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Part I: Minicircle Vector Technology limits DNA size restrictions on ex vivo gene delivery using nanoparticle vectors: Overcoming a translational barrier in neural stem cell therapy

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ABSTRACT

Genetically engineered neural stem cell (NSC) transplant populations offer key benefits in regenerative neurology, for release of therapeutic biomolecules in ex vivo gene therapy. NSCs are 'hard-to-transfect' but amenable to 'magnetofection'. Despite the high clinical potential of this approach, the low and transient transfection associated with the large size of therapeutic DNA constructs is a critical barrier to translation. We demonstrate for the first time that DNA minicircles (small DNA vectors encoding essential gene expression components but devoid of a bacterial backbone, thereby reducing construct size *versus* conventional plasmids) deployed with magnetofection achieve the highest, safe non-viral DNA transfection levels (up to 54%) reported so far for primary NSCs. Minicircle-functionalized magnetic nanoparticle (MNP)-mediated gene delivery also resulted in sustained gene expression for up to four weeks. All daughter cell types of engineered NSCs (neurons, astrocytes and oligodendrocytes) were transfected (in contrast to conventional plasmids which usually yield transfected astrocytes only), offering advantages for targeted cell engineering. In addition to enhancing MNP functionality as gene delivery vectors, minicircle technology provides key benefits from safety/scale up perspectives. Therefore, we consider the proof-of-concept of fusion of technologies used here offers high potential as a clinically translatable genetic modification strategy for cell therapy.

Keywords: Minicircles, magnetic nanoparticles, genetic engineering, ex vivo gene therapy, neural stem cells; regenerative neurology

1. Introduction

Neural stem cell (NSC) mediated gene therapy holds considerable promise for the treatment of diseases, injuries and malignancies of the central nervous system (CNS). NSCs can function as a cell source for 'restorative cell therapeutics' in sites of neuropathology (given their capacity for self-renewal and multipotentiality) [1,2]. They create an environment permissive for repair by releasing pro-regenerative and immunosuppressive proteins, and these cells have rapidly progressed to clinical trials for several neurological disorders [3–7]. NSCs have key properties making them particularly attractive as vehicles for delivery of therapeutic molecules to neurological injury sites, such as the ability to migrate long distances in nervous tissue. Specifically, they show migration towards pathology sites such as tumours/neurodegeneration (termed 'pathotropism') [8,9]. Intravenously delivered NSCs can cross the blood-brain barrier surrounding neural tissue into pathology foci - a phenomenon modulated by inflammation, astrocytosis and blood vessel formation - minimising adverse effects in surrounding normal host tissue [10]. These cells can be propagated for extended periods in culture offering flexibility for genetic modification techniques. Additionally, they show functional integration into the host microenvironment with little evidence of adverse consequences [10]. Therefore, a widely held belief is that these cells could aid in overcoming a major challenge of CNS gene therapy, namely, how to safely and effectively target therapeutic genes to diseased/injured tissue. This is particularly relevant to complex neurological pathologies wherein multiple therapeutic targets must be addressed to achieve repair (i.e. '*combinatorial therapy*') [11].

Several preclinical models of CNS pathology have demonstrated the utility of genetically engineered NSCs for improving functional neurological outcomes [12–16]. Recent data prove that transplantation of NSCs engineered to release insulin-like growth factor (IGF-1) reduced plaque pathology and enhanced learning, cognitive and memory processes in a mouse model

of Alzheimer's disease (www.neuralstem.com). Additionally, retrovirally-transduced cytosine deaminase-expressing NSCs in combination with leucovorin are currently being tested in human clinical trials for treatment of recurrent high grade gliomas (www.clinicaltrials.gov, Clinical trial ID NCT0117296) having successfully passed a pilot feasibility study. It should be noted here that current genetic engineering approaches for NSCs *almost exclusively rely on viral methods* due to the high transduction efficiencies achieved (up to *ca* 97%) [17]. However, viral gene delivery has significant drawbacks restricting clinical use including immunogenicity, toxicity and technical challenges for large scale production [18–20].

A major non-viral alternative for neural gene transfer that has emerged relatively recently is 'magnetofection' wherein transfection-grade iron oxide magnetic nanoparticles (MNPs) are deployed with magnetic fields (static/oscillating) to assist gene delivery. We recently reported technically simple, rapid and safe magnetofection protocols for genetic modification of major neural transplant types including NSCs, with oscillating magnetic fields always outperforming static fields [21–25]. MNPs have emerged strongly as a class of advanced functional materials for neuro-regeneration, serving as 'multifunctional tools' for cell therapy given additional applications for non-invasive cell imaging and magnetic cell targeting, so it is clear that this non-viral gene transfer method offers significant benefits for clinical translation [26]. Despite the critical advantages offered by this technique, we and others have reported that *DNA plasmid size bears an inverse relationship with transfection efficiency* for non-viral methods such as liposome- and nanoparticle- mediated transfection [23,27–29]. For example, using lipofection, a systematic analysis of luciferase expression levels (using reporter constructs of increasing size; 4.8 kb to 10.5 kb) showed reduced transfection with greater plasmid size [29]. This finding was consistent across various promoters (SV40 and γ-globin gene promoter) and cell lines (Jurkat and 293T) tested [29]. The reasons for this differential effect are largely unknown, but the gross structure of plasmid/lipid complexes was ruled out as similar

physicochemical properties (lipoplex structure and size) were observed irrespective of plasmid size (0.9 kb to 52.5 kb) [29]. Similarly, we reported an inverse relationship between transfection efficiency and plasmid size when using reporter plasmids (3.6 kb, transfection efficiency, *ca*. 32.2%) versus therapeutic constructs encoding basic fibroblast growth factor (7.4 kb, transfection efficiency: 13.5%) [23]. As therapeutic DNA constructs inevitably increase DNA size using conventional plasmids, this reduction in transfection efficiency and the functionality of nanoparticle vectors, is a critical translational barrier for *ex vivo* neural gene therapy.

Offering a potential solution, minicircles (mCs) are emerging as highly promising DNA vectors for therapeutic gene delivery. These are small circular vectors devoid of bacterial backbone sequences e.g. antibiotic resistance genes, origin of replication and other inflammatory sequences, intrinsic to bacterial DNA. These minimal expression cassettes are derived from '*parental*' plasmid (pp) DNA, through site-specific recombination (induced via L-arabinose operon system). Their use results in sustained transgene expression due to lower activation of nuclear transgene silencing mechanisms, and reduced immunogenic responses [30]; the latter resulting in higher safety. The clinical potential of mCs has been exploited for non-viral DNA reprogramming to generate human induced pluripotent stem cells [31]. An analysis of mC DNA transfection in a murine stem cell line CGR8-NS using microporation reported up to 60% transfection with high cell survival (~90%) [32]. However, such cell lines have high survival rates given their resistance to nanotoxicity, and behave in a relatively uniform clonal manner. It is therefore critical to evaluate the efficacy of DNA mC technology in *primary* NSC, as these heterogeneous but hard-to-transfect cell populations are more akin to NSCs *in vivo*, and consequently of higher biomedical relevance. Furthermore, it should also be noted that mC use in conjunction with magnetofection has *never been tested in any cell type of primary origin*. Accordingly, our goal here is to provide the first 'proof-of-concept'

that mC DNA vector functionalized MNPs can be used with oscillating magnetofection technology for efficacious genetic engineering of primary NSCs.

2. Materials and Methods

2.1. Reagents / equipment

Cell culture reagents were from Life technologies (Paisley, Scotland, UK) and Sigma (Polle, Dorset, UK). Human recombinant basic fibroblast growth factor (bFGF) was from Sigma (Poole, Dorset, UK) and human recombinant epidermal growth factor (EGF) from R&D Systems Europe Ltd. (Abingdon, UK). Thermo Scientific Nunc culture dishes (non-treated surface) and tissue culture-grade plastics were from Fisher Scientific UK (Loughborough, UK). NeuroMag magnetic particles were from OZ Biosciences, Marseille, France. The magnefect-nano 24-magnet array system was purchased from nanoTherics Ltd. (Stoke-on-Trent, UK) and comprises horizontal arrays of NdFeB magnets (grade N42) onto which 24-well cell culture plates can be placed. Reagents used for the mC DNA vector technology were from System Biosciences (SBI; Mountain View, CA, USA). All electrophoresis reagents were from Life Technologies (Paisley, Scotland,UK). Primary antibodies were directed to: the NSC markers, nestin (clone 25, BD Biosciences, Oxford, UK) and SOX2 (Millipore, Watford, UK); neuronal marker, class III β-tubulin (clone TUJ1, Covance, Princeton, NJ, USA); the oligodendrocyte marker, myelin basic protein (MBP; clone 12, AbD Serotec, Kidlington, UK); the astrocytic marker glial fibrillary acidic protein (GFAP; DakoCytomation, Ely, UK), proliferation marker Ki-67 (Abcam, Cambridge, UK) and GFP (ThermoFisher Scientific, Rockford, IL,USA). Cy-3 and FITC conjugated secondary antibodies were from Jackson Immunoresearch Laboratories Ltd (Westgrove, PA, USA). Vectashield mounting medium with 4',6-diamidino-2-phenylindole (DAPI, nuclear marker) was from Vector Laboratories (Peterborough, UK).

2.2. NSC culture

The care and use of all animals used in the production of cell cultures were in accordance with the Animals Scientific Procedures Act of 1986 (UK).

NSCs were maintained and expanded under growth factor stimulation as free floating cellular aggregates according to the well characterised 'neurosphere' culture method. Briefly, the subventricular zone of neonatal CD1 mice were dissected, dissociated and NSCs were cultured in neurosphere medium (NS-M) comprising a 3:1 mix of DMEM:F12 containing 2% B-27 supplement, 50 U/ml penicillin, 50 mg/ml streptomycin, 4 ng/ml heparin, 20 ng/ml bFGF and 20 ng/ml EGF. Neurospheres were fed every 2-3 days and passaged every 6-7 days using an Accutase-DNaseI mix (up to passage 2).

2.3. Monolayer cultures

To prepare two-dimensional adherent NSC monolayers, neurospheres (passages 0 – 2) were dissociated enzymatically with accutase-DNase I, triturated several times before resuspension at 1.5 x 10^5 cells/ml monolayer culture medium (ML-M; comprising of a 1:1 mix of DMEM:F12 containing 1% N2 supplement, 50 U/ml penicillin, 50 μg/ml streptomycin, 4 ng/ml heparin, 20 ng/ml FGF2 and 20 ng/ml EGF). The cells were seeded on polyornithine/laminin-coated, nitric acid-washed glass coverslips in 24-well plates (0.6 ml suspension/well) and cultured at 37 ºC in 95% air:5% CO_2.

2.4. NSC differentiation

In order to induce NSC differentiation into the three daughter cell types (neurons, astrocytes, oligodendrocytes), ML-M was replaced with differentiation medium (DF-M) consisting of NS-M (without growth factors) and supplemented with 1% fetal bovine serum. Medium was replaced every 2-3 days.

2.5. Minicircle production

A mC system was employed to address the influence of plasmid size and copy number on the efficacy of MNP-mediated gene transfection. This system comprises a parental plasmid pMC.CMV-GFP-SV40PolyA (herein termed pp-GFP; size 5562 bp) from which recombinant mCs (mC-GFP; size 1552 bp) lacking extraneous bacterial backbone sequences were prepared using a specifically engineered E. coli strain (ZYCY10P3S2T) which was induced to express ϕC31 integrase and SceI endonuclease upon addition of arabinose (0.01% final concentration), as per the manufacturer's instructions and previously described protocols [33]. The integrase splits the full size pp-GFP to (i) the backbone sequence containing SceI sites for targeted endonuclease degradation and (ii) mC DNA containing only the expression cassette which was purified. Thus it is possible to obtain DNA preparations which differ markedly in size but contain identical elements for mammalian gene expression, including the reporter gene per se.

Following mC purification using a Qiagen Endofree Maxiprep Kit, restriction enzyme digests of both pp-GFP and mC-GFP were run on an agarose gel in order to demonstrate the absence of pp-GFP contamination in mC-GFP plasmid stocks. In the presence of pp-GFP contamination, mC-GFP was digested with mC-safe DNase according to the manufacturer's instructions.

2.6. MNP characterization, magnetofection and multifection

The Neuromag particles used here for transfection have been extensively characterized previously [34,35]. The formulation of fluorescent Neuromag particles is proprietary and patented by the company Oz Biosciences. The particle size range reported by the company is 140–200 nm, average 160 nm with homogeneity in particle size (polydispersity index 0.027), and the particles carrying a positive charge (zeta potential +40.3 mV) in distilled water.

Dynamic light scattering (DLS) was used to determine the average hydrodynamic diameter and ζ-potential of the MNPs and MNP-DNA complexes which were suspended in DMEM:F12 base medium within which the transfection complexes were formed prior to addition to cells. This was carried out using a Zetasizer Nano ZS (Malvern Instruments Ltd,UK).

In order to achieve the highest possible transfection level, a range of plasmid copy numbers were assessed. At 24 h post-plating, medium was replaced with fresh ML-M (0.225 ml) before addition of transfection complexes. To prepare the particle-DNA complexes, varying amounts of DNA vectors (see below) were diluted with 75 µl DMEM:F12 (1:1) base medium, added to 0.62 µl Neuromag and mixed and incubated for 20 min at room temperature (RT). DNA input was varied to obtain six different copy numbers of each DNA vector (pp-GFP and mC-GFP) per well: 1.2×10^{10}, 2.3×10^{10}, 4.2×10^{10}, 1.5×10^{11}, 3×10^{11} and 6×10^{11}. The mix was added drop-wise to cells whilst gently swirling the plate. Plates were returned to the incubator, and exposed to a magnetic field using the magnefect-nano oscillating magnetic array system, with a 24-magnet array (NdFeB, grade N42; field strength of 421 ± 20 mT) with an oscillating frequency of 4Hz (amplitude = 0.2 mm). The array moves laterally with oscillation frequency and amplitude controlled via a computerised motor. For single transfection, complexes were left for 48 h until fixation. For double transfection, the exact same protocol was followed, with a 24 h time window between the first and second transfection. The following procedures were carried out 48 h after the first transfection: (i) Cells were fixed in PFA for immunocytochemistry (to assess for transfection efficiency, proliferative capacity and stemness); (ii) The media was switched to DF-M and the cells were allowed to differentiate for three days, one week or four weeks (to assess for long term GFP expression, differentiation capacity and quantify transfected differentiated progeny) before PFA fixation. For long-term cultures, media was replaced every two days. (iii) Live cells were

stained for 15 minutes with propidium iodide (PI, prepared in fresh medium at a final concentration 5μM) prior to fixation (for cell death quantification). (iv) DAPI-stained fixed samples were used to corroborate cell pyknosis (counts of small, fragmented nuclei) and total cell number.

2.7. Assessment of DNA vector binding to MNPs

The nanoparticle/DNA reaction was performed as above, in a smaller volume of 20μl base medium. Samples were centrifuged at 14,000 rpm for 5 minutes to remove particle and particle/DNA complexes. The absorbance of the supernatant which contained the free/unbound DNA was measured at 260nm against a DMEM blank. Absorbance was also measured for the particle-free (with equivalent DNA copy number) sample in order to obtain accurate input DNA measurements. The amount of DNA bound to MNPs was calculated as: % MP - bound DNA = 100 – [(total 'free' DNA in supernatant/ total input DNA) x 100].

2.8. Immunocytochemistry

NSC monolayers and differentiated NSCs were fixed in 4% paraformaldehyde (PFA) for 15 min at RT, followed by three washes in PBS. Non-specific binding was blocked using 5% normal donkey serum in PBS-0.3% Triton-X-100 for 30 min at RT, followed by overnight primary antibody incubation at 4°C. The following antibodies in blocking solution were added at the indicated dilutions: Sox2, 1:1000 and Nestin, 1:200; GFAP, 1:500; Tuj1, 1:1000; MBP, 1:200; Ki-67, 1:500 and GFP, 1:1000. The use of the GFP antibody enhanced the detection of GFP in these cells. This is because copGFP (the reporter protein expressed from the parental plasmid and mC vectors), is a natural GFP found in the copepod *Pontellina plumata* whose fluorescence intensity is less than the widely used mutated versions such as TurboGFP and maxGFP. The advantages of using this form of GFP however, is that it is non-toxic and non-aggregating which is important for assessment of GFP expression in long term

cultures, which is carried out in this study. Therefore all samples were co-stained with this antibody. The following day, after several washes in PBS, samples were blocked as above and incubated with either FITC-or Cy3-labelled secondary antibody (1:200) in blocking solution at RT for 2 h. Samples were washed thrice in PBS before mounting with Vectashield mounting medium containing DAPI.

2.9. Microscopic analysis

An AxioScope A1 microscope equipped with an Axio Cam ICc1 digital camera and AxioVision software (version 4.7.1, Carl Ziess MicroImaging GmbH, Goettingen, Germany) was used for fluorescence imaging and obtaining photomicrographs. For all quantitative analyses, a minimum of 2000 cells were counted across 3-4 random fields using Image J software. Cells were identified by fluorescence microscopy using cell-specific immunological markers co-localised with their nuclei using DAPI. Percentage transfection efficiency was determined by: the number of cells expressing GFP/ total number of $DAPI^+$ cells. The fluorescence intensity was determined by calculating corrected total cell fluorescence (CTCF) using the formula: CTCF = Integrated density–(Area of selected cell x Mean fluorescence of background readings) using the ImageJ 1.47 Software (NIH). Quantification measurements performed in the same quadrant of randomly selected fields to avoid cell selection bias; a minimum of 90 cells were counted for each condition.

2.10. Statistical analyses

Treatment groups were analysed either by one-way analysis of variance and Bonferroni's multiple comparison test or an independent sample Student's *t*-tests, using Prism software (version 6.07; Graphpad, USA). Each error bar represents the standard error of the mean of a minimum of three separate experiments. The number of experiments (n) refers to the number of NSC cultures which are each generated from a different mouse litter.

3. Results and Discussion

3.1. High purity derivation of cell cultures and mC DNA vector

The NSC cultures used in this study were of high purity as judged by staining NSC markers Nestin, a cytoskeletal protein (purity: 96.1 ± 2.0%, n = 3) and Sox2, a transcription factor (purity: 96.7 ± 0.8%, n = 3). The two DNA vectors employed in this study (for direct comparative assessment of plasmid size on transfection efficiency) are shown in Fig. 1. The parental plasmid expressing green fluorescent protein (termed pp-GFP, Fig. 1A) is a conventional bacterial plasmid containing a kanamycin resistance gene, with GFP expression driven by a cytomegalovirus (CMV) promoter. It also contains two recombinase target sequences, which through a site-specific recombination event, is used to derive mC DNA (mC-GFP, Fig. 1A) which contains only the expression cassette present in pp-GFP. As mC-GFP is derived from pp-GFP, both vectors express the same variant of GFP. The parental plasmid / mC system is ideal for testing plasmid size relationships as differences in vector sequences and reporter gene variance can be ruled out as confounding variables. The bacterial backbone is excised and degraded in the process (Bb, Fig. 1A). Plasmid digests of both pp-GFP and mC-GFP with NotI (Fig. 1B) revealed the expected fragment sizes by gel electrophoresis, and also demonstrated the absence of parental plasmid contamination in the mC preparation.

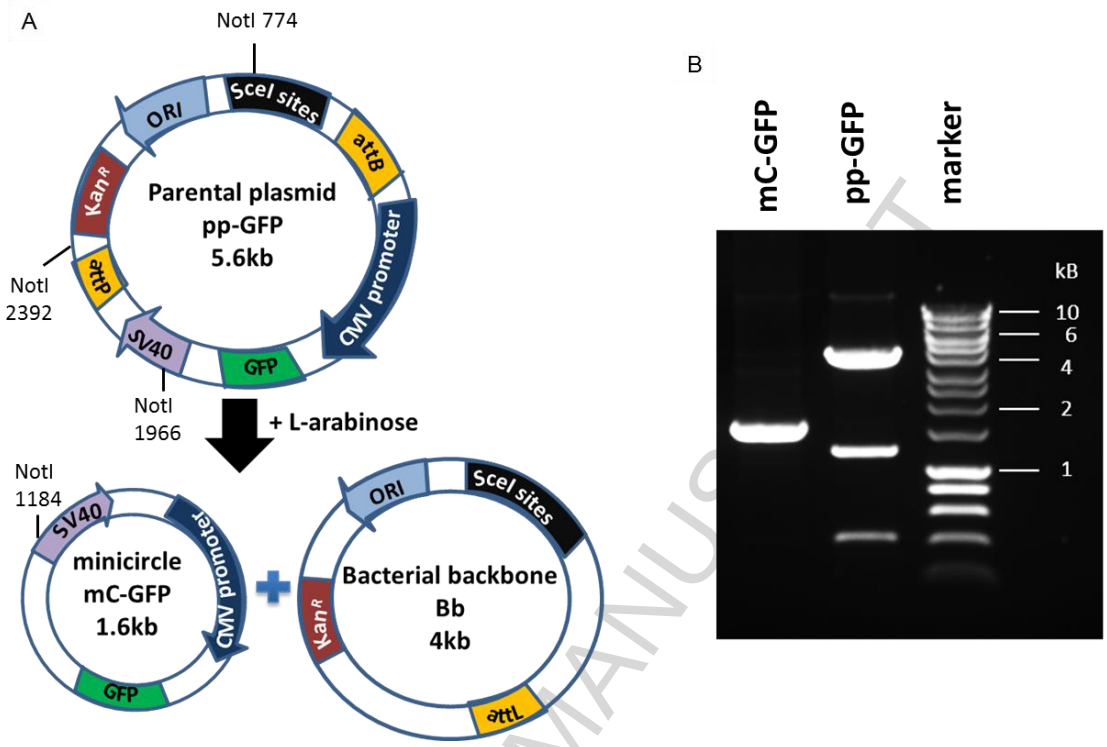


Fig.1. *(A) Schematic showing DNA vector maps and their relative sizes (in kb) of parental plasmid pp-GFP, the corresponding mC derivative, mC-GFP and the excised bacterial backbone (Bb) following mC induction. (B) Agarose gel electrophoresis of NotI restriction digests of pp-GFP and mC-GFP run alongside a 1 kb DNA marker displaying the expected fragment band sizes of both DNA vectors.*

3.2. Minicircle use with magnetofection dramatically enhances NSC gene transfer levels

Most non-viral studies employ DNA vectors expressing variants of GFP that provide enhanced levels of brightness such as TurboGFP and TagGFP [23,36], whereas in this study, pp-GFP and mC-GFP express a non-toxic, non-aggregating but less bright 'copGFP'. The advantage of expressing this natural form of the protein enables analyses of GFP expression over a long period with less protein crystallization and aggregation resulting in lower toxicity in cells. However, to increase detection levels an anti- GFP antibody was used; GFP immunostaining significantly enhanced protein detection as depicted in Fig. 2A compared to

native GFP expression (inset), with protein expression observed throughout the cell body using both plasmids. Hence all samples were analyzed following GFP immunostaining for subsequent experiments.

In order to optimize protocols to achieve high transfection levels, a range of pp-GFP and mC-GFP copy numbers per well were assessed; the oscillation frequency previously determined to be optimal for NSC transfection (4Hz) was used for all experiments [23]. Transfection efficiency was overall lower in cells transfected with pp-GFP compared to mC-GFP, with the highest transfection efficiency observed when using a mC copy number of 6×10^{11}. Transfected cells for pp-GFP and mC-GFP at this copy number are shown in Fig. 2B and C respectively. Standard transfection protocols report MNP:DNA ratios using DNA weight which can conceal differences in the actual copy number of DNA vectors present. Therefore, transfection results here are presented as a function of both plasmid copy number (Fig. 2D) and the corresponding DNA weight (Fig. 2E). At the lowest plasmid copy number (1.2×10^{10} per well), there was no difference in the number of transfected cells between pp-GFP and mC-GFP with mean transfection efficiencies of <10% (Fig. 2D). The highest transfection efficiency achieved with pp-GFP was 15.4% at a copy number of 3×10^{11} and a further increase in copy number resulted in reduced transfection. For mC-GFP, a further increase in copy number to 6×10^{11} yielded a statistically significant and dramatically higher transfection efficiency compared to pp-GFP (transfection efficiency range 42.1 – 45.4% versus 13.4 – 13.8%). Therefore, for subsequent experiments, this copy number was used for double transfection (multifection) in order to yield maximal gene transfer. Double transduction/transfection is routinely used for many viral as well as non-viral genetic modification approaches such as lipid-mediated gene delivery and electroporation in order to enhance transfection efficiency and to prolong gene expression [37–39]. We previously showed that multifection of NSCs (cultured as 3-D neurospheres) resulted in higher

transfection levels (~27%) compared to single transfection (~13%) with no effect on cell physiology and health [22]. NSC monolayers multifected here with mC-GFP showed significantly higher transfection (see Fig. 2F versus Fig. 2C) with a range of 50.7 – 54.4% versus 15.1 – 20.5% for pp-GFP (Fig. 2G). Transfection levels for mC-GFP multifection were also significantly higher than when using single transfection ($p < 0.001$, student's *t*-test, n=3). To our knowledge, these are the highest non-viral DNA transfection levels reported to date for the hard-to-transfect population of primary NSCs. Our data are in line with a previous systematic study in which mC-magnetofection technology applied to HeLa cell lines showed an inverse relationship of DNA size on transfection efficiency [40].

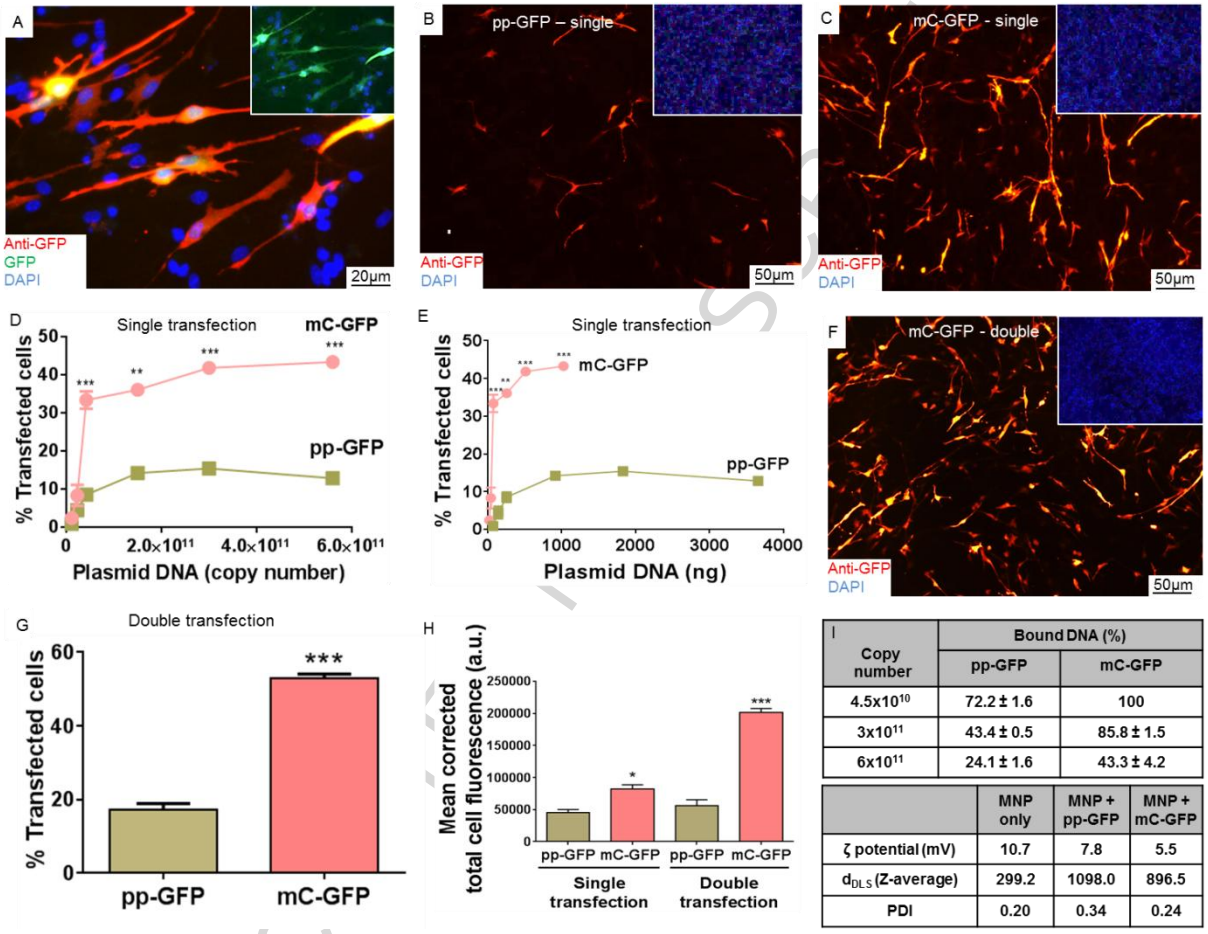
It could be argued that higher numbers of transfected cells can be achieved by selectively enriching for these cells through antibiotic selection or flow cytometry/single-cell sorting. However, both approaches are associated with high costs and additional manipulations of cells can alter physiology and function [41,42]. Further, loss of non-transfected healthy cells (through cell death or discarding associated with these methods) is undesirable given the limited tissue availability and ethical concerns associated with human tissue and embryonic stem cells and that generation and maintenance of primary NSCs is costly and laborious. Therefore the optimal approach currently appears to be transplantation of a *mixed* population of engineered and non-engineered cells.

Total cell fluorescence intensity measurements revealed significantly higher GFP expression in NSCs transfected with mC-GFP compared to pp-GFP (Fig. 2H), suggesting that an increased number of expression cassettes are present per transfected cell for mC-GFP engineered cells; fluorescence intensity per cell was more than doubled using mC-multifection versus single transfection (Fig. 2H). A previous study in which cell sorting of GFP- and luciferase- expressing mouse melanoma cells (B16F10) reported that the 'high expressing sub-population' demonstrated two-fold higher luciferase expression and three

times higher plasmids/nucleus in comparison to their 'low expressing' counterparts [38]. To further explain this phenomenon, our observations in DNA binding experiments showed that, at each copy number assessed, a higher number of mCs copies are bound to MNPs versus parental plasmid (Fig. 2I). Our results suggest that as each MNP can only complex a fixed maximum mass of DNA and given their small size, mCs have different binding properties (enabling efficient binding) compared to larger plasmids (which are approximately 3.5 times larger in size than mCs). Therefore, it is feasible that increased loading of mCs per particle (~ 3.5 times more mCs than parental plasmids) accounts for the higher gene transfer efficiency observed in mC-engineered cells, which was approximately 3.5-fold higher than pp-engineered NSCs. Additionally, differences in physical binding and subsequent cleavage of DNA from the carrier MNP between pp DNA and mC DNA also cannot be ruled out. It should be noted that a previous report where equal copy numbers of both DNA vectors were microporated into cells also suggested higher mC number within nuclei versus the parental counterpart (63.7% *vs* 18.3%) [32]. It has been reasoned that low molecular weight DNA has a 'higher survival rate', making it more likely to enter the nucleus for two reasons: (i) higher mobility, therefore it is less likely to be degraded by host nucleases due to lower time of residence in the cytoplasm [43,44] and (ii) improved release of smaller DNA molecules from nanoparticles allowing for quicker transportation to the nucleus [27]. Together these data indicate that mC use can enhance the level of protein expression per cell, of high relevance to release of therapeutic proteins to promote regeneration.

Furthermore, it is well known that the size and charge of MNP-DNA complexes during magnetofection procedures can impact transfection outcomes. However, in our study the DLS measurements showed only small differences in hydrodynamic diameter and surface charge (Figure 2I) between pp-GFP and mC-GFP functionalised MNPs. These findings suggest that

differences in the physicochemical properties of the particle-plasmid complexes are not likely to be the primary explanation for the differences in transfection levels obtained.



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Copy number	Bound DNA (%)	
	pp-GFP	mC-GFP
$4.5x10^{10}$	72.2 ± 1.6	100
$3x10^{11}$	43.4 ± 0.5	85.8 ± 1.5
$6x10^{11}$	24.1 ± 1.6	43.3 ± 4.2

	MNP only	MNP + pp-GFP	MNP + mC-GFP
ζ potential (mV)	10.7	7.8	5.5
d_{DLS} (Z-average)	299.2	1098.0	896.5
PDI	0.20	0.34	0.24

Fig. 2. Enhanced gene transfer of NSC monolayers with mCs compared to parental plasmid. *(A) Representative triple-merged image of GFP-stained NSC monolayers transfected with mC-GFP showing enhanced GFP detection (equivalent DAPI/GFP double-merged image shown in inset). Representative image of GFP immunostained NSC cultures at 48 h post transfection with (B) pp-GFP and (C) mC-GFP at the same copy number (6 x 10^{11} copies per well); insets show the respective DAPI stained images indicating similar cell densities. (D) Relationship between transfection efficiency and plasmid copy number. (E) Relationship between transfection efficiency and plasmid weight. (F) Representative image showing NSC multifection using mC-GFP at 6 x 10^{11} copies per well, inset showing cell density comparable to that used for single transfections shown above. (G) Bar chart showing quantitative analysis*

*of transfection efficiency following multifection with pp-GFP and mC-GFP. (H) Corrected total cell fluorescence measurements for single and double transfected NSCs at 6 x 10^{11} copies per well of pp-GFP and mC-GFP. A.u. denotes arbitory units. *p < 0.05, **p < 0.01, ***p < 0.001, versus pp-GFP (student's t-test), n=4 cultures for all graphs. (I) Upper panel showing quantitative analysis of DNA bound to equivalent number of MNPs; three identical copy numbers of pp-GFP and mC-GFP were tested. Mean ± SEM from three different experiments are shown. Lower panel displaying hydrodynamic diameter (Z-average) and surface charge (ζ-potential) of naked MNPs and particles functionalized with pp-GFP and mC-GFP. DLS denotes dynamic light scattering.*

3.3. High protocol safety with combined use of minicircle vectors and magnetofection

The effect of multifection on cell health and cell physiology was assessed to evaluate the safety of our protocols. The majority of transfected cells at 48 hours post-transfection, showed normal bipolar morphologies with round healthy nuclei and normal adherence indicating high viability. Multifected NSCs (both pp-GFP and mC-GFP retained their normal morphologies and stem cell marker expression for both Nestin and Sox2 (Fig. 3A and inset). Quantitative analyses revealed comparable numbers of Nestin and Sox2 positive cells for pp-GFP, mC-GFP and non-transfected conditions (Fig. 3B and C respectively). Assessment of proliferative capacity, at 48 hours post-transfection, using Ki-67, an endogenous nuclear marker for cell division (Fig. 3D) revealed no differences between the three groups (Fig. 3E); GFP positive cells showed proliferative ability (inset) with similar proportions of dividing cells as non-transfected cells (63.7 ± 3.6% and 66.7 ± 3.1% respectively). Similarly, negligible differences were observed in cell viability based on cell number (Fig. 3F). Pyknosis/cell death was assessed morphologically by quantifying propidium iodide (PI, a fluorescent intercalating DNA stain that is non-permeant to live cells) positive nuclei (Fig. 3G, arrows and 3H) as well as nuclear shrinkage and fragmentation (Fig. 3G inset and 3I) and

showed no differences between conditions. Although, multifection has previously been used for NSC neurospheres, demonstrating high viability and cell survival [23], these data are the first demonstration of the safety and efficacy of mC transfection in primary NSCs. The high safety of the methods highlights the translational potential of the methodology for clinical cell therapies.

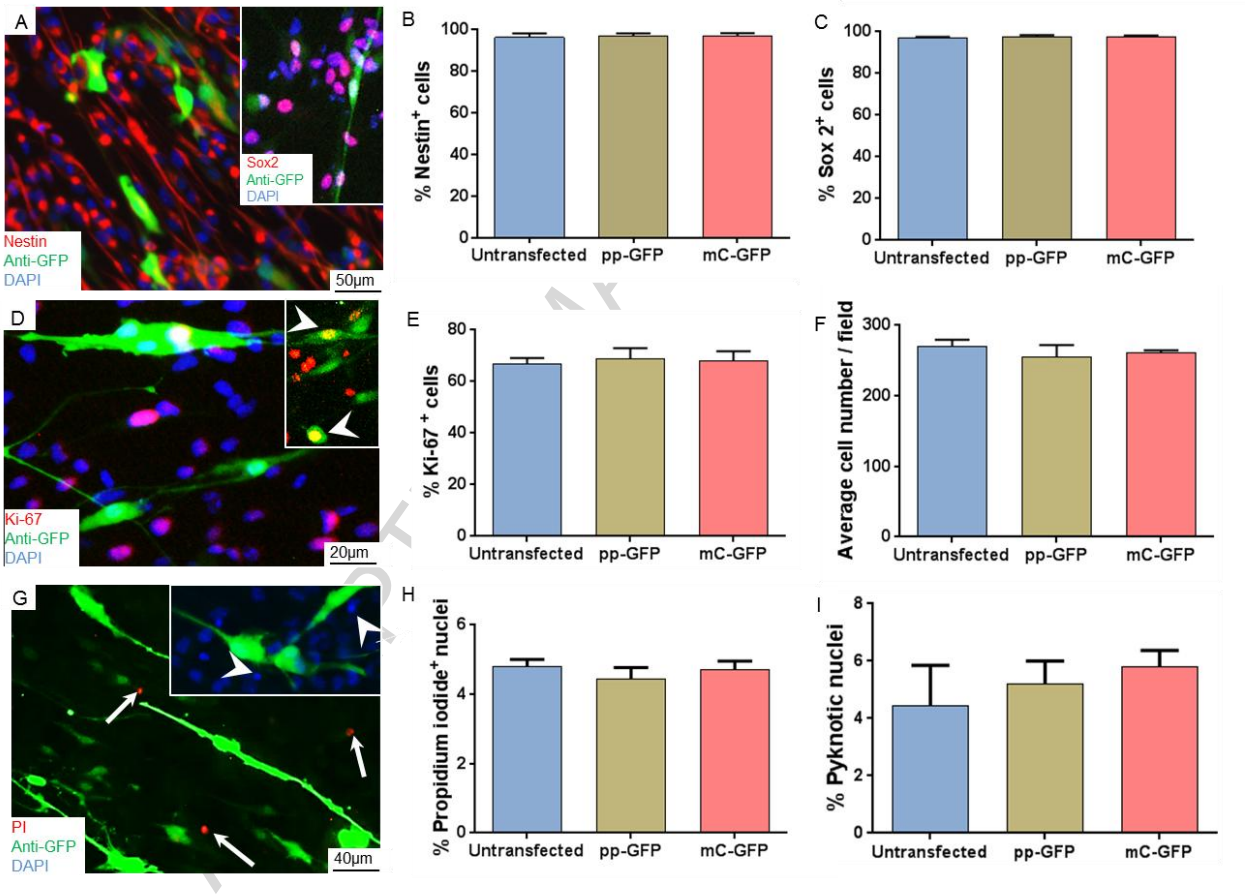


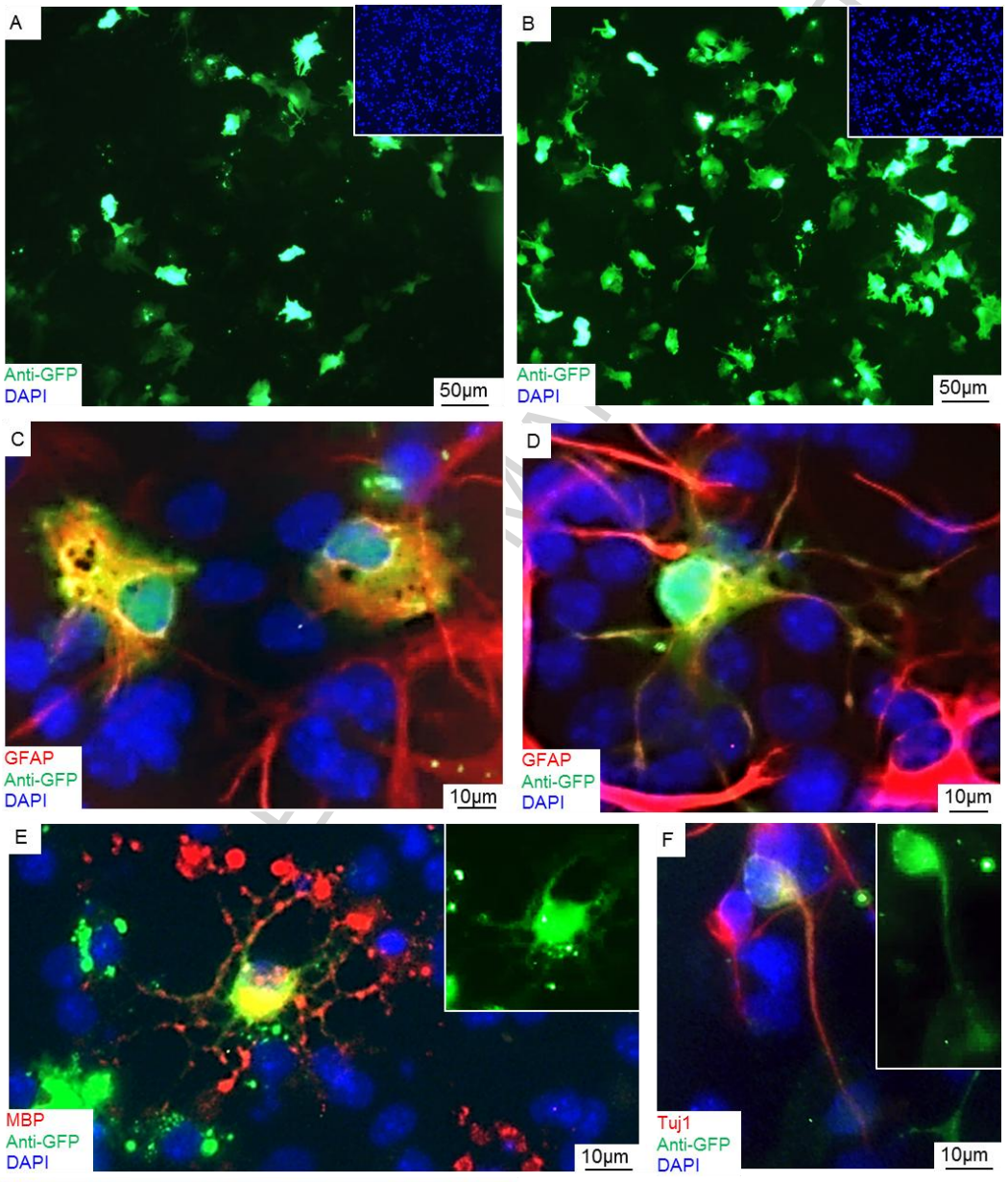
Fig. 3. Safety assessment of multifected NSCs. *Representative merged images of NSCs multifected with mC-GFP showing stem cell marker expression of (A) Nestin and (A – inset) Sox2. Bar charts showing quantification of (B) Nestin$^+$ and (C) Sox2$^+$ cells. (D) Merged image of mC-GFP multifected NSCs with Ki-67, a marker for proliferation, inset showing transfected cells also expressing Ki-67 (arrowheads). Bar chart showing no significant differences in (E) proliferation and (F) average cell number per field between the transfected and non-transfected groups. (G) Merged images of propidium iodide (PI) stained cells*

(arrows) and cell pyknosis (inset, arrowheads). (H) Bar charts displaying PI^+ and (I) pyknotic nuclei counts.

3.4. Minicircle use skews profile of transfected progeny of NSCs towards hard-to-transfect neurons and oligodendrocytes

Genetically engineered NSCs were allowed to differentiate into their three daughter cell types in order to determine whether the protocols can alter the fate or proportion of the daughter cell populations. This is an important parameter to consider for clinical translation as NSC multipotency (ability to generate the three major cell types of the nervous system) is a key element of their therapeutic capacity. Post-differentiation, overall transfection was lower in the progeny of NSCs transfected with pp-GFP versus mC-GFP (Fig. 4A, B and H). Microscopic assessment revealed normal cellular and nuclear morphologies of the daughter populations (Fig. 4C, D, E). The protocols did not alter the relative proportions of each daughter cell type generated demonstrating that following multifection NSCs retained their potential to differentiate. Astrocytes were the main transfected daughter cells for both pp-GFP and mC-GFP, however a higher number of transfected neurons was obtained using mC-GFP. It is noteworthy that using mCs, we have obtained transfected daughter oligodendrocytes for the first time (Fig. 4H); a phenomenon we have never observed using conventional plasmids. Although the numbers of transfected neurons/oligodendrocytes were small, it does appear that use of larger plasmids restricts the profile of transfected progeny towards a single cell type. This restriction appears to be reduced with mC use, or at least increases chances of obtaining in genetically engineered 'hard-to-transfect' cells, which would offer a significant advantage when using mC DNA vectors compared to their larger plasmid counterpart. The reasons for this altered profile are unclear and future work will need to focus on further enhancing the numbers of specific transfected cell types. However, this observation has important implications for cell replacement therapy as targeted cell engineering of daughter cells

especially neurons and oligodendrocytes can significantly impact functional/regenerative outcomes. For example, transfected neurons engineered to express BDNF show increased axonal sprouting and dendrite number in organotypic rat hippocampal explants [45].



H

% *Overall* transfected cells	pp-GFP	mC-GFP
	6.4 ± 0.4	21.4 ± 0.7

% Transfected daughter cells (*of GFP +ve cells*)		
	pp-GFP	mC-GFP
Astrocytes	99.7 ± 0.1	98.3 ± 0.3[b]
Neurons	0.3 ± 0.1	1.7 ± 0.3[a]
Oligodendrocytes	0	0.03 ± 0.03

Fig. 4. Effect of differentiation on transfected NSCs. *NSCs multifected with (A) pp-GFP and (B) mC-GFP post-differentiation. Representative triple merged images of multifected NSCs three days post-differentiation showing (C and D) $GFAP^+/GFP^+$ Type I and Type II astrocytes respectively, (E and inset) MBP^+/GFP^+ oligodendrocytes and (F and inset) $Tuj1^+/GFP^+$ neuron. (G) Bar graph comparing proportions of the daughter cell types of multifected and non-transfected NSCs following differentiation. One way analysis of variance with Bonferonni's post-tests, n = 3. (H) Quantitative analysis showing overall transfection profile and skewed transfection towards neurons and oligodendrocytes using mC-GFP.* ^{a} *$p < 0.05$,* ^{b} *$p < 0.005$ vs pp-GFP (student's t-test, n = 3).*

3.5. Minicircle engineering of NSCs results in sustained gene expression

Microscopic observations revealed no obvious differences in cell morphology and adherence between vector-treated cells and untreated controls over time. Cell viability at four weeks, as judged by proportions of pyknotic nuclei showed no differences between groups (3.9 ± 0.4% for untransfected cells, 4.1±0.4% for pp-GFP and 4.0± 0.3% for mC-GFP). Our data demonstrate the long term protocol safety of the multifection approach.

In our study, gene expression was assessed in differentiated NSCs, as differentiation is the natural fate of NSCs post-transplantation therefore we consider the analysis to be of greater relevance for engineered cell therapy, than studying long term expression in NSCs [43,44]. Fluorescence measurements were made in astrocytes as this was the predominant transfected population at all time points. mC-GFP engineered cells displayed a higher numbers of transfected cells versus pp-GFP, for up to four weeks (Fig. 5A-E). Cell fluorescence intensity indicative of protein expression level (Fig. 5F) also showed significantly greater protein expression in mC-GFP transfected cells. Our finding that mC-engineered cells contain higher

GFP expression cassettes than pp-engineered cells likely explains the higher and sustained levels of protein expression seen in the mC engineered NSC population. Additionally it is possible that these cells have an intrinsic capacity to maintain exogenous DNA for longer periods, or alternatively, generate a greater proportion of stable transfectants.

In our recent study using a conventional pmax-GFP plasmid (3.6 kb), transfection efficiency in magnetofected NSCs was assessed up to 2 weeks reporting 'low' transfection (≤10% cells) [23]. mC transfection with microporation in parent NSCs in a separate study showed transfection efficiency of ca. 20% at 10 days post-transfection, but longer time points were not studied [32]. It is possible that the prolonged gene expression with mC DNA versus conventional plasmids is due to the lack of transcriptional silencing associated with the presence of unmethylated CpG islands [30,46]. The removal of these individual sequences using conventional molecular biology cloning techniques is laborious and expensive; in comparison the mC DNA technology enables a facile, cheaper approach for eliminating bacterial sequences, involving fewer and less time consuming procedures. It must be noted that the viral promoter used to drive the transgene expression (CMV) has been demonstrated in some studies to be a relatively weak promoter and likely to be silenced in undifferentiated mammalian stem cells [47,48]. This highlights the importance of using other effective promoters such as elongation factor 1 alpha (EF1a) in order to achieve higher and prolonged expression *in vivo*, an approach that we have employed in Part II of this study, submitted [49]. Neurorestoration requires long-term therapeutic intervention [50–52] so from a clinical perspective, achieving sustained gene/protein expression can significantly reduce costs and time by reducing the need for repeat administration of therapeutic proteins/cells. Notably, a sustained yet tapered profile of therapeutic biomolecule expression may be beneficial as it mirrors the temporal pattern of molecular expression profiles that are associated with regenerative processes in pathology sites [22]. Indeed, it has been suggested that

overexpression of therapeutic biomolecules for inappropriate periods can have detrimental effects on regenerative processes [53].

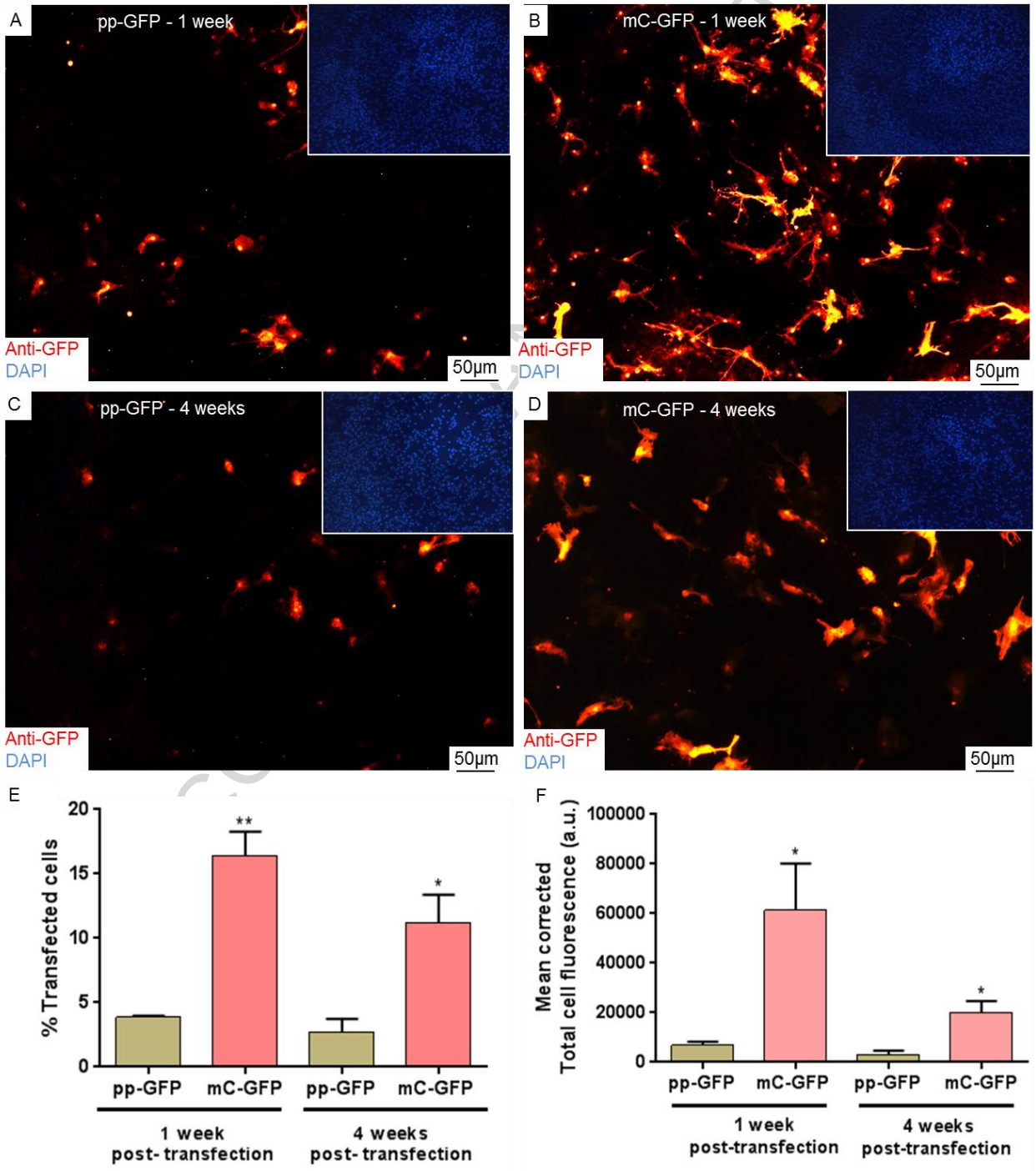


Fig. 5. Sustained gene delivery using mCs. *Representative image of GFP immunostained differentiated NSCs transfected with (A) pp-GFP and (B) mC-GFP (as NSCs) one week post-transfection and four weeks post transfection (C and D respectively). Bar graphs showing (E)*

transfection efficiency and (F) extent of cell fluorescence in pp-GFP and mC-GFP at both time points (students t-test, n = 3). a.u. denotes arbitory units.

3.6. MC DNA vector technology offers key benefits for clinical translation

Based on our findings, we consider that mC vector DNA technology can offer a new solution to a key translational challenge confronting therapeutic gene transfer, with significant implications for safe and effective genetic engineering of neural transplant cells. Our reported level of gene transfer is likely to be sufficient for achieving therapeutic outcomes. For example, a previous study reported that transplantation of vascular endothelial growth factor (VEGF) secreting NSCs (20-30% transfection) promoted angiogenesis following transplantation in the rodent brain [54].

Due to their small size, mCs can enable the incorporation of larger genes such as receptors, or enzymes e.g. chondroitinase ABC, (insert size: 2kb, total mC size 4kb) without compromising transfection efficiency [55]. For transient gene expression *in vivo*, the coding sequence for the fluorescent tag of the mC system (size ca. 756 bp) could be replaced with neurotherapeutic growth factors sequences (whose open reading frames are typically <1.2 kb) without appreciable increases in the overall mC size, representing a realistic solution for safe genetic engineering of neural transplant populations with MNP vectors. While the aim of this study was to provide proof-of-concept that mC vector use is compatible with nanoparticle delivery systems, our functional study on the feasibility of this approach for therapeutic gene delivery indicates that DNA sequences encoding a neurotherapeutic factor (BDNF) can be inserted into mCs for growth factor release by engineered stem cells (see submitted manuscript, part II: Functional Delivery of a Neurotherapeutic Gene to Neural Stem Cells using Minicircle DNA and Nanoparticles: Translational advantages for Regenerative Neurology). This shows the high versatility of the system, which can be used for incorporation of a range of therapeutic sequences.

Of critical importance to clinical translation, is the absence of a bacterial backbone (containing antibiotic resistance genes and an origin of replication) in mCs. Bacterial backbone sequences have been associated with inflammatory responses *in vivo* due to the presence of a high number of unmethylated CpG motifs- structures characteristic of prokaryotic DNA sequences, which induce the innate immune response [46]. Guidance on the use of medicinal products for gene transfer advise great care in the use of selection markers used in such products due to their significant potential to impact human therapy. This is especially pertinent to the presence of antibiotic resistance genes since these can restrict more general treatment options [30,46,56,57]. Indeed, medical and clinical regulatory bodies advise limiting the use of antibiotic resistance genes for therapy where feasible [58]. Further, less mC DNA (<100 ng per well) is required to obtain similar transfection levels as the parental plasmid (~250 ng per well i.e. for *ca* 0.9 X 10^5 cells), so for large scale production of vectors encoding therapeutic genes, this could imply considerable cost and time benefits for cell therapies.

Finally, upscaling of MNPs and mCs for clinical applications appears feasible as MNPs have long been used clinically as contrast agents with several methods currently being employed for large scale manufacture [46,59–61]. It should be noted that given their clinical utility, a novel method involving affinity-based chromatography has been developed for obtaining highly purified mC DNA for gene therapy and vaccination, enabling safe production at an industrial scale [33,62,63]. Together, these factors highlight the critical advantages offered by mC DNA vector technology in conjunction with magnetofection, for clinical translational applications involving genetically engineered neural transplant cells (summarized in Fig. 6). Moreover, mCs are likely to enhance the utility of a wide range of advanced funtional non-

viral gene delivery materials, broadening the scope for safe and efficient genetic engineering of several different cell types for therapeutic applications.

4. Conclusion

As far as we are aware, this is the first reported proof of the fusion of nanovector and mC DNA technologies, for genetic engineering of any cell type of primary origin. We show for the first time that such an approach is entirely feasible for the genetic modification of NSC transplant populations, and that mCs can enhance the functional efficacy of transfection grade nanoparticles. It has become increasingly clear in recent years that it is essential to employ DNA vectors of the minimum size possible, to achieve maximal gene delivery when using nanoparticle based systems to genetically engineer neural cells. We prove here that such DNA size restrictions on nanoparticle based gene transfer can be overcome by deploying mC vectors, whose small size compared with conventional plasmids enables enhanced transfection outcomes to be achieved. Additionally, due to their unique structure, mCs offer a host of advantages for clinical translation, such as reduced immune responses, sustained gene expression and versatility for incorporation of a range of therapeutic sequences. Similarly, MNPs have high translational potential as already demonstrated by their utility as clinical contrast agents. Production of both mCs and MNP is feasible in terms of scale up for human therapies. Further, the methods involved in the genetic modification protocols are simple, cost-effective and reliable, highlighting the key utility of this genetic modification approach for translational applications.

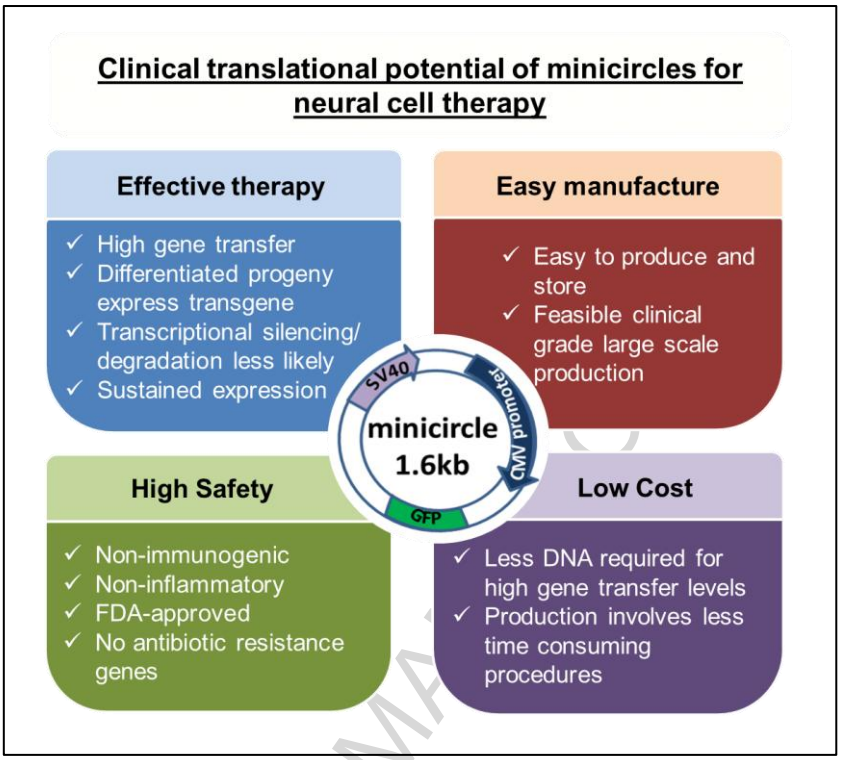


Figure 6. Advantages of mC technology for cell based therapy. *Schematic showing clinical translational potential of mCs.*

Acknowledgements

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Abbreviations

NSCs, neural stem cells; CNS, Central Nervous system; GFP, Green Fluorescence Protein; mC, minicircle; mC-GFP, minicircle-GFP; pp-GFP, parental plasmid-GFP; Bb, backbone; MNP, magnetic nanoparticle; RT, room temperature; PI, propidium iodide; CTCF, corrected total cell fluorescence; PFA, paraformaldehyde; PBS, phosphate buffer saline; NS-M, neurosphere medium; ML-M, monolayer medium; DF-M, differentiation medium, GFAP, glial fibrillary acidic protein; MBP, myelin basic protein, Tuj1, class III β tubulin; DAPI, 4',6-diamidino-2-phenylindole; CMV, cytomegalovirus

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Graphical Abstract

Magnetic nanoparticle-mediated gene delivery to neural stem cells using minicircles (small DNA vectors) achieves significantly higher and sustained gene transfer compared to larger DNA vector counterparts.