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PII: S0168-3659(16)30416-3
DOI: doi: 10.1016/j.jconrel.2016.06.039
Reference: COREL 8348

To appear in: *Journal of Controlled Release*

Received date: 22 April 2016
Revised date: 21 May 2016
Accepted date: 27 June 2016

Please cite this article as: Alinda R. Fernandes, Divya M. Chari, Part II: Functional Delivery of a Neurotherapeutic Gene to Neural Stem Cells using Minicircle DNA and Nanoparticles: Translational advantages for Regenerative Neurology, *Journal of Controlled Release* (2016), doi: 10.1016/j.jconrel.2016.06.039

Part II: Functional Delivery of a Neurotherapeutic Gene to Neural Stem Cells using Minicircle DNA and Nanoparticles: Translational advantages for Regenerative Neurology

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Abstract

Both neurotrophin-based therapy and neural stem cell (NSC)-based strategies have progressed to clinical trials for treatment of neurological diseases and injuries. Brain-derived neurotrophic factor (BDNF) in particular can confer neuroprotective and neuro-regenerative effects in preclinical studies, complementing the cell replacement benefits of NSCs. Therefore, combining both approaches by genetically-engineering NSCs to express BDNF is an attractive approach to achieve combinatorial therapy for complex neural injuries. Current genetic engineering approaches almost exclusively employ viral vectors for gene delivery to NSCs though safety and scalability pose major concerns for clinical translation and applicability. Magnetofection, a non-viral gene transfer approach deploying magnetic nanoparticles and DNA with magnetic fields offers a safe alternative but significant improvements are required to enhance its clinical application for delivery of large sized therapeutic plasmids. Here, we demonstrate for the first time the feasibility of using minicircles with magnetofection technology to safely engineer NSCs to overexpress BDNF. Primary mouse NSCs overexpressing BDNF generated increased daughter neuronal cell numbers post-differentiation, with accelerated maturation over a four-week period. Based on our findings we highlight the clinical potential of minicircle/magnetofection technology for therapeutic delivery of key neurotrophic agents.

Keywords

BDNF, minicircle, magnetofection, neural stem cells, genetic engineering

1. Introduction

Following brain/spinal cord injury, the pathological microenvironment differs substantially from that of the normal endogenous tissue, with detrimental effects at the local tissue level, resulting in little or no tissue regeneration. Therefore, in order to stimulate neural repair, the injury microenvironment critically requires manipulation towards a pro-regenerative profile, through strategies such as induced expression of neurotrophins e.g. brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF) and nerve growth factor (NGF) [1–4]. In this context, genetically engineered stem cell-based strategies offer both direct (donor cells for repopulation of lost cells) and indirect (support via secretion of neurotrophic factors) advantages to facilitate and accelerate cell repair and regeneration. This combinatorial approach (i.e. fusion of gene therapy and cell-based therapeutics) is considered to be an attractive and clinically relevant approach for repair of complex pathologies such as injuries of the brain and spinal cord [5].

Of the heterogenous population of cells present in the central nervous system (CNS), neurons are particularly challenging to replace and repair following injury. BDNF has been widely validated as a therapeutic target given its beneficial effects in several neurological conditions such as Alzheimer's Disease, Parkinson's Disease, epilepsy and brain/spinal cord injuries [6,7]. For example, intravenous BDNF administration has been shown to reduce infarct size of cerebral ischemia in a rat stroke model [8]. Intrathecal delivery of recombinant methionyl human BDNF for treatment of Amyotrophic Lateral Sclerosis has progressed to clinical trials, and appears to be well-tolerated and feasible. However, investigations of a sufficient number of cases is required before conclusions can be drawn regarding the clinical efficacy of this therapy [9]. These studies highlight the benefits of a combinatorial approach for neurological repair and neurorestoration; transplantation of genetically engineered NSCs can facilitate such an approach. Indeed, promising results were observed using cell-based therapies, for example, grafted BDNF-expressing NSCs in a murine model of stroke enhanced neurogenesis, showed neuroprotective properties (reduced apoptosis) and induced angiogenesis with functional recovery demonstrated by improved locomotor function [10].

These BDNF delivery studies almost exclusively employ virus mediated gene delivery but safety and scale-up issues pose major barriers to the clinical translation of this approach [11–14]. Non-viral magnetofection methods, using magnetic nanoparticles (MNPs) in conjunction

with oscillating magnetic fields can offer a safe solution for gene delivery in neural cells [15-23] but a major challenge encountered has been reduced transfection associated with increased size of therapeutic plasmids [23-27] (discussed in detail in Part I).

In this context, we have proved (using reporter gene sequences) that minicircles (mCs) are ideal DNA constructs for neural gene delivery in combination with magnetofection (see part I of manuscript). These mini-DNA vectors are devoid of the bacterial backbone structure typically present in all plasmid vectors (making them safer), but also increasing gene transfer efficacy (up to *ca.* 54%) due to their small size, both of critical importance for clinical translation. [28] We and others have reasoned that the superior transfection efficiency is likely due to mC engineered cells containing higher transgene copies per cell *versus* conventional plasmids. The mC vectors are also compliant with regulations on the use of medicinal products for gene transfer which restrict the use of selection markers due to their substantial potential to impact therapeutic outcomes [29–33]. Despite the obvious clinical translational benefits offered by this genetic modification methodology for regenerative neurology, *the feasibility of mC based delivery of therapeutic genes to neural transplant populations has never been evaluated*. Accordingly, the objectives of this study are to: (i) demonstrate the feasibility of using mC-functionalized MNPs for BDNF gene delivery into primary NSCs (ii) assess the safety profile of this approach, (iii) validate the short-term and long term functional effects of BDNF using an *in vitro* neuronal cell-based assay.

2. Methods

2.1. Materials

Cell culture reagents were obtained from Invitrogen and Sigma. Cell culture grade plastics were from Fisher Scientific. Human recombinant epidermal growth factor (EGF) and human BDNF Quantikine ELISA kit were from R&D Systems Europe Ltd. (Abingdon, UK). Transfection-grade magnetic particles, NeuroMag were from OZ Biosciences, Marseille, France. The magnefect-nano 24-magnet array system was purchased from nanoTherics Ltd. (Stoke-on-Trent, UK). Reagents used for the mC DNA production were obtained from System Biosciences (SBI; Mountain View, CA, USA). Kits for plasmid and mC DNA vector purification (both minipreps and maxipreps were from Qiagen, UK. All restriction/cloning enzymes were purchased from Promega, UK. Primary antibodies were: NSC markers, nestin (clone 25, BD Biosciences, Oxford, UK) and SOX2 (Millipore, 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), GFP (ThermoFisher Scientific, Rockford, IL,USA), Ki-67 (Abcam, UK) and BDNF (Promega, UK). Secondary antibodies (either Cy-3 and FITC-conjugated) were from Jackson Immunoresearch Laboratories Ltd (Westgrove, PA, USA). Vectashield mounting medium with the nuclear stain 4',6-diamidino-2-phenylindole (DAPI) 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 generated from CD1 mice (postnatal days 1-3). Briefly, whole brains were harvested and the subventricular zone was microdissected, dissociated into a single-cell suspensions. NSCs were cultured as neurospheres (free floating cell clusters) in neurosphere medium (NS-M) comprising a DMEM:F12 (3:1 mix) containing B-27 supplement (2%), penicillin (50 U/ml), streptomycin (50 μg/ml), heparin (4 ng/ml), bFGF and EGF (20 ng/ml each). Neurospheres were fed every 2-3 days and passaged every 6 days using an Accutase-DNaseI mix.

2.3. Monolayer cultures

To prepare two-dimensional adherent NSC monolayers, neurospheres (passages 0 – 2) were dissociated with accutase-DNase I, cells resuspended at 1.5 x 10^5 cells/ml monolayer culture medium (ML-M; comprising of a DMEM:F12 (1:1 mix) with N2 supplement (1%), penicillin (50 U/ml), streptomycin (50 μg/ml), heparin (4 ng/ml), FGF2 and EGF (20 ng/ml each). NSCs were plated on polyornithine/laminin-coated 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

NSCs were differentiated to the three major daughter cell types (neurons, astrocytes, oligodendrocytes) by replacing ML-M with differentiation medium (DF-M) consisting of NS-

M (without growth factors) and supplemented with fetal bovine serum (0.5%). Medium was changed every 2-3 days. Cells were maintained in the differentiated state for up to 4 weeks.

2.5. Construction of BDNF overexpression parental and mC DNA

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 of a parental plasmid pMC.EF1α-MCS-IRES-GFP-SV40PolyA (herein termed pp-BDNF-GFP; size: 8084 bp) from which the mC DNA vector is derived through the mC induction protocol described below. Such IRES vectors allow for the expression of the gene of interest and reporter gene separately but simultaneously facilitating the detection of the expression of the insert.

BDNF coding sequence was amplified by PCR from a plasmid containing human BDNF coding sequence (Dharmacon Research Inc, GE Healthcare Life Sciences, UK) using the following primers:

Forward: 5' GCGAATTCATGACCATCCTTTTCCTTACTATGG and

Reverse: 5' GGGATCCTATCTTCCCCTTTTAATGGTCAATGTACAT.

The EcoRI and BamHI restriction sites are underlined respectively. The PCR products were cloned into the EcoRI and BamHI sites of pp-BDNF-GFP. Recombinant mCs (mC-BDNF-GFP; size 4075 bp) lacking extraneous bacterial backbone sequences were prepared from pp-BDNF-GFP, which were transformed into specifically engineered *E. coli* strain which was induced to express ϕC31 integrase and SceI endonuclease upon addition of arabinose (0.01% final concentration), as per the manufacturer's instructions. The integrase splits the full size pp-BDNF-GFP construct 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 vectors that are markedly reduced in size whilst retaining the expression cassette containing both the therapeutic gene and the reporter gene. Endotoxin-free plasmids were purified according to the manufacturer's instructions (Qiagen, UK). Following mC purification, restriction enzyme digests of both pp-BDNF-GFP and mC-BDNF-GFP were run on a 1% agarose gel in order to demonstrate (i) the presence of the BDNF insert in both vectors and (ii) the absence of pp-BDNF-GFP contamination in mC-BDNF-GFP stocks. In the presence of contamination, mC-BDNF-GFP was digested with mC-safe DNase according to the manufacturer's instructions. Cloned inserts were sequenced (outsourced to Source Bioscience, UK) to verify the integrity of BDNF within the construct.

2.6. Magnetofection of NSC cultures

For magnetofection experiments of NSC monolayers, medium was replaced with fresh ML-M (0.225 ml) before addition of transfection complexes. The particle-DNA complexes were prepared by mixing 170 ng of mC-BDNF-GFP and 0.62 µl Neuromag in 75 µl DMEM:F12 (1:1) base medium for each well. Following incubation for 20 min at room temperature (RT), the entire mixture was added to the cells. 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 same protocol as above was followed, with a 24 h time window between the first and second transfection. Cells were either fixed (to assess transfection efficacy, 'stemness' and viability), or differentiated (to assess NSC differentiation potential) 48 h after the first transfection.

2.7. Immunocytochemistry

Paraformaldehyde-fixed cells were blocked (5% normal donkey serum in PBS-0.3% Triton-X-100) for 30 min at RT, followed by primary antibody incubation overnight at 4°C. The following antibodies (diluted in blocking solution) were added: Glial fibrillary protein (GFAP for astrocytes), 1:500, beta-Tubulin (Tuj1 for neurons), 1:1000, myelin basic protein (MBP for oligodendrocytes), 1:200 and GFP 1:1000. GFP immunostaining enhanced GFP detection in all cell types as previously reported (see manuscript part I). Secondary antibodies (either Cy3- or FITC-labelled) were diluted in blocking solution at 1:200 and were added to cells and incubated for 2h at RT. Following PBS washes, coverslips were mounted with Vectashield mounting medium containing DAPI.

2.8. Enzyme linked immunosorbent assay (ELISA)

At specific time points (48h, 5 days, 2 weeks and 4 weeks post transfection), the supernatant was collected, centrifuged (to remove cells and cell debris) and processed for ELISA. BDNF protein concentration in cell supernatants was determined using an ELISA kit specific for human BDNF (Quantikine® ELISA, R&D Systems) according to the manufacturer's instructions.

2.9. Microscopic analysis

For fluorescence imaging, 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 quantitative analyses, a minimum of 200 cells were counted across random fields using Image J software. For assessing neurite outgrowth, NeuronJ was used. Cells were identified by fluorescence microscopy using cell-specific immunological markers co-localised with their nuclei using DAPI. Percentage transfection efficiency was determined by counting the number of cells co-expressing GFP and cell-specific markers. NSC proliferation was assessed by counting cells positive for Ki-67 (a proliferation-specific marker), as well as, by quantifying their absolute numbers per field. Neuronal morphometric quantification and analysis was carried out using NeuronJ. Neurite length was measured at the 2 week post-transfection time point, i.e. the condition at which neurites can be measured without interference from overlapping neurites.

2.10. Statistical analyses

Treatment groups were analysed by either independent sample *t*-tests or one-way analysis of variance and Bonferroni's multiple comparison test using Prism software (version 4.03; Graphpad, USA). Each error bar represents the standard deviation of a minimum of three separate experiments. The number of experiments denoted (n) refers to the number of NSC cultures, each generated from a different CD1 mouse litter.

3. Results and Discussion

3.1. High purity of NSC primary cultures and DNA constructs encoding BDNF

Nestin- and Sox2-specific immunostaining of NSCs demonstrated high culture purity (95.2 ± 1.1% and 97.9 ± 0.5% respectively, n=3 cultures). The two DNA constructs employed in this study were of high purity and encoded the full length human BDNF protein. The IRES-containing bicistronic parental plasmid (pp-BDNF-GFP, Fig. 1A) was engineered to incorporate the BDNF insert resulting in co-expression of BDNF and GFP, driven by the Elongation Factor 1α (EF1a) promoter. Systematic studies investigating the quantitative comparison of constuitive promoters using lentiviral vectors in various cell types (including mesenchymal stem cells and embryonic stem cells) report that the EF1a promoter is a strong

promoter in cell lines derived from rodents, humans and macaques [34–36]. pp-BDNF-GFP was used to generate two circular DNA entities (i) mC DNA (mC-BDNF-GFP, Fig. 1A) which was purified for use in the study and (ii) the bacterial backbone which is excised and degraded during the process of mC induction. EcoRI and BamHI restriction digests of both constructs (Fig.1B) revealed the expected fragment sizes (the high molecular weight fragment corresponds to the vector and low molecular weight fragment corresponds to the BDNF insert) and also demonstrated the absence of parental plasmid contamination in the mC preparation. Comparable results were obtained from two separate preparations of mC-BDNF-GFP demonstrating the high reproducibility of the mC induction protocol employed in the study (Fig. 1B).

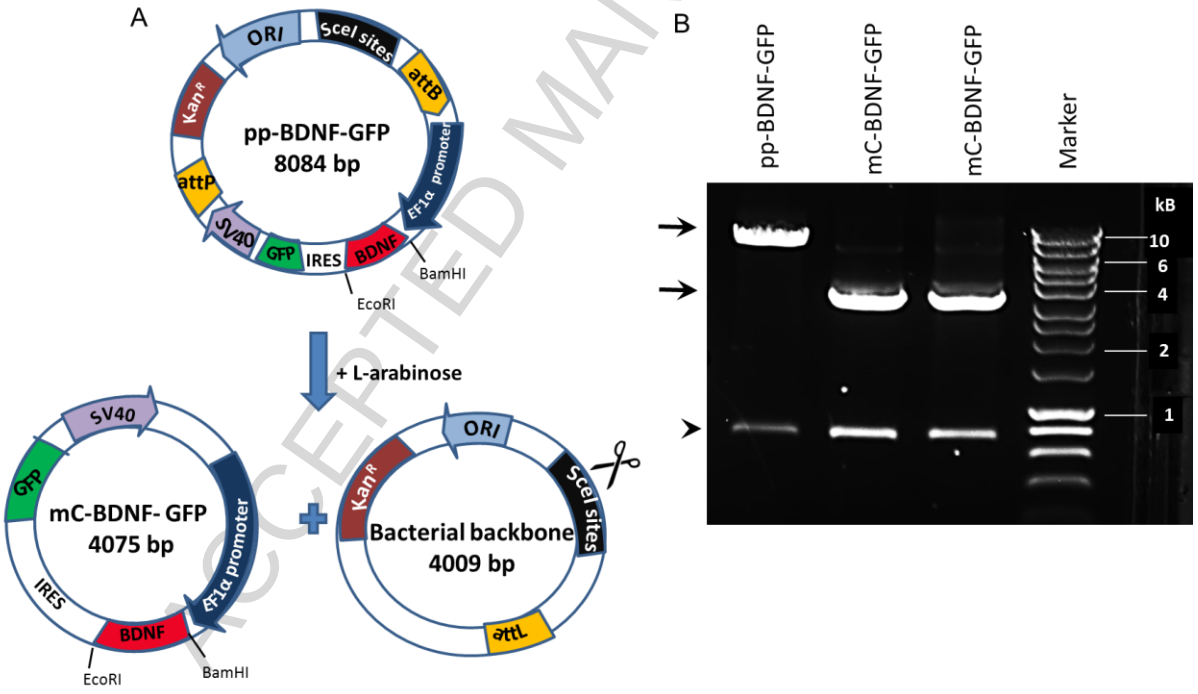

Fig. 1. Minicircle derivation, DNA vector maps and purity of vector preparations. *(A) Schematic showing the generation of mC-BDNF-GFP from pp-BDNF-GFP following L-arabinose induced (i) recombination between attB and attP sites (present in pp-BDNF-GFP) and (ii) SceI endonuclease initiated degradation of the bacterial backbone.(B) Agarose gel electrophoresis micrograph of EcoRI and restriction BamHI digests of pp-BDNF-GFP (Lane 1) and two independent preparations of mC-BDNF-GFP (Lane 2 and 3) run alongside a 10 kB DNA marker (Lane 4). The DNA fragment corresponding to the DNA vector (i.e. parental*

plasmid or minicircle) is denoted by arrows, arrowheads denote the BDNF insert. (bp = base pairs; kB = kilobases).

3.2. Minicircle DNA exhibits dramatically superior NSC transfection efficiencies compared to its corresponding parental plasmid

Immunostaining for GFP significantly enhanced protein detection as depicted in Fig. 2A; native GFP expression is shown in the inset. Therefore all samples were processed for GFP immunostaining prior to analysis for transfection efficiency. Transfection with both constructs, pp-BDNF-GFP and mC-BDNF-GFP, resulted in GFP expression throughout the cell body including cell processes. GFP^+ cells appeared to express elevated levels of BDNF expression (Fig. 2B) compared to untransfected cells. Microscopic observation at high magnification revealed a punctate staining profile of BDNF throughout the cell body, indicative of BDNF packaged within vesicles. Dense perinuclear localization of BDNF was observed in most cells representing the highly active endoplasmic reticulum/Golgi apparatus, for protein synthesis and packaging (Fig. 2C) [37]. Double transfection (multifection) was carried out in all experiments as it results in higher transfection efficiencies than single transfection in experiments using reporter plasmids [38]. NSCs multifected with mC-BDNF-GFP reported higher transfection efficiencies compared to pp-BDNF-GFP (transfection efficiency range 28.9 – 21.8% *vs* 4.4 - 3.9% respectively) (Fig.2 D -F). Having determined that BDNF is expressed within cells, BDNF secretion was verified at 24 hours following the second transfection. mC-BDNF-GFP engineered cells showed approximately a 20-fold increase in secretion levels compared to untransfected NSCs (Fig. 2G). In comparison, pp-BDNF-GFP transfection resulted in a two-fold increase in BDNF secretion.

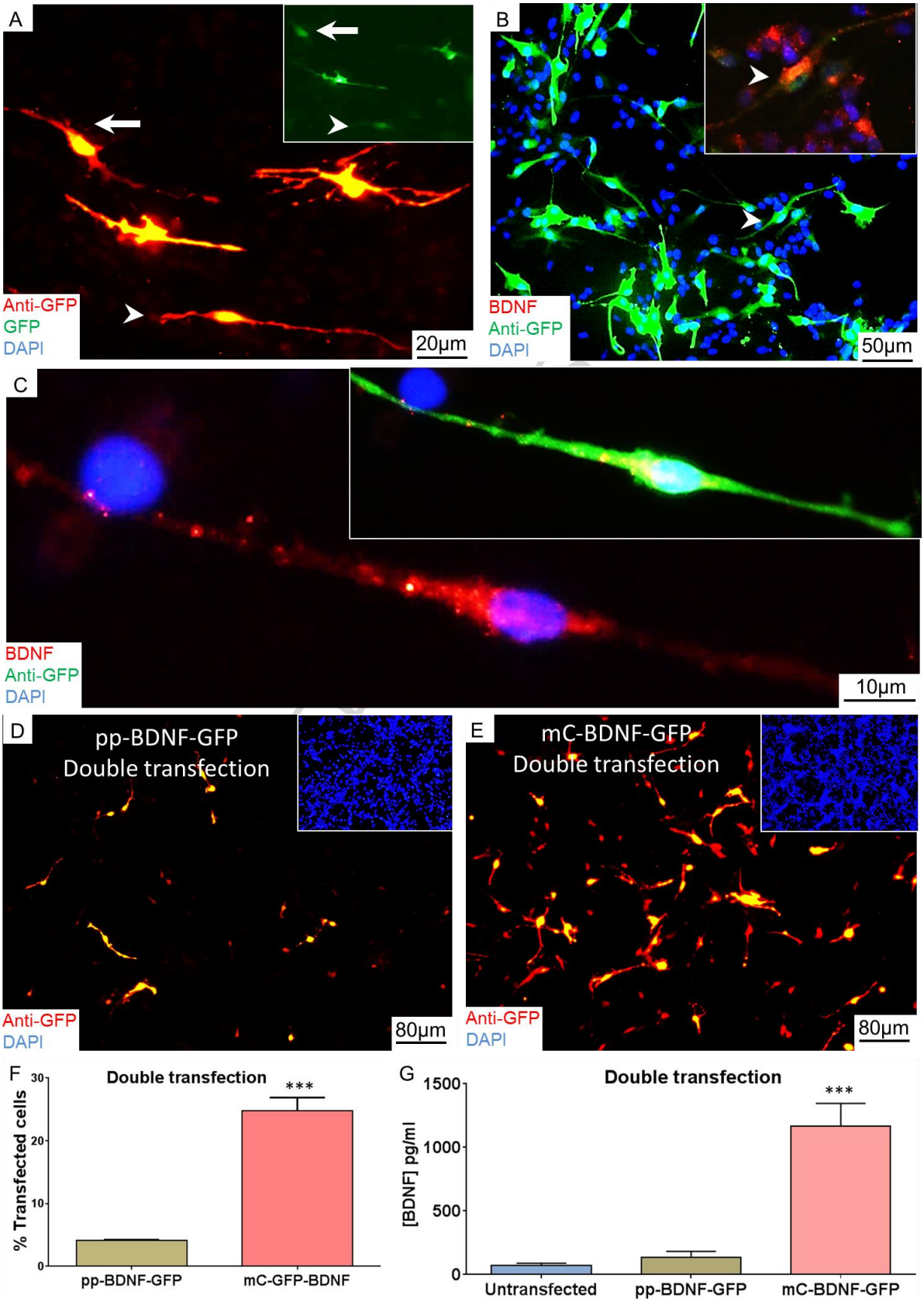

Fig. 2. Enhanced therapeutic gene transfer in NSC monolayers using minicircles versus parental plasmid. *(A) Representative image of GFP immunostained NSC monolayers transfected with mC-BDNF-GFP demonstrating enhanced GFP detection (equivalent*

*unstained image is shown in inset with corresponding cells shown by arrows/arrowheads). (B) Representative double-merged image of GFP-immunostained mC-BDNF-GFP engineered NSC monolayers (equivalent BDNF expression is shown in inset, arrow points to same cell in both images). (C) High magnification micrograph displaying BDNF$^+$ dense punctate staining within the cell cytoplasm (corresponding GFP staining shown in inset). Representative image of GFP immunostained NSC cultures multifected with (D) pp-BDNF-GFP and (E) mC-BDNF-GFP; insets show corresponding DAPI labelled cells, indicating similar cell densities across conditions (F) Bar graph showing comparative transfection efficiencies between pp-BDNF-GFP and mC-BDNF-GFP following double transfection in NSCs. (G) BDNF secretion levels in media (measured by ELISA) following double transfection of NSCs with pp-BDNF-GFP and mC-BDNF-GFP in comparison to untransfected controls. Error bars represent standard error of the mean; ***p < 0.001, versus pp-BDNF-GFP (student's t-test), n=3 cultures for both graphs.*

3.3. BDNF overexpression promotes NSC proliferation

BDNF overexpression resulted in a significant increase in NSC number compared to non-transfected cells as judged by DAPI staining (average cell number per field: 224 ± 14 for untransfected *vs* 288 ± 12 for mC-BDNF-GFP) (Fig. 3A, B and G). An increased number of Ki-67$^+$ NSCs was observed in parallel in the mC-BDNF-GFP condition compared to untransfected counterparts (Fig. C-F and H). It is important to note that the NSC monolayer media contains both EGF and bFGF implying that in combination with these mitogenic factors, BDNF may have a synergistic effect on NSC proliferation [39]. Due to this overall proliferative effect of BDNF on NSCs, the actual transfection efficiency reported here is therefore likely to be an underestimate of the initial transfection levels. The result here demonstrates the functional effects of the encoded BDNF as it is known to be involved in NSC proliferation, among many other cellular processes such as neural differentiation and maturation [40]. Our study shows that the BDNF levels induced are sufficient to have a functional biological effect on NSCs. As an example, transplantation of a mixed population of VEGF-engineered NSCs (transduction efficiency of *ca* 20-30% obtained in this study) demonstrated neuroprotective effects and increased angiogenesis in intact non-disease brains [41]. The proliferative effects of BDNF on NSCs can be predicted to be advantageous for *ex vivo* gene therapy, resulting in genesis of increased numbers of progenitor populations that can in turn result in enhanced production of neurons [40]. While such enhanced division does

raise issues related to safety of the procedures, we consider that the observed enhancement of proliferation is unlikely to be an issue of major clinical concern given that the default fate of NSCs has repeatedly been shown to be differentiation towards the neuronal and glial phenotypes, along with a loss of proliferative capacity [42]. However, this will need to be robustly tested using transplantation of mC engineered neural transplant cells delivered to *in vivo* models of neurological injury.

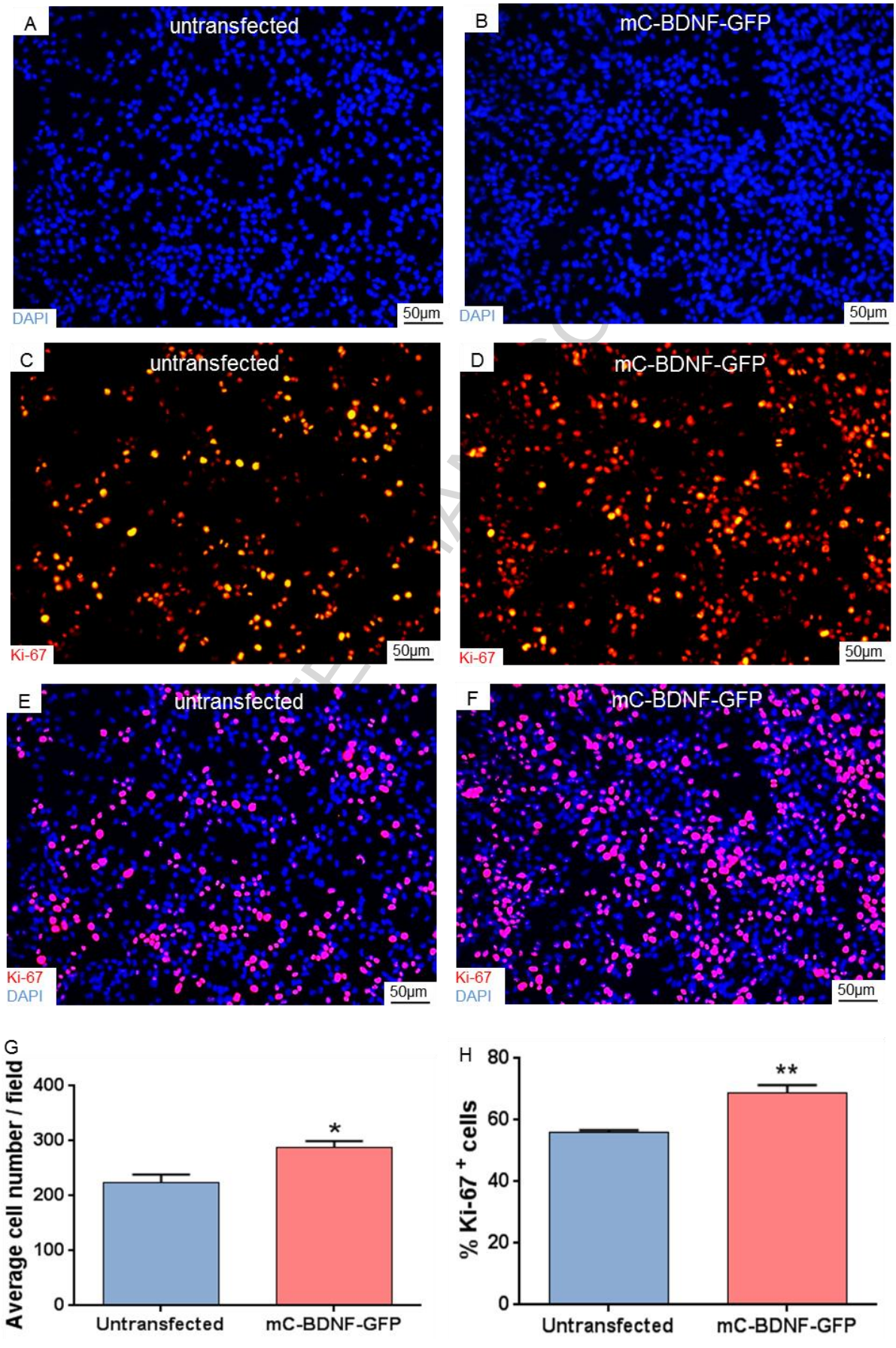
A
untransfected
DAPI
50μm
B
mC-BDNF-GFP
DAPI
50μm
C
untransfected
Ki-67
50μm
D
mC-BDNF-GFP
Ki-67
50μm
E
untransfected
Ki-67
DAPI
50μm
F
mC-BDNF-GFP
Ki-67
DAPI
50μm
G
Average cell number / field
400
300
200
100
0
*
Untransfected
mC-BDNF-GFP
H
% Ki-67+ cells
80
60
40
20
0
**
Untransfected
mC-BDNF-GFP

Fig. 3. BDNF overexpression results in increased NSC proliferation. *Representative micrographs of untransfected and mC-BDNF-GFP engineered NSCs respectively showing (A, B) DAPI$^+$ staining, (C, D) corresponding immunostaining for proliferation marker Ki-67 and (E, F) DAPI$^+$/ Ki-67$^+$ double merged images. Bar graphs showing quantification of (G) average cell number per field and (H) proportion of Ki-67$^+$ cells for both groups. Error bars represent standard error of the mean; *p < 0.05, **p < 0.01 versus untransfected NSCs (student's t-test), n=3 cultures for both graphs.*

3.4. mC-BDNF-GFP engineered NSCs exhibit normal cell stemness and viability

No observable differences in cell morphology and adherence were identified between the untransfected and mC-BDNF-GFP transfected conditions. Normal bipolar cell morphologies with round nuclei were observed indicating healthy cells (Fig. 4A) under both conditions. Quantification of propidium iodide (a fluorescent marker for cell death) stained nuclei (indicated by arrows in Fig. 4A) demonstrated no differences in cell viability between the two conditions (Fig. 4B). Transfected NSCs also retained their stem cell specific marker expression profile for Nestin, a cytoskeletal protein (Fig. 4C) similar to untransfected cells (Fig. 4D). Similarly assessment of another marker for undifferentiated stem cells, Sox2 transcription factor (Fig.4E) showed no differences in the proportions of Sox2$^+$ cells between the two groups (Fig. 4F). These results demonstrate that NSC multifection with mC-BDNF-GFP does not affect NSC cellular health and physiology. We consider that this high safety profile is of critical importance, as evidenced here and in our previous study (see manuscript part I), emphasizing the translational potential of mC mediated engineering for cell-based therapies. Although BDNF is known to drive differentiation towards neuronal phenotypes, our data suggests that BDNF in the presence of mitogenic factors which maintain pluripotency such as EGF and bFGF (present at high concentration in the media) does not induce differentiation [43].

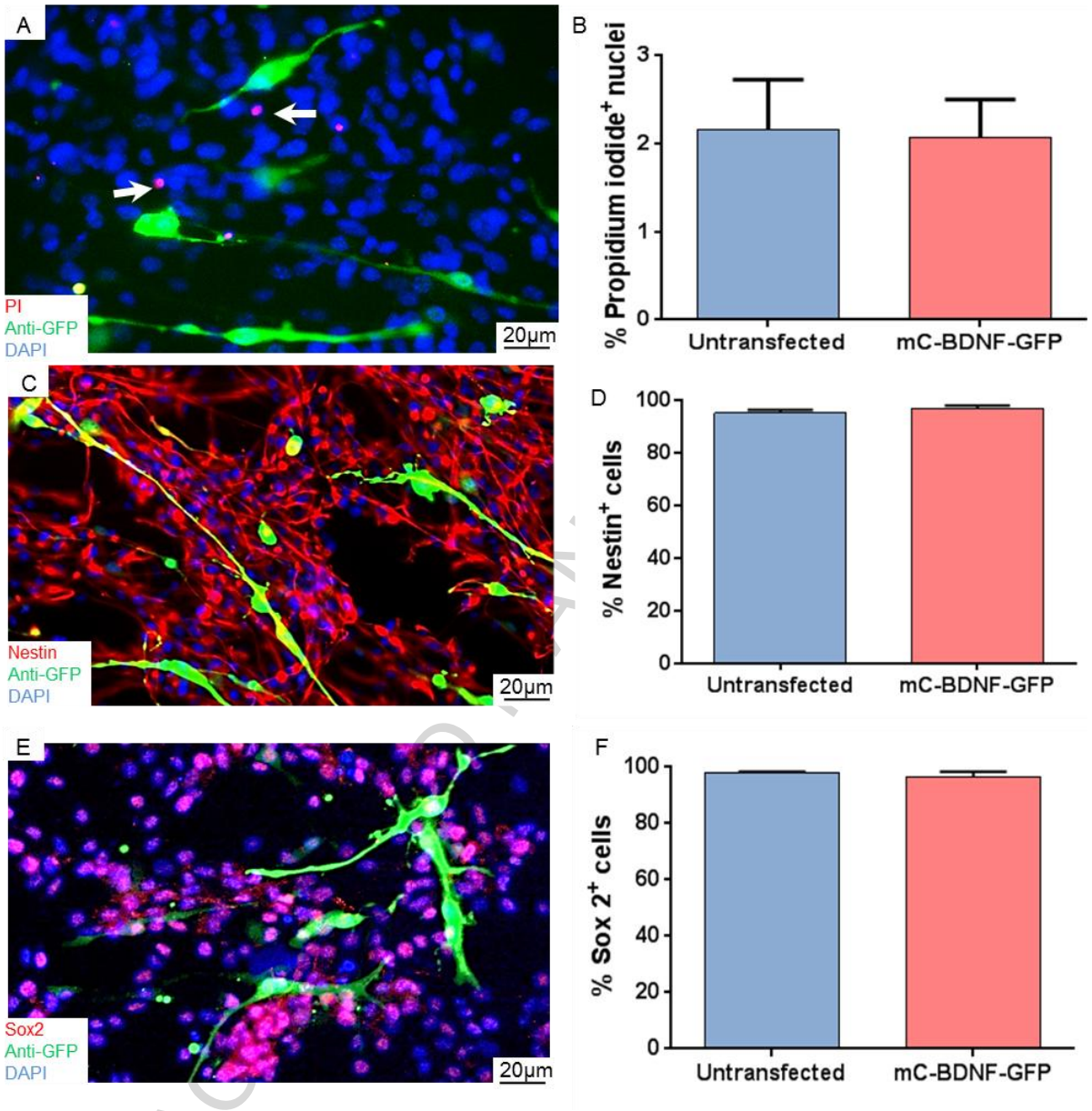

Fig.4. Safety assessments of mC-engineered NSCs expressing BDNF. *(A) Representative triple merged image of mC-BDNF-GFP multifected NSCs showing propidium iodide (PI) staining, a nuclear marker for cell death. (B) Bar graph quantifying the proportion of PI$^+$ nuclei in untransfected and mC-BDNF-GFP multifected groups. (C) Representative triple merged image of mC-BDNF-GFP multifected NSCs showing immunostaining for Nestin- a cytoskeletal NSC marker. (D) Bar graph quantifying proportion of Nestin$^+$ cells in untransfected and mC-BDNF-GFP transfected groups. (E) Representative triple merged image of mC-BDNF-GFP engineered NSCs showing immunostaining for Sox2- an NSC specific transcription factor. (F) Bar graph quantifying proportion of Sox2$^+$ nuclei between untransfected and mC-BDNF-GFP transfected groups. Error bars represent standard error of the mean.*

3.5. The differentiation potential of BDNF transfected NSCs is skewed towards increased neurogenesis

Following transplantation *in vivo*, it is well established that NSCs spontaneously differentiate into neuronal and glial lineages [38]. Following multifection, NSCs were induced to differentiate (by removal of growth factors from the medium) in order to examine the potential of NSCs to give rise to the three main daughter cell types (neurons, astrocytes and oligodendrocytes; the relative cell proportions usually generated are *ca.* 20%, 75% and 5% respectively). Microscopic assessment of the daughter cells showed normal morphologies, adherence and staining profiles. Untransfected NSCs generated *ca.* 18% neurons while BDNF engineered NSCs showed approximately two-fold increase in neurons generated (up to *ca.* 38%) (Fig. 5A-C). A previous study using SVZ-derived NSCs from rats reported that addition of BDNF in the media generated a marked increase (approximately 14-fold) in the number of neurons (2.6% *vs* 35.3%, control vs BDNF-containing differentiation media) [44]. It is important to note that the amount of BDNF administered in the previous study was 10ng/ml, ~10x higher than the concentration measured from the media supernatants in our study (*ca.*1ng/ml). Our data is in line with previous studies where BDNF induction led to a two-fold increase in neuronal numbers [45]. A possible explanation for this phenomenon is that the presence of BDNF influences the fate of these uncommitted progenitor cells, therefore favouring differentiation towards neurons [45]. Moreover, several studies have also reported that BDNF promotes the survival of neurons, which could account for the significant increase in neuronal numbers in the mC-BDNF-GFP condition [46–48].

In parallel with increased genesis of neurons, the number of astrocytes generated was reduced significantly while oligodendrocyte numbers were similar between the two groups in our study (Fig. 5C). Of the differentiated progeny, astrocytes were the predominant GFP^+ cell types (Fig.5D) as reported earlier (see manuscript part I) [49–51]. GFP expression was co-localised with extensive BDNF expression within cells (Fig. 5E). Neurons (Fig. 5F) and oligodendrocytes (Fig.5G) were rarely observed to be GFP^+. This finding is in contrast to our previous report using magnetofection/mC mediated reporter gene delivery using mC-GFP which resulted in relatively higher numbers of GFP^+ neurons and oligodendrocytes. This highlights the potential significance of using smaller size DNA vectors for cell specific transfection since mC-GFP (1.5kb in size) is approximately 2.6 smaller than mC-BDNF-GFP (4kb) used in this study. These findings also highlight the importance of using separate mC

constructs for delivery of multiple therapeutic genes instead of multicistronic constructs (multi-gene co-expression vectors resulting in increased construct size) for cell specific engineering applications.

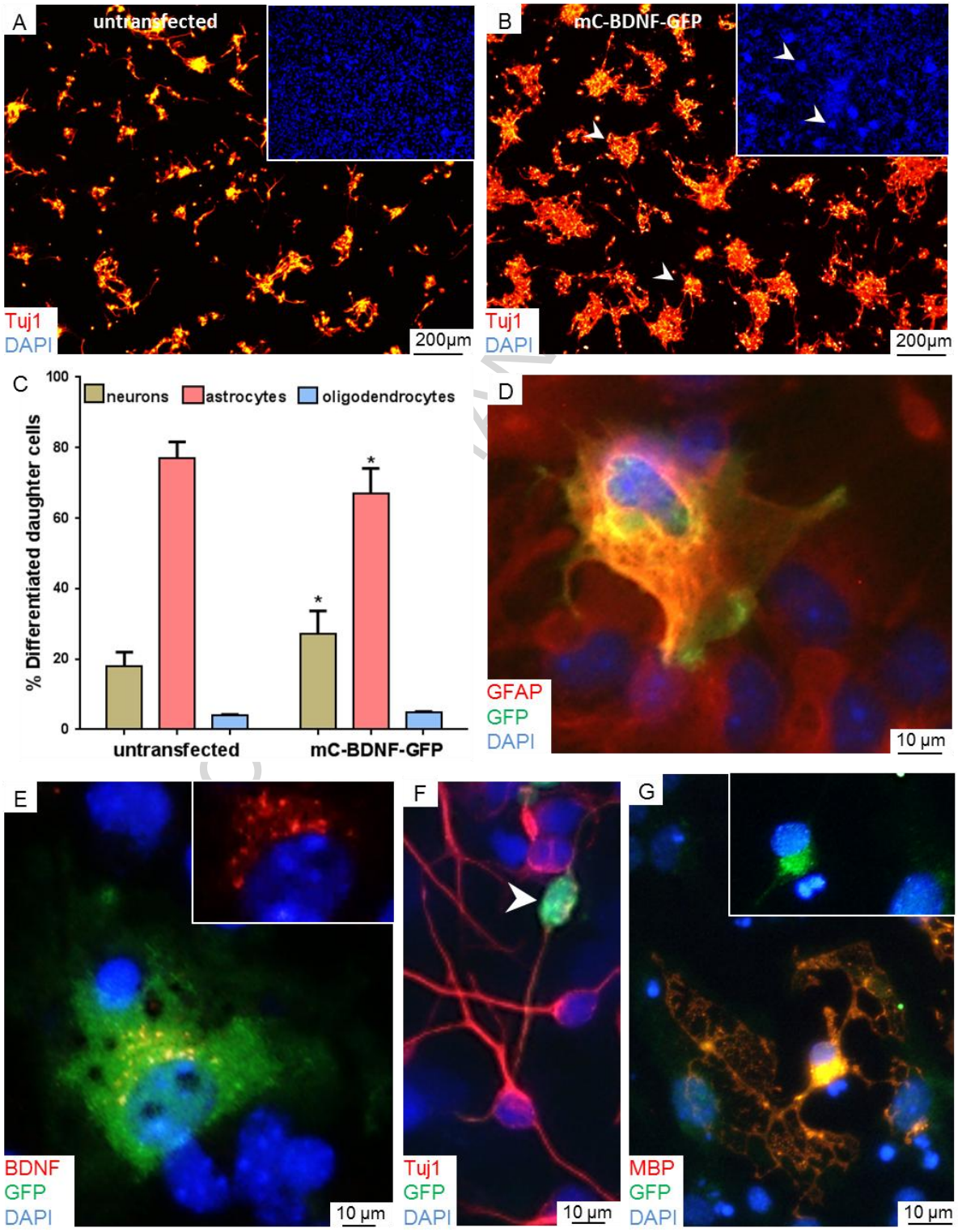

Fig. 5. BDNF overexpression results in increased neuronal number following differentiation of mC-BDNF engineered NSCs. *Micrographs showing Tuj1 immunostained*

*differentiated NSCs in (A) untransfected and (B) mC-BDNF-GFP engineered groups at 5 days post-transfection. Insets show corresponding DAPI-stained cells, arrowheads in (B) point to distinct neuronal clusters. (C) Bar graph displaying the proportions of neurons, astrocytes and oligodendrocytes generated from differentiated untransfected and mC-BDNF-GFP engineered NSCs. (D) Representative triple merged micrograph of a GFP^+ astrocyte- the predominant daughter cell population observed to be transfected post-differentiation. (E) GFP^+ cells (displaying an astrocytic morphology) were $BDNF^+$ (inset shows BDNF accumulation within the same cell) demonstrating that astrocytes derived from mC-engineered NSCs express the therapeutic protein. Representative triple merged micrographs of (F) $Tij1^+$ neurons and (G) an MBP^+ oligodendrocyte derived from mC-engineered NSCs; both were rarely observed to be transfected (an example each shown by the arrow in (F) and G inset). *$p < 0.05$, versus untransfected NSCs (student's t-test), n=3 cultures.*

3.6. BDNF overexpression enhances neurite outgrowth

Augmented BDNF levels also resulted in enhanced neurite outgrowth. The need for accelerated neurite outgrowth is key as mature neurons are required in order to facilitate the establishment and integration of functional neuronal networks with the host tissue.[52] Morphological observations showed obvious differences in neurite length at the 2 week and 4 week time points (Fig. 6 A-B, D-E). Neurite length assessments were carried out at the 2 week time point as optimal measurements for neurite length were obtained for both conditions (i.e. no overlapping neurites). Neurite length was dramatically increased in mC-BDNF-GFP treated cells compared to untransfected cells (Fig. 6C). Experimental evidence of BDNF-mediated neurite outgrowth has been shown in both NSC derived neurons as well as mature neuronal cultures [45,53]. Though BDNF levels continually decreased over the four week time period (Fig. 6F), it is important to note that this is an underrepresentation of *in vivo* BDNF levels as media was changed every few days, therefore diluting the actual BDNF levels in this *in vitro* assay. In the case of therapeutic gene delivery, a tapered profile of therapeutic expression is favoured following transplantation as (i) the functional outcome can be achieved early on i.e. increased neuronal number and enhanced maturation (ii) molecular expression of neurotrophic factors profiles change over time therefore reducing the need for specific therapeutic factors.

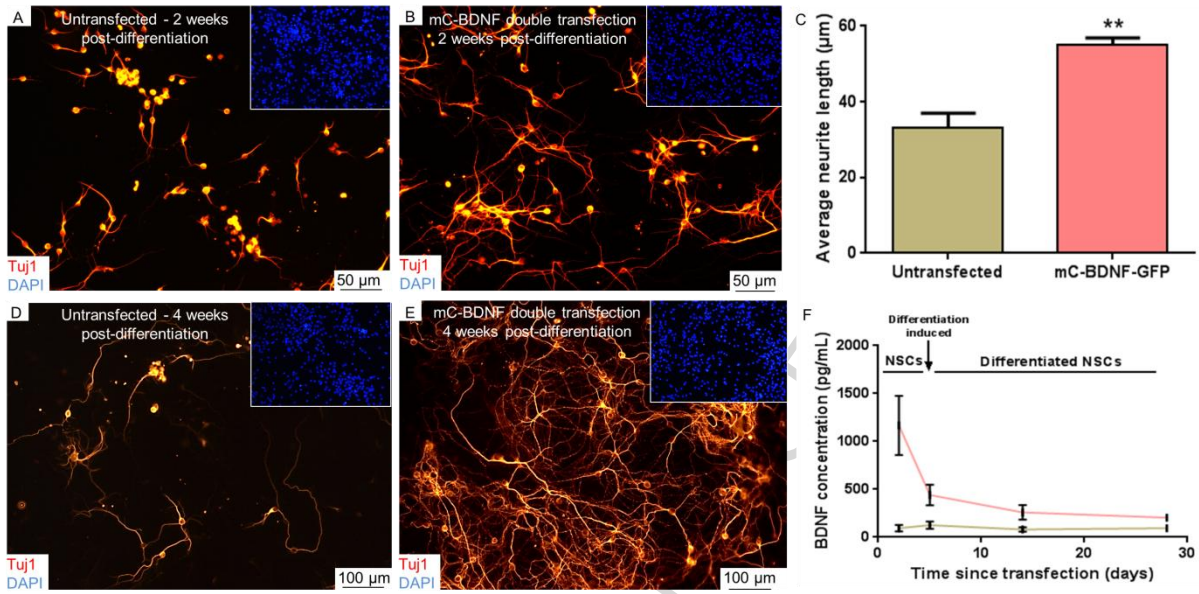

Fig. 6. Augmented BDNF levels in mC-engineered NSCs accelerate neuronal maturation. *Representative micrographs of Tuj1-immunostained differentiated NSCs for (A) untransfected and (B) mC-BDNF-GFP engineered cells, 2 weeks post-transfection. (C) Bar graph showing quantitative analysis of average neurite length between both groups at 2 weeks post-transfection. Representative micrographs of Tuj1-immunostained differentiated NSCs for (D) untransfected and (E) mC-BDNF-GFP engineered cells, 4 weeks post-transfection. (F) Temporal secretion profile of BDNF levels (measured by ELISA) in media supernatants over 4 weeks post-transfection. The insets in A-B and D-E show corresponding DAPI-stained images. **$p < 0.01$ versus untransfected control (student's t-test), n=3 cultures.*

3.7 Clinical implications of minicircle/magnetofection engineered NSCs expressing BDNF for regenerative neurology

This is the first demonstration of mC technology used in conjunction with MNPs for delivery of a neurotherapeutic biomolecule. Using reporter gene sequences, we previously suggested that such an approach could be of high clinical relevance in terms of safety, transfection efficiency and sustained gene transfer (Part I). The target protein, BDNF, is a major therapeutic candidate for a number of neurological disorders and injuries such Alzheimer's disease, stroke and spinal cord injury as demonstrated in rodent and primate preclinical models [6,54–56]. The CNS has a limited capacity to generate neurons following injury, as

the microenvironment within the site of pathology is unfavourable for the survival and differentiation of neurons. BDNF overexpression (through the paracrine effect conferred by the predominantly engineered astrocytic population) can address this issue in three ways. 1) generation of higher neuronal numbers 2) neuroprotection of differentiated neurons and 3) accelerated levels of neuronal maturation. We show the potential of mC-engineered NSCs to contribute to such regenerative events, findings that are in line with several in vitro, *ex vivo* and *in vivo* studies. We consider the combination of approaches used here to be safer, cost-effective and less time-consuming for the clinic, in comparison to viral-mediated gene delivery approaches and systemic BDNF administration. Further, neurotrophin production has high manufacturing costs [57] and systemic neurotrophic factor administration including BDNF has been shown to be less efficient as only a small amounts of the protein can cross the blood brain barrier and can result in undesirable side effects. [58] Given the pathology–homing nature of NSCs towards a site of injury, our combinatorial approach could enable a more localised delivery of the therapeutic gene enabling *in situ* biomolecule expression. In the context of improved safety and efficacy of gene delivery, the entire mC construct used in this study is biocompatible including the promoter (human EF1a) as opposed to the widely used virus-derived promoters such as CMV. Additionally, using mCs without the reporter gene (further reducing size) could enable much higher and sustained levels.

Accelerated and effective functional therapeutic outcomes require delivery of multiple genes simultaneously. For example, to further increase neuronal yield, a combination of BDNF, b-FGF and IGF1 may be required [59]. The mC system can enable a versatile yet flexible therapeutic approach in which a number of different neurotrophic factors can be delivered simultaneously to NSCs in order to achieve optimal therapeutic outcome *in vivo* [23]. This is especially pertinent in the context of addressing the complexity of neural pathology where a combinatorial therapy can also allow multiple genes to be expressed simultaneously to address *multiple* regenerative targets, in other words a '*molecular cocktail*'. As an example, these could include BDNF for axonal outgrowth, chondroitinase ABC for breakdown of repair-inhibitory matrix glycoproteins and VEGF to promote cell survival and angiogenesis (potentially via use of multicistronic plasmids), without greatly increasing vector size. We have previously demonstrated that MNPs can mediate delivery of two different reporter genes (GFP and RFP) to NSC neurospheres and monolayers (with up to 90% of transfected cells co-expressing both markers) [23] indicating that delivery of multiple therapeutic genes using

mC technology is entirely feasible. This can offer flexibility for '*tailor-made gene therapy*' depending on type and severity of the pathology being treated.

Further advantages of our approach have been discussed previously (see manuscript Part I). Briefly, both mCs and MNPs can be economically produced at a large scale at high purity with the development of novel technologies such as affinity-based chromatographic purification and flame spray method [60,61]. Additionally, the robust one-step protocol used here requires minimal training and basic laboratory equipment/containment level. mC/magnetofection technology could be also be exploited for the delivery of recombinant proteins such as Tau-neutralising antibodies [62], the prevalent component in neurofibrillary tangles, brain-penetrating biologic TNF-inhibitor in Parkinson's disease [63] and other emergent *biologics* currently being developed for effective neuro-therapeutics.

4. Conclusion

Our findings provide the first proof that mC DNA vectors in conjunction with nanoparticle carriers, can mediate delivery of a gene encoding a major neurotrophic factor, BDNF, which enhances neurogenesis and exerts neuroprotective effects. The mC DNA vector system allows for the restrictions imposed by increased plasmid size (leading to decreased nanoparticle-mediated cellular transfection) to be reduced. Our procedures were associated with high safety, and clear neuroregenerative outcomes could be observed in terms of increased genesis of neurons/enhanced neuronal maturation, consistent with the reported role of BDNF. Based on these findings, we consider that a fusion of mC DNA vector technology with nanoparticle vehicles significantly enhances the functionality of nanoparticle vector platforms, and could come to represent the genetic modification method of choice for clinical cell therapies. Additionally, given the progression of both neurotrophins and NSCs to clinical trials, we suggest that mC based engineering for translational applications appears realistic.

Acknowledgments

This research has been supported by the Biotechnological and Biological Science Research Council BB/JO17590/1.

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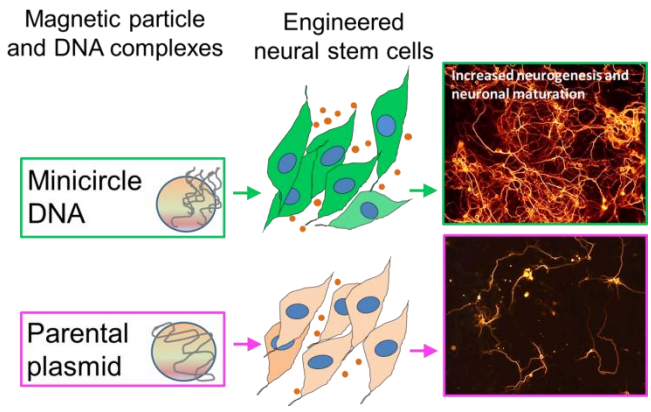
Graphical abstract



Genetic engineering of neural stem cell transplant populations to overexpress brain derived neurotrophic factor using minicircle DNA vectors and nanoparticle vehicles appears feasible and safe. The procedure results in enhanced genesis of neurons and neuronal maturation.