Magnetic Nanoparticle-Mediated Gene Transfer to Oligodendrocyte Precursor Cell Transplant Populations Is Enhanced by Magnetofection Strategies

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ABSTRACT This study has tested the feasibility of using physical delivery methods, employing static and oscillating field "magnetofection" techniques, to enhance magnetic nanoparticle-mediated gene transfer to rat oligodendrocyte precursor cells derived for transplantation therapies. These cells are a major transplant population to mediate repair of damage as occurs in spinal cord injury and neurological diseases such as multiple sclerosis. We show for the first time that magnetic nanoparticles mediate effective transfer of reporter and therapeutic genes to oligodendrocyte precursors; transfection efficacy was significantly enhanced by applied static or oscillating magnetic fields, the latter using an oscillating array employing high-gradient NdFeB magnets. The effects of oscillating fields were frequency-dependent, with 4 Hz yielding optimal results. Transfection efficacies obtained using magnetofection methods were highly competitive with or better than current widely used nonviral transfection methods (*e.g.*, electroporation and lipofection) with the additional critical advantage of high cell viability. No adverse effects were found on the cells' ability to divide or give rise to their daughter cells, the oligodendrocytes—key properties that underpin their regeneration-promoting effects. The transplantation potential of transfected cells was tested in three-dimensional tissue engineering models utilizing brain slices as the host tissue; modified transplanted cells were found to migrate, divide, give rise to daughter cells, and integrate within host tissue, further evidencing the safety of the protocols used. Our findings strongly support the concept that magnetic nanoparticle vectors in conjunction with state-of-the-art magnetofection strategies provide a technically simple and effective alternative to current methods for gene transfer to oligodendrocyte precursor cells.

KEYWORDS: oligodendrocyte precursor cells · magnetic nanoparticles · magnetofection · transplantation · organotypic slice culture · gene therapy · transfection

Oligodendrocyte precursor cells (OPCs) are a major transplant population to promote repair following myelin damage (as occurs in neurological diseases such as multiple sclerosis) and in spinal cord injury (SCI).[1−5] The beneficial effects of OPCs are primarily due to the production of new myelin by oligodendrocytes, the daughter cells of OPCs.[6] Myelin is the lipid-based insulating sheath covering nerve fibers that aids in the efficient conduction of electrical signals and exerts neuroprotective effects on axons.[7,8] Cell replacement studies have noted a down-regulation of genes associated with tissue damage and inflammation following OPC transplantation, indicating additional mechanisms by which OPCs may exert tissue-sparing effects.[9] These cells are abundant in fetal and adult brain, and relatively pure OPC populations can be obtained from CNS tissue using FACS/antibody selection; protocols have also been established to derive OPCs from rodent/human embryonic stem cells (hESCs), highlighting their translational potential for clinical cell transplantation therapies.[9−11] The major therapeutic and translational potential of OPCs is evidenced by the commencement of human clinical trials in regenerative medicine, for example that by Geron Corporation (CA, USA) of their product GRNOPC1, consisting of hESC-derived OPCs for repair of SCI.[12]

While cell transplantation therapies have major potential to mediate neural repair, successful therapeutic interventions are likely to require so-called "combinatorial" approaches (*e.g.*, delivery of cells *plus* therapeutic factors to injury foci), as neural repair is a complex process requiring multiple goals to be achieved for successful regeneration.[13−17] Consequently, engineering the repair properties of transplanted OPCs, for example by introducing genes encoding therapeutic factors, constitutes a key strategy to improve neural repair. OPCs can be modified to deliver therapeutic proteins such as fibroblast growth factor 2 (FGF2), ciliary neurotrophic factor

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(CNTF), and neurotrophin 3 (NT3) following transplantation.[14,15,18,19] Such interventions make lesion environments more conducive to repair, enhance both graft-mediated and endogenous cellular repair, encourage axonal outgrowth in lesions, and promote angiogenesis.[20−24]

From a technical perspective, it is important to note that previous studies have relied heavily on viral methods to achieve gene transfer to OPCs.[14,15,25,26] Viral methods are efficient and have yielded valuable information in experimental studies on neural repair. However, despite recent technological advances, there are still significant risks and disadvantages associated with viral-mediated gene transfer, primarily safety issues such as toxicity, nonspecific cellular uptake, inflammatory responses, and oncogenic effects leading to abnormal cellular growth.[25,27−32] Concerns have been raised that viral vectors may themselves cause alterations in OPC proliferation and differentiation, oligodendrocyte death, and myelin damage.[27] There are also major limitations with viral delivery in terms of limited plasmid insert size and achieving large-scale production for clinical applications, leading to a major international drive for the development and evaluation of nonviral transfection methods.[31,32] Despite this, many of the nonviral methods used for neural gene transfer also have major associated disadvantages. For example, nonviral vectors have lower transfection efficacies than their viral counterparts, leading to the adoption of physical gene delivery methods, such as biolistic transfection and electroporation, which can be associated with adverse effects such as membrane damage, abnormal cell physiology, and substantial cell death (up to 80% cell loss).[30,33]

In this context, magnetic nanoparticle (MNP)-based vector platforms have emerged as an essential tool for advanced biomedical technology, due to progress in recent years in both the large-scale synthesis and complex surface functionalization of such particles.[29,34] These next-generation nanomaterials are used in major areas of biological research and medicine, including drug/gene therapy, magnetic targeting (*e.g.*, cancer therapies), and diagnostic imaging (as contrast enhancers).[35−40] The particles consist of a superparamagnetic core, within a biocompatible surface coating, allowing attachment of specific functional groups to the surface coating (Figure 1). MNP size and surface chemistry can be adapted to suit the "functionalizing" molecule, and a tailored combination of surface functionalizations can potentially be achieved to construct complex particles that can mediate multiple applications (*e.g.*, real-time imaging of neural transplant populations plus drug/gene therapy) while retaining nanoscale dimensions.[34,40,41] For example, MNP platforms have been shown to have key applications for noninvasive MR imaging of a range of transplant cells, notably, for tracking of myelin-generating cell populations: MNP-labeled primary Schwann and olfactory ensheathing cells (OECs),[42] as well as CG4 cells

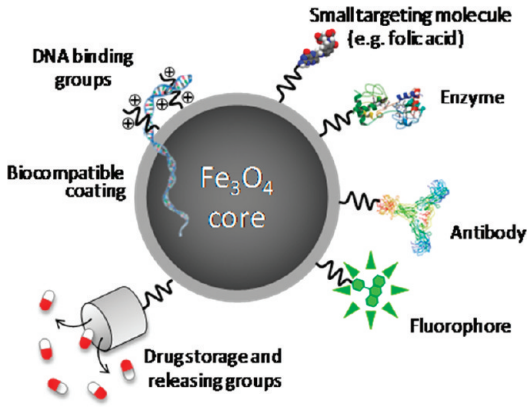

Figure 1. Schematic diagram depicting the multilayered design of magnetic nanoparticles, illustrating the magnetic iron oxide (magnetite) core and biocompatible coating, with examples of potential functionalization for biomedical applications.

(an OPC cell line),[43] could all be detected by MRI following transplantation into areas of brain and spinal cord pathology, without adverse effects on transplant cell migration, cell fate, or repair potential.

In terms of MNP-based transfection, a key feature of MNP vectors is their compatibility with novel magnetofection techniques, *viz.*, the application of magnetic fields to assist gene transfer.[44−47] These methods have the major safety advantage that they exploit the natural uptake pathways (endocytotic mechanisms) of cells during the transfection process, without disrupting the cell membrane, resulting in high cell viabilities post-transfection.[48−52] We have recently demonstrated the significant potential of MNPs to mediate gene transfer to key neural transplant cell populations [such as astrocytes[53] and neural stem cells (NSCs)].[54] We proved that transfection efficacies could be dramatically improved using magnetofection approaches, the latter including use of an oscillating array system utilizing high-gradient NdFeB magnets applied at a range of frequencies.[53] To date, however, the use of MNPs to mediate safe gene transfer to the major transplant population of OPCs has not been assessed. The current study aims to address this issue by employing OPCs derived from primary rat mixed glial cell cultures to assess (i) whether OPCs can be transfected using MNP vectors, (ii) if novel static and oscillating field magnetofection techniques can enhance transfection efficiencies, (iii) whether these transfection protocols affect the survival or differentiation potential of OPCs, and (iv) the transplantation potential of MNP-transfected OPCs using organotypic cerebellar slice cultures as host tissue.

RESULTS AND DISCUSSION

Cell Cultures. These experiments were performed using untransformed OPCs, derived from primary rat mixed glial cultures, to eliminate the potential problems commonly encountered with the use of cell lines, which include transformation-induced abnormalities

in cell physiology, microbial contamination, karyotyping necessitated by the risk of cellular aneuploidy, and genetic instability. Highly pure cultures of OPCs were consistently obtained, as judged by cellular morphology and A2B5 (OPC marker) staining (94.3 ± 2.3%; $n = 5$). Light microscopic observations showed that cells displayed an elongated bipolar morphology with oval cell bodies characteristic of OPCs.

Choice of Particles and Particle Concentrations. Neuromag particles are transfection-grade MNPs that were originally designed for use with primary neurons and in which they have a history of "safe" usage. We have utilized these particles to demonstrate MNP-mediated transfection and the efficacy of magnetofection approaches, in a range of neural cell types [astrocytes,[53] NSCs,[54] OECs, and oligodendrocytes (unpublished data)], making them the nanoparticle of choice in our laboratory. In terms of incubation times required to achieve optimal transfection, removal of particle–plasmid complexes after 1 h incubation (as per the protocol developed by ourselves for transfection of astrocytes)[53] resulted in negligible transfection (typically <1%) over a 48 h monitoring period, for all field conditions. By contrast, increasing the length of OPC exposure to complexes to 48 h resulted in a dramatic increase in reporter protein expression over a similar monitoring period. This exposure time was not associated with toxicity, and qualitative microscopic observations on the extent of green fluorescent protein (GFP) expression in OPCs over a three-week observation period revealed that protein expression peaks at 48 h, consistent with the timing of peak expression reported earlier for plasmid-based transfection.[19,53] Therefore, in all experiments, toxicity and transfection efficiency were routinely assessed by fluorescence microscopy at 48 h.

Establishment of Safe Particle–Plasmid Concentrations and Field Conditions. As cell survival and phenotypic stability are key considerations in cell replacement strategies, a battery of assays was used to assess the toxicity of Neuromag–*gfp* (pmaxGFP plasmid) complexes and magnetofection techniques on OPCs. At every concentration and applied magnetic field condition, plasmid alone controls were found to be without any effect on OPC morphology or survival and never resulted in GFP expression (Figure 2A). The particle concentration routinely used to safely transfect neurons (1.0×) as recommended by the manufacturer resulted in the obvious presence of rounded, detaching cell profiles and dramatic loss of OPCs. Therefore, lower concentrations (0.5× and 0.1×) of Neuromag–*gfp* were evaluated; irrespective of the applied magnetic field, treatment with 0.5× Neuromag–*gfp* was also found to result in a decrease in cell number per field, the presence of nonadherent cells, and an increase in pyknotic nuclei (Figure 2A and B). In contrast, 0.1× Neuromag–*gfp* had no statistically significant effects on any of these parameters under all magnetic field conditions used (Figures 2A). It was deemed unnecessary to test for

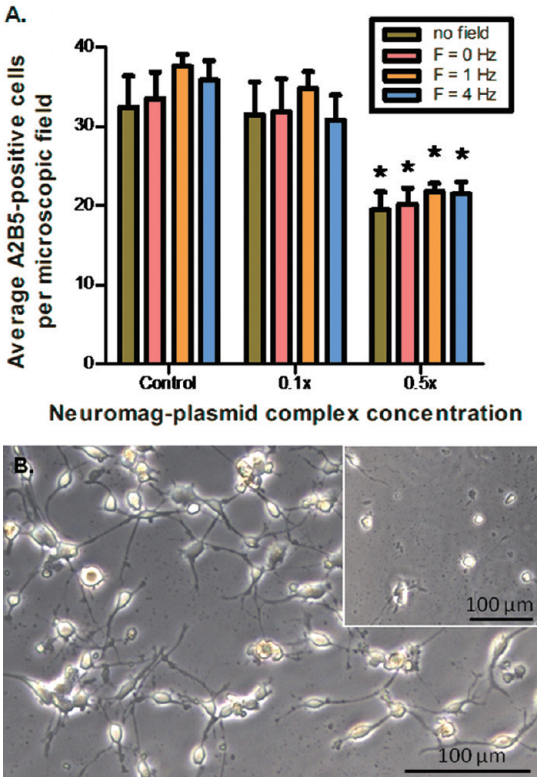

Figure 2. Establishment of safe particle dose and magnetic field conditions for OPC magnetofection. (A) Bar chart showing average number of cells stained for the OPC marker A2B5 per microscopic field, 48 h post-transfection with Neuromag–plasmid complexes. For all magnetic field conditions tested and the no field condition, the number of A2B5$^+$ cells per field was markedly reduced at 0.5× complex concentration, but not at 0.1×, compared to control cultures exposed to plasmid alone. *$p < 0.05$ *versus* control, no field; $n = 5$ cultures. (B) Representative phase-contrast micrograph of OPCs, 48 h post-transfection with 0.1× Neuromag–plasmid complexes. Note phase-bright cells with mostly bipolar morphologies. Inset shows OPCs 48 h post-transfection with 0.5× Neuromag–plasmid complexes. Note reduced density of cells and lack of processes.

toxicity of the particles alone, as Neuromag MNPs have been specifically developed as transfection grade particles and, as such, have no intended usage without conjugation of nucleic acids. However, in pilot experiments, 0.1× Neuromag particles alone were not found to be associated with any adverse effects on OPC viability or proliferative/differentiation potential. Additionally, OPCs could be incubated with 0.1× Neuromag–*gfp* complexes for up to 48 h without induction of significant toxic effects, and no alterations were observed in the morphology or A2B5 staining profiles of OPCs at this concentration. This was therefore deemed the "safe" dose for transfection applications.

These data highlight the need for rigorous dose-optimization prior to MNP use. When considering the development of optimal magnetofection methodologies for cells of neural origin, it is of note that experimental protocols required to prevent toxicity vary between individual neural cell types. This underscores

the critical importance of tailoring particle dosing regimens on a cell-by-cell basis with respect to (i) particle concentration and (ii) incubation time, in order to develop safe protocols for biomedical applications. First, the particle concentration routinely used to safely transfect neurons is toxic not only to OPCs, as shown here, but also to astrocytes[53] and NSCs,[54,55] necessitating the use of lower MNP concentrations for these non-neuronal cell types. Second, at a given particle concentration, the duration of particle exposure that can be considered "safe" also varies between cell types. For example, in a previous study, it was necessary to limit astrocyte incubation time with 0.1× Neuromag−*gfp* complexes to 1 h to prevent toxicity,[53] whereas in the present study, OPCs could be safely incubated with the same particle−plasmid complex concentration for a prolonged period of time (48 h).

It is not clear what specific cellular mechanisms account for these intercellular differences. As uptake of MNPs is known to occur *via* endocytotic mechanisms such as macropinocytosis and receptor-mediated uptake (for example clathrin- and caveolin-mediated), we can speculate that differences in the mechanisms and/or activity of endocytosis between astrocytes and OPCs account for differences in the extent of particle uptake and toxicity.[48,50,52] This may in turn be related to the specific neurophysiological functions performed by the cell type in question (*e.g.*, homeostasis, immune surveillance).

Assessment of Magnetofection Techniques for Transfection of OPCs. *OPC Transfection Studies and Effects of Applied Magnetic Fields (Magnetofection).* Transfection efficacy was assessed at 0.1× Neuromag−*gfp* concentrations, with and without application of static and oscillating magnetic fields. Basal GFP expression was observed, *i.e.*, in the absence of a magnetic field (mean transfection efficiency: 6.1 ± 1.0%; range: 3.1−8.1%; Figure 3A). All magnetofection conditions produced transfection efficiencies significantly higher than basal levels (Figure 3A). Specifically, the application of a static magnetic field resulted in a transfection efficiency that was approximately 2-fold that in the absence of a field (F = 0 Hz: 12.5 ± 1.2%; range 10.8−17.0%). The oscillating magnetic field conditions resulted in transfection efficiencies that were approximately 2.5-fold (F = 1 Hz: 15.5 ± 1.9%; range 9.3−19.9%) and 3.5-fold (F = 4 Hz: 20.6 ± 2.2%; range 15.9−26.3%) that in the absence of a field. Importantly, the 4 Hz oscillating magnetic field condition produced significantly greater transfection efficiency than the static magnetic field condition (Figure 3A). However, no significant difference was found between the 1 Hz oscillating field condition and either the static or 4 Hz oscillating field conditions. In all experiments, transfected OPCs were observed to be phase bright and exhibited normal cell adherence. Cells expressed GFP throughout the cell body and processes (Figure 3B). Transfected cells retained their characteristic bipolar phenotypes and exhibited normal staining profiles for the OPC marker A2B5, and DAPI-stained nuclei appeared healthy with no evidence of increased chromatin condensation or pyknosis (Figure 3C).

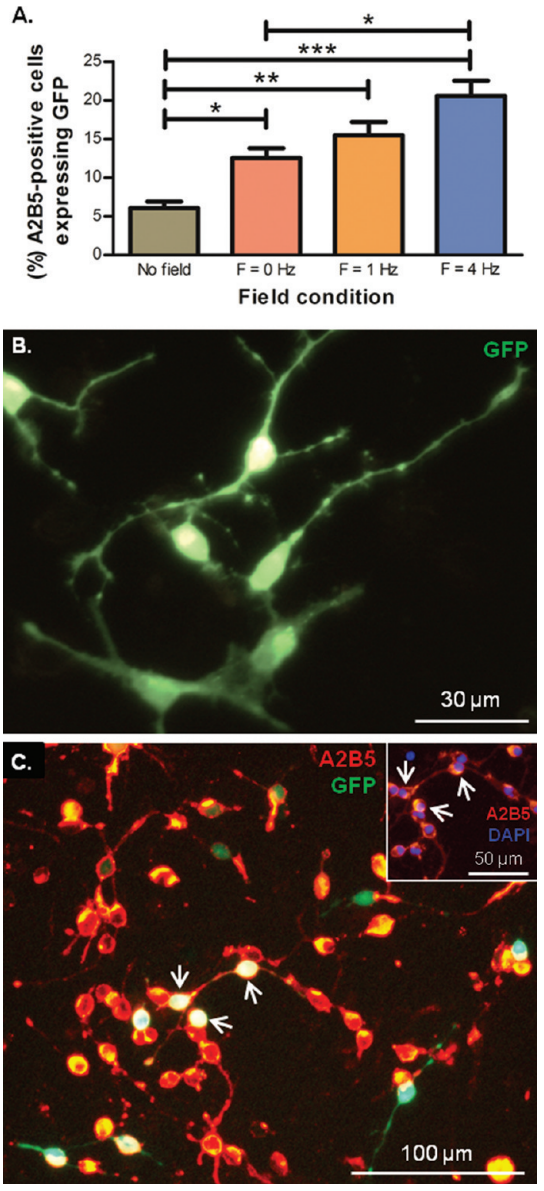

Figure 3. Application of static or oscillating magnetic arrays (magnetofection) enhances MNP-based OPC transfection without affecting cellular morphology or staining profiles. (A) Bar chart showing transfection efficiencies achieved in OPCs 48 h post-transfection using Neuromag−*gfp* complexes (0.1× complex concentration), as judged by the percentage of A2B5$^+$ cells expressing GFP (A2B5 is an OPC marker). Static and oscillating magnetic field conditions significantly enhanced transfection efficiency compared to the basal level (no field). Importantly, application of the 4 Hz oscillating magnetic field significantly enhanced transfection efficiency compared to the static magnetic field condition. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; $n = 5$ cultures. (B) Representative image of transfected OPCs with bipolar morphologies, expressing GFP throughout the cell body and processes. (C) Representative image of transfected OPCs. Note co-localization of GFP expression with A2B5 staining, and (inset) morphologically normal DAPI-stained nuclei. Arrows indicate same transfected cells in main image and inset.

TABLE 1. Comparative Transfection Efficiencies in Rat OPCs for Viral and Nonviral Vectors[a]

transfection method	gene	transfection efficiency	viability	ref
Viral Methods				
retrovirus (MoMuLV)	β-Gal	0.005−0.5%	not reported	56
retrovirus (LZRS)	D15A/EGFP	60%	not reported	14
retrovirus (LZRS)	CNTF/EGFP	60−70%; adult OPCs	not reported	15
adenovirus (AdLacZ)	β-Gal	>50%; did not distinguish OPCs and astrocytes	not reported	38
Nonviral Methods				
magnetic nanoparticles	GFP	21%	no significant toxicity	current study
lipofection	β-Gal	1−3%	not reported; toxic at even low doses (<2 μg)	30
calcium phosphate precipitation	β-Gal	3−5%	~10%	56
calcium phosphate precipitation	β-Gal	<2%	not reported; induced morphological changes	30
electroporation	EGFP	~43%	60%	57[b]
electroporation	β-Gal	10−15%	20−25%	30

[a] β-Gal = β-galactosidase; D15A = multineurotrophin, with brain-derived neurotrophic factor (BDNF) and neurotrophin 3 (NT3) activity; EGFP = enhanced green fluorescent protein; CNTF = ciliary neurotrophic factor; GFP = green fluorescent protein. [b] Company Web site.

A range of nonviral methods have been tested previously for OPC transfection (Table 1). Our data show that magnetofection strategies compare favorably with such methods, in terms of both transfection efficiency and cell viability. There is considerable variability in transfection efficiencies reported previously for OPCs, and all nonviral alternatives have resulted in reduced cell viability. For example, calcium phosphate-based transfection of OPCs produced <5% transfection efficiency and resulted in abnormal morphological changes in cells.[56] The lipofection agents Lipofectamine, Lipofectin, and Cellfectin were found to be toxic to OPCs, even at low concentrations, and resulted in <3% of cells being transfected,[30] while electroporation has been reported to result in efficiencies ranging from 10% to 15% (20−25% cell viability)[30] to approximately 43% (60% viability).[57] In terms of virus-mediated gene delivery, although retroviral transduction has been reported to yield a gene transfer efficiency of up to 70% in OPCs,[15] results can vary greatly, with another study reporting only 0.005−0.5% transfection using retroviral methods[56] (OPC viability not reported in either study). Irrespective of the efficiencies of other techniques, it must be stressed that MNPs offer a combination of advantages for neural cell transplantation therapies (compared with existing vector platforms), owing to their unique multifunctionality for "theragnostic" applications, *i.e.*, the fusion of therapeutic approaches, such as gene/drug delivery, with imaging methods such as MRI.[29,34−36,38,41]

In keeping with our previous findings,[53,54] the efficacy of oscillating fields was found to be frequency-dependent (optimal frequency = 4 Hz for OPCs). We have described previously the efficacy of magnetofection strategies in NSCs[54] and astrocytes[53] and discussed the basis for their effects; this finding is in contrast to the optimal oscillation frequency for astrocyte transfection (F = 1 Hz) but identical to that for NSC monolayers. It is not clear what mechanisms are responsible for the cell-to-cell variation in optimal transfection-promoting frequencies. The oscillating movements may serve to reduce biases in magnetic field strength across the cellular monolayer, thereby improving cellular access to particles and hence transfection levels. The lateral motion imparted by oscillating magnetic fields may also increase the likelihood of particle−cell contact and stimulate endocytosis; we cannot rule out the induction of pro-transfection uptake mechanisms such as macropinocytosis by the oscillating fields.[38,53,59] If this is the case, differences in cell size/plating density and extent of membrane elaboration by cells may account for such frequency-dependent effects. Combined with the significant variations in the timing of particle exposure and extent of transfection between cells, these results highlight further the need to tailor MNP transfection protocols for individual cell types, as protocols for one neural cell type cannot be extrapolated to another. Further analyses using blockers of specific endocytotic pathways and detailed TEM analyses to systematically compare MNP uptake are necessary to establish the basis of intercellular variability in the efficacy of magnetofection methods.

Effects of Magnetofection on OPC Fate (Proliferation and Differentiation). MNP-mediated transfection did not alter the proliferative capacities of OPCs, as comparative counts of OPC densities per field between cultures treated with particle−plasmid complexes and plasmid only controls revealed no significant differences at 48 h (Figure 2A). Additionally, transfected progeny (immature oligodendrocytes) with daughter nuclei in close apposition and therefore appearing to result from recent OPC divisions could be observed (Figure 4A), supporting the finding that MNP-based transfection does not alter the proliferative potential of OPCs. Furthermore, transfected OPCs differentiated into GFP^+/MBP^+ oligodendrocytes (myelin basic protein, MBP, is a late-stage oligodendrocyte marker) with mature, highly branched morphologies, comparable to that observed in control cultures (Figure 4B). Counts of MBP^+ cells from untreated cultures *versus* MNP transfected

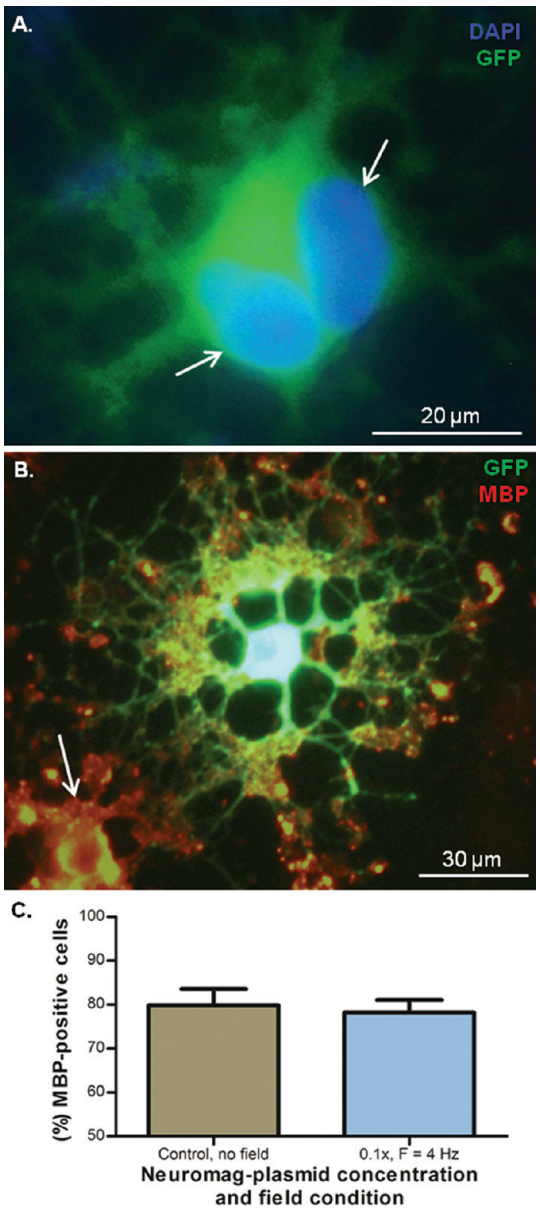

Figure 4. Transfection of OPCs using magnetofection does not affect proliferation or differentiation potential. (A) Following culture in Sato differentiation medium, transfected OPCs developed multipolar morphologies typical of early-stage oligodendrocytes. Arrows indicate two nuclei, suggestive of a recent cell division, and therefore normal proliferative activity. (B) Following culture in Sato differentiation medium, transfected OPCs developed morphologies typical of mature oligodendrocytes, expressed GFP throughout their processes, and stained for myelin basic protein (a late-stage oligodendrocyte marker). Arrow indicates MBP^+/GFP^- cell. (C) Bar chart showing percentage of MBP^+ cells, 12 days post-transfection. The percentage of MBP^+ cells in transfected cultures (0.1× complex concentration; $F = 4$ Hz) was not significantly different from controls (without complexes or magnetic field). $n = 4$ cultures.

cultures ($F = 4$ Hz) (cultured in differentiation-promoting medium) revealed no significant difference in the percentage of MBP^+ cells at 12 days post-transfection (Figure 4C). Transfected OPCs and their progeny were observed to display the full range of morphological

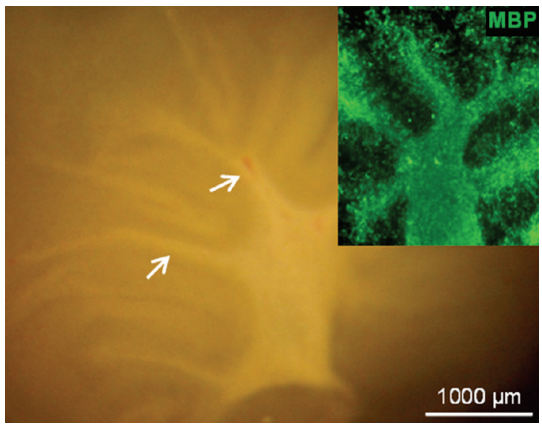

Figure 5. Cerebellar slice cultures (10 days in culture) retain structural and anatomical organization. (A) Representative image of slice culture, with clearly visible white matter tracts (arrows) branching from the base. Inset illustrates that these white matter tracts are distinctly revealed by MBP staining, a marker for mature oligodendrocytes.

phenotypes observed as standard within differentiating cultures of the oligodendroglial lineage, from bipolar unbranched cells to multipolar, highly branched, complex forms with extensive membrane elaboration, lending further support to the finding that the differentiation potential of OPCs is unaffected by the transfection protocols used in this study. Longer term monitoring of cultures revealed that expression of GFP persisted within oligodendrocytes for up to 22 days (the latest time point observed). However levels of expression declined with time, and at 22 days approximately 5% of peak levels of transfection were observed. Such transient expression is especially desirable in the context of myelin repair, where regeneration-promoting molecules are expressed in a temporally specific pattern, such that each stage of the repair process occurs in the appropriate sequence; maintaining pro-proliferation or pro-differentiation environments for inappropriate periods has been suggested to be detrimental to repair.[6,7,17,60–62] For example, platelet-derived growth factor (PDGF-AA) and FGF2 induce proliferation and migration of OPCs, but also inhibit late-stage oligodendrocyte differentiation, and so their upregulation should not be prolonged beyond the "recruitment" phase of repair.[18,63]

Evaluation of Transplantation Potential of Transfected OPCs. *Organotypic Cerebellar Slice Cultures As Host Tissue.* The study has employed organotypic models for the introduction and subsequent monitoring of OPC transplant cells. Such three-dimensional, *in vitro* models are widely used in experimental neurology research, providing an excellent bridge between isolated cell cultures (which lack tissue organization and environmental cues that can influence transplant cell behavior) and *in vivo* investigations that are typically expensive, time-consuming, and difficult to monitor in real time.[64–67] Slice cultures demonstrated high viability, as judged by

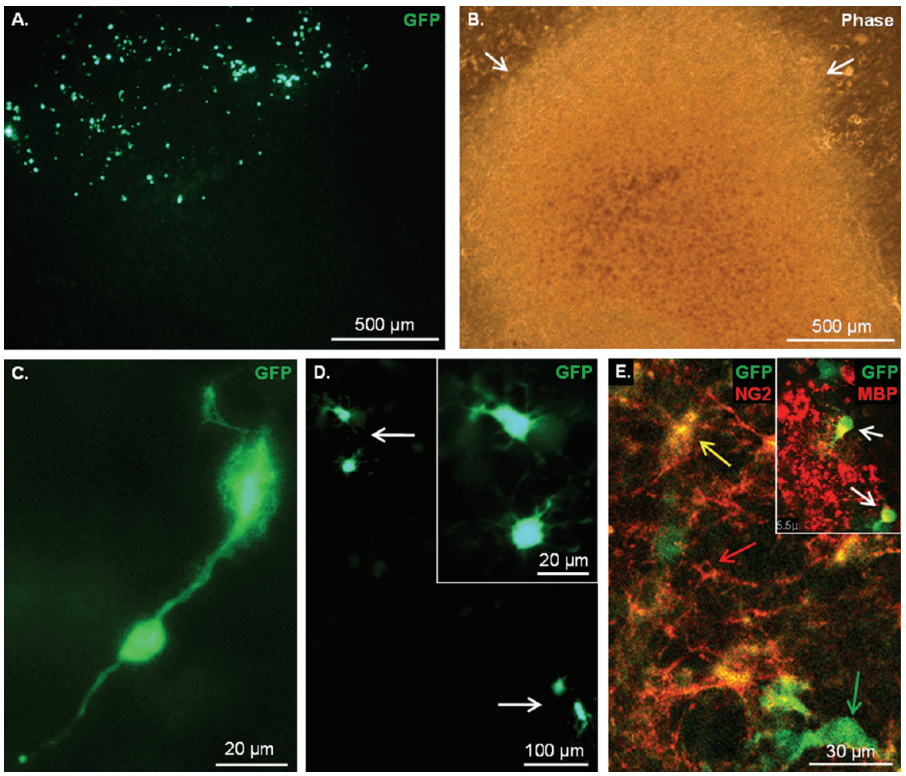

Figure 6. Transfected OPCs survive, migrate, proliferate, and differentiate following transplantation into organotypic slice cultures. (A) Transfected OPCs could be focally delivered to slices, as evidenced by fluorescence microscopy immediately post-transplantation. GFP$^+$ transplanted cells exhibited the rounded morphology typical of enzymatically detached cells. (B) Counterpart phase image to (A), illustrating slice margin (arrows). Post-transplantation, GFP$^+$ cells were observed with bipolar morphologies typical of migrating OPCs (24 h; C), in pairs, suggestive of proliferation (48 h; D), and with multipolar morphologies, indicative of normal differentiation (D, inset). (E) When slices were stained for the OPC marker NG2, 48 h post-transplantation, confocal microscopy revealed GFP$^+$/NG2$^+$ transplanted OPCs (yellow arrow) against a background of red NG2$^+$ host OPCs (red arrow). Some GFP$^+$ cells were NG2$^-$ (green arrow). Inset shows confocal image of GFP$^+$ cells stained for the oligodendrocyte marker MBP 48 h post-transplantation (arrows), indicating OPC differentiation within the slice.

live/dead staining (Viability kit, Invitrogen, UK; data not shown). Well-preserved white matter (WM) tracts could be clearly observed using phase contrast microscopy (Figure 5). This was confirmed by fluorescence microscopy following MBP immunostaining (Figure 5, inset), and WM tracts showed similar anatomical organization to that found *in vivo*.[66]

OPC Transplantation onto Cerebellar Slices. Transplants were performed with 15 slices in total, from four animals. Slices imaged immediately post-transplantation showed that focal delivery of OPCs could be achieved. Delivery of the cells was by simply pipetting onto the slices, rather than by invasive injection. From an experimental perspective, this approach has the major advantage of greatly reduced trauma to both cells and slice host tissue. The transplantation procedure produced passive spread with a radius of 700 μm (Figure 6A and B), due to the force of the transplantation procedure alone. Immediately after transplantation, OPCs were clearly observed to express GFP and displayed rounded morphologies characteristic of enzymatically detached cells (Figure 6A). Post-transplantation, cells were observed to recover their characteristic cellular morphologies. At 24–48 h post-transplantation, GFP$^+$ cells showed evidence of (1) migration, as indicated by the presence of cells with elongated bipolar morphologies that are characteristic of migratory OPCs (Figure 6C), and (2) proliferation, indicated by the striking occurrence of GFP$^+$ cells in pairs (Figure 6D). Slices were stained for either NG2, an OPC marker, or MBP, a late-stage oligodendrocyte marker, to establish the cellular identity of transplant cells. Confocal microscopy revealed the presence of GFP$^+$/NG2$^+$ cells against a background of host NG2$^+$ cells, confirming the OPC identity of the transplanted cells (Figure 6E). There was also clear evidence of OPC differentiation in slices, as demonstrated by the presence of multipolar cells (Figure 6D, inset), and GFP$^+$/MBP$^+$ cells, detected in slices using confocal microscopy (Figure 6E, inset). Transplant populations replated into chamber slides with Sato differentiation medium also retained GFP expression and exhibited normal oligodendroglial morphologies, supporting the observations that MNP transfection protocols do not alter the differentiation capabilities of the transfected, transplanted cells.

These results demonstrate the transplantation potential of MNP-transfected OPCs. They further prove

that cellular properties of OPCs that are key to their regenerative potential, such as the ability to divide, migrate, and give rise to myelin-generating cells, are unimpaired following the transfection protocols. These findings emphasize the safety of the protocols developed and suggest that the MNP platform has considerable potential for clinical translational OPC applications involving *ex vivo* CNS gene transfer. In terms of the transplantation methods used here, tissue engineering approaches using brain and spinal cord slice cultures to evaluate cell replacement therapies represent a key recent advance in the reduction, replacement, and refinement (3Rs) of experimental animal usage in neurological research. We have extensively documented the suitability of organotypic model systems for assessing the transplantation potential of key neural cells, including NSCs and astrocytes, using high-throughput analyses.[50,53,55] Our current data lend support to our previous work and further demonstrate that the slice model can be successfully used to monitor the survival and fate of transplanted OPC populations. It is important to note that cerebellar slice models offer unique advantages as host tissue within which to evaluate OPC transplantation approaches, as these retain large, well-defined, axonal (WM) tracts that undergo myelination *in vitro*.[66,67] Additionally, slices can be prepared from dysmyelinating mutants (including those with limited postnatal survival), and focal/disseminated demyelinating lesions can be induced in normal slice WM tracts, highlighting the translational potential of cerebellar slices for evaluating the regeneration-promoting effects of OPC transplantation therapies.[66−68]

MNP-Mediated Delivery of Plasmid Encoding Therapeutic Growth Factor. The preceding experiments were conducted with the pmaxGFP plasmid, which encodes a reporter gene only. To assess the potential of the optimized magnetofection protocol for functional gene delivery, OPC cultures ($n = 4$) were transfected using MNPs conjugated with a plasmid encoding the therapeutic protein FGF2 tagged with GFP (FGF2-GFP; 7.4 kb); FGF2 is a major growth factor that promotes neural regeneration due to its mitogenic properties (including for OPCs) and angiogenesis-promoting effects. A recommended plasmid (AN-GFP; 6.6 kb) lacking the FGF2 insert was used as a control. It should be noted that both these plasmids are considerably larger than the pmaxGFP plasmid (3.5 kb) used in earlier experiments. Magnetofection of OPCs with these plasmids, using the optimized protocol (oscillating magnetic field of $F = 4$ Hz), revealed cellular GFP expression using both the therapeutic and control plasmids (Figure 7A−C). For the FGF2-encoding plasmid, GFP expression was largely restricted to the nucleus (Figure 7A and B). These findings are consistent with several reports, demonstrating endogenous expression of nuclear isoforms of FGF2 in glia, notably astrocytes.[69,70] Occasional rounded profiles consistent with dividing OPCs could be observed

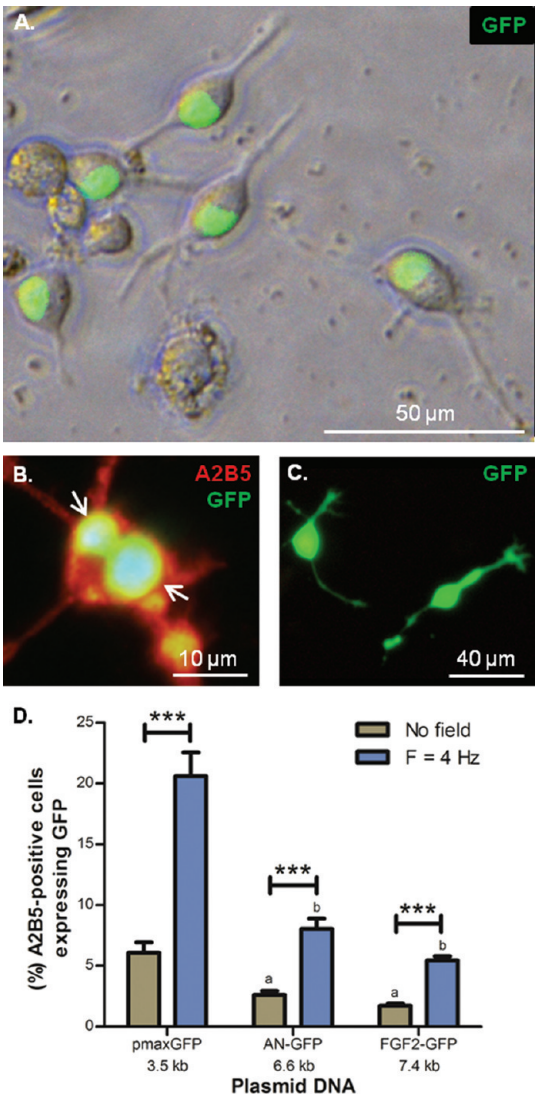

Figure 7. MNP-mediated delivery of therapeutic genes to OPCs. (A) Representative phase image of live cells transfected using the FGF2-GFP plasmid, which encodes fibroblast growth factor 2 (FGF2) tagged with green fluorescent protein (GFP) at 24 h post-transfection. Transfected cells exhibit bipolar morphologies typical of OPCs with GFP expression prominently localized to the nucleus. (B) Fluorescence image of a proliferating OPC transfected with the FGF2-GFP plasmid and stained for the OPC marker A2B5; both daughter cells appear to express GFP (arrows indicate daughter nuclei). (C) Representative fluorescence image of cells transfected using the AN-GFP control plasmid. Note that GFP expression is present throughout the cell body and processes. (D) Bar chart illustrating transfection efficiencies achieved in OPCs using Neuromag (0.1×) conjugated to various plasmids, as judged by percentage of $A2B5^+$ cells expressing GFP. Data are shown for transfection without a magnetic field and with applied oscillating field ($F = 4$ Hz). For each plasmid, the 4 Hz oscillating field significantly enhances transfection efficiency over the no field condition (*$p < 0.001$). Transfection efficiency for both the AN-GFP and FGF2-GFP plasmids is significantly lower compared to pmaxGFP, for the corresponding field conditions ($^a p < 0.001$ compared to pmaxGFP no field condition; $^b p < 0.001$ compared to pmaxGFP $F = 4$ Hz). $n = 5$ cultures for pmaxGFP studies; $n = 4$ cultures for AN-GFP and FGF2-GFP studies. Data in B−D were at 48 h post-transfection.**

with both daughter cells appearing to inherit the introduced gene (Figure 7B). For the control plasmid, the pattern of GFP expression (Figure 7C) was similar to that seen in earlier experiments with the pmaxGFP plasmid (Figure 3B and C), *i.e.*, with GFP expression evident throughout the cell body and extending into the processes of cells. In immunostaining studies, all transfected cells were found to clearly express the OPC-specific marker A2B5 (for example see Figure 7B). Determination of transfection efficiencies yielded values of $5.5 \pm 0.4\%$ for the FGF2-GFP plasmid and $8.1 \pm 1.0\%$ for the AN-GFP control plasmid with applied oscillating magnetic fields, both of which were significantly higher than the corresponding values determined in the absence of a magnetic field (Figure 7D).

It is clear from these data that use of larger plasmids results in a systematic decrease in MNP-mediated transfection efficacy. This strongly indicates that experimental protocols will need to be modified to account for variations in plasmid size. This issue could be addressed using a range of strategies, including (i) reduction of overall plasmid size by removal of the reporter protein component and other elements of the constructs; (ii) use of particles with different polymer coatings/sizes to further enhance the DNA binding capacity of the particles; and (iii) testing of different oscillation frequencies to determine if the optimal frequency differs with plasmid size, with additional optimization of the duration of field application.

In terms of the translational applications of our findings to clinical cell transplantation therapies, a number of studies have demonstrated that repair mechanisms can be enhanced by genetic manipulation of OPC transplant populations.[14,15,18,19] The optimal transfection efficiency for neural repair following *ex vivo* gene delivery will ultimately need to be determined by evaluation of functional recovery post-transplantation of transfected OPCs in a range of experimental pathologies; such effects can be predicted to be dependent on the nature of the pathology, the functional gene under study, and extent of the neural injury. Cao *et al.* (2010) retrovirally transfected adult rat primary OPCs (transfection efficacy approximately 70%) with CNTF, then transplanted them into adult spinal cord, 9 days post-contusion.[15] At 7 weeks post-transplantation, rats that received transplants of CNTF-OPCs demonstrated 4-fold greater survival of transplanted OPCs, a greater number of myelinated axons, and a greater level of functional recovery, compared to rats that received transplants of unmodified OPCs. Previous studies suggest that transfection of 20–50% of a neural progenitor transplant population can help to promote effective functional recovery (*via* angiogenesis and neuroprotection, for example).[22,23] In addition, the unmodified OPC transplant populations *per se* have an intrinsic repair capability;[9,13] therefore our results indicate that the OPC transfection levels obtained here (with protocol optimization to account for plasmid size variations as described above) may be sufficient to promote neural repair; this will need to be tested using functional genes in neural injury models. Further work is being carried out to measure the migratory capacity and long-term integration of transfected OPCs following transplantation into slice cultures.

In summary, MNPs can be used to transfect OPCs, with transfection efficiency enhanced by application of a static/oscillating magnetic field. Transfected OPCs can be successfully transplanted into cerebellar tissue slices, where they survive, migrate, proliferate, and differentiate into oligodendrocytes, highlighting the translational potential of the MNP platform for OPC transplantation therapies.

EXPERIMENTAL SECTION

Reagents and Equipment. Tissue culture-grade plastics, media, and media supplements were from Fisher Scientific (Loughborough, UK) and Sigma-Aldrich (Poole, UK). Recombinant human platelet-derived growth factor (PDGF-AA) and basic fibroblast growth factor (FGF2) were from Peprotech (London, UK). Polyclonal rabbit anti-NG2, Omnipore membrane (JHWP01300), and Millicell culture inserts were obtained from Millipore (Watford, UK). Monoclonal rat antimyelin basic protein was from Serotech (Kidlington, UK), monoclonal mouse anti-A2B5 was from Sigma-Aldrich (Poole, UK), and secondary antibodies (Cy3-conjugated) were from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA). Mounting medium with DAPI (4′,6-diamidino-2-phenylindole) was from Vector Laboratories (Peterborough, UK). The pmaxGFP plasmid (3.5 kb) was from Amaxa Biosciences (Cologne, Germany), and both the pCMV6-FGF2-GFP plasmid (encodes the open reading frame of human FGF2 with a carboxy-terminal turboGFP tag; 7.4 kb, FGF2-GFP) and the recommended control plasmid, pCMV6-AN-GFP (6.6 kb, AN-GFP), were from OriGene Technologies (Rockville, MD, USA).

Neuromag transfection-grade MNPs were purchased from Oz Biosciences (Marseille, France). These positively charged particles have an average diameter of 160 nm (range 140–200 nm). DNA binding curves showed that these particles bind DNA effectively with DNA binding increasing particle size by 35%, assessed using a Malvern Zetasizer 3000. The MAGNEFECT-NANO oscillating magnetic array system was from NanoTherics Ltd. (Stoke-on-Trent, UK). This system allows 24-well plates to be placed over a horizontal array of 24 neodymium (NdFeB) magnets, grade N42, which match the plate configuration. The magnetic array can be programmed to oscillate laterally beneath the culture plate *via* a computerized motor; both the frequency and the amplitude of oscillation can be varied. The field strength at the face of each magnet is 421 ± 20 mT (NanoTherics Ltd., personal commun.).

Cell and Tissue Culture Procedures. The care and use of animals was in accordance with the Animals (Scientific Procedures) Act of 1986 (United Kingdom) with approval by the local ethics committee.

OPC Cell Cultures. Primary mixed glial cell cultures were first prepared from cerebral cortex of Sprague–Dawley rats at postnatal day 1–3 (P1–3), using an established protocol.[71] Medium (D10) was DMEM supplemented with 10% fetal bovine serum, 2 mM glutaMAX-I, 1 mM sodium pyruvate, 50 U/mL penicillin, and 50 μg/mL streptomycin. After 8–10 days culture at 37 °C in 5% CO_2/95% humidified air, flasks were shaken for 2 h on a

rotary shaker at 200 rpm, and the medium, containing mainly microglia, was discarded. Fresh D10 medium was then added, and the flasks were shaken overnight at 200 rpm. This medium, containing mainly OPCs, was transferred to nontissue culture grade Petri dishes, to which microglia (but not OPCs) will readily attach in order to reduce microglial contamination. After 30 min, unattached cells were resuspended at 2×10^5 cells/mL in OPC maintenance medium (OPC-MM, comprising DMEM, 2 mM glutaMAX-I, 1 mM sodium pyruvate, 10 nM biotin, 10 nM hydrocortisone, 30 nM sodium selenite, 50 μg/mL transferrin, 5 μg/mL insulin, 0.1% BSA, 50 U/mL penicillin, 50 μg/mL streptomycin, 10 ng/mL PDGF-AA, 10 ng/mL FGF2). OPCs were plated (0.3 mL/well of a 24-well plate) either on PDL-coated coverslips for assessment of transfection efficiency or on PDL-coated wells for transplantation experiments. This plating density (3×10^4 cells/cm^2) yields optimal cell survival, as lower OPC densities can result in significant cell death, likely due to loss of paracrine effects.

Organotypic Cerebellar Slice Cultures. Brains were extracted from P9−14 rats into ice-cold slicing medium (EBSS buffered with 25 mM HEPES). Cerebella were dissected and meninges removed; then 350 μm parasagittal slices were prepared using a McIlwain tissue chopper. Slices were incubated on ice for 30 min, then transferred to pieces of Omnipore membrane on Millicell culture inserts in six-well plates containing slice culture medium (50% MEM, 25% heat-inactivated horse serum, 25% EBSS, supplemented with 36 mM D-glucose, 50 U/mL penicillin, and 50 μg/mL streptomycin). Slices were incubated at 37 °C in 5% CO_2/95% humidified air for at least 8 days before transplantation, with medium changes every 2−3 days.

Toxicity Experiments. It was necessary to determine the maximum safe dose of Neuromag−plasmid complexes for transfection and to determine whether magnetofection conditions affected toxicity. Neuromag MNPs are recommended for use with neurons at a final concentration of 7 μL/mL in medium (referred to here as 1.0×), but lower concentrations have been required for safe use with other neural cells (astrocytes, NSCs).[53−55] Control cultures were treated with pmaxGFP plasmid alone, without Neuromag. The DNA-binding capacity of Neuromag MNPs is maximal at a ratio of 3.5 μL Neuromag to 1 μg DNA;[53] this ratio was therefore routinely used in the present study. Plasmid-based transfection is often reported to result in peak expression at 48 h,[19,53] and the results of preliminary experiments were consistent with this timing; therefore in all cases transfection efficiency was assessed at 48 h. Accordingly, Neuromag−*gfp* toxicity was assessed by incubating OPCs with various concentrations of complexes (1.0×, 0.5×, and 0.1×), with and without exposure to magnetic fields (no field, static field, 1 Hz oscillating field, and 4 Hz oscillating field). Exposure to a magnetic field was for 30 min, and total incubation time for all conditions was 48 h, at which point cells were fixed. Several parameters of cytotoxicity were assessed by microscopy, specifically (i) whether cells were processed, phase bright, and adherent; (ii) the presence of pyknotic nuclei, as judged by DAPI staining; (iii) expression of the OPC marker A2B5; and (iv) cell counts per microscopic field, which are indicative of proliferative capacity.

Transfection (Magnetofection) Experiments. Transfection protocols were based on those supplied with the Neuromag MNPs; however the specific protocols used in this study were tailored for the OPC densities used (3×10^4 cells/cm^2). In preliminary experiments, Neuromag−*gfp* concentrations at 0.1×, 0.5×, 1.0×, 2.0×, and 4.0× were tested rather than simply adopting the 0.1× dose devised for use with astrocytes as reported by ourselves previously.[53] In terms of DNA quantity, we have previously generated DNA binding curves for Neuromag MNPs to determine the particle:DNA ratio that results in maximal binding;[53] this ratio was maintained constant while varying particle concentrations. The combination of parameters resulting in optimal OPC survival and maximal DNA binding that yielded transfection without cytotoxicity were used in this study. For the transfection step, at 2 h after OPC plating, medium was replaced with 0.225 mL of fresh antibiotic-free OPC-MM. After a further 24−48 h, transfection complexes (per well, at 0.1×) were prepared by mixing 60 ng of pmaxGFP in 75 μL of DMEM base medium, then gently mixing this with 0.21 μL of Neuromag. For control wells, pmaxGFP was mixed in DMEM, but no Neuromag was included. After 20 min incubation, the entire complex mix was added dropwise to cells, while gently swirling the plate. For magnetofection, immediately following application of complexes to cells, the 24-well plate was placed above a 24-magnet array on the MAGNEFECT-NANO device, which had been prewarmed in the incubator. The array either remained static or was programmed to oscillate with an amplitude of 0.2 mm and a frequency (F) of 1 or 4 Hz. After 30 min, the plate was removed from the magnetic array. For the no field condition, plates were returned to the incubator without application of a magnetic field. Cells were incubated with complexes for 48 h in total, then fixed. Experiments to assess the delivery of the plasmid FGF2-GFP (which encodes the functional gene FGF2 tagged with turboGFP) and the appropriate control plasmid AN-GFP (which encodes turboGFP alone) were conducted in an identical manner. Transfections were performed either without application of a magnetic field or with the initial application of an oscillating magnetic field of $F = 4$ Hz.

Differentiation Potential of Transfected OPCs and Long-Term GFP-Expression in Oligodendrocyte Progeny. At 48 h post-transfection, cells were washed with PBS, then switched to Sato medium (DMEM supplemented with 2 mM glutaMAX-I, 1 mM sodium pyruvate, 30 nM triiodothyronine, 30 nM thyroxine, 1% N2 supplement, 50 U/mL penicillin, and 50 μg/mL streptomycin) to induce oligodendrocyte differentiation. Cells were monitored to assess the extent of cellular expression of GFP and to evaluate potential adverse effects on OPC differentiation for up to 3 weeks. Medium was changed every 2−3 days.

Transplantation of Transfected OPCs onto Slice Cultures. At 24 h post-magnetofection ($F = 4$ Hz), OPCs were washed with PBS, then detached using accutase-DNase I. Cells were resuspended at 2×10^7 cells/mL Sato medium, and 0.5 μL was pipetted onto cerebellar slice cultures. Passive spread of cells due to the pipetting procedure was assessed by transferring cerebellar slices to a small quantity of PBS on a microscope slide, then transplanting cells onto these slices and viewing immediately using fluorescence microscopy. At the end of the transplant procedure, remaining cells were also plated at 2×10^5 cells/mL Sato medium directly on PDL-coated chamber slides. Samples were fixed and stained 24−48 h post-transplantation to assess the survival and morphology of the transplanted and replated cells.

Immunocytochemistry and Immunohistochemistry. Cells and slices were washed with PBS, fixed with 4% paraformaldehyde [room temperature (RT); 25 min], then washed again. For staining, samples were incubated with blocking solution (5% serum in PBS, with 0.3% Triton X-100 for NG2 and MBP; RT; 30 min), then with primary antibody in blocking solution (A2B5 1:200, NG2 1:150, MBP 1:200; 4 °C; overnight). The A2B5 antibody recognizes cell surface ganglioside epitopes, while the NG2 antibody recognizes a cell surface chondroitin sulfate proteoglycan; MBP is a major protein constituent of myelin and is used to detect late-stage oligodendrocytes. All the above markers are widely used to label cells of the oligodendrocyte lineage. Samples were then washed with PBS, incubated with blocking solution (RT; 30 min), and then incubated with the appropriate Cy3-conjugated secondary antibody in blocking solution (1:200; RT; 2 h). Samples were then washed with PBS and mounted with DAPI.

Fluorescence Microscopy and Image Analysis. Samples were imaged using fixed exposure settings on an Axio Scope A1 fluorescence microscope (Carl Zeiss MicroImaging GmbH, Goettingen, Germany), and the images merged using Adobe Photoshop CS3 (version 10.0.1). For toxicity and transfection experiments, a minimum of 200 nuclei per treatment per culture were scored for association with A2B5 staining (for culture purity) or for GFP expression (for transfection efficiency). For OPC differentiation experiments, a minimum of 100 nuclei per treatment per culture were scored for MBP expression. Slices were imaged post-transplantation using the AxioScope and, in some experiments, using a BioRad MRC1024 confocal laser scanning microscope.

Statistical Analysis. Data were analyzed using GraphPad Prism statistical analysis software. Data are expressed as mean ± SEM. The number of experiments (n) refers to the number of OPC

cultures used, each from a different rat litter. To determine whether transfection conditions affected toxicity or transfection efficiency, data (the number of $A2B5^+$ cells per field and the percentage of $A2B5^+$ cells expressing GFP, respectively) were analyzed by one-way ANOVA, with Bonferroni's multiple comparison test for *post hoc* analysis. To determine whether transfection conditions affected the differentiation of OPCs into MBP^+ oligodendrocytes, the percentage of MBP^+ cells in cultures without Neuromag and without application of a magnetic field was compared to magnetofected cultures using a two-tailed paired *t*-test. To compare the transfection efficiencies for the pmaxGFP, FGF2-GFP, and AN-GFP plasmids, with and without the application of an oscillating magnetic field, the percentage of $A2B5^+$ cells expressing GFP was analyzed by one-way ANOVA, with Bonferroni's multiple comparison test for *post hoc* analysis.

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