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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).

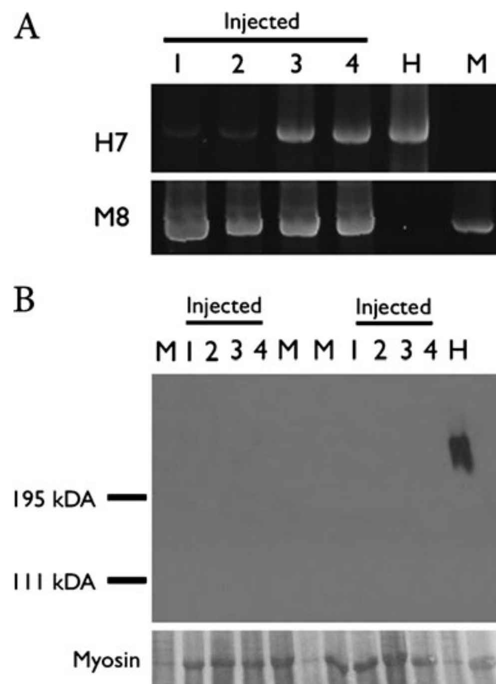

Fig. 1 **a** Polymerase chain reaction analysis for human chromosome 7 α-satellite sequences (H7) and mouse chromosome 8 centromeric repeat sequence (M8) of *SJL* mice. Muscles of the injected *SJL* mice, samples shown are the following: 1–Distal foreleg muscle; 2–Proximal foreleg muscle; 3–Distal hindleg 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: Time hanging (seconds)		Ambulation (cm)		Inclined plane (degrees)		Wire hanging: Time hanging (seconds)		Ambulation (cm)	
	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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