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TISSUE ENGINEERING AND REGENERATIVE MEDICINE

Survival, Differentiation, and Neuroprotective Mechanisms of Human Stem Cells Complexed With Neurotrophin-3-Releasing Pharmacologically Active Microcarriers in an Ex Vivo Model of Parkinson's Disease

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ABSTRACT

Stem cell-based regenerative therapies hold great potential for the treatment of degenerative disorders such as Parkinson's disease (PD). We recently reported the repair and functional recovery after treatment with human marrow-isolated adult multilineage inducible (MIAMI) cells adhered to neurotrophin-3 (NT3) releasing pharmacologically active microcarriers (PAMs) in hemiparkinsonian rats. In order to comprehend this effect, the goal of the present work was to elucidate the survival, differentiation, and neuroprotective mechanisms of MIAMI cells and human neural stem cells (NSCs), both adhering to NT3-releasing PAMs in an ex vivo organotypic model of nigrostriatal degeneration made from brain sagittal slices. It was shown that PAMs led to a marked increase in MIAMI cell survival and neuronal differentiation when releasing NT3. A significant neuroprotective effect of MIAMI cells adhering to PAMs was also demonstrated. NSCs barely had a neuroprotective effect and differentiated mostly into dopaminergic neuronal cells when adhering to PAM-NT3. Moreover, those cells were able to release dopamine in a sufficient amount to induce a return to baseline levels. Reverse transcription-quantitative polymerase chain reaction and enzyme-linked immunosorbent assay analyses identified vascular endothelial growth factor (VEGF) and stanniocalcin-1 as potential mediators of the neuroprotective effect of MIAMI cells and NSCs, respectively. It was also shown that VEGF locally stimulated tissue vascularization, which might improve graft survival, without excluding a direct neuroprotective effect of VEGF on dopaminergic neurons. These results indicate a prospective interest of human NSC/PAM and MIAMI cell/PAM complexes in tissue engineering for PD. STEM CELLS TRANSLATIONAL MEDICINE *2015;4:1–15*

SIGNIFICANCE

Stem cell-based regenerative therapies hold great potential for the treatment of degenerative disorders such as Parkinson's disease (PD). The present work elucidates and compares the survival, differentiation, and neuroprotective mechanisms of marrow-isolated adult multilineage inducible cells and human neural stem cells both adhered to neurotrophin-3-releasing pharmacologically active microcarriers in an ex vivo organotypic model of PD made from brain sagittal slices.

INTRODUCTION

Parkinson's disease (PD) is an incurable neurodegenerative disorder, characterized by the progressive degeneration of the nigrostriatal dopaminergic system, that leads to a severe dopamine (DA) deficiency in the striatum required for motor control [1]. Clinical trials with dopaminergic fetal tissue transplantation have shown the efficacy of this strategy but also its limits, in particular, poor cell availability [2, 3]. Among the different stem cell sources, neural stem cells (NSCs), which differentiate into neuronal cells and improve the motor behavior of parkinsonian rats, hold great promise for PD cell therapy [4–7]. Another possible cell source is an easily accessible homogeneous, human mesenchymal stromal cell (MSC) subpopulation named marrow-isolated adult multilineage inducible (MIAMI) cells that express pluripotent stem cell markers, have

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a potential for neuron-like differentiation, and secrete tissue repair factors [8–12]. NSC and MSC populations have been evaluated in PD clinical trials that demonstrated the feasibility, safety, and efficacy of the therapeutic strategy [13–16]. However, despite these encouraging results, stem cell-based therapies for PD face low cell survival, differentiation, and engraftment, limiting their effectiveness and use [17, 18].

The development of new tools able to enhance cell therapy potential is needed. In this context, pharmacologically active microcarriers (PAMs), which provide a three-dimensional biomimetic support to the cells and mediate sustained release of cytokines, have been shown to be beneficial in several animal models of different brain disorders, including PD and cerebral ischemia [12, 19–22]. We have previously demonstrated that the striatal implantation of MIAMI cells, precommitted toward a dopaminergic phenotype and adhering to laminin-coated PAMs releasing neurotrophin-3 (LM-PAM-NT3), induced significant nigrostriatal pathway repair and functional recovery in a rat model of PD, partly by their increased survival and tyrosine hydroxylase (TH) expression [12]. However, to be successfully translated to the clinic, additional studies are needed to fully understand the MIAMI cells' and NSCs' mode of action and to elucidate the contribution of PAMs to neural repair and regeneration.

Organotypic cultures made from brain slices, which can be maintained in culture for several weeks, can model the pathological neurodegenerative disease state, allowing one to test the stem cell potential in a PD context (reviewed in [23]). Our group has previously developed a novel organotypic PD model from the rat brain that includes all the areas involved in the nigrostriatal pathway in a single slice preparation, without using neurotoxins to induce the dopaminergic lesion. This physiologically relevant model allows the observation of cellular interactions and mechanisms, using small amounts of material, and represents a promising tool to better understand and refine new therapeutic approaches [23, 24].

In the present work, we aim at elucidating the survival, differentiation, and neuroprotective mechanisms of both MIAMI cells and NSCs and the contribution of NT3-releasing PAMs when the cell/PAM three-dimensional complexes are transplanted in an ex vivo model of nigrostriatal degeneration. We first assessed the protection/repair potential of NSCs and MIAMI cells, associated or not with PAMs, after grafting in the ex vivo model of PD. Stem cell survival and proliferation and their neuronal and dopaminergic differentiation capacity were next studied with the cells administered alone or combined with PAMs, to evaluate their mechanisms of action and the contribution of PAMs. Finally, neuroprotective mechanisms were clarified by studying the secretory phenotype of the cells and the paracrine-mediated impact on the organotypic slice microenvironment.

Materials and Methods

An extended section is available in the supplemental online data.

Ethics

To overcome the ethical limitations associated with human embryonic NSCs, a cell line of human NSCs, namely hNS1, was used. MIAMI cells were obtained from a human postmortem donor organ (with agreement from the French Agency of Biomedicine). Animal care and use were in strict accordance with the regulations of the French Ministry of Agriculture. All animal procedures were approved by animal experimentation ethic committee of Pays de la Loire.

Cell Culture

Culture of MIAMI Cells

Human MIAMI cells were isolated from a 20-year-old male donor and expanded in vitro, as previously described [8, 25].

Culture of NSCs

hNS1 cells, a cell line of human NSCs of forebrain origin expressing green fluorescent protein (GFP) [26, 27], were expanded in vitro from passage 10 to 15 on poly-D-lysine (PDL, Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com)-coated flasks [26]. This cell line spontaneously generates neuronal cells after mitogen removal and intracerebral transplantation [28, 29] and differentiates into dopaminergic neurons when micrografted into rat striatal brain slices [30].

Obtaining and Culturing Parkinson's Disease Organotypic Slices

Organotypic cultures were prepared as previously described [24].

PAM Formulation and Characterization

Formulation of PAMs

NT3-loaded microspheres were prepared using a solid/oil/water emulsion solvent extraction-evaporation method [12, 31]. A poly (lactic-coglycolic acid) (PLGA) copolymer with a lactic/glycolic ratio of 37.5:25 (molecular weight, 25,000 Da) (Phusis, Saint Ismier, France) was used.

To obtain LM-PAMs, PLGA microspheres were coated by incubation with a combination of LM and PDL molecules (both from Sigma-Aldrich) at a final concentration of 6 μg/ml PDL and 9 μg/ml LM [12, 32, 33].

Release Profile and Bioactivity of Released Protein From LM-PAM-NT3

To assess NT3 release kinetics, 5 mg of LM-PAM-NT3 was dispersed on 500 μl of medium. Every day from day 1 to day 5 and thereafter every 3 days, the sample was centrifuged at 295*g* for 10 minutes. The supernatants were collected and stored at −20°C before bioassay.

The bioactivity of NT3 released from the LM-PAM-NT3 was evaluated in vitro by determining the extension of the cellular process of dorsal root ganglion (DRG) cells after NT3 treatment (n = 2).

Formation of PAM/Stem Cell Complexes

Cell/PAM complexes were prepared as previously described [32–35]. Cell adhesion to the PAM surface was assessed by microscopic observation, and the cells adhering to PAMs were quantified using a Cyquant cell proliferation assay kit (Invitrogen, Carlsbad, CA, http://www.invitrogen.com). Complexes were studied further using fluorescence microscopy for NSCs and scanning electron microscopy for MIAMI cells [20].

Injection of Stem Cell/PAM Complexes Into Organotypic Slices

Two days after organotypic slice preparation, the cells/complexes constituting different treatment conditions were injected into the striatum using a 22-gauge needle (Hamilton, Bonaduz, Switzerland, http://www.hamiltoncompany.com) connected to a micromanipulator (Fig. 1A). Nine experimental groups were evaluated. For each experimental group, three independent experiments were performed, unless otherwise stated. The total injection volume consisted of 2 μl of culture medium containing approximately 50,000 cells alone or adhering to 0.1 mg of PAMs. The injections were done at an infusion rate of 0.5 μl/minute. The needle was left in place for 5 minutes before removal to avoid the cells being expelled from the organotypic slices.

Histological Study

At 1 and 14 days after injection (Fig. 1A), the organotypic slices were fixed with 5 ml of ice cold 4% paraformaldehyde (Sigma-Aldrich) in phosphate-buffered saline (PBS) (Lonza, Walkersville, MD, http://www.lonza.com) (pH 7.4) for 2 hours at 4°C. Next, the slices were washed with PBS. Nonspecific sites were blocked with PBS, Triton 1% (PBS-T, Triton X-100; Sigma-Aldrich), bovine serum albumin (BSA) 4% (Fraction V; PAA Laboratory), and normal goat serum 10% (Sigma-Aldrich) for 4 hours at room temperature under agitation. For CD31 study, 0.05% Triton was used.

Tyrosine Hydroxylase Immunochemistry

Two antibodies (mouse anti-rat TH [10 μg/ml in PBS-T, clone 6D7, Covance, Emeryville, CA, http://www.covance.com], for endogenous nigrostriatal pathway analysis, and polyclonal rabbit anti-human TH [1:100, AS-DOUB-LX immunization program, Liège, Belgium], to assess the catecholaminergic/dopaminergic differentiation of grafted human stem cells) were used as described in the supplemental online data. TH-positive rat fiber and human TH-positive fiber quantification was performed using Metamorph software (Molecular Devices, Sunnyvale, CA, http://www.moleculardevices.com).

Anti-Mitochondria, β3-Tubulin, Ki67, and CD31 Immunofluorescence

MIAMI cells were detected using a mouse anti-human mitochondria antibody (10 ng/ml, mitochondrial cytochrome C oxidase subunit II; Abcam, Cambridge, U.K., http://www.abcam.com). GFP-NSCs were directly observed under fluorescence microscopy. Mouse anti-human β3-tubulin (2 ng/ml, SDL.3D10; Sigma-Aldrich) or rabbit monoclonal anti-β3-tubulin (1:400, EP1331Y; Abcam), mouse anti-human CD31 (300 ng/ml, WM59; BD Pharmingen, Franklin Lakes, NJ, http://www.bdbiosciences.com) and monoclonal mouse anti-human Ki67 (350 ng/ml, MIB-1; Dako, Glostrup, Denmark, http://www.dako.com) antibodies were used to characterize grafted cell differentiation. Mouse anti-rat CD31 antibody (10 μg/ml, TLD-3A12; Abcam, Paris, France) was used to analyze brain slice vascularization. The slices were incubated for 48 hours with the first antibody in PBS-T, BSA 4% at 4°C. The washed slices were incubated with the corresponding biotinylated mouse or rabbit second antibody (7.5 μg/ml; Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com). The slices were washed and incubated with streptavidin fluoroprobes R488 or R547H (Interchim, Montluçon, France, http://www.interchim.com) diluted 1:200 in PBS for 2 hours before mounting with a fluorescent mounting medium. Isotypic controls and/or primary antibody omission were performed to assess specificity.

The intensity and density of the positive stained cells were quantified using Metamorph software. Colocalizations were confirmed using confocal microscopy.

Molecular Study

Dopamine Quantification by Mass Spectrometry

Quantification of DA in the striatum at days 0 and 16 was performed as previously described [24] and was expressed as the DA content per milligram of striatum.

Laser Microdissection

At 24 hours after implantation (Fig. 1A), cells grafted alone or complexed with PAMs were isolated from the striatum using laser microdissection (LMD). Organotypic slices were dehydrated by 4 successive 4-minute ethanol baths (70°, 90°, 100°, and 100°). Immediately after slice dehydration, microdissection was performed at room temperature with the LMD6000 microdissection system and software from Leica (Leica Microsystems, Wetzlar, Germany, http://www.leica-microsystems.com), using a UV laser with a wavelength of 355 nm. The microdissection settings were as follows: power 128; speed 1; specimen balance 0; and objective 6.3×. Areas of approximately 12 mm^2, corresponding to the whole striatum, were collected in 70 μl of RNA Later (Life Technologies, Carlsbad, CA, http://www.lifetechnologies.com). Striatum from 2 organotypic slices were pooled, centrifuged, and kept at −80°C until RNA extraction.

Reverse Transcription and Real-Time Quantitative Polymerase Chain Reaction

The design of primers specific for human genes and polymerase chain reaction (PCR) were performed as described previously [10, 12] (Table 1). Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed as previously described [10, 12, 35]. Several housekeeping genes were tested for normalization. The relative transcript quantity was determined using the ΔCt method, and relative quantities were normalized using the multiple normalization method [36].

Enzyme-Linked Immunosorbent Assays

Every 2 days, from day 0 to day 16, slice culture media were collected and stored at −80°C until use. Enzyme-linked immunosorbent assays (ELISAs) were performed to detect grafted cell secretion of human stanniocalcin-1 (hSCT-1) and human vascular endothelial growth factor (DuoSet ELISA and hVEGFA, specific to the 121, 165a, and 165b forms, DuoSet ELISA; both from R&D Systems, Minneapolis, MN, http://www.rndsystems.com), following the manufacturer's instructions. Three independent samples were tested in triplicate.

Statistical Analysis

The data are presented as the mean value of three independent experiments ±SD, unless otherwise stated. Significant differences between the samples were determined using an analysis of variance test, followed by a Scheffé post hoc test, which indicated whether the conditions were significantly different. The p value was $<.05$, unless otherwise stated.

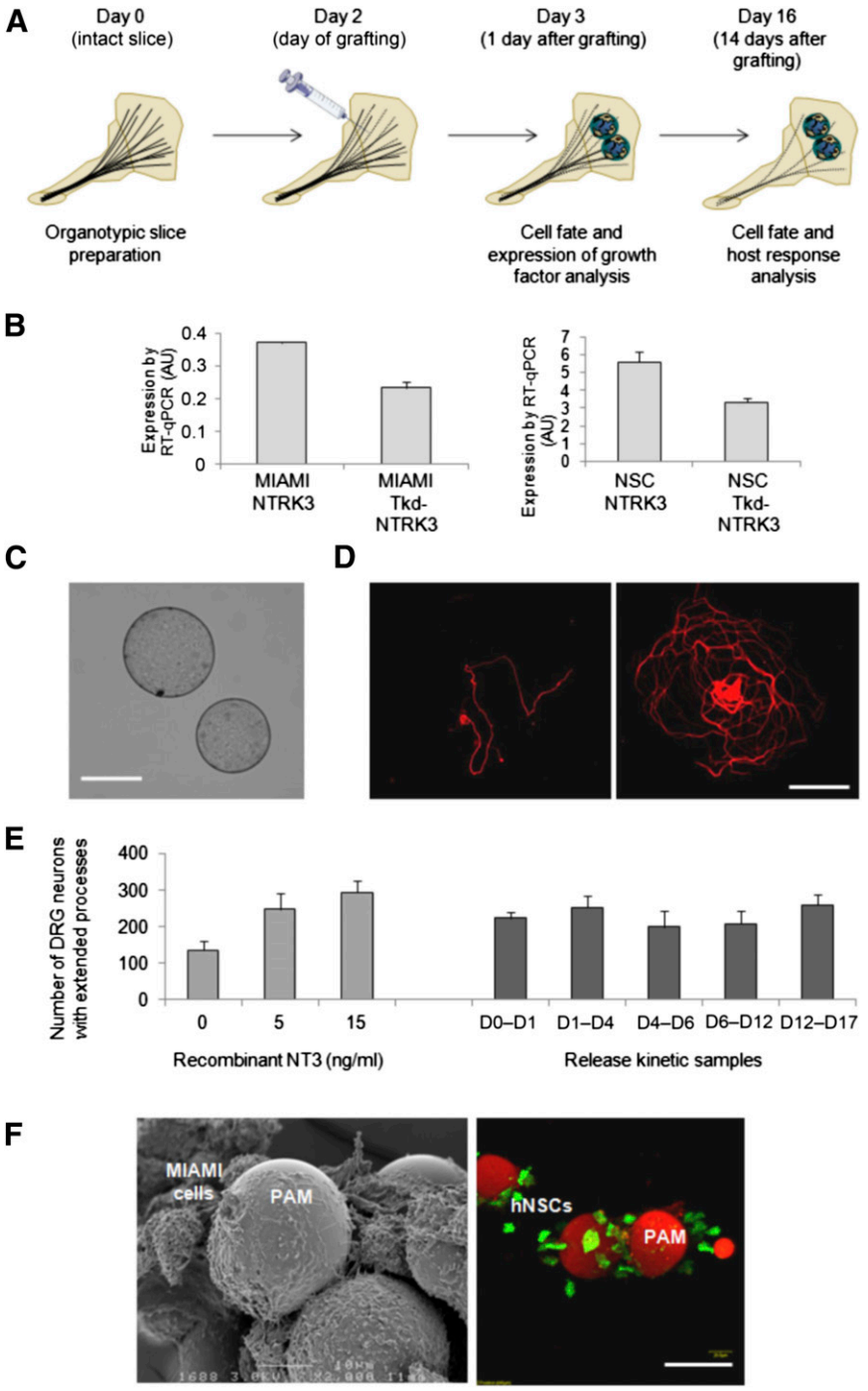

Figure 1. Experimental protocol and characterization of stem cells and PAMs alone and associated. **(A):** Experimental protocol. At day 0, the nigrostriatal pathway remains intact but degeneration is initiated. Two days after slice preparation, cell-PAM complexes were transplanted in the striatum. At day 3 (1 day after grafting), the grafted cells' fate and growth factor expression were analyzed. At day 16 (14 days after grafting), dopaminergic degeneration is complete. The grafted cells' fate and host tissue responses were also studied at this time point. **(B):** RT-qPCR against NT3 receptors for MIAMI cells and NSCs in vitro. Both types of cells express NT3 receptor mRNA, in particular, NSCs. Housekeeping genes *GAPDH*, *HPRT1*, *ACTB*, *RSP18*, and *HSPCB* were used for normalization (supplemental online data). **(C):** Observation of microparticles by bright field microscopy. Microparticles were perfectly spherical with a smooth surface and a mean diameter of 60 μm. Scale bar = 50 μm. **(D):** DRG neurons observed by immunofluorescence against β3-tubulin. (Left) A nontreated DRG neuron and (Right) a DRG neuron cultured with 5 ng/ml NT3, exhibiting extended processes. Scale bar = 30 μm. **(E):** Number of DRG neurons with extended processes. The released NT3 was bioactive and was equivalent to approximately 5–10 ng/ml compared with the recombinant control. **(F):** Observation of PAMs by scanning electronic microscopy when complexed with MIAMI cells and by fluorescence microscopy when complexed with GFP-positive NSCs. Scale bars = 10 μm (MIAMI-PAM) and = 60 μm (NSC-PAM). Abbreviations: D, day; DRG, dorsal root ganglion; GFP, green fluorescent protein; hNSCs, human neural stem cells; MIAMI, marrow-isolated adult multilineage inducible (cell); NSC, neural stem cell; NT3, neurotrophin-3; NTRK3, neurotrophic kinase receptor, type 3; PAM, pharmacologically active microcarrier; RT-qPCR, reverse transcription quantitative polymerase chain reaction; Tkd-NTRK3, tyrosine kinase-deficient isoform of NTRK3.

Table 1. Gene primers used for RT-qPCR

Gene	Full name	Accession number	Sequence
BDNF	Brain-derived neurotrophic factor	000011.9	Qiagen, reference no. QT00235368
GDNF	Glial cell line-derived neurotrophic factor	011675.2	Qiagen, reference no. QT00001589
HGF	Hepatocyte growth factor	001010934	F = 5′-TCTGGTTCCCCTTCAATAGC-3′
			R = 5′-GTGTTCGTGTGGTATCATGG-3′
NT3	Neurotrophin-3	001102654.1	Qiagen, reference no. QT00204218
NGF	Nerve growth factor	002506	Qiagen, reference no. QT00043330
VEGFA	Vascular endothelial growth factor A	001204384	F = 5′-CAGCGCAGCTACTGCCATCCA-3′
			R = 5′-CAGTGGGCACACACTCCAGGC-3′
STC1a	Stanniocalcin-1	003155	F = 5′-AGGCAAGGCTGACTTCTCTG-3′
			R = 5′-AACTACTTGTCGCATTGGGG-3′
NTRK3	Neurotrophic tyrosine kinase, receptor, type 3, Variant A	AF058389.1	F = 3′GAACCTCTACTGCATCAACG-5′
			R = 3′ACTATCCAGTCCACATCAGG-5′
TKd-TRK3	Neurotrophic tyrosine kinase, receptor, type 3 transcript variant 3 (isoform C)	AF058390.1	F = 3′AGCCGGACACGTGGGTCTTTT-5′
			R = 3′CTTGGAATGTCCGGGAAGGCTTA-5′

Abbreviations: F, forward; R, reverse.

RESULTS

Stem Cell NT3 Receptor Expression

Neurotrophic kinase receptor type 3, which represents all NT3 receptor variants and the tyrosine kinase-deficient isoform, were expressed in both NSCs and MIAMI cells, suggesting that both stem cells might respond to this neurotrophin and confirming previous reports [12]. Furthermore, NT3 receptor expression was 10-fold higher in NSCs than in MIAMI cells (Fig. 1B).

Characterization of PAMs

The mean LM-PAM size was 60.92 $\pm$ 20.04 μm. Observation of microspheres and PAMs under bright field and scanning electron microscopy showed that they were perfectly spherical, with a smooth surface and no pores on their surface (Fig. 1C). The NT3 microencapsulation yield was approximately 100%, determined using the NanoOrange total protein assay (Life Technologies).

The amount of bioactive NT3 released from PAMs was quantified using a bioassay based on rat DRG cell process extension (Fig. 1D). The released NT3 was bioactive and was equivalent to approximately 3.5–10 ng/ml compared with the recombinant control. After considering the dilution of the collected samples, this corresponded to approximately 185 ng NT3 per milligram PAM during the first day, constituting the burst release. Next, approximately 11–30 ng of NT3 per milligram of PAM was released for each time period examined (Fig. 1E). Furthermore, NT3 contained within the PAMs was bioactive 8 months after PAM preparation when conserved lyophilized at -20°C (data not shown).

The stem cells adhered to PDL/LM PAMs as observed by fluorescence microscopy and scanning electron microscopy and formed 3D cell/PAM complexes (Fig. 1F). The percentage of cells adhering to PAMs' surface at the end of the cell attachment protocol was approximately 94%.

Effects of Grafted Stem Cell/PAM Complexes on the Organotypic Nigrostriatal Pathway

Nigrostriatal Pathway Repair/Protection

Immunohistochemistry against rat TH was used to assess both nigrostriatal lesions in the organotypic slice and therapeutic benefits of treatment strategies (Fig. 1A). A decrease in TH fiber density in the striatum of nontreated slices was observed over time (Fig. 2A). A day 0 slice exhibited a TH density of 100%. The density had reached 40% at day 3 and 0% at day 16, confirming the findings of a previous report [24]. Implantation of LM-PAM-NT3 or NSC/LAM-PAM-NT3 induced only a slight recovery of TH expression, which was greater after MIAMI cell/LM-PAM-NT3 grafting (Fig. 2A). The quantification indicates that, after implantation of MIAMI cells/LM-PAM-NT3, the TH fiber density significantly increased (Fig. 2C) and reached 77% of the intact slice compared with the degenerated slice. For all other conditions tested, the density varied from approximately 30% to 65% (Fig. 2C). The regions of interest can be observed in supplemental online Figure 1A.

In the nontreated slices, TH-positive fibers within the medial forebrain bundle (MFB) were observed 1 day after organotypic slice preparation. However, no TH staining within the MFB could be detected after 16 days in culture, showing the dopaminergic neuron degeneration. Fourteen days after NSCs or MIAMI cell transplantation, both were adhering to LM-PAM-NT3, the dopaminergic fiber degeneration within the MFB was less significant, and dopaminergic fibers with some varicosities could be observed. In contrast, LM-PAM-NT3 injected alone did not seem to protect those dopaminergic fibers (Fig. 2B).

Striatal Dopamine Levels

The striatal DA level was quantified to assess the grafted cells striatal DA content restoration. A statistically significant reduction of 80% in striatal DA content (from 17.9 to 3.3 pg/mg tissue) was detected 3 days after slice culture compared with the day 0 intact slices. The DA content remained low until day 16, confirming our previous results [24]. The injection of MIAMI cells alone or complexed to LM-PAM-NT3 induced a slight DA recovery, which was not different compared with a negative control. However, the striatal DA content of an organotypic model of PD grafted with NSCs, adhering or not to PAMs, had remarkably reached normal levels 14 days after grafting, with a dopamine concentration of 18 pg/mg tissue (Fig. 2D).

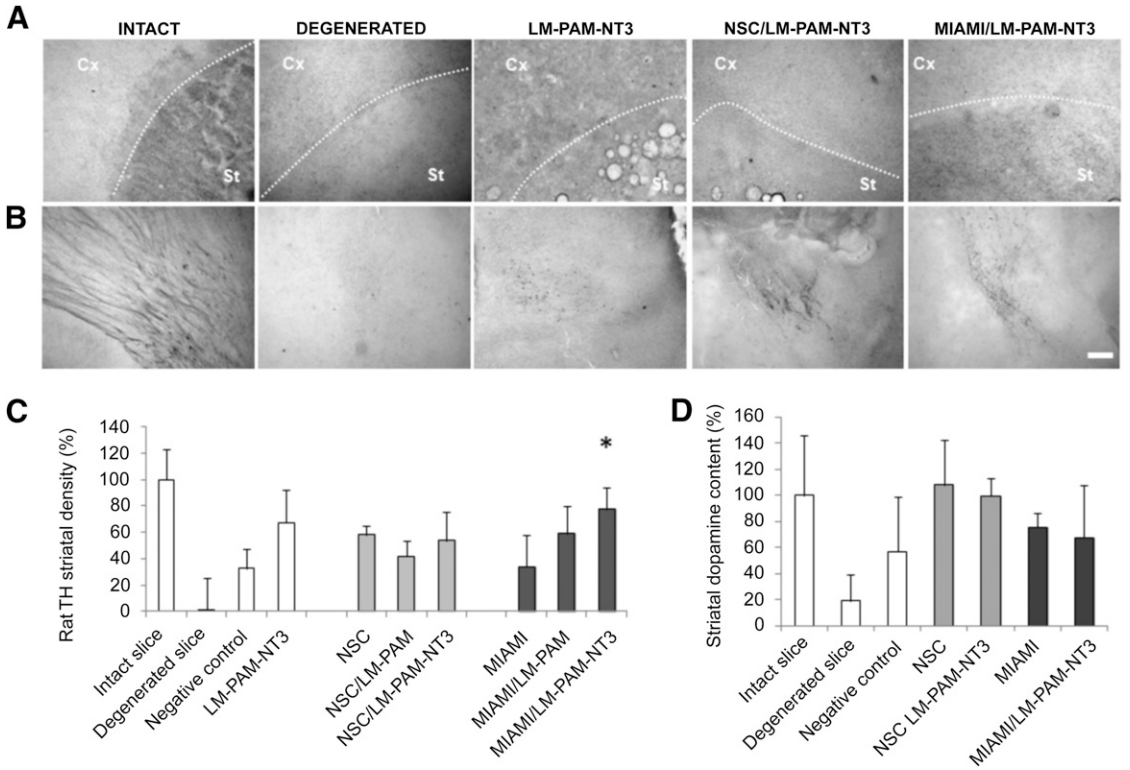

Figure 2. Protection/repair of the nigrostriatal pathway at day 16. **(A):** TH immunochemistry of St/Cx tissue. In intact slice, strong staining is observed compared with Cx, which represents the background. A total disappearance of TH staining was observed for the negative control, and injection of LM-PAM-NT3 injected alone or NSC/LM-PAM-NT3 induces only a slight recovery. For MIAMI/LM-PAM-NT3 injections, a TH staining can be observed highlighting a protection of the dopaminergic fibers. Scale bar: 100μm. **(B):** TH immunochemistry of MFB dopaminergic fibers at day 16. A total disappearance of the fibers was observed for negative control and for LM-PAM-NT3 injected alone while injection of MIAMI/LM-PAM-NT3 complexes or NSC/LM-PAM-NT3 complexes protected TH^+ fibers in comparison with degenerated slice. Scale bar = 100 μm. **(C):** Quantification of rat TH density in the organotypic striatum. A significant increase of rat TH^+ fiber density was observed after MIAMI/LM-PAM-NT3 treatment compared with the degenerated slice (n = 3; $p < .05$). **(D):** Quantification of the striatal dopamine content per mass of tissue in percentages. A slight increase in dopamine content was observed after MIAMI cell injection, and a remarkable increase was observed after NSC implantation. Dopamine content was quite similar when adherent cells were implanted alone or combined with LM-PAMs or LM-PAM-NT3 (n = 2). Abbreviations: LM, laminin; MFB, medial forebrain bundle; MIAMI, marrow-isolated adult multilineage inducible (cell); NSC, neural stem cell; NT3, neurotrophin-3; PAM, pharmacologically active microcarrier; St/Cx, striatal/cortex; TH, tyrosine hydroxylase.

Fate of Stem Cell/PAM Complexes After Injection in Organotypic Cultures

Morphology

Human-specific mitochondria (hMito) for MIAMI cells and GFP expression for NSCs were used to evaluate the fate, survival, and integration of stem cells injected alone or combined with PAMs in the organotypic model of PD (Fig. 1A). One day after intrastriatal implantation, the NSCs and MIAMI cells exhibited a round-shaped morphology typical of undifferentiated stem cells (supplemental online Fig. 1B, 1C, left). Fourteen days after grafting, the stem cells remained adhered to PAMs and circumscribed within the striatum (Fig. 3B, 3E). At this time point, the NSCs showed a neuron-like morphology with significant neurite outgrowth, and the MIAMI cells exhibited a bipolar neuron-like morphology (supplemental online Fig. 1B, 1C, right).

Survival and Proliferation of Grafted Cells

At 24 hours after implantation, a slight increase in GFP-expressing NSCs was observed in the slices grafted with NSC/PAM complexes compared with NSCs grafted alone (Fig. 3A, 3C). After 14 days, no differences in the density of GFP-expressing NSCs were found when the cells were grafted alone or combined with PAMs (Fig. 3A, 3C). In contrast, more hMito-expressing cells were observed when the MIAMI cells adhering to PAMs were grafted at both time points (Fig. 3E, 3F). Quantification of hMito-positive cells within the striatum showed that both types of PAMs significantly increased, by 2-fold and 3.3-fold, stem cell survival 1 day and 14 days after transplantation, respectively (Fig. 3F).

One day after grafting, many of the GFP-expressing NSCs were double-labeled for Ki-67, a cell cycle-related nuclear protein, indicating that a proportion of the cells continued to proliferate at a similar proportion whether adhering or not to PAMs (Fig. 3B, 3D). However, a strong decrease in cell number and Ki-67 expression was observed for all conditions 2 weeks after injection (Fig. 3B, 3D). No significant differences were found for these parameters when the cells were adhered to LM-PAMs or LM-PAM-NT3. These results indicate that most of the cells stopped proliferating, which suggests that cells can enter into a process of differentiation over time. For the MIAMI cells, no evident cell proliferation was detected at both time points (data not shown).

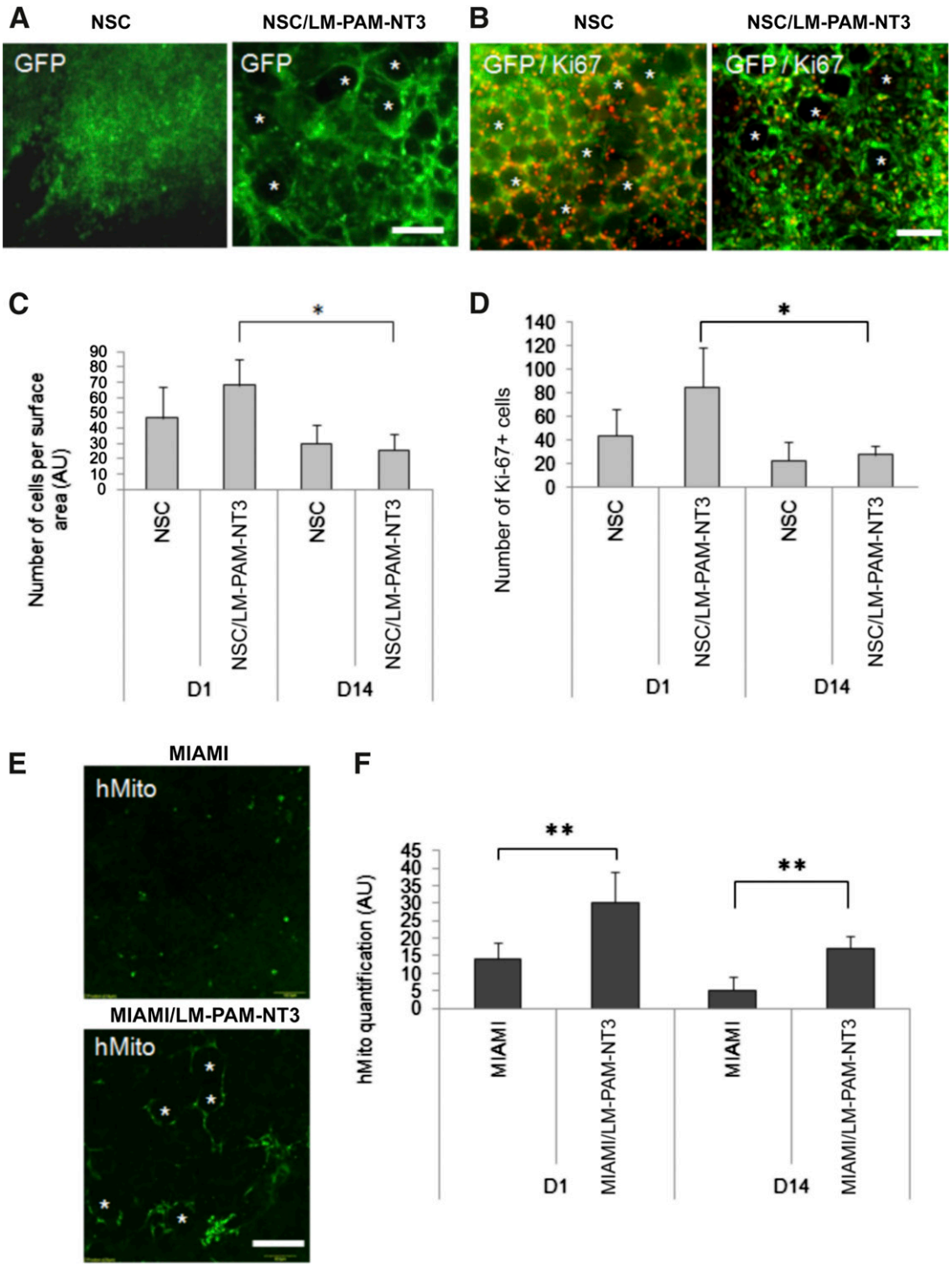

Figure 3. Stem cell survival and proliferation. **(A):** GFP-expressing NSCs, day 16, survival. hNS1 cells observed by fluorescence microscopy 14 days after grafting alone (left) or complexed with PAMs (right). No significant differences in GFP expression were noticed. Scale bar = 100 μm. **(B):** GFP expressing NSCs, days 1 and 16, Ki-67 expression. Immunofluorescence against human Ki-67 (red), highlighting the proliferation of grafted hNS1 cells 1 day (left) and 16 days (right) after grafting when complexed to PAMs. A significant decrease in Ki-67 expression was observed over time. PAMs are indicated by asterisks. Scale bar = 100 μm. **(C):** Quantification of GFP expression by hNS1 cells. A significant decrease in GFP expression was noticed 14 days after grafting when NSCs were complexed to PAMs. $*$, $p < .05$. **(D):** Quantification of Ki-67 expression by hNS1 cells. A significant decrease in Ki-67 expression was noticed 14 days after grafting when NSCs were complexed to PAMs. $*$, $p < .05$. **(E):** MIAMI cells, day 16, survival. Immunofluorescence against hMito 14 days after grafting MIAMI cells alone (above) or combined with PAMs (below). An increase in hMito-positive cells was noticed when cells were adhering to PAMs. PAMs are indicated by asterisks. Scale bar = 100 μm. **(F):** Quantification of hMito-positive MIAMI cells, day 16. After 1 and 14 days in situ, the MIAMI cell number increased 2 or 3 times when adhering to PAMs. $**$, $p < .01$ ($n = 3$). For all those experiments, similar results were obtained when cells were implanted with LM-PAMs or LM-PAM-NT3. Abbreviations: D, day; GFP, green fluorescent protein; hMito, human-specific mitochondria; LM, laminin; MIAMI, marrow-isolated adult multilineage inducible (cell); NSC, neural stem cell; NT3, neurotrophin-3; PAM, pharmacologically active microcarrier.

Differentiation of Grafted Cells to a Neuronal Dopaminergic Phenotype

At any time point and for any treated group, the number of β3-tubulin-positive cells, a neuronal marker, was 10 times higher for NSCs than for MIAMI cells (Fig. 4A, 4B). Notably, at day 14, for both types of stem cells, the fraction of β3-tubulin-positive cells increased when the cells were adhering to LM-PAMs and was even higher when adhering to LM-PAM-NT3 compared with cells grafted alone. A significant ninefold increase was observed when the MIAMI cells were adhering to LM-PAM-NT3. Finally, for NSCs, the fraction of β3-tubulin-positive cells also increased within the NSC/LM-PAM-NT3 grafted cells between days 1 and 14 (Fig. 4A–4C).

Immunohistochemistry against human-specific TH (hTH), the rate-limiting enzyme in DA synthesis, illustrates the differentiation of grafted cells toward a neuronal dopaminergic-like phenotype (Fig. 4D, 4E; negative control in supplemental online Fig. 1B). Quantification of hTH^+ cells 1 day after injection only showed a small number of positive cells at this early time point. However, an overall trend toward a dopaminergic-like phenotype was observed when stem cells were complexed to LM-PAM-NT3. Stem cell dopaminergic-like differentiation was more evident 14 days after cell injection, where the number of dopaminergic-like differentiated cells had significantly increased by threefold for cells alone (Fig. 4F). Human TH expression increased twofold when both stem cells were administrated combined with LM-PAM-NT3 (reached more than 300 TH-positive for NSCs and 250 TH-positive for MIAMI cells), although the differences were not statistically significant for the MIAMI cells.

Repair and Protection Mechanisms

In Vitro and Ex Vivo mRNA Expression of Tissue Repair Factors

In vitro, NSCs mainly expressed hVEGFA and brain-derived neurotrophic factor (BDNF), and nerve growth factor (NGF) was expressed at a lower level (data not shown) (supplemental online Fig. 2A). The MIAMI cells expressed very high levels of hVEGFA (about 20-fold more than that of NSCs) and BDNF and low levels of glial cell line-derived neurotrophic factor (GDNF) and NGF (supplemental online Fig. 2B), confirming previous results [12]. The hNS1 cell hVEGFA expression was slightly increased 24 hours after adhesion to LM-PAMs and was almost doubled for MIAMI cells (supplemental online Fig. 2A, 2B).

At 24 hours after implantation in the lesioned striatum, a complementary study was performed ex vivo, also with two supplemental primers, hepatocyte growth factor (HGF) and STC-1, after laser microdissection of grafted complexes. Just as observed in vitro, NSCs expressed hVEGFA and STC-1. A slight increase in hVEGFA expression was observed when NSCs were adhering to PAMs (Fig. 5A). MIAMI cells expressed hVEGFA, HGF, and GDNF, with a slight decrease in expression when complexed with PAMs (Fig. 5B). MIAMI cells expressed hVEGFA mRNA, almost 4- to 10-fold more than that of NSCs (Fig. 5C), although NSCs expressed 40- to 100-fold more STC-1 mRNA than did the MIAMI cells (Fig. 5D).

Analysis of Secreted STC-1 and hVEGFA Proteins in Slice Cultures

NSCs secreted 2,350 $\pm$ 170 pg/ml STC-1 at day 3 and 310 $\pm$ 70 pg/ml at day 16 (Fig. 5E). Because the MIAMI cells expressed very low STC-1 mRNA levels, no ELISAs were performed on those cells for this protein. However, 103 $\pm$ 20 pg/ml hVEGFA was secreted by MIAMI cells at day 5, which had significantly decreased to 54 $\pm$ 7 pg/ml at day 16. In addition, hVEGFA secretion decreased when the MIAMI cells adhering to LM-PAM-NT3 were implanted compared with cells grafted alone at all time points. hVEGFA secretion was very low for NSCs (almost sixfold lower than that of MIAMI cells) and increased slightly when adhering to LM-PAM-NT3 (Fig. 5F).

CD31 Expression and Blood Vessels Counting in Organotypic Slices

The paracrine stem cell-mediated effect of hVEGFA on endothelial cells around the graft area was assessed by CD31 immunofluorescence (Fig. 6A). $CD31^+$ blood vessels and their branches were quantified in the nigrostriatal slices from day 0 to day 16 of culture (Fig. 6B). At day 0, the number of blood vessels was approximately 7 $\pm$ 3 and had decreased to almost 0 from day 4 to day 16, suggesting the disappearance of vascularization in the organotypic slices when no treatment was administered. Medium injection did not induce an increase in blood vessel numbers nor did LM-PAM-NT3 when grafted alone (data not shown). In contrast, grafted MIAMI cells induced an increase of CD31 expression, suggesting angiogenesis and blood vessel maintenance. Moreover, the number of vessels increased when MIAMI cells were adhering to PAMs, becoming statistically significant when the cells were adhering to LM-PAM-NT3 at days 3 and 16. Therefore, the treatment with MIAMI cell LM-PAM-NT3 promoted the normalization of blood vessel number in the organotypic model of PD. This effect however, was not observed with NSCs alone or when complexed to PAMs (Fig. 6A, 6B).

Discussion

Cell therapies have failed to demonstrate consistent benefits in clinical trials of PD. In addition, combinatorial cell and drug delivery strategies still need to be refined to improve cell engraftment and differentiation. Therefore, efforts at understanding stem cell mechanisms, including angiogenesis, neuroprotection, modulation of inflammation, among others, are essential to successfully translate tissue-engineering approaches into the clinic. In the present study, the biological mechanisms by which MIAMI cells and NSCs, complexed or not with LM-PAM-NT3, induce neuroprotection were elucidated in part using an ex vivo model of PD. We showed that LM-PAM-NT3 enhanced the dopaminergic differentiation of NSCs secreting DA, which possessed a modest neurorepair/protection potential, In contrast, LM-PAM-NT3 enhanced survival and differentiation of neural-specified MIAMI cells into dopaminergic-like cells, inducing a strong neurorepair that appears to be mediated at least by VEGFA secretion.

MIAMI cells were chosen for our study because they can be used for autologous implantation directed at the repair of damaged tissues. These cells have been previously characterized in 3- to 72-year-old donors, and it has been shown that MIAMI cell properties (e.g., proliferation, differentiation) were minimally affected by donor age and gender [8], making them an ideal candidate for neuron replacement therapy. Human NSCs represent an interesting therapeutic alternative. For the present study, an immortalized lineage of hNSCs expressing GFP, with a normal karyotype and no signs of transformation, disruption,

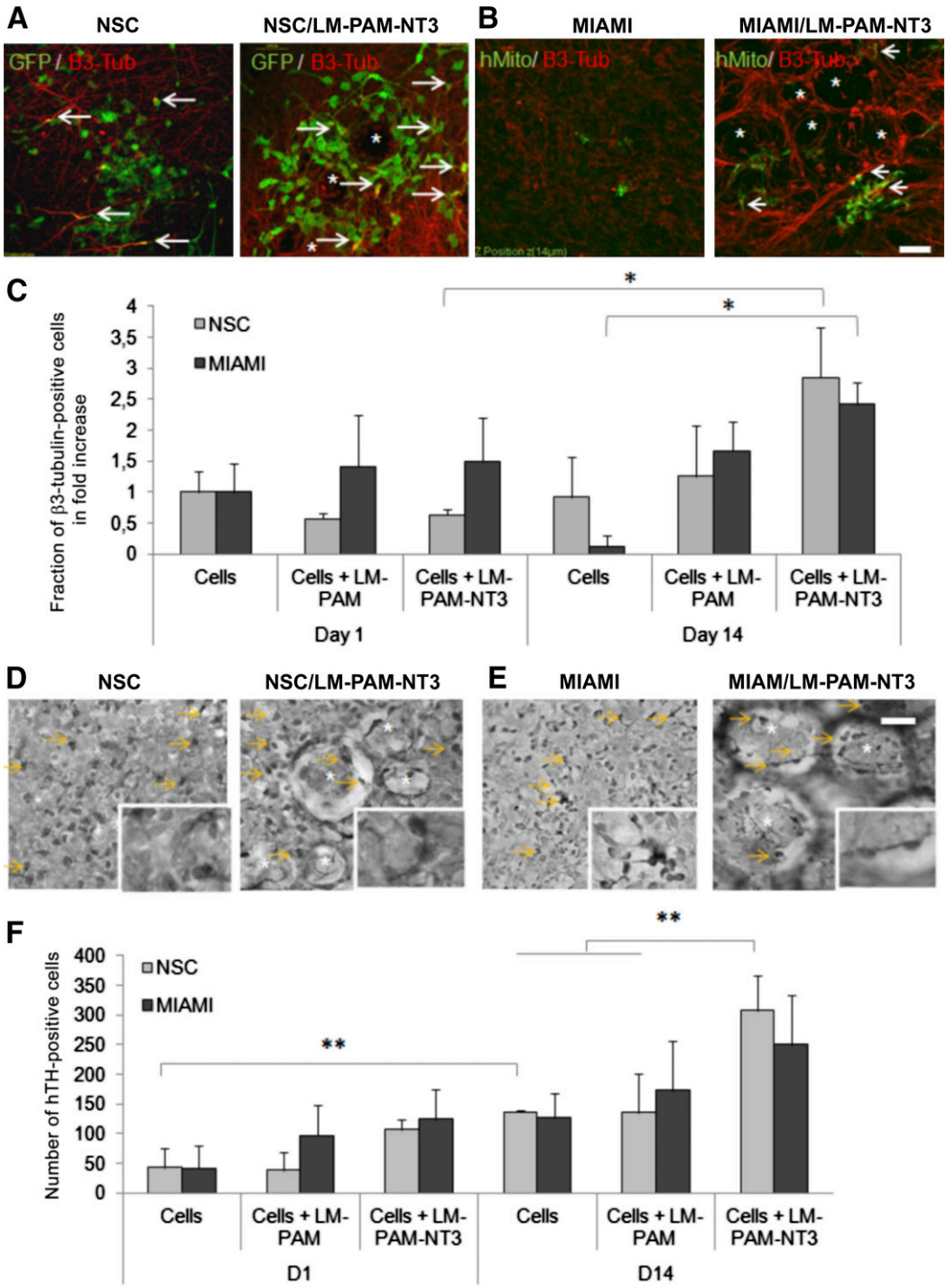

Figure 4. Differentiation potential of grafted cells. β3-tubulin expression of GFP expressing hSN1 **(A)** and hMito-counterstained MIAMI cells **(B)** at day 16. Both cell types were implanted alone (left) and adherent to LM-PAM-NT3 (right) and observed by immunofluorescence 14 days after grafting. White arrows indicate β3-tubulin-positive cells. Scale bar = 50 μm. **(C):** β3-Tubulin expression quantification of grafted NSCs and MIAMI cells 1 and 14 days after grafting when adherent or not to PAMs on fold increase against cells grafted alone at day 1. hNS1 as MIAMI cells showed a significant expression of β3-tubulin after 14 days in situ when complexed with LM-PAM-NT3 (n = 3; $*$, $p < .05$). Human TH expression of NSCs **(D)** and MIAMI cells **(E)** at day 16. Both cell types were grafted alone (left) and when adherent to LM-PAM-NT3 (right) and observed by immunohistochemistry 14 days after grafting. Yellow arrows indicate hTH-positive cells. Inset represents magnification of TH-positive cell to observe morphology. Scale bar = 50 μm. **(F):** Quantification of TH expression 1 and 14 days after grafting of NSCs and MIAMI cells alone and combined with PAMs. TH expression is very weak 24 hours after grafting for NSCs and MIAMI cells. A slight increase in expression was observed when cells were adherent to LM-PAM-NT3. At 14 days after grafting, TH expression had increased and was significantly different for NSC/LM-PAM-NT3 compared with the 2 other conditions at this time point. An increase in TH expression was also observed for MIAMI cells when adherent to LM-PAM-NT3 (n = 3; $*$, $p < .01$). Abbreviations: D, day; GFP, green fluorescent protein; hMito, hMito, human-specific mitochondria; hTH, human-specific tyrosine hydroxylase; LM, laminin; MIAMI, marrow-isolated adult multilineage inducible (cell); NSC, neural stem cell; NT3, neurotrophin-3; PAM, pharmacologically active microcarrier.

or tumorigenesis, was used, because primary NSCs of human origin are difficult to obtain and face significant ethical barriers [26, 29].

In the present study, we chose a new ex vivo model of PD able to mimic the early and late disease stages to better understand the efficacy and mode of action of stem cells to neuroprotect the nigrostriatal pathway [23, 24]. The novelty of the present work resides, in part, in using organotypic cultures to elucidate mechanisms of neuroprotection or repair mediated by stem cells alone or combined with PAMs, because only a small number of studies have explored this possibility. To be able to implant PAM/cell complexes in the brain slices, Stoppini's membrane interface air-medium method was selected. This method allows better access and observation of the cultured tissue but also a more limited survival time [37]. The degeneration of the nigrostriatal pathway obtained here was not accompanied by the formation of Lewy bodies in the dying neurons. Despite those limitations, organotypic cultures can be very useful to better elucidate the mechanisms by which stem cell therapy, combined or not, with drug delivery can exert beneficial effects, because cell graft-host tissue cross-talk is present, and they can be kept in culture for a few weeks, maintaining the cytoarchitecture of the original tissue [23].

In the present study, we first showed that released NT3 from PAMs was bioactive. The released NT3 possessed a strong neuronal differentiation capacity on DRG cells at a low dose (approximately 5 ng/ml), confirming that sufficient amounts of NT3 were released to induce stem cell survival/differentiation. Moreover, cell differentiation was maintained until the end of the experiment in the presence of released NT3. This allowed an important therapeutic window for the MIAMI cells and NSCs to differentiate toward a neuronal phenotype and to explore repair mechanisms.

Stem cell transplants were performed 2 days after organotypic slice preparation (Fig. 1A), when approximately 80% of the TH-positive neurons in the substantia nigra and 30% of dopaminergic fibers in the striatum had degenerated [24], mimicking an early stage of PD. We first confirmed our previous in vivo observations, showing a significant striatal dopaminergic fiber protection/repair after MIAMI/LM-PAM-NT3 grafting [12]. Although the amount of PAMs and cells injected was less in this ex vivo paradigm, clear protection/repair was observed. Moreover, the effect was observed 14 days after grafting, which is a short time window to observe a complete regenerative process (usually evaluated 4 [38] and 8 [12] weeks after implantation in hemiparkinsonian rats). Furthermore, neuroprotection of the MFB fibers was also observed after MIAMI/LM-PAM-NT3 grafting. The degree of protection was, however, less prominent after NSCs/LM-PAM-NT3 grafting, which was somewhat similar to that of LM-PAM-NT3 not conveying any cells. The NT3 antiapoptotic role in 6-OHDA-treated PC12 cells [39] and its neuroprotective effects on the nigrostriatal pathway in the adult rat model of PD [40] have been previously reported. Although NT3 can increase the survival of grafted ventral mesencephalic cells in rat striatum, it does not significantly increase host TH staining, which was confirmed in our study by the lack of TH staining in the MFB after LM-PAM-NT3 grafting [41]. In addition, a weak spontaneous dopaminergic reinnervation of the striatum was also observed, as previously described [42].

The grafted cells acquired a neuronal-like morphology 2 weeks after grafting, suggesting integration into the host tissue. Nevertheless, β3-tubulin expression by NSCs was higher than that by MIAMI cells at all times, confirming that NSCs spontaneously differentiate into neuronal cells in vivo [28, 29]. The neuronal differentiation was further enhanced for both types of cells by LM-PAM-NT3. These results confirm the combined action of laminin and NT3 on the increase of β3-tubulin MIAMI cell expression ex vivo, as previously observed in vitro [9]. A significant increase in TH expression was noticed for NSCs when they were grafted alone in the organotypic model of PD at day 14. Furthermore, LM-PAM-NT3 enhanced TH expression for NSCs and MIAMI cells, and for the latter, NT3 release was an essential factor. This differentiation potential of stem cells has been observed despite a short time window and should be further explored to understand their degree of commitment toward the neuronal lineage. RT-qPCR performed on MIAMI cells after epidermal growth factor (EGF)/basic fibroblast growth factor (bFGF) treatment indicates that MIAMI cells express Nurr1 mRNA during proliferation (data not shown). These data, together with the previously published data in which we observed increased Nestin, Pax 6, and Ngn2 expression [10], suggest that EGF/bFGF pretreated cells might be engaged into a "neuronal dopaminergic precursor" phenotype. Furthermore, slight hTH protein expression has been found by Western blot in hNS1 cells cultured under proliferative conditions, in particular, in low oxygen tension conditions (data not shown), confirming that hNS1 have a slight basal expression of TH in proliferation [27]. However, injection of the cells within the striatum, in particular, in the damaged striatum, can accelerate this TH induction. This dopaminergic inductive effect of the striatum has also been described both in vitro and in vivo [30, 43–46]. In a previous study using hNS1 cells, 25% of mature dopaminergic neurons able to secrete dopamine were found after only 12 days of coculture with lesioned striata [30].

A remarkable increase of DA content in the striatum, which reached normal levels, was observed after NSC/LM-PAM-NT3 grafting. In this case, DA was most likely released by differentiated, more mature NSCs, because a significant restoration of the host dopaminergic pathway was not observed with these cells. The high NSC survival rate might have contributed to the striatal DA restoration observed in our study. All these data suggest that NSC grafts combined with LM-PAM-NT3 exert their beneficial effect by a cell replacement mechanism. In contrast, after MIAMI/LM-PAM-NT3 grafting, a slight increase in DA content in the striatum occurred that could have resulted from the significant repair/protection of the host dopaminergic pathway. MIAMI cells and, in particular, MIAMI cell/LM-PAM-NT3 complexes might exert neuroprotective effects on the nigrostriatal pathway through their potential to secrete tissue repair factors [12, 19]. Our study demonstrates that MIAMI cells expressed BDNF and GDNF and high levels of hVEGFA mRNA in vitro and ex vivo, confirming our previous results showing elevated levels of hVEGFA, significantly higher than for hMSCs [11]. All three dopaminotrophic factors are known for their neurotrophic properties and their ability to act on nigrostriatal DA neurons [47–49]. NSCs expressed lower levels of hVEGFA and mainly secreted hSTC1 in the organotypic culture model of PD. Moreover, in our paradigm, MIAMI cells secreted approximately 100 pg/ml VEGFA; thus, it has been proved that neuroprotection in PD was more efficient with a low secretion of VEGFA (close to 1 ng/ml) and can even be deleterious at higher doses (close to 100 ng/ml) [50]. In addition, a graft of an hVEGFA-expressing cell line in parkinsonian rats induced strong

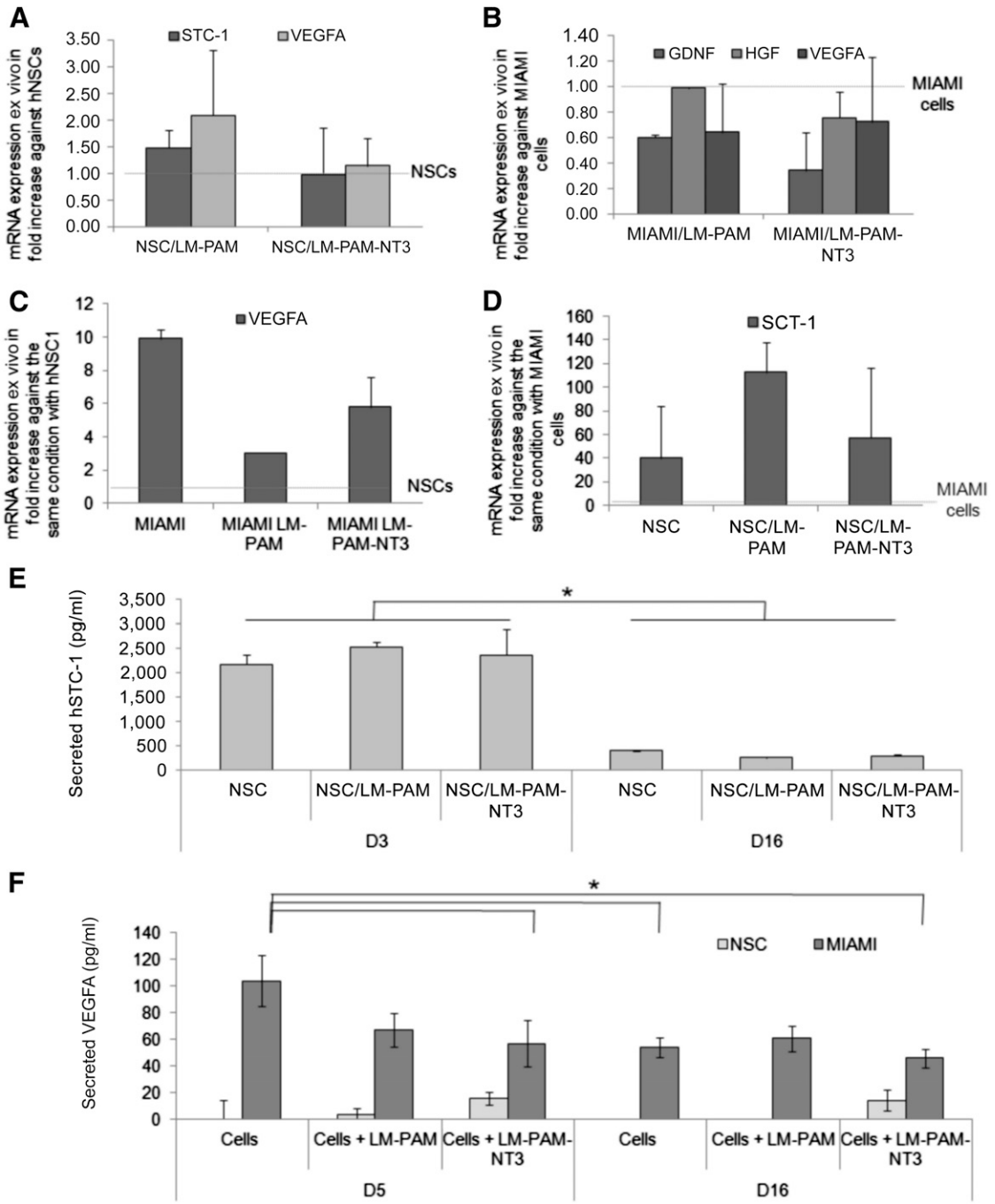

Figure 5. Ex vivo mRNA and protein expression of cells grafted alone or adhering to PAMs. **(A):** hNS1 cells complexed to PAM mRNA expression in fold increase against hNS1 cells implanted alone. GDNF, NGF, and tumor necrosis factor-α-stimulated gene 6 (TSG6) were not expressed. **(B):** mRNA expression in fold increase of MIAMI cells complexed to PAMs compared with MIAMI cells implanted alone. TSG6 was not expressed, and STC-1 was only slightly expressed. **(C):** VEGFA mRNA expression in fold increase of MIAMI cells compared with NSCs in the same conditions. MIAMI cells expressed 4–10-fold more VEGFA mRNA than did hNS1 cells. **(D):** STC-1 mRNA expression in fold increase of hNS1 compared with MIAMI cells in the same conditions. hNS1 cells expressed 40–100-fold more STC-1 mRNA than did MIAMI cells. **(E):** Enzyme-linked immunosorbent assay (ELISA) quantification of secreted STC-1 in culture medium of stem cell-grafted organotypic slices. STC-1 secretion decreased significantly over time ($n = 3$; $*$, $p < .05$). **(F):** ELISA quantification of secreted VEGFA in culture medium of stem cell-grafted organotypic slices. For MIAMI cells, VEGFA secretion decreased over time and was lower with PAM adherence compared with cells alone. For NSC cells, VEGFA secretion remained very low ($n = 3$; $*$, $p < .05$). Abbreviations: D, day; GDNF, glial cell line-derived neurotrophic factor; HGF, hepatocyte growth factor; hNSCs, human neural stem cells; LM, laminin; MIAMI, marrow-isolated adult multilineage inducible (cell); NGF, nerve growth factor; NSC, neural stem cell; NT3, neurotrophin-3; PAM, pharmacologically active microcarrier; SCT-1, stanniocalcin-1; VEGFA, vascular endothelial growth factor A.

behavioral recovery and protection of the nigrostriatal pathway [47, 51, 52]. It was observed that VEGFA secretion is significantly reduced over time after LM-PAM-NT3 adhesion of MIAMI cells, but this condition gives the most significant neuroprotection of the striatal dopaminergic fibers. These results can be explained by a probable cumulative effect between hVEGFA secreted by MIAMI cells and NT3 released by PAMs, because both have neuroprotective and proangiogenic properties [53–55]. It can

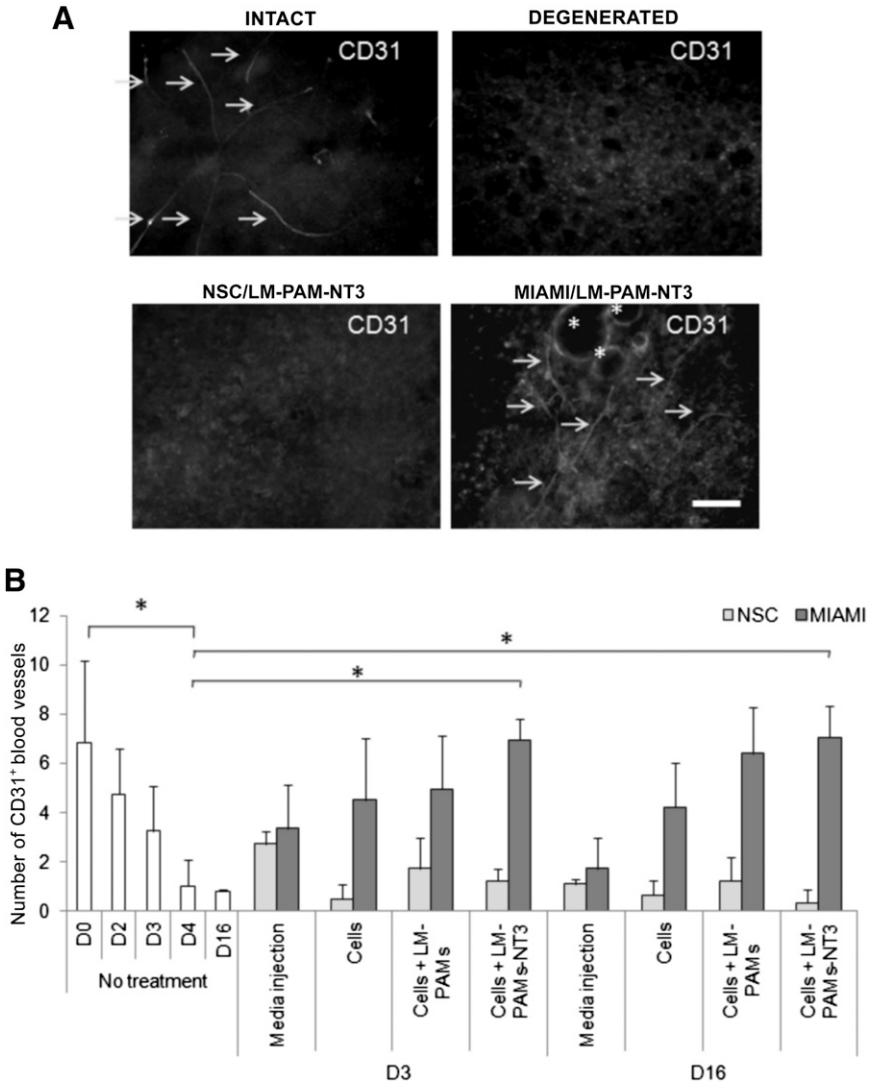

Figure 6. Paracrine stem cell-mediated effect of human vascular endothelial growth factor A on rat endothelial cells. **(A):** Immunofluorescence against rat-specific CD31 in the striatum. A disappearance of blood vessels was observed in degenerated slices and after NSC/LM-PAM-NT3 implantation compared with intact slices. After NSC/LM-PAM-NT3 implantation, no blood vessels were observed. The blood vessel number increased after MIAMI/LM-PAM-NT3 implantation. White arrows show the blood vessels, and asterisks indicate PAMs. Scale bar = 50 μm. **(B):** Quantification of blood vessels in the slice. At day 4, no blood vessels were detected. No amelioration was observed after grafting NSCs even when complexed with PAMs, but a significant protection of blood vessels was observed after MIAMI cell implantation, particularly when adherent to LM-PAMs-NT3 (n = 3; $*$, $p < .05$). Abbreviations: D, day; LM, laminin; MIAMI, marrow-isolated adult multilineage inducible (cell); NSC, neural stem cells; NT3, neurotrophin-3; PAM, pharmacologically active microcarrier.

also be assumed that VEGFA binds to its own receptors and thus will no longer be found in the culture media.

The importance of LM-PAMs has been highlighted in the present study. MIAMI cells implanted alone induced a recovery of 30% of rat striatal TH. The association of MIAMI cells with LM-PAMs doubled survival and, as a consequence, doubled the level of striatal rat TH. This showed that the MIAMI cells themselves induce protection/repair of the TH^+ fibers by VEGFA secretion. Furthermore, MIAMI/LM-PAM-NT3 induced an increase of 24% of the rat TH level compared with MIAMI/LM-PAM, indicating that NT3 and VEGFA might act in association to give the 77% TH^+ fibers of the nigrostriatal pathway detected in our study, because NT3 alone can only give 67%.

No change was found in the blood vessel number over time in the organotypic slices treated with NSCs, which did not secrete hVEGFA. NSCs differentiated toward TH-expressing cells able to secrete DA, which can block hVEGFA expression [56]. In contrast, vascularization was enhanced by VEGF-secreting MIAMI cells, particularly when the cells were complexed to LM-PAM-NT3. Angiogenesis induction by VEGFA in hippocampal slice cultures had been previously described, suggesting that angiogenesis might occur in our paradigm, parallel to maintenance of the blood vessel number [57]. However, hVEGFA secretion decreased over time when the cells were grafted alone or were adherent to PAMs, in which case they already expressed lower hVEGFA levels, probably owing to MIAMI cell differentiation into neuron-like cells expressing TH [56]. Significant expression of the angiogenic factor HGF was detected in MIAMI cells, indicating that this factor might also mediate the increase of CD31-expressing cells. In our model,

VEGFA will mainly act by a direct neuroprotective effect on the cells and will probably have a protective effect via angiogenesis on grafted cells in in vivo models. Indeed, vascularization of grafted tissue and its integration into the host circulatory system is needed for graft survival [58, 59]. However, certain studies have shown that VEGFA can also be effective in later disease stages [47, 60]. Although it is still controversial, a blood vessel defect might be an additional contributing factor to the development of PD [61]. In addition, increased angiogenesis can be associated with an improvement of motor capacity in in vivo PD models [62]. The increased number of cells observed 1 day after grafting when complexed to PAMs might also explain the neuroprotective effects of these complexes, because more neuroprotective factors might be secreted at this early time point. The increased survival induced by PAMs might be also result from the three-dimensional scaffolds [22]. The enhancement of survival when cells are attached to a three-dimensional substrate has been reported previously for retinal pigmental epithelium cells [63], which led to a clinical trial in a PD paradigm [64]. In contrast, no significant increase in NSC survival was detected with the 3D scaffold. However, NSC survival remained higher than that of MIAMI cells at any time. STC-1 highly secreted by NSCs might act by autocrine mechanisms to induce cell protection against the mitochondrial membrane defects induced by reactive oxygen species, such as has been reported with neural crest-derived cells [65]. Moreover STC-1 can increase microtubule-associated protein 2c expression, an axon specific marker, in neuroblastoma cells. Thus, STC-1 could be in part responsible for NSC neuroprotection but also could increase neuronal differentiation by an autocrine action [66]. Although the molecules we examined represent attractive mediators of the mechanisms discussed, our studies do not exclude the possibility that other molecules might also be playing mechanistic roles.

Taken together, those findings show that those stem cells have different modes of action that are clearly complementary. It would be interesting to study the beneficial effect of both types of cells grafted together with LM-PAM-NT3, because it has previously been reported that hMSCs can increase survival of NSCs and neuroprotection in a Huntington's disease paradigm [67] and that MSCs might protect NSCs from oxidopamine-induced apoptosis [68].

Conclusion

Using an ex vivo model of PD, we showed that PAMs enhanced the survival and neuronal differentiation of NSCs and MIAMI cells to induce neuroprotection of the nigrostriatal pathway. Moreover, NSCs might act through direct replacement of loss/damaged cells for PD treatment, and MIAMI cells might protect the host's remaining dopaminergic cells by a paracrine mechanism that could involve support both to the neural and to the vascular compartments. Our findings have improved our understanding of the stem cell neuroprotection mechanisms. Taken together, our findings show a prospective interest of both types of cells combined with LM-PAM-NT3 in tissue engineering for PD.

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Author Contributions

N.D.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; E.G.: data analysis and interpretation, final approval of manuscript; L.S.: collection and assembly of data; A.M.-S. and P.C.S.: final approval of manuscript; C.N.M.-M.: conception and design, data analysis and interpretation, administrative support, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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