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Comparative characterization of bone marrow-derived mesenchymal stromal cells from four different rat strains

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Background aims

Bone marrow (BM) multipotent mesenchymal stromal cells (MSC) hold great potential for cell-based regenerative medicine. Because of the growing use of autologous rat MSC transplantation in various rat models, there is a need to establish minimal criteria for rat MSC characterization independent of the specific strain employed in each study. We aimed to compare the phenotypic and functional traits of BM MSC from the four strains of rats commonly used in research: Fisher, Lewis, Sprague–Dawley and Wistar.

Methods

Rat MSC were isolated from the BM of the four different rat strains in an identical fashion. Cells were characterized for their cell-surface phenotype in early and late passage. Functional mesenchymal differentiation capacities were examined following adipogenic and osteogenic inductions. Population doubling times were determined across the four strains throughout 10 passages. In vitro *proliferation assays of immune cells were conducted following co-culture of spleen cells and MSC of the four different strains.*

Results

We found that rat MSC from different strains exhibited similar cell-surface phenotype. Expansion rates and differentiation capacities of the MSC were also similar across the different strains. Co-culture of rat MSC with spleen cells obtained from rats of a different strain did not induce proliferation of immune cells.

Conclusions

Our findings suggest that BM-derived MSC from different strains share similar characteristics, in contrast to the variations previously described in the characterization of mice MSC from different strains.

Keywords

Mesenchymal stromal cells, differentiation, Fisher, Lewis, splenocytes, Sprague–Dawley, Wistar.

Introduction

Bone marrow (BM) multipotent mesenchymal stromal cells (MSC) have been reported for their multilineage differentiation potential [1,2], and have been shown to be clinically effective when transplanted into animal models of various diseases [3–6]. One of the prominent advantages of MSC for future cell-based regenerative medicine is the prospect of enabling autologous transplantation. Hopefully, the future of MSC-based therapy will include autologous human to human transplantations [7]. In the mean time, because of the convenience and availability of rat animal models for various diseases, rat MSC have been used widely over the last few years as an autologous cell source for the treatment of diseases affecting the bones, cartilage, nervous system, heart, liver, blood vessels, gastric system and kidneys [8–24].

Human MSC have been studied extensively and are characterized in accordance with clear criteria agreed between researchers [25]. Mice BM-derived MSC have been described previously to differ in their cell-surface phenotype, proliferation rates and differentiation potential between different inbred strains [26]. Rat MSC from

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different strains have been characterized in some detail as a part of studies performed with Fisher [27], Lewis [15,24], Sprague-Dawley [12,13,20,21,28] and Wistar strains [19,29]. However, no report has been made regarding the comparative characterization of rat MSC derived from different strains under the same experimental conditions.

This study describes the functional and phenotypic comparison of BM MSC derived from four different rat strains commonly employed in medical research. The characteristics evaluated in this study were the ones most often used to fulfill the minimal criteria for defining cells as MSC [25], namely cell-surface epitope expression, mesenchymal differentiation capacity and expansion rate. The results indicate that rat MSC isolated from all four strains share similar characteristics and do not differ significantly from one another in the examined traits. Moreover, *in vitro* analysis demonstrates that the MSC do not induce immune cell proliferation when co-cultured with spleen cells of a rat from a different strain.

Methods

Animals

Male Fisher, Lewis, Sprague–Dawley and Wistar rats (Harlan, Jerusalem, Israel) at 8–12 weeks of age were used. Each primary culture was pooled from two to four different animals from the same stain. All experimental protocols were approved by the University Committee of Animal Use for Research and Education. Every effort was taken to reduce the number of animals used and minimize their suffering.

Isolation of rat MSC

Following killing of the rats with CO_2, tibiae and femora were dissected and the bones placed in Hanks' balanced salt solution (HBSS; Biological Industries, Beit HaEmek, Israel). The epiphyses of the bones were removed, and the marrow was flushed out using a syringe filled with HBSS. Low-density BM mononuclear cells were separated, based on density gradient, using UNISEP maxi tubes (NovaMed, Jerusalem, Israel). Next, the mononuclear cells (at a density of approximately 250,000/cm^2) were plated in a polystyrene plastic flask (75 cm^2; Corning, NY, USA) in a growth medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal calf serum (FCS), 2 mM L-glutamine, 100 μg/mL streptomycin, 100 U/mL penicillin, 12.5 U/mL nystatin (SPN) (all from Biological Industries) and 0.001% 2-mercaptoethanol (Sigma-Aldrich, St Louis, MO, USA). The next day, non-adherent cells were removed with medium replacement. Cells were then cultured for 2 weeks; the medium was changed twice a week and cells maintained at 37°C in a humidified 5% CO_2 incubator. Confluent cultures (passage 0) were subcultured according to experimental requirements.

Growth rate analysis

Passage 0 cells were trypsinized and reseeded at a density of 5000 cells/cm^2. Following 7 days, cells (passage 1) were trypsinized, counted and subcultured onwards in the same manner up to passage 10. The medium was changed twice a week throughout all experiments.

Mesenchymal differentiation

For adipogenic induction, cells (passage 5) were grown to confluence and treated with adipogenic differentiation medium for 21 days. The medium contained DMEM, L-glutamine (12 mM), SPN (as in the growth medium), FCS (10%) (all from Biological Industries), insulin (5 μg/mL), indomethacine (50 μm), dexamethasone (1 μm) and 3-isobutyl-1-methylxanthine (IBMX; 0.5 μm; all from Sigma-Aldrich). Oil Red O staining was conducted at the end of the differentiation protocol to assess lipid accumulation, as a marker for adipogenic differentiation. For osteogenic induction, cells (passage 5) were plated on wells coated with vitronectin (12.5 μg/mL; Millipore, Billerica, MA, USA) and collagen 1 (12.5 μg/mL; Sigma), grown to confluence and treated with osteogenic differentiation medium for 21 days. The medium contained DMEM, L-glutamine (12 mM), SPN (as in the growth medium), FCS (10%) (all from Biological Industries), dexamethasone (0.1 μm), ascorbic acid (0.2 mM) and glycerol 2-phosphate (10 mM) (all from Sigma-Aldrich). Alizarin red staining was conducted at the end of the differentiation protocol to detect calcium deposits as a marker for osteogenic differentiation.

Flow cytometry analysis of cell-surface markers

Rat MSC were harvested at the second or seventh passage. Cells were trypsinized, washed, suspended in phosphate-buffered saline (PBS) and distributed into duplicate samples containing $2–5 \times 10^5$ cells in 50 μL. Cells were incubated with appropriate antibodies for 45 min on ice, washed twice in flow-buffer consisting of 5% FCS and 0.1% sodium azide in PBS, and centrifuged for 10 min. The cells were resuspended in 0.2 mL PBS and studied with a

FACSCalibur™ flow cytometer using an argon laser at 488 nm. An appropriate isotype control was included in each experiment. Specific staining was calculated by subtracting the isotype controls from the positive cells observed. The cells were examined for CD29 expression [phycoerythrin (PE)–anti-mouse/rat CD29, hamster IgG; 1:50] with isotype control (PE–anti-rat, hamster IgG; 1:50), CD90.1 [flourescein isothiocyanate (FITC)–anti-rat/mouse CD90.1, mouse IgG1K; 1:125] and CD45RA (FITC–anti-rat/mouse CD45RA, mouse IgG1K; 1:50) with isotype control (FITC–anti-rat, mouse IgG1K; 1:125), CD11b/c (FITC–anti-mouse/rat CD11b/c, mouse IgG2α,K; 1:25) with isotype control (FITC–anti-rat, mouse IgG2α,K; 1:125), and CD45 (PE–anti-rat CD45, mouse IgG1K; 1:25) and CD106 (PE–anti-rat CD106, mouse IgG1K; 1:50) with isotype control (PE–anti-rat, mouse IgG1K; 1:50) (all from BioLegend, San Diego, CA, USA). Cells were also examined for CD44 expression (FITC–anti-rat CD44, mouse IgG2α; 1:25) and major histocompatibility complex (MHC) II (FITC–anti-rat MHC class II, mouse IgG1; 1:25) (both antibodies purchased from Serotec, Oxford, UK) with a suitable FITC-conjugated isotype control.

Spleen cell proliferation assay

Rats were killed with CO_2, and the spleens removed and mechanically dissociated. Splenocytes were washed twice with PBS and placed for 1 h in RPMI-1640 medium supplemented with 2 mM glutamine, 50 μM 2-mercaptoethanol SPN (as above), and 10% heat-inactivated FCS (all from Biological Industries) at 37°C. Splenocytes were then counted and plated at a concentration of 3×10^5 cells/well in wells inhabiting confluent adherent MSC cultures in quintuplets. The cells were incubated for 72 h at 37°C in humidified air containing 5% CO_2. [^{3}H]Thymidine (1 μCi/well) was added for the last 16 h of incubation and the cultures were harvested and counted using a Matrix 96 Direct beta counter (Packard Instruments, Meridien, CT, USA). The proliferative response was measured using [^{3}H]thymidine incorporation expressed as mean counts per minute (c.p.m.) of quintuplet wells.

Statistics

The results are expressed as means ± standard error. For comparisons between two groups the Student's *t*-test was employed. Several group comparisons were done by ANOVA with Scheffe post-hoc analysis. Statistical calculations were performed using SPSS v. 13.

Results

Growth rates of MSC of do not vary between the different strains and along multiple passages

Analysis of the proliferation capacities of MSC derived from the four different strains revealed no statistically significant differences between them. Throughout 10 cell passages conducted once a week, accumulative population doublings (PD) were as follows: 22.37 PD in Fisher MSC (2.49 ± 0.09 mean PD/week), 21.81 PD in Lewis MSC (2.42 ± 0.14 mean PD/week), 23.16 PD in Sprague–Dawley MSC (2.57 ± 0.2 mean PD/week) and 20.49 PD in Wistar MSC (2.28 ± 0.31 mean PD/week) (Figure 1A).

Analyzing the mean PD time along the different passages, we found inconsistent, small but statistically significant changes in the proliferation rates of the cells throughout passages 1–10 (Figure 1B). Specifically, we found that in the first passage the Wistar MSC were statistically significant different from the Fisher MSC, and the last were also statistically significant different from the Lewis MSC; In the second passage, the Wistar MSC were

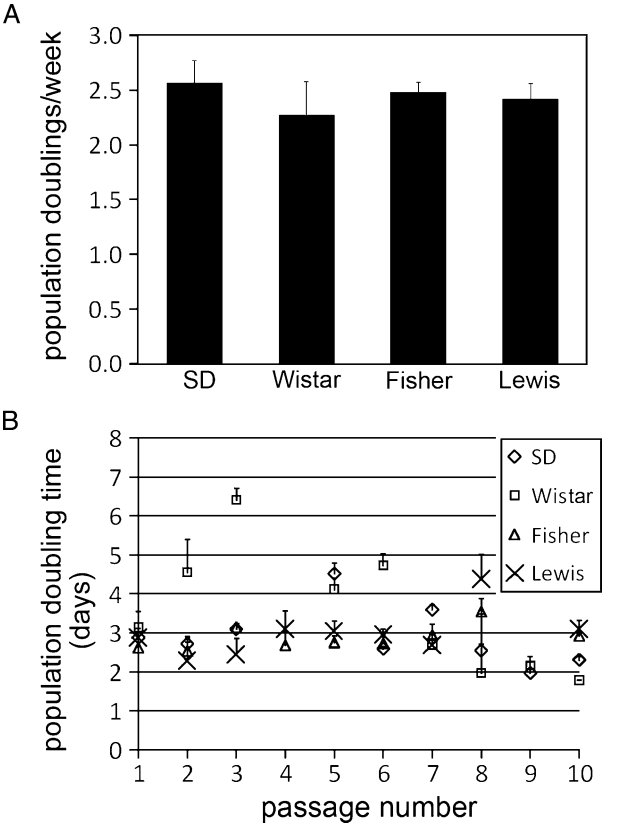

Figure 1. *Expansion rate of MSC from four different strains. (A) Mean number of PD observed over 10 cell passages. (B) PD as observed across 10 cell passages. Cells were passaged once a week.*

statistically significant different from the Fisher and Lewis MSC; In the third, sixth and tenth passages, the Wistar MSC were statistically significant different from all other stains; In the fourth, fifth and ninth passages, the Wistar MSC and the SD MSC were statistically significant different from both Lewis and Fisher MSC; In the tenth passage SD MSC were statistically significant different from all other strains. However, as the majority of PD times were 2–4 days, it is probable these changes are not biologically significant.

Mesenchymal differentiation capacities

Under similar conditions, MSC derived from the different strains exhibited similar morphology and similar differentiation potential toward adipogenic and osteogenic fates (Figure 2). Consistent with previous reports, we observed both spindled fibroblast-like cells and flat cells in cultures obtained from all strains (data not shown).

Cell-surface phenotyping reveals existence of similar, homogeneous cell populations

MSC from different strains were characterized for their cell-surface phenotype at early passage [2] and late passage [7]. In all experiments, MSC were found to express ubiquitously CD29 (β-integrin) and CD90 (Thy-1), independent of passage number and rat strain. On the other hand, MSC did not express the hematopoetic markers CD45 (common leukocyte antigen), CD11b (macrophage marker), CD45RA (B-cell marker) and MHC class II (always $< 2\%$ of cells), again independent of passage number and rat strain. One exception was the presence of small numbers of $CD45^+$ and $CD11b^+$ ($< 10\%$) in Sprague–Dawley and Wistar MSC at passage 2, which were absent upon analysis in passage 7 (Figure 3). Other markers often expressed in MSC, CD44 and CD106, displayed irregular expression patterns that varied both between strains and early and late passages (Figure 3A, D, G, J).

Co-culture of MSC and spleen cells does not result in immune cell proliferation

In an attempt to determine whether MSC act as immunogens that induce immune cell proliferation when exposed to cells from different strain, we co-cultured MSC from each strain with spleen cells derived from rats of the different strains. The results revealed that immune cell proliferation was not induced in any of the co-culture

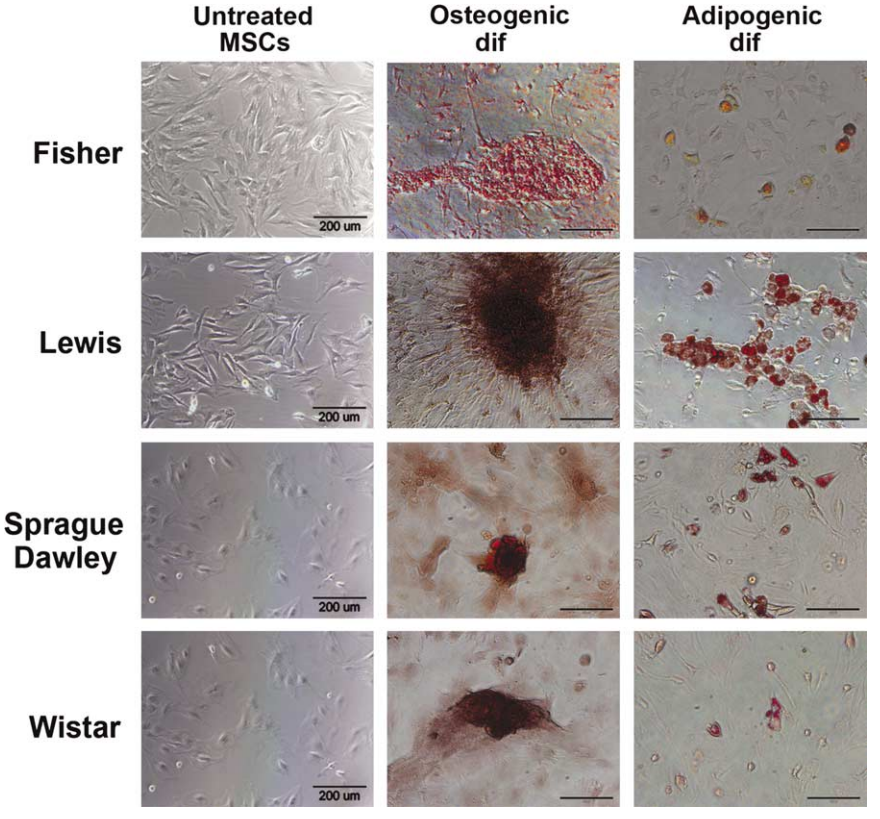

Figure 2. *Differentiation of MSC from four different strains toward osteogenic and adipogenic fates.*

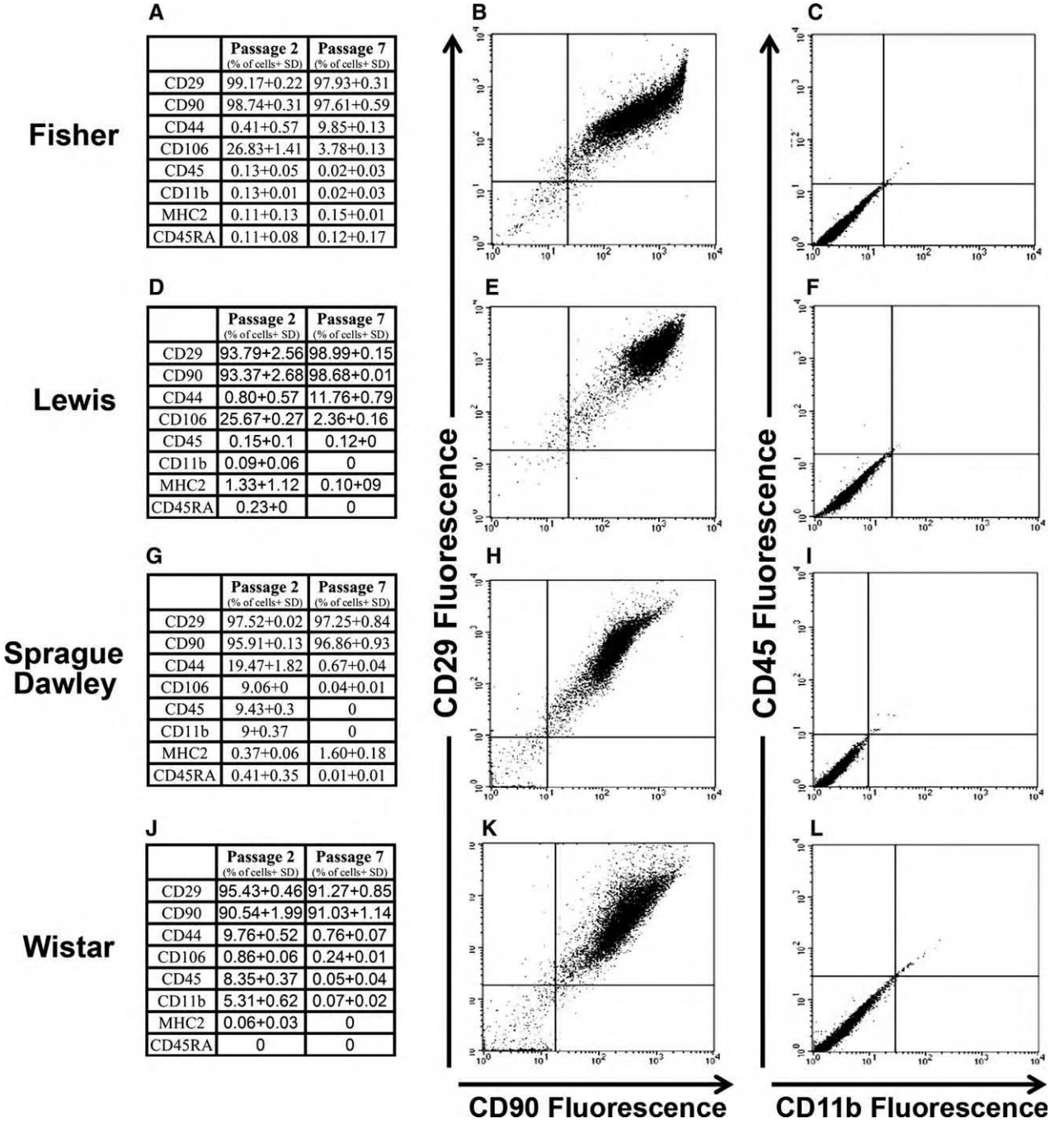

Figure 3. *Cell-surface phenotyping of rat MSC from four different strains as observed using flow cytometry. (A, D, G, J) Summary of CD marker expression levels at the second and seventh passages. (B, E, H, K) Double staining for mesenchymal markers CD29 and CD90 at the seventh passage MSC. (C, F, I, L) Double staining for the common leukocyte antigen CD45 and macrophage CD11b markers at the seventh passage.*

combinations compared with the basal spleen cell proliferation rate observed in the absence of MSC. However, we did observe that the MSC obtained from the Lewis rat reduced the proliferation capacity of spleen cells from all of the strains upon co-culture (Figure 4).

Moreover, we observed that MSC obtained from the Lewis rat reduced the proliferation capacity of spleen cells from all of the strains upon co-culture in a statistically significant manner compared with untreated splenocytes. MSC derived from Wistar and Fisher rats reduced the

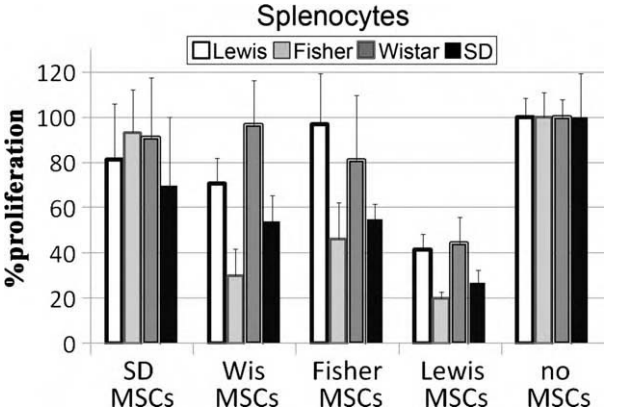

Figure 4*. Spleen cell proliferation rates following co-culture with MSC from a different rat strain. Spleen cells were cultured in suspension on MSC-coated wells for 72 h. Labeled thymidine was added 16 h prior to harvesting and analysis. The percentage is presented in comparison with splenocyte proliferation with no MSC.*

spleen cell proliferation rate in a statistically significant manner only when co-cultured with Fisher- and Sprague–Dawley-derived splenocytes. Interestingly, Sprague–Dawley MSC did not produce a statistically significant reduction in spleen cell proliferation regardless of the spleen cell origin.

Discussion

We provide descriptive data regarding the characteristics of BM-derived MSC from the four most common strains of rats employed in research. We show that, under the same experimental conditions, MSC can be easily isolated and expanded from Fisher, Lewis, Sprague–Dawley and Wistar rats. These cells represent a homogeneous population, as observed by their typical morphology, osteogenic and adipogenic differentiation capacity, proliferation rate and cell-surface phenotype. Between strains, minimal differences were observed, although two strains (Fisher and Lewis) are inbred and the other two (Sprague–Dawley and Wistar) outbred. Overall, the results represent a pooling of BM from several animals in each stain. By pooling we focused the research on interstrain differences and not on intrastrain differences. As the variance was minimal in most parameters from each strain, it is fair to assume that the intrastrain variance is low in the parameters tested. These results were not anticipated, as mice MSC have been characterized extensively across different strains and been found to vary between strains in various traits, such as cell-surface epitopes, proliferation rates and differentiation potentials [26]. In that sense, it would be fair to state that rat MSC are more similar to human MSC (in the traits evaluated in the current study) than to mice MSC.

One of the hallmark advantages of MSC is that they enable autologous transplantation rather than allogenic transplantation that can be performed using embryonic stem cells. Transplantation of human MSC to rats adds the somewhat unexpected factor of an immune response, as some reports indicate that rejection takes place [30]. Some studies employ immune-suppression in human MSC transplantations to rats that may also hamper the cell differentiation potential [31]. In our laboratory, we found far greater survival rates of autologous rat MSC transplantation to the rat's brain in comparison with human MSC transplantation to immunocompromised rats [29]. Although the ultimate goal is to treat human patients with their autologous human MSC in the clinic, the only way to truly imitate this strategy in animal studies is by transplanting animals with MSC derived from a peer animal of the same species and strain. In that sense, MSC derived from mice and rats provide a huge experimental tool.

The growing interest in MSC as key players in stem cell regenerative medicine, and the fact that there is no one 'gold standard' marker for these cells, has urged researchers to define strict criteria for characterization of these cells. These criteria are clearly defined for human MSC [25] and have also been investigated in MSC derived from different mice strains [26]. In this study, we wanted to compare the same traits between rat strains and establish a basic characterization procedure for rat MSC. To date, few studies have investigated rat MSC traits. Those studies describe the effect of cell-density seeding on proliferation rate (conducted in Fisher rats) [32], the effect of rat age on MSC yield (conducted in Wistar rats) [33,34] and comparison of MSC derived from different tissues (conducted in Sprague–Dawley and Fisher rats) [35,36]. A single study has provided extensive immunophenotyping of rat MSC derived from Sprague–Dawley rats [37]. However, no report has been made comparing MSC from different strains. As different laboratories employ different rat strains for specific animal models, we thought it would be useful to compare the MSC derived from different strains.

As some strains carry specific genetic traits that make them more suitable to be used as a specific model, or carry a transgene that provides a useful research tool (like GFP transgenic rats), it is probable that one would consider transplanting rat MSC from one strain to a different host

strain. In this study, we conducted *in vitro* analysis of the immunogenic response induced by spleen cells when co-cultured with MSC from a different strain. Our results indicate that MSC across strains do not induce spleen cell proliferation and some MSC act as immunosuppressants and down-regulate the spleen cell proliferation. However, few studies involving MSC transplantations across different strains report that, *in vivo*, transplanted cells can be immunogenic [38]. The data also suggest that the different strains of rat react differently in terms of splenocyte proliferation, hence possess different immunogenic responses.

In conclusion, our results indicate that, independent of the specific strain, rat MSC provide a homogeneous population throughout 10 passages and 20 PD. These cells are capable of differentiating to fat and bone cells under specific conditions. Moreover, throughout the different strains, the MSC population ubiquitously co-expresses CD90 and CD29 (more than 95%) and is devoid of cells expressing CD45 (common leukocyte antigen) and CD11b (macrophage marker). Thus our study provides the basic criteria for characterization of rat MSC, independent of the strain used.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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