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Human Adipose Tissue Derived Pericytes Increase Life Span in *Utrn*^{tm1Ked} *Dmd*^{mdx}/J Mice

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Abstract Duchenne muscular dystrophy (DMD) is still an untreatable lethal X-linked disorder, which affects 1 in 3500 male births. It is caused by the absence of muscle dystrophin due to mutations in the dystrophin gene. The potential regenerative capacity as well as immune privileged properties of mesenchymal Stem Cells (MSC) has been under investigation for many years in an attempt to treat DMD. One of the questions to be addressed is whether stem cells from distinct sources have comparable clinical effects when injected in murine or canine muscular dystrophy animal models. Many studies comparing different stem cells from various sources were reported but these cells were obtained from different donors and thus with different genetic backgrounds. Here we investigated whether human pericytes obtained from 4 different tissues (muscle, adipose tissue, fallopian tube and endometrium) from the same donor have a similar clinical impact when injected in double mutant *Utrn*^{tm1Ked} *Dmd*^{mdx}/J mice, a clinically relevant model for DMD. After a weekly regimen of intraperitoneal injections of 10^6 cells per 8 weeks we evaluated the motor ability as well as the life span of the treated mice as compared to controls. Our experiment showed that only adipose tissue derived pericytes are able to increase significantly (39 days on average) the life span of affected mice. Microarray analysis showed an inhibition of the interferon pathway by adipose derived pericytes. Our results suggest that the clinical benefit associated with intraperitoneal injections of these adult stem cells is related to immune modulation rather than tissue regeneration.

Keywords Pericytes · Stem Cells · DMD · Life Span · Adipose Tissue

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Introduction

Duchenne Muscular Dystrophy (DMD) is a lethal genetic muscle degenerative disorder which affects about 1:3500 male newborns [1] and for which there is still no effective treatment. It is caused by mutations in the DMD gene, which leads to the absence of muscle dystrophin. Although the current management, mostly based on corticosteroids, can improve patient's ambulatory capacity and life expectancy there are several side effects related to their long-term use [2]. The reason why corticosteroids have a clinical impact in DMD is partially elusive, though the most accepted hypothesis is that it

diminishes the chronic inflammation of the muscle triggered by the breaking down of muscle fibers as a result of the dystrophin gene mutation.

Among the different models for DMD [3] the mdx mice has been the most widely used, although it does not faithfully recapitulate the severity of the human clinical condition. For this reason, other models with a more severe phenotype were developed, including the double mutant *Utrn*^{tm1Ked} *Dmd*^{mdx}/J (also referred to as DKO) mouse, which shows extensive muscle wasting from birth, kyphosis, limb paralysis, chronic inflammation and early death [4].

The potential regenerative capacity as well as immune privileged properties of mesenchymal Stem Cells (MSC) for the treatment of degenerative disorders have been under investigation for many years [5–8]. In order to assess the effect of these cells in injected animals it is paramount to have clinical objective parameters such as the impact on lifespan and to isolate well characterized MSC because the in vivo identity of these cells has yet to be clearly defined. Various pieces of evidence point out to pericytes as the in vivo counter part of the heterogeneous fibroblast-like plastic adherent population derived from the digestion of adult vascularized tissues [9–12].

The isolation and myogenic potential of pericytes from various tissues such as adipose, brain, muscle and pancreas obtained from different donors has been reported [9]. More recently, several characteristics such as the proliferation rate, cell surface markers, myogenic genes expression, as well as the in vitro and in vivo myogenic potential of mesenchymal stromal cells (MSC) from adipose tissue, synovial membrane and bone marrow, from 3 different donors were compared [13]. The authors concluded that there were differences according to the donors (such as population doubling time) and according to the tissue of origin (myogenic contribution in vivo). However, few studies have compared and assessed the effect of these cells on the survival of animal models with a short lifespan (such as the DKO mice). The comparison of cells from different sources but the same donor is also very limited in the literature. The obstacles for such studies are the obtainment of enough cells from each source as well as experiments including a significant number of animals in different groups with a robust clinical read-out.

In the present study, we addressed the question whether pericytes obtained from different sources could increase life expectancy when injected in a large group of dystrophic double mutant *Utrn*^{tm1Ked} *Dmd*^{mdx}/J mice, which have an average lifespan of about 5 months [4].

Material and Methods

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

Tissue and Cell Preparation

Four tissue specimens from the same donor, namely: adipose tissue, muscle, fallopian tubes and endometrium were obtained from total hysterectomy procedures. Tissues were stored in PBS or DMEM with 4 % antibiotics at 4 °C and processed within 24 hours after harvest. Tissue digestion was performed as described elsewhere [9], but with smaller quantities of material from each tissue (adipose tissue: ~10 mg; endometrium: ~3 mg; fallopian tubes: ~2 mg; muscle tissue: ~1 mg). Tissues were minced with scalpels and incubated with 1 mg/mL of collagenase type II (Sigma Aldrich) in a 1:3 ratio (tissue weight:collagenase volume) diluted in DMEM-F12 with 20 % fetal bovine serum (FBS), 1 % non-essential amino acids (GIBCO) and 1 % of antibiotics/antimycotics (GIBCO) at 37 °C for 30–40 minutes in a shaker, at 250 RPM. Cells were then passed through a 70 μm strainer and incubated in blood lysis solution for 5–10 minutes. PBS was added in a ratio 2:1 and the solution was filtered again through a 40 μm strainer. Later, cells were incubated with conjugated antibodies against CD-34 (Percp-Cy5.5), CD-45 (FITC), CD-56 (APC) and CD 146 (PE), all from BD Biosciences. DAPI was added just before the analysis, and all cells positive for DAPI were excluded. The gating strategy was performed according to Crisan et al.[9]. All pericyte populations were confirmed to have at least 75 % purity after sorting. Cells (CD146+/CD34-/CD45-/CD56-) were sorted into a 24 well plate at a density of 20.000 cells/cm^2 and cultivated until the first passage in EBM-2 complete medium (Lonza). After the first passage, all cells were cultivated in DMEM F-12 medium with 20 % FBS, 1 % NEAA and 1 % antibiotics/antimycotic up to passage four and then frozen. After thawing, cells were always cultivated in EBM-2 medium and were injected between passages 7–10. All cells used in the in vivo experiments were derived from a single 46-year-old healthy female donor.

Animals

Double knockout mice (*Utrn*^{tm1Ked} *Dmd*^{mdx}/J dystrophin and utrophin negative), were purchased from the Jackson Laboratory (Bar Harbor, ME http://www.jax.org) and bred in collaboration with IPEN (Instituto de Pesquisas Energéticas e Nucleares) to form our colony. Animal care and experiments were performed in accordance with the animal research ethics committee of the Biosciences Institute, University of São Paulo. Animals, 48 days old on average, were separated in seven groups (n=20 per group on average). The mean age of treatment onset did not differ between groups (ANOVA p=0.99). They were injected intraperitonially with 1 million viable

cells (or vehicle), once a week, for a total of 8 weeks, without any immunosuppression. These seven groups were comprised by animals receiving either: vehicle (HBSS), fibroblasts (isolated in our laboratory from skin biopsy explant from another normal control), myoblasts (from a commercial myoblast human cell line), pericytes derived from endometrium, pericytes derived from fallopian tubes, pericytes derived from adipose tissue and pericytes derived from muscle. Additionally, we have followed the natural clinical course of 36 untreated DKO mice.

Physical Performance Tests

Three sets of tests, before and after treatment, were done to evaluate physical performance: (a) the ambulation test to determine the mean length of a step measured in hind foot ink prints while mice ran freely in a corridor (length, 50 cm; width, 8 cm; height of lateral walls, 20 cm) [14]; (b) the grip test to measure the force against which the animal resisted while being dragged through a grid; (c) the rotarod test to evaluate motor coordination and fatigue resistance.

Myogenic Differentiation

For myogenic differentiation, cells were plated in T25 flasks (BD) coated with collagen-1 and allowed to reach confluence of 80-90 %. At this point, the proliferation media was changed to induction media, which consisted of DMEM-F12 with 2 % horse serum (HS) for 15 days. Cells were then analyzed for expression of myogenic markers.

In vitro conditioning

Fibroblasts, myoblasts and pericytes were incubated for 24 hours in either HBSS or peritoneal wash (PW) from 7–8 pooled 2 months old *Utrn*^{tm1Ked} *Dmd*^{mdx}/J mice. After incubation, cell culture media were kept at −20 °C until analyzed in MagPix Luminex equipment with the Human 8-plex Kit (Bio Rad). Total RNA was also isolated from cells as described before and hybridized in a microarray human expression chip (Affymetrix HuGene 1.0). We performed a comparative analysis of differentially expressed genes (at least a 10-fold change by the t-Students test) among PW-conditioned cells (pericytes derived from all tissues against non-pericytes), using the Ingenuity Systems Software.

Real-time PCR

Total RNA from all cell lineages were extracted using miRNeasy Kit (Quiagen) following manufacture's protocol at three different time points of the myogenic induction protocol (T0, T8 and T15). In short, 1 μg of RNA 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 5 ng of cDNA and SYBR Green 1 Master (Roche) in a Light Cycler 480 II equipment (Roche). The PCR conditions were 95 °C for 5 min (activation), 95 °C for 10 sec, 62 °C for 10 sec, 72 °C for 5 sec (40 cycles).

Human DNA Analysis

For human DNA PCR we generated a new protocol based on two published articles [15],[16] using 3 sets of primers: (1) amplicon of 215 bp resulting from both human and mouse DNA [15]; (2) amplicon of 189 bp resulting from mouse DNA only [15]; (3) amplicon of 141 bp resulting from human DNA only [16]. This procedure allowed to identify, in a single reaction, the presence of foreign human DNA in five distinct mice tissues, namely: muscle (gastrocnemius), lungs, liver, kidneys and spleen.

Western Blot

Proteins were extracted using a hot extraction buffer containing SDS (1 %) and centrifuged 13.000 RCF at 4 °C for 15 minutes. Afterwards, equivalent amounts of proteins, measured by BCA (Thermo) in Nanodrop (Thermo), were resolved in a 10 % SDS-polyacrilamide gel and transferred to nitrocellulose membranes (Hybond; Amersham Bio-sciences, Piscataway, NJ, http://www.amersham.com). 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: A4.1056 against the myosin heavy chain 2× (1:1000) and DYS-1 against dystrophin (Vector). For secondary antibodies we used mouse conjugated with HRP (Thermo) and incubated membranes for 1 hour. Immune reactive bands were detected with ECL Plus (GE Healthcare) in a Kodak film.

Immunocytochemistry

Cells were fixed in cold methanol for 5 minutes and then were blocked with PBS containing 10 % SFB and 5 % BSA for 1 hour and then incubated for 1 hour at 37 °C with mouse anti-myosin heavy chain2x (DHSB - MF20/1:500) and rabbit anti-dystrophin (Abcam 15277/1:200). After 3 washes with PBS for 5 min, cells were incubated with secondary antibody against mouse (Alexa 488 – Invitrogen/1:400) and rabbit (Alexa 594 – Invitrogen/1:400). After incubation, cells were washed 3× with PBS and incubated with Hoechst 33342 (Sigma-Aldrich) for 5 min to visualize the nuclei. Fluorescence signal was examined in Axiovert 200 (Carl Zeiss, Jena, Germany, http://www.zeiss.com) and in AxioImager Z1 (Carl Zeiss).

Muscle Biopsy Analysis

Several qualitative aspects of the muscle tissue such as caliber variation, atrophic fibers, regeneration, collagen in the perimysium and endomysium, nuclei centralization and inflammatory infiltration were analyzed by an independent investigator (HS) in blind test regarding the animals from different experimental groups.

Statistical Analysis

All data are expressed as the mean±SD. Performance tests were analyzed using ANOVA multiple tests. Survival curve data was analyzed by the following tests:

1) One-way ANOVA test: to confirm that there was no difference of age between groups when the treatment was initiated
2) log-rank trend test: to verify whether there was a trend in the survival curves (indicating a difference between them), the DKO mice were divided in 3 groups: Non pericytic cells treated group n=41, vehicle treated group n=21, pericytic treated group n=81
3) log-rank (Mantel-Cox) test and Gehan-Breslow-Wilcoxon test: to ask whether the survival curves after administration of the specific cells were significantly different ($p<0.05$), the pericytic treated group was divided in four different sub-groups according to their tissue origin, namely: endometrium derived pericytes (n=21), fallopian tubes derived pericytes (n=21), adipose tissue derived pericytes (n=20) and muscle derived pericytes (n=18). If the test indicated a statistical difference among the curves, subsequently, the survival curves were analyzed in a two-by-two comparison and considered significantly different if the p-value was less than 0.05 by the Gehan-Breslow-Wilcoxon test;
4) Cox proportional-hazards regression test: To analyze whether the age of onset of the cell injections had an effect on animal survival within each group.

All data used in the survival curves were measured in days after the onset of treatment. Outliers within each group were excluded using Grubbs' test (ESD method) considering the value for alpha as 0.05.

Results

Pericyte Sorting

We successfully isolated pericytes from four donors. We established cell lines from all tissues (endometrium, fallopian tubes, muscle and adipose tissue) from one of them and we obtained 3 tissues from the other three donors. Since muscle is the scarcest source of pericytes (~0,7 % of the total) isolating cell lines from this tissue was more difficult as compared to endometrium, fallopian tube and adipose tissue (with ~6,5 %, ~4,3 % and ~10,7 % respectively) although, interestingly, this particular donor had a relatively higher pericytes percentage in the muscle tissue than average (~2 % - Fig. 1). After sorting, cells from different tissues did not have the same morphology or proliferation rate. The population doubling time (PDT) of the cells after passage 5 was calculated and compared to fibroblasts and a commercially available myoblast lineage (Invitrogen). The results are summarized in Table 1. Notably, pericytes from endometrium and fibroblasts have a much rapid PDT than the others.

We also evaluated whether the markers used for sorting were maintained in our culture system after 8 passages. Most of the mesenchymal stem cells markers (CD90, 105, 73) were expressed. However, the percentage of CD146 positive cells fell to approximately 35 % in all lineages (data not shown). Other markers of pericytes like NG2 and alpha-actin were absent. ALP, another pericyte marker [9, 10] stained all cells including fibroblasts and myoblasts.

Pericytes do not commit to myogenic differentiation

Before mice injections, we analyzed the myogenic potential of the sorted and ex vivo-expanded pericytes. However, we were unable to differentiate pericytes from any of the four tissues into skeletal muscle cells in vitro. To make sure our sorting strategy was being done properly, we analyzed a myoblast population (CD34-CD45-CD56+CD146-) derived from the same donor tissue. As expected, myoblasts could fuse and form multinucleated syncytium. We analyzed both mRNA expression (qPCR) (Fig. 2 – primers listed in Table 2) and protein expression (immunofluorescence – SM-2) and both proved the lack of myogenic commitment of pericytes as compared to myoblasts. Fibroblasts also did not show any myogenic potential as expected.

Human Derived Pericytes have a Beneficial Impact on Survival in DKO Mice Without Immunosuppression

The survival curve of non-injected DKO animals was not different from the vehicle treated animals (p=0.7) and none of *mdx* littermate controls died throughout the whole time they were followed (10 months).

After the injections, mice were followed until natural death and all the data was plotted in a survival curve. A significant trend, measured by the log-rank trend test, was observed in the survival curves obtained from non-pericytes, vehicle and pericytes treated animals during treatment time (Fig. 3a; p=0,048) and until death (Fig. 3b; $p<0,0001$).

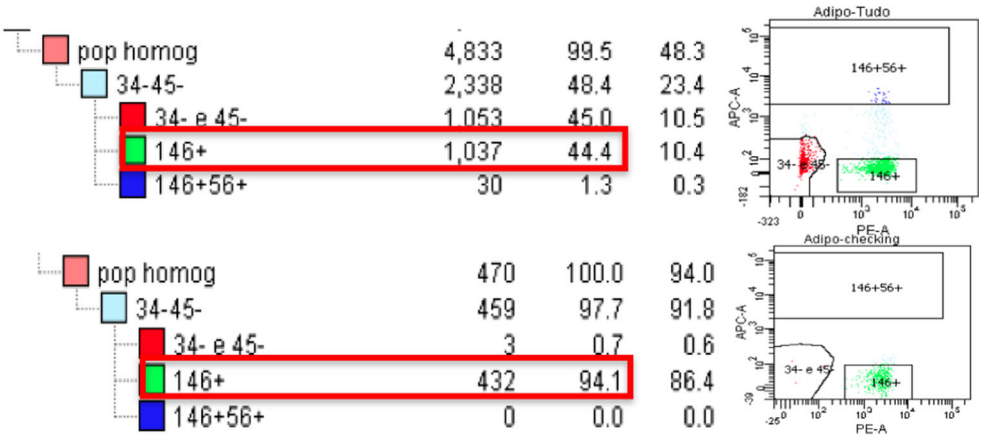

Fig 1 Cytometry analysis of the muscle single cell population of the 19 F sample just before sorting (upper image) and right after sorting (lower image)

Only adipose tissue derived pericytes increase life expectancy in *Utrn*^{tm1Ked} *Dmd*^{mdx}/J mice

Surprisingly, pericytes from distinct sources did not have the same effect on lifespan of *Utrn*^{tm1Ked} *Dmd*^{mdx}/J treated mice (p<0,0001). Only animals injected with pericytes from adipose tissue lived significantly longer as compared to vehicle treated (39 days more, in average; p=0,03) and non-pericytes treated mice (57 days more, in average; p=0,005). There was also significant change in survival in animals treated with adipose tissue derived pericytes as compared to muscle derived pericytes group (p=0,02) and fallopian tube derived pericytes group (p=0,01) (Fig. 4a). These differences were observed irrespectively from animal age.

Although we observed a significantly longer survival curve in the adipose derived pericyte injected group, none of the physical tests revealed differences between the groups. We also could not find human cells in any of the analyzed tissues (Fig. 5). Moreover, we could not observe any difference in HE stained sections of the gastrocnemius muscle. All of them showed signs of muscle wasting, inflammation, fiber size variations and fibrosis (Fig. 6).

We also analyzed the expression of 8 human cytokines, namely IL-2, IL-4, IL-6, IL-8, IL-10, GM-CSF, INF-gamma and TNF-alpha, in the media after a 24-h incubation with either HBSS or PW. None of them differed significantly in the longer survival group as compared to the others. We then investigated differential gene regulation expression through microarray analysis. In adipose derived pericytes PW conditioned media we observed that the interferon pathway was highly activated when compared to fibroblast and myoblasts (Table 3) while the insulin pathway was down regulated in the former and activated in the latter.

Table 1 Time in hours taken by each population to double

Lineage	PDT (hrs)
Fibroblast	33,6
Myoblast	63,6
Pericytes from Endometrium	35
Pericytes from Fallopian Tubes	56,2
Pericytes from Fat	64,2
Pericytes from Muscle	67,2

Endometrial-derived pericytes injections showed an age dependent difference on lifespan

The differently shaped survival curve of the endometrium derived pericytes injected mice led us to investigate it further. It did not differ significantly from vehicle, non-pericytes or adipose tissue derived pericytes treated mice curves (Fig. 4b). There was a significant effect related to the age of onset of endometrial derived pericytes injections (p=0,03). The younger the DKO mice started being treated, the better the survival (SM-1). This observation was not seen in any of the other treated groups.

Discussion

Although it has been more than 20 years since the discovery of the DMD gene, and innumerous approaches aiming the cure for this devastating disorder have been attempted, corticoids are still the standard treatment so far despite their many side effects [17–19]. The possibility to treat muscular dystrophies with stem-cell therapy has been the subject of many investigations. One of the questions to be addressed is whether stem cells from distinct sources have comparable clinical effects when injected in murine or canine muscular dystrophy animal models [20, 21].

Following the work of Pittenger and colleagues [22] describing a cultivation strategy for human bone marrow derived mesenchymal stem cells (hBMMSC), MSC have been

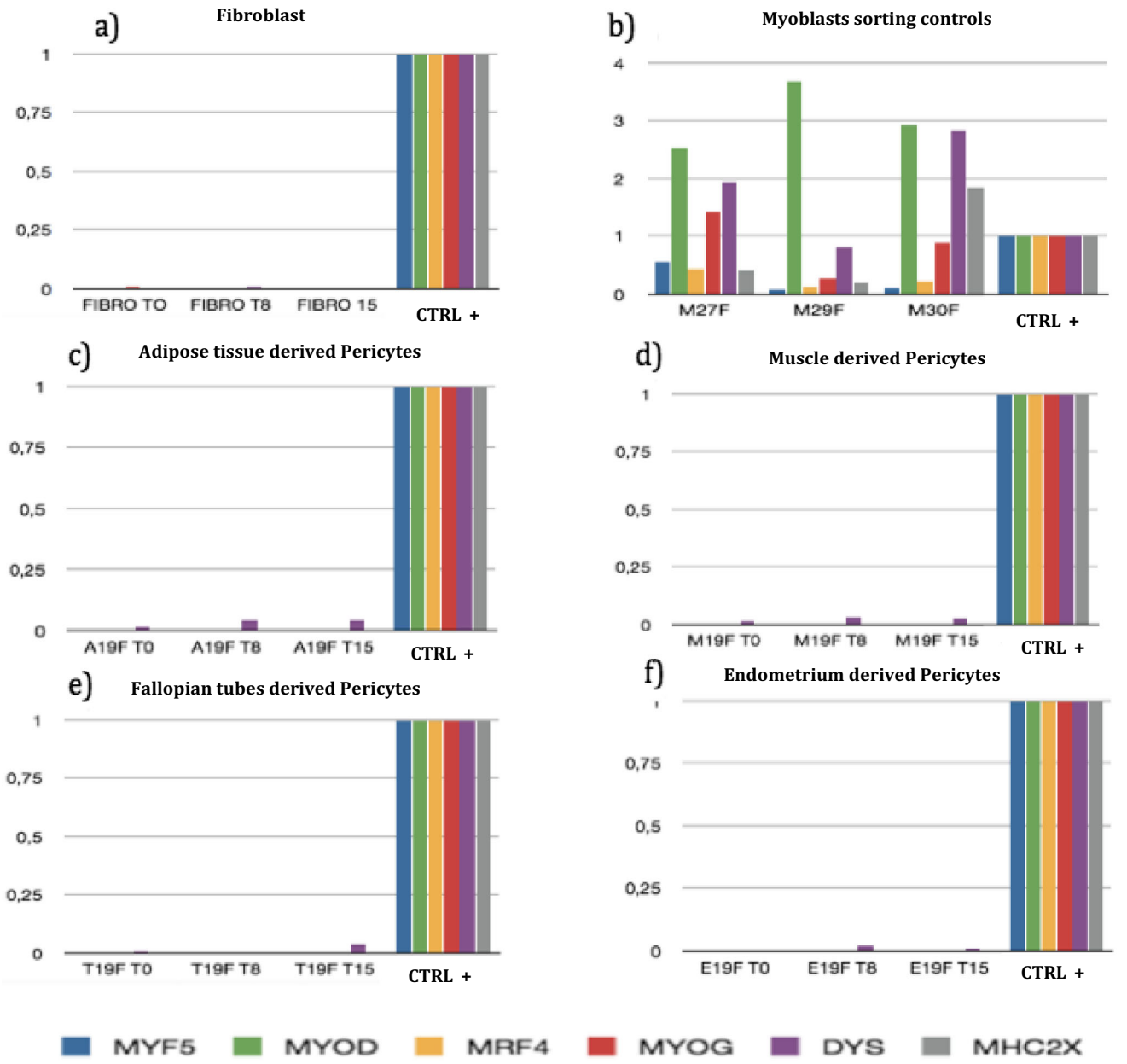

Fig 2 Expression of myogenic markers throughout the differentiation. None of the lineages were able to express the markers at any time point when compared to the positive control (commercial and FACS isolated myoblasts). A19F – Adipose derived pericytes; E19F – Endometrial derived pericytes; M19F – muscle derived pericytes; T19F – fallopian tubes derived pericytes

Table 2 Primers used to assess myogenic differentiation

Gene	Forwarded (5'-3')	Reverse (5'-3')
Gapdh	TCTTTTGCGTCGCCAGCCGA	CCAGGCGCCCAATACGACCA
Myf5	CTGCCGGGACAGAGCTGCTC	GGACTGTTACATTCGGGCATGCC
Myod	GCCCAGCGAACCCAGGCC	GCGGAGGCGACTCAGAAGGC
Mrf4	TGGGGGTGGACCCCTTCAGC	ACTTGCTCCTCCTTCCTTAGCCGT
Myog	AGGCCCTGCTCAGCTCCCTC	CATTCGCTGGGCACCCCTGG
Dystrophin	TCACGGTCAGTCTAGCACAGGGA	GGAGCTTCCAAATGCTGTGAAGGA
Mhc2×	GGTCGCATCTCTACGCCAGGGTCC	GGAGGAAAGGAGCAGCCTCCCCA

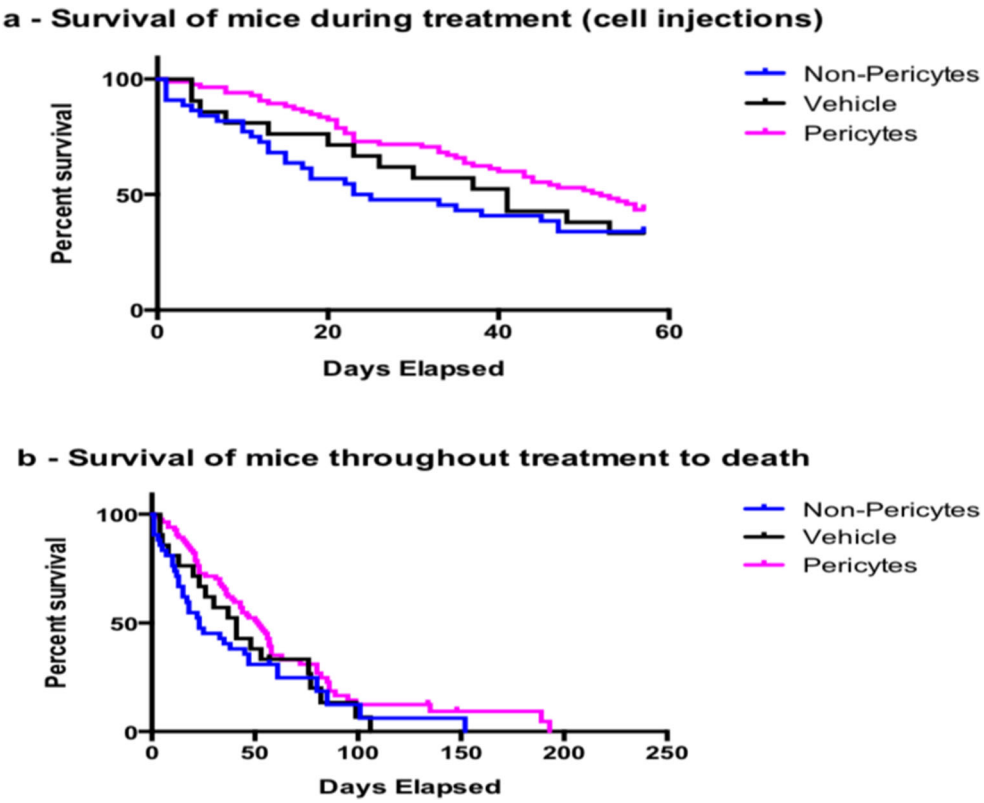

Fig 3 Survival curves plotted considering 2 time points: during cell injection only (**a**) e and throughout the whole life of the injected mice (**b**). Both indicated a trend with different levels of significance (p=0,04 and p<0,0001, respectively)

isolated from various tissues such as: decidual teeth [23], adipose tissue [24], synovium [25], umbilical cord [26], menstrual blood [27], fallopian tubes [28] and others. Many studies comparing MSC from various sources were also reported [23, 29–31] but isolated from different donors. The in vitro expression profile of MSC comparing paired umbilical cord blood and tissue was reported [26, 32, 33]. However, comparative analysis of MSC obtained from distinct sources from the same donor and therefore the same genetic background are very difficult to perform due to tissue availability. To our knowledge [13] there is only one reported study that has compared MSC from different tissues from the same donor for therapeutic purposes. Here, we have isolated pericytes from four different biological sources (Fig. 1), from a single donor, and expanded them for over 10 passages to analyze their in vitro and in vivo therapeutic potential in a severely affected murine model of muscular dystrophy.

Crisan et al.[10] showed, through a series of cell sorting strategies from crude tissue, that MSC could have a perivascular location in vivo. They observed that pericytes from different tissues could be sorted out using the specific markers (CD34-Cd45-CD56-CD146+) and, after ex vivo expansion, they expressed regular MSC markers. The isolation strategy used in the present work, allows the establishment of an identity of cells among different tissues and thus comparisons between pericytes from distinct sources of the same person, which was our goal.

However, differently from these [10] and other [11] authors we could not observe in vitro myogenic potential in pericytes derived from any of the tissues, although myoblasts isolated from the same muscle showed strong myogenic activity (Fig. 2). One possibility to explain this discrepancy was that in the paper of Crisan and colleagues, the myogenic potential of pericytes was analyzed within the first passage after sorting (10 days of proliferation) while we investigated the myogenic potential of the cells after 7 passages (which was required in order to reach a therapeutic relevant amount of cells). Another possibility would be that the myogenic potential of these cells were no longer preserved due to differences in culture conditions [11].

According to a recent report [34], within the mouse muscle pericyte population, we can find two different populations with different contribution to the muscle environment. One of them has an exclusive adipose fate and the other a myogenic fate. This observation has yet to be reported in human tissue, but in this study, cytometry analysis did not identify markers that were exclusively enriched in a specific population (data not shown).

Here we show for the first time that human ex vivo expanded pericytes have a significant impact in overall survival rate when injected without any immunosuppression in DKO mice (that normally have a short life span of about 5 months) [4]. We show that during the treatment, the plotted survival curves show a statistically significant trend (Fig. 3a; p=0.04)

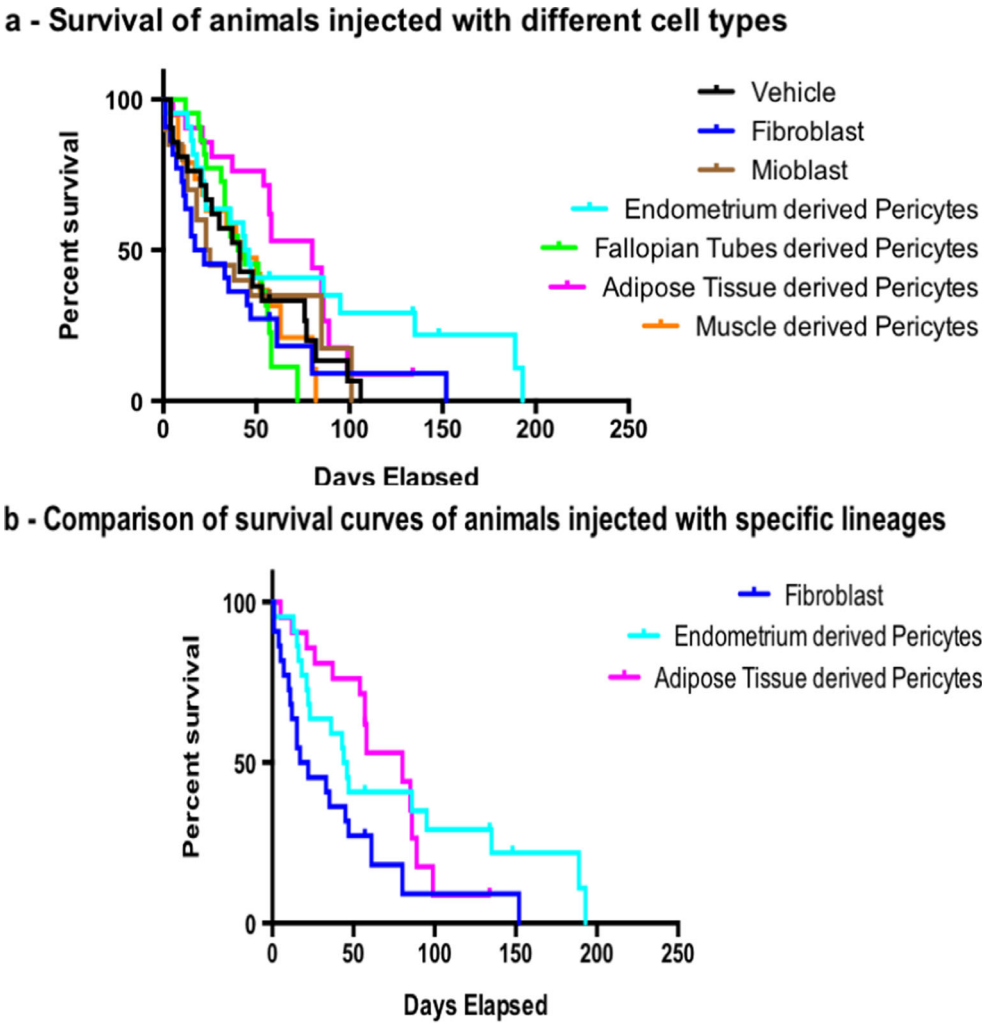

Fig 4 Survival curves of (**a**) all injected cells until death of all animals and (**b**) fibroblast, adipose and endometrium derived pericytes until death of all animals. Notice to the abrupt change on the endometrium survival curve, highlighting the age dependent correlation

and the follow-up of the animals until death showed a highly significant trend ($p<0{,}0001$; Fig. 3b).

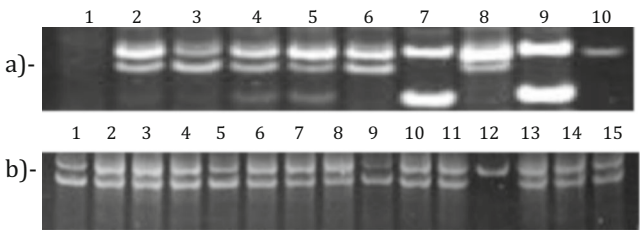

Fig 5 Multiplex PCR to search for human DNA in mouse tissues extracted from injected animals. In (**a**) controls of the reaction: lane 1: water; lanes 2 and 3: 0,1 % human mixed with mouse DNA; lanes 4 and 5: 2 % human DNA mixed with mouse DNA; lanes 6 and 8: mouse DNA; lanes 7 and 9: human DNA; lane 10: ladder. PCR was done in 5 different tissues of each dissected mouse (spleen, lungs, liver, kidneys and muscle). In (**b**) representative gel of the PCR done with the DNA derived from the tissues of the adipose tissue derived pericytes injected mice. Lane 1: muscle of the animal 1; lanes 2–6: all the tissues from animal 4; lanes 7–11: all tissues from the animal 5; lane 12: ladder; lanes 13–15: DNA from spleen, lungs and liver of the animal 24

Secondly, we evaluated whether all pericytes contributed equally to the increased survival rate of the DKO treated animals. We observed that (with exception of endometrium derived pericytes; $p=0{,}26$), adipose derived pericytes is the only group that is significantly different from all other groups, namely: fibroblasts ($p=0{,}0036$), myoblast ($p=0{,}026$), vehicle ($p=0{,}03$), fallopian tube derived pericytes ($p=0{,}017$) and muscle derived pericytes ($p=0{,}02$; Fig. 4a). This observation supports the hypothesis according to which only pericytes derived from adipose tissue truly influences animal survival, independently of the genotype of the donor or the age of animals when the injections were started.

Interestingly, despite the apparent higher survival of some animals in the injected endometrium group (illustrated in Fig. 4) the survival curve did not differ significantly from any other groups. The different shape curve (showing an abrupt change in survival, Fig.4b) in this group is reflected by the mice age when the treatment was initiated. However this age related phenomenon, which was not observed in the other groups, should be interpreted with caution due to the

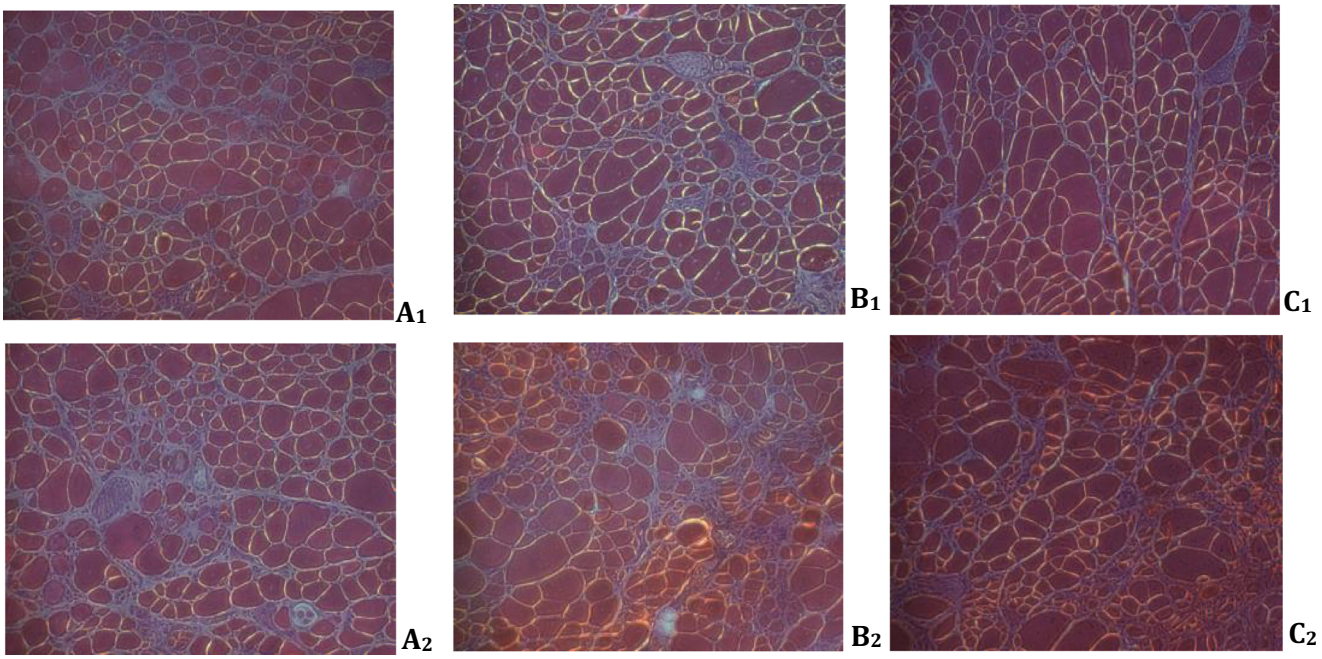

Fig 6 Histological representation of the muscle tissue of two animals in three different groups (vehicle – a1-2, fibroblast – b1-2, and adipose tissue derived pericytes – c1-2). No difference was found in a blind qualitative analysis

intrinsic variability of the endometrium in different phases of the menstrual cycle. If confirmed it would indicate that for some types of cells, the age when the treatment starts could be very important.

In an attempt to explain our findings we next evaluated whether there was any correlation between increased life span and pericyte contribution in five collected tissues (liver, lungs, spleen, muscle and kidneys). However, one week after the eight injections or at the time of death we could not find any human DNA in any of the analyzed tissues (Fig. 5). Previous work related to ageing has also reported a beneficial effect of stem cell transplantation without the presence of donor cells in recipient animals. Lavasani et al.[35] conducted a series of experiments with a progeria murine model that showed a significant beneficial effect of wild type Muscle Derived Stem Cells (MDSCs – probably perivascular located cells) transplantation in the life span of the progeroid mice. The effects observed were systemic and promoted animal growth, decreased muscle atrophy and neovascularization even in tissues in which cells were not found such as muscle and brain. According to these authors, there were many evidences of a paracrine function induced by the injected cells into the host tissue.

Since we also did not find human cells, even among animals with increased life span, we investigated whether a possible paracrine effect might explain our results. The expression profile and cytokines that were released from the pericyte population (when conditioned with peritoneal wash of double mutant 2 month old mice) showed no significant differences among all cell types for the 8 human specific cytokines released in the medium (IL-2, IL-4, IL-6, IL-8, IL-10, TNF-alpha and INF gamma). However, the gene expression profile analysis showed that the inflammatory pathway was inhibited by the adipose tissue derived cells, which could have resulted in an increased life span (Table 3 and Fig. 7). This observation supports the current view according to which these cells are believed to act modulating immune responses. What is surprising is the fact that only the adipose tissue derived pericytes seem to release the factors in a quantity large

Table 3 List of the ten first genes that are up regulated in pericytes derived from fat related to fibroblasts and myoblasts when incubated for 24 h in peritoneal wash

Symbol	Entrez Gene Name
HERC5	HECT and RLD domain containing E3 ubiquitin
RSAD2	radical S-Adenosyl methionine domain containing 2
SLC7A2	solute carrier family 7 (cationic amino acid
F2RL2	coagulation factor II (thrombin) receptor-like 2
IFIH1	interferon induced with helicase C domain 1
UPK1B	uroplakin 1B
IFIT1	interferon-induced protien with tetratricopeptide
NPTX1	neuronal pentraxin I
IFI44L	interferon-induced protien 44- like
IGFBP2	insulin growth factor binding protien 2, 36 kDa

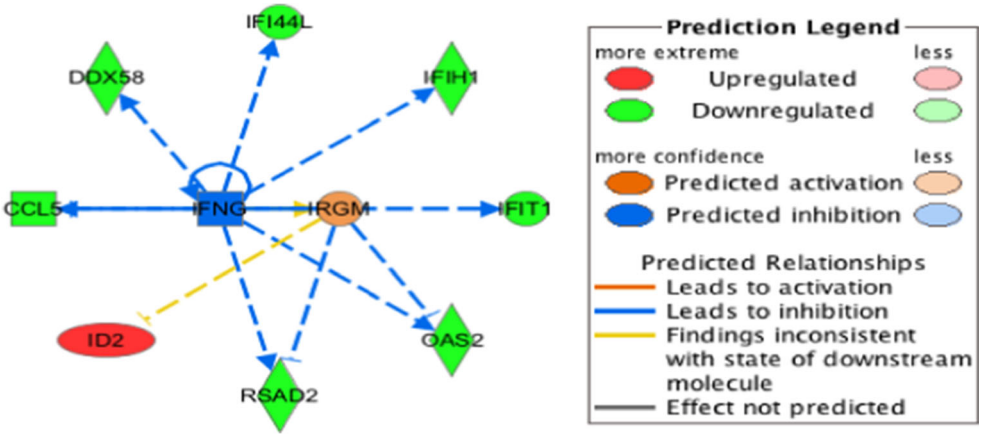

Fig 7 Integrated analysis of the interferon pathway indicating down regulation of many genes in the non-pericytic lines (fibroblasts and myoblasts) in comparison to adipose derived pericytes

enough to have a significant impact in the life span of this mouse model.

In short, the present study indicates that pericytes from distinct sources obtained from the same donor and, therefore the same genetic background, have a different clinical impact when injected in a murine double mutant model for Duchenne dystrophy. It also suggests that adipose tissue derived pericytes seems to have a clinical advantage over other types of pericytes when injected in *Utrn*^{tm1Ked} *Dmd*^{mdx}/J mice activating immune modulating pathways in contact with the host peritoneal environment and not by grafting in the diseased tissues. These findings support previous observations from our group showing a beneficial effect of human adipose mesenchymal cells injected in murine and dog models without immunosuppression. Although these results are encouraging, further studies should evaluate the effect of single versus multiple injections as well as the route of administration (intravenously or arterial). It is also worthy to clarify if continued injections (longer than 2 months) would increase the benefit evaluated in these mice.

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Conflict of Interest We declare no conflict of interest.

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