffer containing 10mM Tris-HCl (pH 8.0), 150mM NaCl, 5mM EDTA, 1% Triton X-100 and 60mM octylglucoside. Samples were centrifuged at 13,000 x *g* for 10 minutes to remove insoluble debris. Soluble proteins were resolved by 6% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membranes (Hybond; Amersham). All membranes were stained with Ponceau (Sigma) to evaluate the amount of loaded proteins. Blots were blocked for 1 hour in Tris-buffered saline Tween (TBST) containing 5% powdered skim milk and reacted overnight with the following primary antibody: anti human-dystrophin Mandys1062C6(1:100) kindly provided by Dr. Glenn E. Morris at Center for Inherited Neuromuscular Diseases, Oswestry, Shropshire, UK. Blots were incubated one hour with secondary antibodies. Immunoreactive bands were detected with ECL chemiluminescence detection system (GE Healthcare).

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Figures Legend

Figure 1 – Polymerase chain reaction analysis for human chromosome 7 -satellite sequences H) and canine dystrophin gene C) of biopsied muscle DNA samples. a) Muscle samples of systemically injected animals B-before A-after the injections; II-6 and II-8 samples were taken 2-months after the last injection; II-7 and II-11 died before this period, samples shown are form necropsied muscle; H) Human DNA; C) canine DNA. b) Muscle samples of locally injected animals. Samples: 1 and 2) locally injected females; H) Human DNA; C) canine DNA.

Figure 2– Western blot analysis for human-dystrophin of the muscles of injected animals. a)Muscle samples of systemically injected animals B-before A-after the injections; II-6 and II-8 samples were taken 2-months after the last injection; II-7 and II-11 died before this period, samples shown are form necropsied muscle; G) GRMD muscle protein; H) Human muscle protein; C) canine muscle protein. b) Muscle samples of still alive systemically injected animals B-before, 2m- 2months after the last injection, 6m - 6months after the last injection, 1y – 1year after the last injection; C) canine muscle protein. c) Muscle samples of locally injected animals. Samples: 1 and 2)inejected females H) Human muscle protein; C) canine muscle protein. Hu-Dyst = Specific anti-human-dystrophinMandys 106 2C6 Antibody. Myosin = myosin band detected in the Ponceau S pre-stained blot, for the evaluation of loaded muscle proteins.

Figure 3 -Expression of dysferlin and human-dystrophin in the muscle from human and dog normal controls, in the systemic injected animals.Immunofluorescence analysis of dystrophin, human-dystrophin and human nuclei of the biceps femuralis muscle of the injected and control. Histopathological analysis in skeletal muscles from injected dogs showed comparable alterations including size variation among individual muscle fibers, fiber splitting, small regenerated basophilic fibers, numerous fibers with centrally located myonuclei and significant connective tissue replacement.

Figure 4 – Pedigree of the systemic injected dogs.

Figure 5 – Dolar and Yuan. 1 week and 1 year after the last injection.

Figure 1

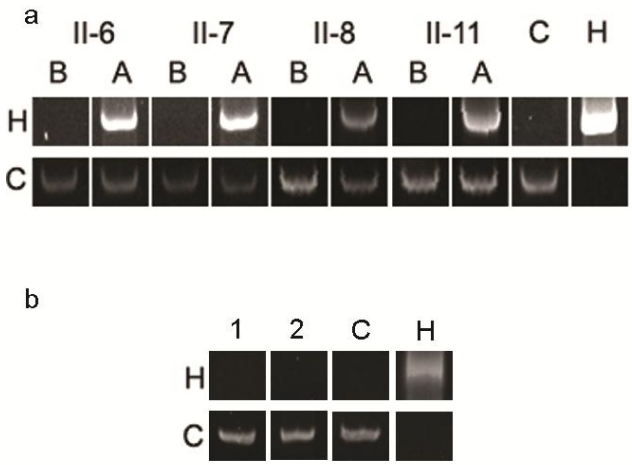


Figure 2

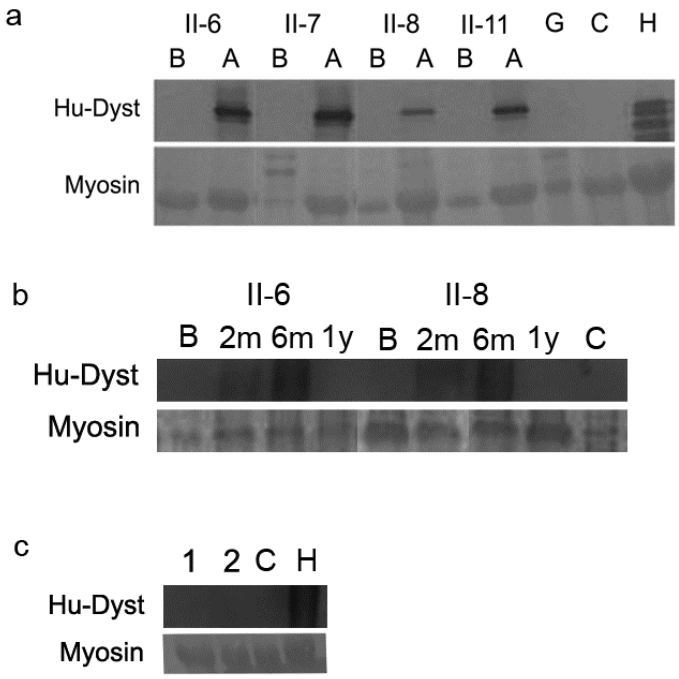


Figure 3

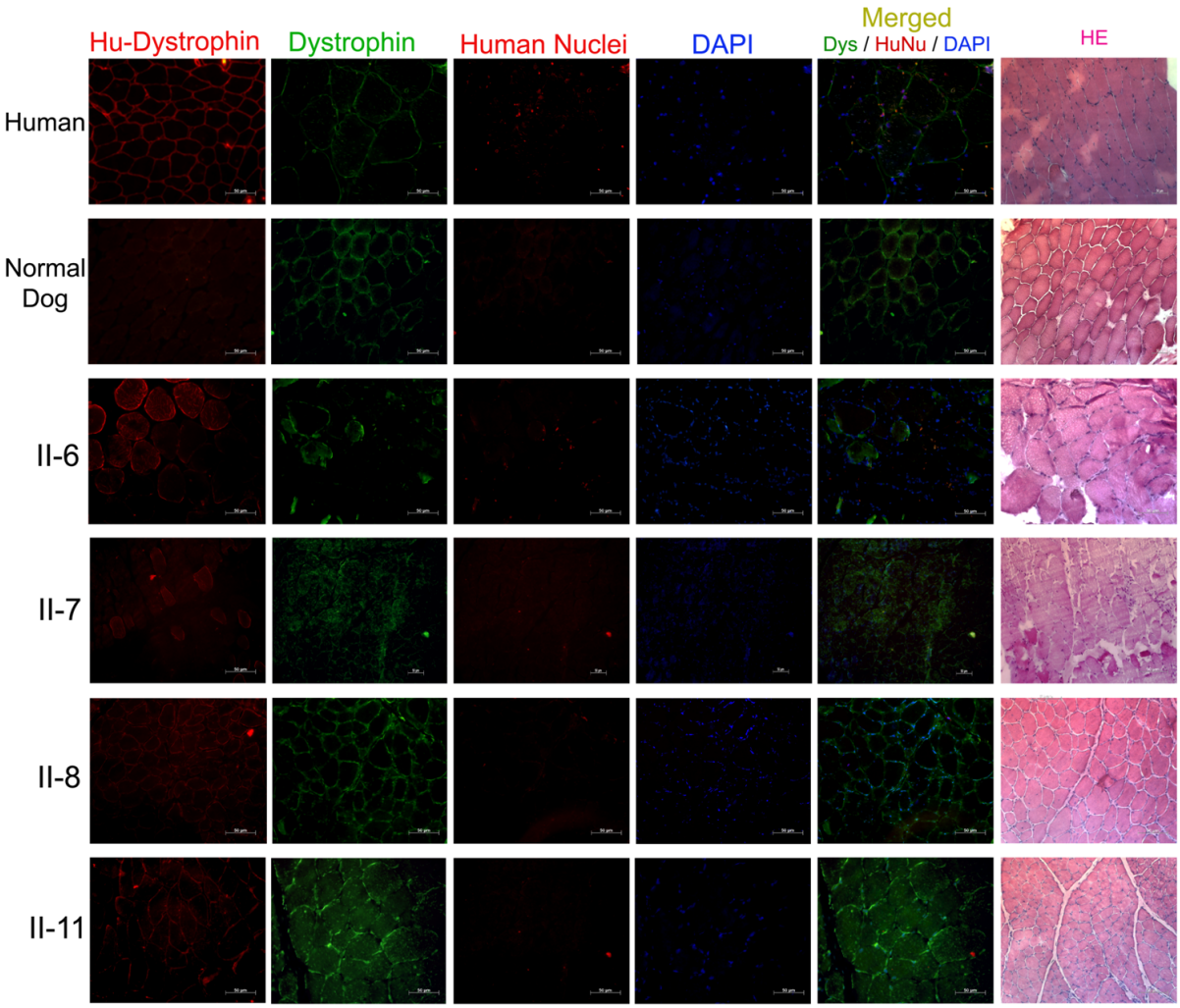


Figure 4

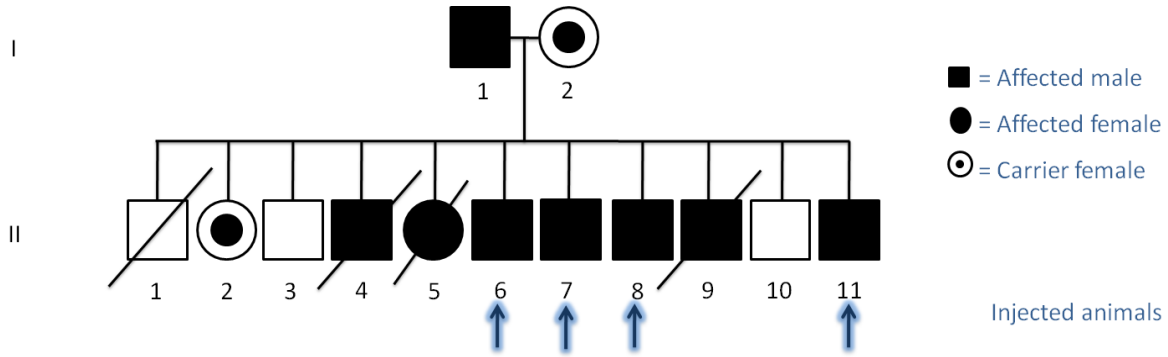
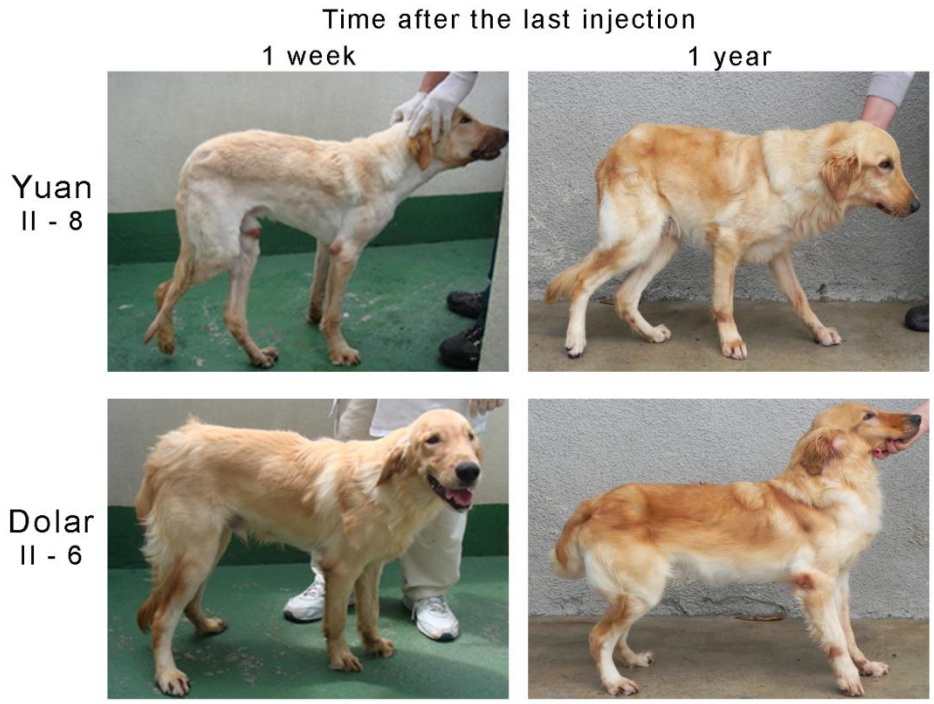


Figure 5


Time after the last injection
1 week
1 year
Yuan
II - 8
Dolar
II - 6

Discussão Geral

O tecido muscular, em resposta a uma lesão, ativa as células satélites que se dividem e fundem-se a fim de reparar as fibras musculares danificadas. No entanto, a população de células satélites representa apenas de 1 a 5 por cento do total de células musculares e seu potencial de auto-renovação diminui com a idade do indivíduo. Em pacientes portadores de DMPs a degeneração intensa que ocorre nas fibras musculares leva ao esgotamento da capacidade de proliferação das células satélites e assim do seu potencial de substituir/reparar as fibras danificadas[1]. A possibilidade de aumentar o reparo do tecido muscular via terapia celular tem sido foco de diversos estudos, principalmente visando sua aplicação em DMPs.

Entretanto, antes de se propor uma terapia celular para DMPs é necessário encontrar uma fonte de células-tronco ideal para este fim. Esta fonte teoricamente deve ser de fácil obtenção, pouco invasiva, rica em células-tronco, que possam ser expandidas mantendo suas características originais e que apresentem potencial de se infiltrar nos tecidos, multiplicar-se, diferenciar-se e manter-se após o transplante. Em uma terapia celular para DMPs é esperado que estas células sejam capazes de: a) interagir com as células musculares (mioblastos e/ou miotubos); b) atingir o tecido muscular se injetadas por via sistêmica sem serem rejeitadas; c) se diferenciar em tecido muscular, e somente neste, *in vivo* quando em contato com músculo ou diminuir a degeneração das fibras distróficas.

Zuk et al. (2001)[2] demonstrou que hASCs são capazes de diferenciar em músculo *in vitro* quando expostas a fatores de indução. Estas células são capazes de se fundir, formar miotubos multinucleados e expressar proteínas musculares[3].

Como as hASCs são capazes de se diferenciar em células musculares *in vitro,* verificar seu potencial de interação com células musculares (mioblastos e miotubos), principalmente com células de pacientes portadores de DMPs era o primeiro passo. Neste sentido analisamos o potencial das hASCs de interagir com células musculares

humanas de pacientes DMD e de pessoas normais *in vitro*. hASCs foram co-cultivadas com mioblastos e com miotubos a fim de avaliar se estas células eram capazes de interagir *in vitro,* e se, quando co-cultivadas com células de pacientes DMD, eram capazes de restaurar a expressão de distrofina *in vitro*. Observamos que as hASCs foram capazes de fundir com os mioblastos, bem como com miotubos e expressar distrofina em ambas as situações[4].

Para serem boas candidatas para terapia celular em DMPs as hASCs devem ser capazes de interagir com o músculo distrófico *in vivo*. Dois diferentes autores relataram que hASC são capazes de diferenciar *in vivo* quando injetadas diretamente no músculo do camundongo *mdx*[5,6]. No entanto, um obstáculo significativo na elaboração de um protocolo de terapia celular para DMPs é a necessidade de atingir a musculatura do corpo inteiro, um problema que não pode ser facilmente superado sem métodos eficazes de entrega sistêmica das células.

Para verificar se essas células têm o potencial de atingir o músculo distrófico após injeções sistêmicas, injetamos hASCs via endovenosa em camundongos *SJL*, sem imunossupressão. Além de atingir o músculo distrófico após injeção sistêmica essa pesquisa teve como objetivo verificar se as CT injetadas eram capazes de formar fibras musculares quiméricas (humano/camundongo); expressar proteínas musculares humanas no músculo dos camundongos e melhorar o seu desempenho. Observamos que hASCs não são rejeitadas após a injeção sistêmica, mesmo sem imunossupressão, são capazes de fundir-se ao músculo do camundongo, expressar uma quantidade significativa de proteínas musculares humanas e melhorar a habilidade motora dos animais injetados[7]. Foram injetados também animais normais, e nestes não foram encontradas células nos músculos, indicando que fatores que influenciam o *homing* das células estão sendo secretados pelo músculo distrófico.

Diferentes fontes de células-tronco mesenquimais apresentam o mesmo potencial de diferenciação miogênico *in vitro*. Para avaliar se estas apresentam o mesmo potencial *in vivo* ou se este depende do nicho de onde foram retiradas, comparamos o potencial de hUCT MSC com as hASCs no modelo *SJL*. Foram injetadas hUCT MSCs na veia caudal dos camundongos *SJL* obedecendo ao mesmo

protocolo utilizado anteriormente para injeção de hASCs. Avaliamos a capacidade dessas células de atingir o músculo dos camundongos após injeção sistêmica, de expressar proteínas musculares humanas e seu efeito no desempenho funcional dos animais. Vimos que as hUCT MSCs foram capazes de atingir o músculo porém não de se diferenciar em células musculares. Diferentemente dos animais injetados com hASCs, além de não encontrarmos expressão de proteínas musculares humanas, não foi observada melhora funcional. Entretanto, o grupo tratado com hUCT MSCs apresentou um desempenho físico significantemente melhor que o grupo não tratado. Essa observação sugere que o potencial terapêutico destas células não está restrito ao reparo do tecido danificado, pois mesmo sem se diferenciar no tecido alvo, as CTMs podem secretar fatores que levem a uma melhora funcional[8].

O melhor modelo animal para DMPs é o cão GRMD que reproduz todas as características da DMD humana. Visto que a terapia celular para DMD não pode ser autóloga, os estudos pré-clínicos com células-tronco heterólogas em modelo animal de maior porte para distrofias musculares são de extrema importância antes de iniciar os ensaios clínicos. Antes de testar a eficiência do transplante de células-tronco mesenquimais de tecido adiposo (*Adipose-derived Stem Cells – ASCs*) nos cães GRMD caracterizamos as células ASCs caninas (cASCs) para verificar se estas possuem o mesmo potencial de diferenciação *in vitro* que as hASCs. Se houvesse uma reação imunológica ao xenotransplante com células humanas, seria interessante injetar cASCs nestes animais. Entretanto vimos que o cultivo das cASCs é muito mais difícil que das hASCs. As cASCs mostraram ter o mesmo potencial das hASCs[9] mas, como não houve reação imunológica quando injetamos hASCs, não foi necessária seu uso nos cães GRMD. Talvez, futuramente sejam realizadas tais injeções visando comparar a eficiência com o xenotransplante realizado neste trabalho.

Para avaliar a segurança e o potencial da utilização de hASCs para terapia celular de DMPs utilizamos nos cães GRMD o mesmo protoloco de injeções utilizados nos camundongos distróficos. Além disso comparamos o sucesso de injeções locais *versus* sistêmicas (intravenosa) de hASCs, sem imunossupressão. Observamos que as hASCs injetadas na veia cefálica dos cães foram capazes de atingir e expressar

distrofina humana no músculo distrófico dos cães GRMD, o que não aconteceu após as injeções locais. Embora tenhamos encontrado um pequeno número de fibras marcadas por imunoflourescência, uma banda forte de distrofina humana foi encontrada por WB até 6 meses após o transplante. Devido à grande variabilidade fenotípica observada em cães GRMD é difícil analisar o impacto funcional deste procedimento. Entretanto foi possível demonstrar que a injeção de uma enorme quantidade de células mesenquimais em um modelo animal de grande porte, sem imunossupressão, é um procedimento seguro.

Os resultados de ensaios pré-clinicos e clínicos envolvendo o potencial terapêutico das células-tronco mesenquimais para distrofias musculares progressivas ainda são muito controversos. Segundo alguns autores, as CTMs poderiam mediar o reparo do tecido por meio de seu potencial de diferenciação, realizando assim a substituição das células danificadas. Outros sugerem que este não é o principal mecanismo de ação das CTMs, que estas atuam liberando fatores que possam modificar o nicho modulando o potencial de reparo do próprio organismo. Estes fatores poderiam ser fatores de crescimento, citocinas e antioxidantes, e agentes imunomodulatórios[10].

Yuan e Dólar, dois cães injetados com hASCs, atualmente com 26 meses de idade, estão sendo acompanhados em nosso canil. Um deles, Dólar, sempre teve um fenótipo mais leve, enquanto Yuan, que é mais afetado, mostrou uma melhora aparente. No entanto, é difícil saber se isso ocorreu como resultado do transplante de células, ou a um melhor manejo dos animais. Portanto, será extremamente importante repetir este protocolo em um número maior de cães GRMD seguidos por um longo período de tempo após o transplante.

As abordagens terapêuticas para a DMD têm analisado o seu sucesso com base na restauração da expressão de distrofina no músculo. No entanto, recentemente, diversos estudos têm mostrado que o resgate da expressão de distrofina pode não estar associado a uma melhora clinica[11,12]. Ou seja, o quadro clínico pode não ser distrofina dependente. Uma ilustração deste fato são os camundongos *mdx,* que são praticamente assintomáticos apesar da ausência de distrofina muscular[13]. Um fenótipo

mais leve pode também ocorrer excepcionalmente em cães GRMD, apesar da ausência completa de distrofina no músculo[14]. Mais recentemente foram encontrados cães sem distrofina e com fenótipo normal, da raça Labrador Retriever (LRMD) (Diane Shelton, comunicação pessoal). Estas observações indicam que o sucesso dos ensaios terapêuticos não deve basear-se unicamente na análise da restauração da expressão da proteína defeituosa no músculo.

A possibilidade de encontrar um fenótipo tão leve em modelos animais de grande porte indica que outros fatores, ainda desconhecidos, além de distrofina muscular, podem desempenhar um papel fundamental na função do músculo. Entender quais são estes fatores será fundamental na validação das tentativas terapêuticas em DMPs além de poderem representar uma terapia para este grupo de doenças.

Sendo assim este trabalho, apesar de apresentar resultados animadores para desenvolvimento de uma terapia celular para DMPs, que devem ser confirmados e aprofundados em amostras maiores, mostra outras perspectivas que podem levar ao tratamento dessas doenças.

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Conclusões

1. As células-tronco mesenquimais de tecido adiposo (*human Adipose-derived Stem Cells*, hASCs) são capazes de se diferenciar em músculo *in vitro* quando em contato com mioblastos e miotubos.
2. hASCs são capazes de chegar ao músculo distrófico após injeção sistêmica endovenosa
3. hASCs são capazes de interagir com o músculo de camundongos distróficos *in vivo*, expressar proteínas musculares humanas sem imunossupressão
4. hASCs não foram encontradas no músculo de camundongos normais após injeções sistêmicas o que sugere que o músculo distrófico libera fatores que influenciam no *homing* de CTMs.
5. hASCs podem ser injetadas em grandes quantidades, sem imunossupressão, repetidas vezes.
6. Quando injetadas em modelo animal de grande porte (cães) estas células são capazes de chegar ao músculo e se diferenciar, expressando proteínas musculares humanas
7. Mais testes devem ser realizados em cães GRMD antes do início de testes clínicos.

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