

## SHORT COMMUNICATION

# Dose-dependent effect of mesenchymal stromal cells co-grafted with dopaminergic neurons in a Parkinson's disease rat model

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## Abstract

A major limiting factor for cell therapy in Parkinson's disease is the poor survival and reinnervation capacity of grafted dopaminergic neurons, independently of the cell source. Mesenchymal stromal cells (MSCs) have high capability to regulate the local environment through the release of trophic, antiapoptotic and immunomodulatory factors. In this work, we investigated whether co-grafting of MSCs could improve the survival and reinnervation ability of dopaminergic precursors transplanted in animal models of Parkinson's disease. Rats with total unilateral dopaminergic denervation were grafted with a cell suspension of rat dopaminergic precursors (500,000 cells) with or without a high (200,000 cells) or low (25,000 cells) number of MSCs. Eight weeks after grafting, rats were tested for motor behaviour and sacrificed for histological analysis. Our results showed that the survival of dopaminergic neurons and graft-derived striatal dopaminergic innervation was higher in rats that received co-grafts containing a low number of MSCs than in non-co-grafted controls. However, the survival of dopaminergic neurons and graft-derived dopaminergic reinnervation was lower in rats receiving co-grafts with high number of MSCs than in non-co-grafted controls. In conclusion, co-grafting with MSCs or MSCs-derived products may constitute a useful strategy to improve dopaminergic graft survival and function. However, a tight control of MSCs density or levels of MSCs-derived products is necessary.

## KEYWORDS

cell death, cell therapy, dopaminergic neuron, Parkinson, stem cells, transplantation

## 1 | INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disorder caused by the selective degeneration of dopaminergic cells in the substantia nigra leading to major problems in the motor system.

One promising therapeutic approach for PD is dopaminergic cell-replacement therapy, in which dopaminergic precursors are grafted into the striatum to restore the lost dopaminergic neurotransmission. Previous clinical trials based on foetal dopamine neuron transplantation have shown promising results,<sup>1</sup> but also

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significant limitations including the survival of grafted dopaminergic neurons, which is very poor independent of the source of dopaminergic neurons.<sup>2,3</sup> Dopaminergic cells from induced pluripotent stem cells (iPSCs) or embryonic stem cells are being investigated as alternative cell sources for PD patients.<sup>4,5</sup> However, strategies to improve the survival of grafted cells and improve host reinnervation must be developed.

Mesenchymal stromal cells (MSCs) are multipotent cells originally isolated from the stroma of the bone marrow.<sup>6</sup> Different studies have shown their capability to regulate the local environment through the release of immunomodulatory, antiapoptotic and trophic factors. These properties make them an attractive cell source for repairing damaged tissue.<sup>7</sup> Consistent with this, we have previously observed that conditioned medium derived from bone marrow MSCs increases the viability of dopaminergic cells of rat and human origin in cultures.<sup>8</sup> However, possible benefits of MSCs on dopaminergic graft survival have not been investigated. In this work, we used mixed suspensions of dopaminergic cells and MSCs (ie co-grafts) in a rat model of PD to study the possible positive effects of MSCs on survival and functionality of grafted dopaminergic neurons and their possible use for improving the results of transplantation therapy in PD.

## 2 | MATERIALS AND METHODS

Young adult rats were subjected to maximal unilateral dopaminergic denervation with 6-hydroxydopamine (6-OHDA). One month after 6-OHDA injections, rats with maximal lesions (dopaminergic depletion >90%) were identified in a rotometer and confirmed with the cylinder test. One week later, rats showing behavioural criteria for maximal dopaminergic denervation were selected for transplantation (ie to investigate the effect of MSCs on the survival of grafted dopaminergic precursors). In a first series of experiments, rats with maximal dopaminergic denervation were grafted with dopaminergic precursors derived from foetal ventral mesencephalon (500,000 cells; 13 days of gestation, E13; VM;  $n = 5$  rats, control group) or co-grafted ( $n = 6$  rats) with 500,000 cells from the same VM suspension and a high number (200,000 cells) of MSCs (VM + high MSC-group). The number of MSCs was determined on the basis of previous studies (see Appendix S1). In a second series of experiments, rats with maximal dopaminergic denervation were grafted with 500,000 cells from a VM suspension ( $n = 5$  rats; control group) or co-grafted ( $n = 5$  rats) with 500,000 cells from the same VM suspension and a low number (25,000 cells) of MSCs (VM + low MSC-group). MSCs were from femur bone marrow and labelled with the live-cell fluorescent dye Cell Tracker™ Orange (CMTMR, Thermo Fisher Scientific). Eight weeks after grafting, the rats were tested again in the cylinder and the rotometer and then killed for histological analysis. Rats from each series (co-grafts and the corresponding controls) were always processed in the same batch. Additional details on materials and methods are provided as supplementary information (see Appendix S1).

## 3 | RESULTS

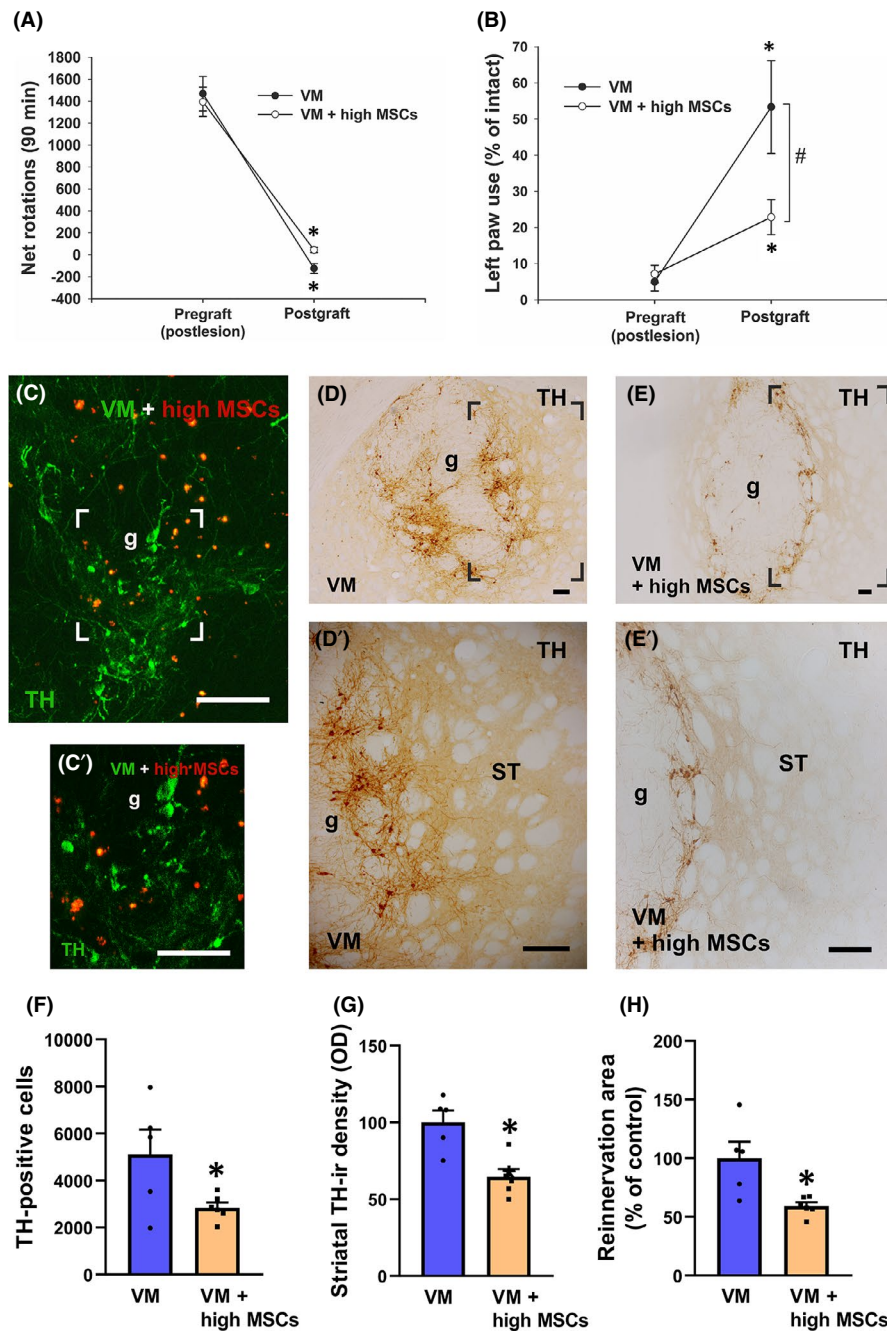
### 3.1 | Effects of co-grafting of VM cell suspensions and a high number of MSCs

Drug-induced rotational behaviour was used as an *in vivo* indicator of graft survival. The lesion induced a strong rotational asymmetry, with ipsilateral turning (ie towards the denervated side) after amphetamine administration, both in VM-grafted rats ( $1468.2 \pm 156.0$  turns) and in VM + high MSC-grafted rats ( $1393.7 \pm 133.8$  turns) (Figure 1A). Eight weeks after transplantation, both groups of grafted rats (ie VM and VM + high MSCs) showed a marked decrease in amphetamine-induced rotations ( $-124.8 \pm 44.4$  turns in VM-grafted animals and  $44.5 \pm 23.7$  in VM + high MSC-grafted rats) (Figure 1A). The cylinder test was used to obtain further information on behavioural recovery (Figure 1B). In the cylinder test, spontaneous paw use is approximately 50% for each of the forepaws in non-lesioned controls. After 6-OHDA lesion, the use of the left/impaired forepaw was reduced to  $5.0 \pm 2.6\%$  in the VM-grafted group and  $7.1 \pm 2.4\%$  in the VM + high MSC-grafted group (Figure 1B). Eight weeks after transplantation, both groups of grafted rats showed a significant improvement in use of the lesioned forepaw, although the improvement was significantly lower in VM + high MSC-grafted rats ( $53.3 \pm 12.8\%$  left paw use in the VM-grafted group and  $22.9 \pm 4.9\%$  in the VM + high MSC-grafted group) (Figure 1B).

As detailed in previous studies,<sup>9</sup> intrastriatal grafts of foetal VM contained numerous TH-immunoreactive (-ir) neurons, which were not evenly distributed within the graft. Most cells were grouped in patches located at the periphery of the graft, whereas the central area of the grafts was usually TH-negative (Figure 1C,D,E,C',D',E'). Cells showing red fluorescence revealed the presence of MSCs in the grafts (Figure 1C,C'). Surprisingly, the total number of TH-ir neurons was significantly lower in VM + high MSC-grafted rats than in the rats transplanted with VM tissue alone (Figure 1F). However, the average graft volume in the VM + high MSC-grafted group was not significantly different from that observed in VM-grafted rats ( $0.351 \pm 0.023$  mm<sup>3</sup> and  $0.454 \pm 0.065$  mm<sup>3</sup>, respectively). The density of TH-ir fibres in the reinnervation area (Figure 1G) and the size of the reinnervation area (Figure 1H) were reduced in the VM + high MSC-grafted group compared to VM-grafted group. Altogether (ie similar graft volume with decrease in TH-ir neurons and fibres) suggests an increase in selective loss of dopaminergic neurons in the VM + high MSC-grafted group.

### 3.2 | Effects of co-grafting of VM cell suspensions and a low number of MSCs

As described above, dopaminergic lesion induced a strong rotational asymmetry, and amphetamine-induced ipsilateral turning (ie towards the denervated side) both in the VM-grafted group ( $1530.8 \pm 179.9$  turns) and in VM + low MSC-grafted group ( $1023.4 \pm 141.5$  turns) (Figure 2A). Amphetamine-induced rotational behaviour was



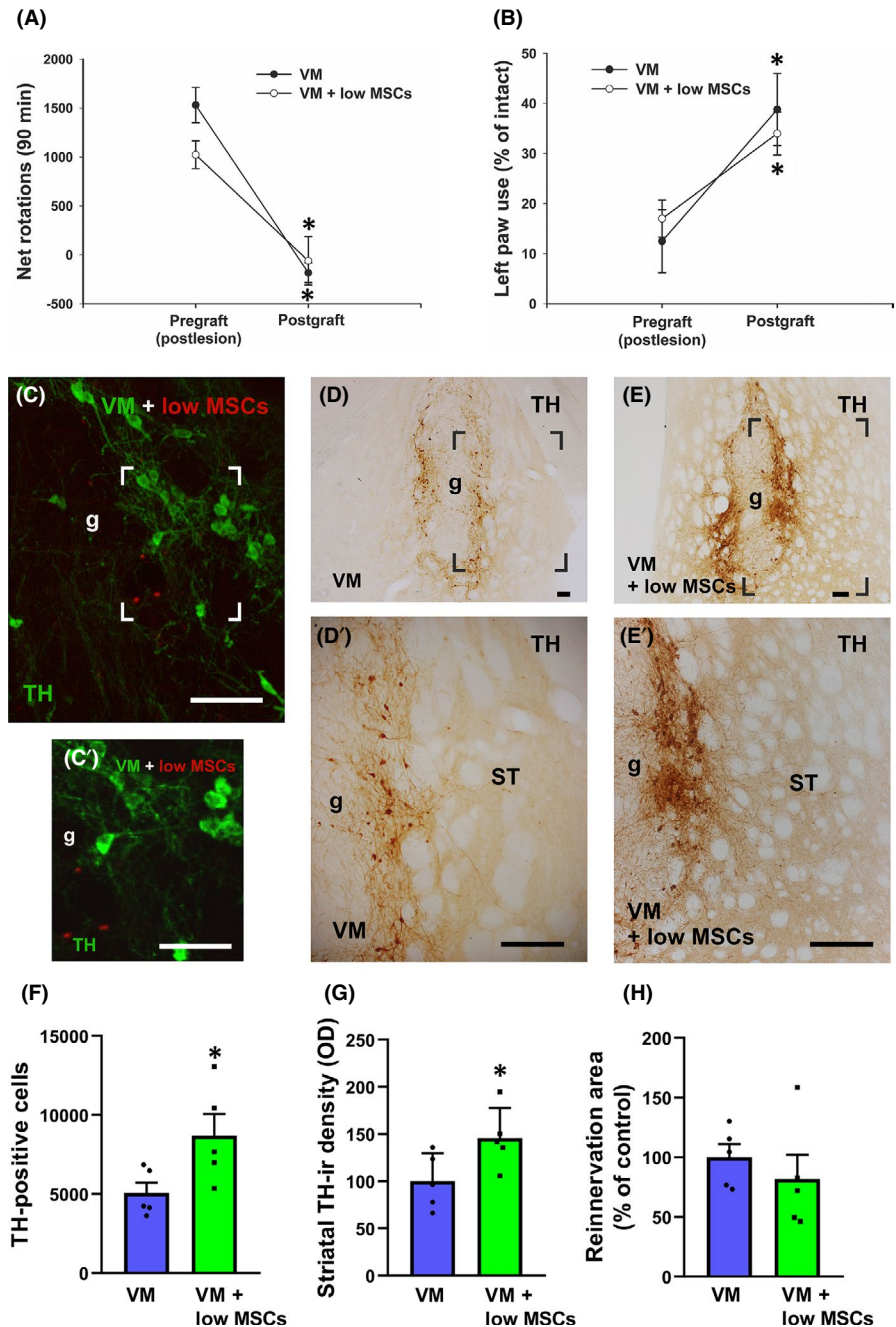
**FIGURE 1** Behavioural (A, B) and histological (C–H) analysis of co-grafts of ventral mesencephalic (VM) cell suspensions and a high number of mesenchymal stromal cells (MSCs). Rotational values (A) for both grafted groups did not differ significantly between groups. In the cylinder test (B), VM-grafted rat scores were significantly better than in the VM + high MSC-grafted group. Immunoreactivity for tyrosine hydroxylase (TH) in rats subjected to dopaminergic denervation and co-grafting (C–C', E–E';  $n = 6$ ) or grafted with VM cells alone (D–D';  $n = 5$ ), and showing green fluorescence from TH-ir dopaminergic cells and red fluorescence from grafted MSCs (C–C'). Areas boxed in C–E are magnified in C'–E'. F, Number of TH-positive (dopaminergic) neurons in both groups. G, Density of TH-immunoreactive (-ir) fibres in the reinnervation area, estimated as optical density (OD) and expressed as a percentage of the value obtained in the control group (VM-grafted group). H, Reinnervation area (ie striatal host area reinnervated by graft-derived TH-ir fibres) in the mentioned groups. Data represent mean  $\pm$  standard error of the mean (SEM). In A, B,  $*p < 0.05$  vs lesioned rats (ie postlesion and pregrafting) and  $^{\#}p < 0.05$  between grafted groups (ie VM and VM + high MSCs) (Student's  $t$  test). In F–G,  $*p < 0.05$  vs VM-grafted rats (Student's  $t$  test). Scale bar = 100  $\mu$ m and 50  $\mu$ m for C'. ST, striatum; g, graft

completely reverted by grafts, both in rats receiving VM transplants and rats co-grafted with VM + low number of MSCs ( $-182.5 \pm 100.7$  turns and  $-60.4 \pm 246.0$  turns, respectively) (Figure 2A). After the 6-OHDA lesion, the use of the left/impaired forepaw was reduced to  $12.5 \pm 6.3\%$  in the VM-grafted group and  $17.0 \pm 3.7\%$  in the VM + low MSC-grafted group (Figure 2B) in the cylinder test. After grafting, a significant improvement in the cylinder test was observed in both groups of transplanted rats (Figure 2B) although no significant difference in the recovery was detected between both groups using this test ( $38.8 \pm 7.2\%$  left paw use in the VM-grafted group and  $34.0 \pm 4.3\%$  in the VM + low MSC-grafted group).

Histological analysis showed that the total number of TH-ir neurons was significantly higher in animals transplanted with VM + low

MSC than in VM-grafted animals (Figure 2C–F and C'–E'). Cells showing red fluorescence revealed the presence of MSCs in the grafts (Figure 2C,C'). No significant difference in the volume of the graft was observed between groups ( $0.455 \pm 0.064$  mm<sup>3</sup> in VM-grafted rats and  $0.510 \pm 0.089$  mm<sup>3</sup> in VM + low MSC-grafted rats). However, the density of TH-ir fibres in the reinnervation area was higher in the rats that received co-grafts of VM and low number of MSCs compared to animals that received suspensions of VM cells alone (Figure 2G). The size of the reinnervation area in the VM + low MSC-grafted group was similar to that observed in the VM-grafted group (Figure 2H). Altogether (ie a similar graft volume with increase in TH-ir neurons and fibres) suggests a decrease in selective loss of dopaminergic neurons in the VM + low MSC-grafted group. The lack of significant differences in

**FIGURE 2** Behavioural (A, B) and histological (C–H) analysis of co-grafts of ventral mesencephalic (VM) cell suspensions and a low number of mesenchymal stromal cells (MSCs). Rotational values (A) and performance of the cylinder test (B) did not differ significantly between groups. Immunoreactivity for tyrosine hydroxylase (TH) in rats subjected to dopaminergic denervation and co-grafting (C–C', E–E';  $n = 5$ ) or grafted with VM cells alone (D–D';  $n = 5$ ), and showing green fluorescence from TH-ir dopaminergic cells and red fluorescence from grafted MSCs (C–C'). Areas boxed in C–E are magnified in C'–E'. F, Number of dopaminergic (TH-positive) neurons in both groups. G, Density of TH-immunoreactive (-ir) fibres in the reinnervation area, estimated as optical density (OD) and expressed as a percentage of the value obtained in the control group (VM-grafted group). H, Reinnervation area (ie striatal host area reinnervated by graft-derived TH-ir fibres) in both groups. Data represent mean  $\pm$  standard error of the mean (SEM). In A, B,  $*p < 0.05$  vs lesioned rats (ie postlesion and pregrafting), and  $\#p < 0.05$  between grafted groups (ie VM and VM + low MSCs) (Student's *t* test). In F–G,  $*p < 0.05$  vs control VM-grafted rats (Student's *t* test). Scale bar = 100  $\mu\text{m}$  and 50  $\mu\text{m}$  for C'. ST, striatum; g, graft



motor behaviour suggests that the observed histological improvement is not enough to induce functional differences or, more probably, that the sensitivity of these basic behavioural tests is not sufficient to detect a difference in the behavioural improvement.

## 4 | DISCUSSION

The lack of alternative sources to dopaminergic neuroblasts from the foetal substantia nigra and the high rate of dopaminergic cell loss during the transplantation procedure and the early post-transplantation period have been two major limitations for cell therapy as possible strategy for PD treatment. Recent advances with alternative sources of dopaminergic

neurons such as iPSCs and embryonic stem cell dopaminergic differentiation have led to the beginning of several promising clinical trials.<sup>4,5</sup> In addition to a number of specific limitations related to transplantation of iPSCs or embryonic-derived dopaminergic neurons,<sup>5,10</sup> the poor survival and integration with the host brain observed in grafts of foetal neuroblasts remain or is even more problematic in the case of grafts from the above-mentioned alternative dopaminergic neuron sources.<sup>1,11</sup>

Major factors responsible for the high levels of dopaminergic cell loss during the transplantation process and early post-transplantation period have not been totally clarified. However, grafting-related cell trauma, lack of growth factors, poor vascularization, neuroinflammation and other factors have been involved.<sup>12,13</sup> MSCs have been effective against several of the

above-mentioned factors,<sup>7,8</sup> and a neuroprotective effect on grafted dopaminergic neurons could be expected. The number of grafted MSCs may decrease over the time,<sup>14,15</sup> and part of the CMTMR-labelled cells may correspond to death MSCs phagocytosed by glial cells. However, it is well known that practically all the dopaminergic cell loss occurs during the first few days after the transplantation,<sup>16,17</sup> when most grafted MSCs are present. In the present study, we showed the capacity of co-grafted MSCs to induce beneficial effects on survival and reinnervation ability of grafted dopaminergic neurons. Surprisingly, the increase in the number of co-grafted MSCs led to detrimental effects. The mechanisms responsible for this effect are still unclear. A high concentration of MSCs may induce MSC senescence, damaged mitochondrial transfer or dysregulation of pro-inflammatory cytokine production.<sup>18-20</sup> In any case, the results reveal that further studies are necessary to know the exact proportion of dopaminergic/MSCs that leads to the highest neuroprotective effects. In addition, future studies are necessary to identify MSCs-derived products that are responsible for the positive effects and may be used as a substitute for MSCs. However, the present results suggest that the dose must be tightly adjusted to the number of grafted dopaminergic cells.

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#### CONFLICT OF INTEREST

The authors declare that there are no competing interests associated with the manuscript.

#### AUTHOR CONTRIBUTIONS

**Jannette Rodriguez-Pallares:** Conceptualization (equal); investigation (equal); writing–review and editing (equal). **Maria Garcia-Garrote:** Formal analysis (equal); investigation (equal); methodology (equal); writing–review and editing (equal). **Juan A. Parga:** Data curation (equal); formal analysis (equal); investigation (equal); writing–review and editing (equal). **Jose Luis Labandeira-Garcia:** Conceptualization (equal); formal analysis (equal); supervision (equal); writing–review and editing (equal).

#### DATA AVAILABILITY STATEMENT


Data are available from the corresponding authors upon reasonable request.

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#### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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