Magnetic Mechanoactivation of Wnt Signaling Augments Dopaminergic Differentiation of Neuronal Cells

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Wnt signaling is a key developmental pathway that regulates dopaminergic progenitor cell proliferation and differentiation during neuronal development. This makes Wnt signaling an important therapeutic target for neurodegenerative conditions such as Parkinson's disease. Wnt signaling can be modulated using peptides such as UM206, which bind to the Wnt receptor Frizzled. Previous work has demonstrated remote activation of the Wnt pathway through Frizzled using peptide-functionalized magnetic nanoparticles (MNPs) with magnetic field stimulation. Using this technology, Wnt signaling is remotely activated in the neuronal cell line SH-SY5Y, and the phenotypic response to stimulation is assessed. Results indicate β-catenin translocalization and activation of TCF/LEF responsive transcription in response to MNP and magnetic fields, which result in dopaminergic marker expression when synergistically combined with differentiation factors retinoic acid and the phorbol ester phorbol 12-myristate 13-acetate. This approach is translated into ex vivo postnatal rat brain slices modeling the developing nigrostriatal pathway. Dopaminergic marker expression is maintained in MNP-labeled SH-SY5Y cells after injection and magnetic stimulation. These results demonstrate the translational value of remote control of signal transduction for controlling neuronal precursor cell behavior and highlight the potential applications for controlled cell differentiation as part of cell therapies for neurodegenerative disease.

1. Introduction

Wnt signaling has many effects on cell behavior and controls fundamental cellular functions such as cell proliferation, migration, differentiation, and directs cell polarity. These pleiotropic effects are highly context dependent with outcomes dependent on a host of factors such as the host species, cell type, lineage, and which Wnt member or receptor is initiating signaling.[1–7] During neuronal development, Wnt signaling is a critical pathway controlling and tightly regulating development and patterning of the forebrain, midbrain, and hindbrain.[8–10] However, the nuances of regulated Wnt signaling during brain development are highlighted in other studies which have found varied effects of Wnt signaling during neurogenesis. For instance, Wnt stimulation has been shown to either defer neurogenesis or indeed promote it.[11] This is also evident by loss of Wnt pathway regulation via the Wnt messenger β-catenin through adenomatous polyposis coli (APC), which has been shown to decrease neuroblast and neural stem cell populations in the developing cortex.[12] Other studies have found that Wnt stimulation enhances neurogenesis. Hirabayashi et al. in 2006 reported that increased expression of Wnt7a induced neurogenesis of neural progenitor cells (NPCs) in vitro.[13]

The Wnt signaling cascade is of significant interest to Parkinson's disease, where there is a compelling clinical need for new cell or pharmacological therapies to restore or replace dopaminergic cell populations lost to the disease. Parkinson's disease (PD) arises from the progressive degeneration of midbrain dopaminergic (mDA) neurons in the substantia nigra pars compacta (SNc). Wnt signaling is known to play a prominent role in dopaminergic neuron formation. Several studies suggest that mid brain dopaminergic (mDA) neurons require Wnt signaling for their generation.[14–17] In the midbrain, β-catenin signaling inhibits Sonic hedgehog (Shh) signaling and causes the induction of dopaminergic differentiation through the expression of DA markers.[16,17] Prakash et al. also showed that Wnt1 preserved *Otx2* levels in the ventral midbrain, which is required to generate dopaminergic neurons.[18] Castelo-Branco et al. showed that Wnt-1 enhances the proliferation of *Nuur1+* expressing precursors, while Wnt-5a enhanced the number of *Nuur1+* precursors with a dopaminergic phenotype.[14]

SH-SY5Y is a well-established model cell line in Parkinson's disease research. When cultured with neurotrophic factors, SH-SY5Y may differentiate into neuron-like cells with neuronal

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morphology while expressing typical neuronal markers such as betaIII-tubulin and MAP2.[19,20] Furthermore, SH-SY5Y may be specified toward dopaminergic-like neurons when cultured with the phorbol ester phorbol 12-myristate 13-acetate (PMA).[21,22] SH-SY5Y have since become a popular cell line for in vitro modeling of neurodegenerative diseases such as Parkinson's disease. Recent work has demonstrated a role for Wnt signaling during SH-SY5Y differentiation[23] and has shown that Wnt activation can protect SH-SY5Y from the toxic effects of 6-[OHDA].[24,25] In contrast, inhibition of Wnt signaling was shown to reduce differentiation and increase susceptibility to the cancer drug doxorubicin.[26]

Altogether this suggests that the Wnt pathway could be a key target for therapeutic intervention in Parkinson's disease. However, the clinical application of Wnt proteins have been hampered due to their complex biochemical nature and extensive post-translational modifications which confers hydrophobic properties and further complicates pharmaceutical production.[27,28] Some of these issues may be circumvented by using MNP to target and activate Wnt receptors directly.

We have previously demonstrated the feasibility of remotely targeting and activating Wnt signaling in stem cells using peptide-functionalized MNP targeted to Frizzled receptors, while using oscillating magnetic fields to control and amplify signaling. We have also shown the potential applications of this approach in a tissue engineering context where Wnt-activating MNP was shown to initiate bone formation in an ex vivo chick femur model of bone development.[29,30] The efficacy of magnetic activation of receptors for tissue engineering is further demonstrated with other known and putative mechanosensitive receptors such as the TREK1 ion channel,[31,32] PDGF receptor,[33] activin A type II receptor,[34] as well as integrins.[35] In this study, we report on the feasibility of using peptide–MNP conjugates for remote mechanoactivation of Wnt signaling and augmentation of neuronal differentiation and dopaminergic marker expression in SH-SY5Y cells. Expression of target Frizzled receptors was first confirmed in the SH-SY5Y cell line. Next, MNP were functionalized with a synthetic peptide known as UM206, the structure of which is based on a conserved fragment of Wnt3a and Wnt5a. UM206 specifically binds to Frizzled 1 and 2 receptors and is able to trigger canonical Wnt signaling. UM206 exists in two conformations, a linear and a cysteine bridged cyclic conformation, both of which can exhibit different effects on signaling activity in vitro.[36] Wnt pathway activation using either linear or cyclic UM206-conjugated MNP and oscillating magnetic fields was demonstrated in SH-SY5Y using Wnt reporter systems. The effect of remote Wnt pathway activation on SH-SY5Y dopaminergic differentiation was then explored in monolayer cultures in the presence of the dopaminergic differentiation factor PMA. Finally, the translation of this approach was demonstrated in ex vivo postnatal rat brain slices, where MNP-labeled cells were injected into the striatum and stimulated over 7 days. The overall aim of this research was to demonstrate that remote MNP activation of Wnt signaling pathways in neuro-progenitor cells can regulate and fine tune neuronal marker expression. This technique has clear applications for in vitro developmental research and may be a useful signaling tool for future cell therapies.

2. Results

2.1. Frizzled Receptor Expression in SH-SY5Y Cells

Target receptor expression was first probed in SH-SY5Y cells cultured in basal using rt-PCR. A base level of expression for Frizzled 1 and 2 receptors was observed in SH-SY5Y cells (**Figure 1**A) with both receptors shown to be well expressed in comparison to the house-keeping gene GAPDH. SH-SY5Y was then subjected to RA and brain-derived neurotrophic factor (BDNF)-mediated neuronal differentiation, and q-rt-PCR was used to map changes in Frizzled gene expression during differentiation. Expression of both Frizzled 1 and Frizzled 2 were found to increase after 4 days (trend only) and remained relatively constant up to day 12 where peak expression was observed with a significant increase in Frizzled 2 expression observed at this time point (Figure 1B). Immunocytochemistry was used to assess neuronal differentiation and Frizzled expression of differentiated SH-SY5Y cells. BetaIII-tubulin staining confirmed neuronal differentiation with most betaIII-tubulin positive cells also co-expressing Frizzled 1 and Frizzled 2 receptors (Figure 1C).

2.2. Wnt Regulates Dopaminergic Gene Expression

The role of canonical Wnt signaling during RA- and BDNF-induced neuronal differentiation was explored by assessment of dopaminergic gene markers after culture in different neuronal induction media formulations containing RA, BDNF, and recombinant human Wnt3A. Addition of BDNF to RA media had minor stimulatory effects on dopamine receptor D2 (DRD2) and TH expression, respectively, and had no overall effect on dopamine receptor D3 (DRD3) and DAT expression. Incorporation of rhWnt into RA and BDNF media further increased DRD2, DRD3, and TH expression, respectively, with a significant increase for DRD2. However, no observable effect was observed on DAT expression. Replacement of BDNF with rhWnt reduced DRD2 expression to base levels, maintained TH expression, and caused a variable increase in DAT expression (**Figure 2**).

2.3. SH-SY5Y Labeling with MNP

Dextran-MNP with a diameter of 250 nm was functionalized with the Frizzled targeting peptide UM206 either in the linear conformation (Linear-UM206-MNP) or cyclic conformation (Cyclic-UM206-MNP). Dextran-coated particles were used in this study, as the dextran matrix protects the magnetite core, has low toxicity, confers bio-compatibility, and is amenable for clinical translation.[37–40] SH-SY5Y cells were then labeled with either UM206-MNP types or non-specific control (IgG)-MNP. Immunocytochemistry to stain the MNP-dextran matrix was then performed to qualitatively assess MNP association with the cells. Fluorescent microscopy confirmed Linear-UM206-functionalized MNP (L-UM206-MNP), Cyclic-UM206-MNP (C-C-UM206-MNP), and Control (IgG)-MNP, all bound to cells 3 h after labeling. Labeling efficiency was similar across all groups (**Figure** 3A). Confocal microscopy was then used to determine
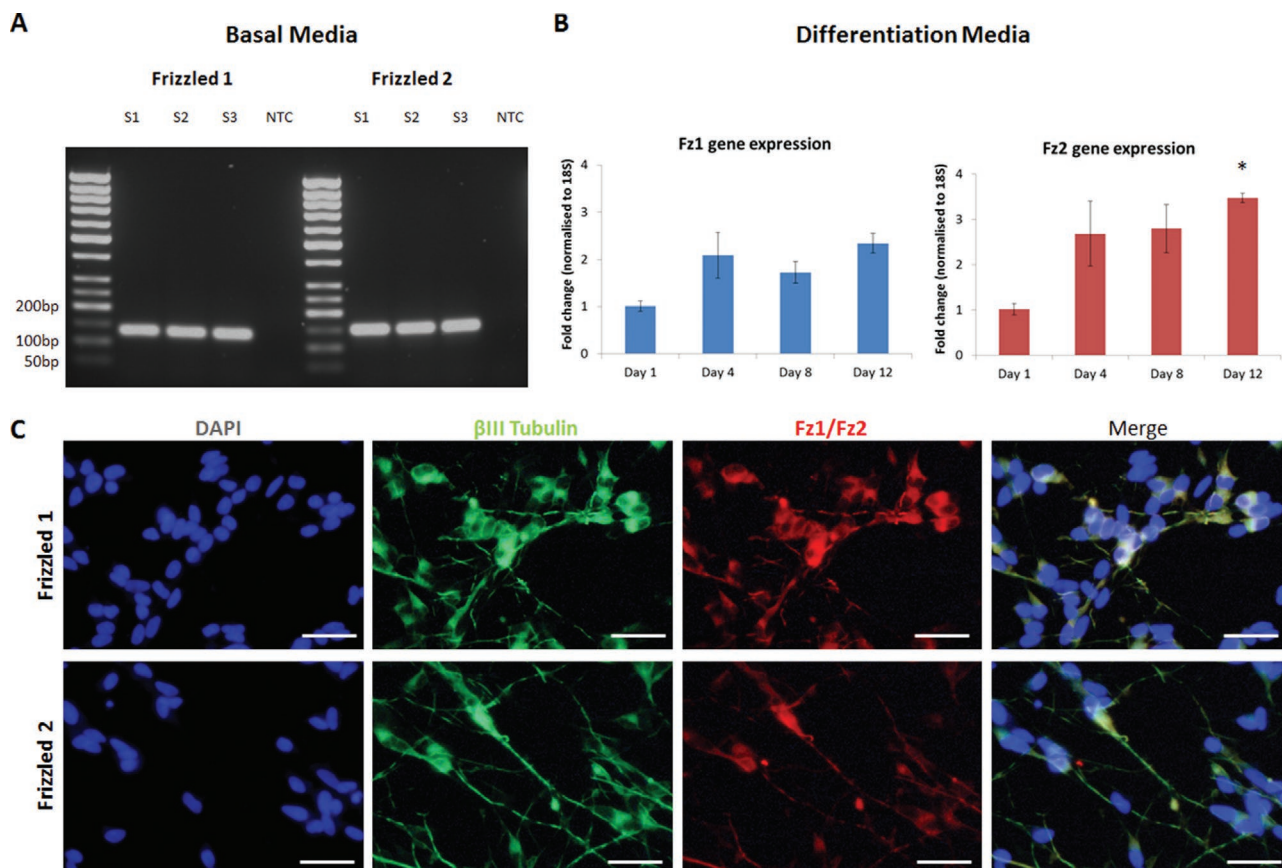
Figure 1. Frizzled receptors are expressed by SH-SY5Y during neuronal differentiation. Reverse transcription-PCR confirmed Frizzled 1 and 2 expression in SH-SY5Y in basal conditions, $n = 3$ (S1–3 = sample 1–3, NTC = no template control) (A). Frizzled 1 and 2 were both up-regulated during SH-SY5Y neuronal differentiation using RA and BDNF as shown by quantitative reverse transcription-PCR; $n = 4$; error bars represent SEM; * represents $p < 0.05$ (B). RA and BDNF differentiated cells expressing β3-tubulin also co-expressed Frizzled 1 and 2 (C); representative images of $n = 3$; bar represents 25 µm.

the sub-cellular location of MNP. Post labeling, MNP remained predominantly bound to the cell membrane (Figure 3B(i–iv)).

2.4. UM206-MNP Initiate β-Catenin Activation

The effect of both Linear and Cyclic-UM206-MNPs on downstream Wnt pathway activity in SH-SY5Y cells was then assessed. The mobilization of the Wnt pathway messenger β-catenin in response to UM206-MNP was first assessed using immunocytochemistry. Cells were treated with UM206-MNP (linear or cyclic) or Control (IgG)-MNP and stimulated with or without magnetic field stimulation for 3 h. β-catenin activation was found to be elevated after treatment with Cyclic UM206-MNP alone (C-C-UM206-MNP) to a similar level as Wnt-CM after 24 h, while Linear-UM206-MNP (L-UM206-MNP) appeared to have a negligible effect (non-significant). An added effect was observed when either Linear or Cyclic-UM206-MNPs were applied in conjunction with magnetic field stimulation. In contrast, control MNP conjugated with non-specific-IgG (Control (IgG)-MNP) had a negligible effect on β-catenin activation with or without magnetic field treatment (**Figure** 4A). The trend was confirmed after the percentage of activated cells (cells containing active β-catenin) was quantified. Significant

increases in β-catenin-positive cells were observed after treatment with L-UM206-MNP only when combined with magnetic field stimulation, C-C-UM206-MNP (with or without magnetic field), or Wnt-CM. In contrast control groups treated with magnetic field alone or Control (IgG)-MNP had no effect over the non-treated cells only control (Figure 4B).

2.5. UM206-MNP Induce TCF/LEF Signaling

SH-SY5Y was then transfected with a TCF/LEF-responsive luciferase reporter and treated with UM206-MNP with or without magnetic field stimulation. Reporter activity was elevated after treatment with either Linear UM206-MNP (L-UM206-MNP) or Cyclic UM206-MNP (C-C-UM206-MNP) after 6h with increased activity observed with C-C-UM206-MNP, thereby confirming UM206-MNP-mediated activation of Wnt signaling in the SH-SY5Y cell line (**Figure** 5A(i)). An added effect was then observed when either UM206-MNP types were applied in conjunction with magnetic field stimulation, suggesting a beneficial effect of magnetic-mediated mechanoactivation on Wnt pathway activity. In contrast, treatment with Wnt-CM caused a mild (non-significant) activation at this time point. After 24 h, reporter activity remained elevated in response to UM206-MNP
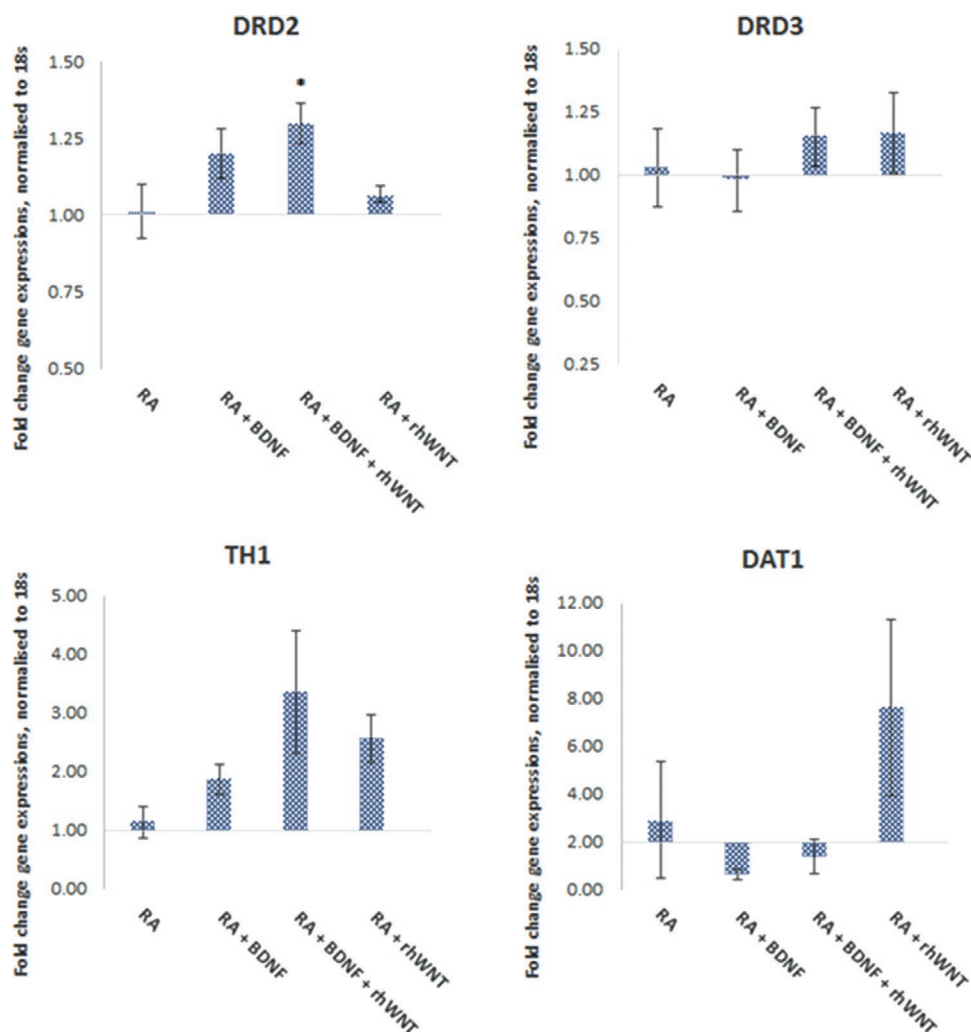
Figure 2. Wnt3a synergizes with RA or BDNF to regulate dopaminergic gene expression. Gene expression analysis of dopaminergic genes (DRD2, DRD3, TH, and DAT) in response to different serum-free media formulations incorporating RA, BDNF, and rhWnt3a. Addition of BDNF to RA media led to minor (trend only) increases in DRD2 and TH expression and had no overall effect on DRD3 and DAT expression. Incorporation of rhWnt into RA and BDNF media further increased DRD2, DRD3, and TH expression (significant increase in DRD2) but had no observable effect on DAT expression. Replacement of BDNF with rhWnt reduced DRD2 expression to base levels, maintained TH levels, and caused variable increases in DAT expression. Figures show mean fold change in gene expression compared to RA alone; expression was normalized to 18S; error bars represent SEM; $n = 4$; *denotes $p < 0.05$.

with similar responses to both UM206-MNP types observed. The activation response by UM206-MNP was also found to be comparable to Wnt-CM (also significant) at this time point (Figure 5A(ii)). In contrast to the Frizzled targeted MNP, control-MNPs functionalized with RGD peptide (RGD-MNP) or non-specific IgG (Control (IgG)-MNP) were used to induce generic membrane stimulation. Both of these control MNPs caused non-significant fluctuations on reporter activity with no overall effect observed (Figure 5B(i–ii)).

2.6. UM206-MNP Enhance Dopaminergic Marker Expression in Dopaminergic Media

The effect of Wnt-targeted MNP on dopaminergic differentiation was then evaluated using immunocytochemistry to assess expression of key dopaminergic markers DAT and TH. Cells were cultured in reduced serum neuronal induction media (RA media) for 3 days, then labeled with MNP. Media was then switched to dopaminergic induction media containing PMA for further 4 days with intermittent magnetic stimulation. Overall expression of dopaminergic markers DAT and TH was maintained by Wnt pathway activating MNP (UM206-MNP) with or without magnetic field stimulation, with localized increases in marker expression observed in some cell populations. In contrast, non-specific Control (IgG)-MNP (either with or without magnetic stimulation) and Wnt-CM had no discernible effect on dopaminergic marker expression, which remained comparable to base levels observed in the control groups (**Figure 6**A). Quantification of the TH/DAT-positive cell numbers confirmed this trend. An overall marginal increase in TH/DAT-positive cell numbers was observed in response to either L-UM206-MNP
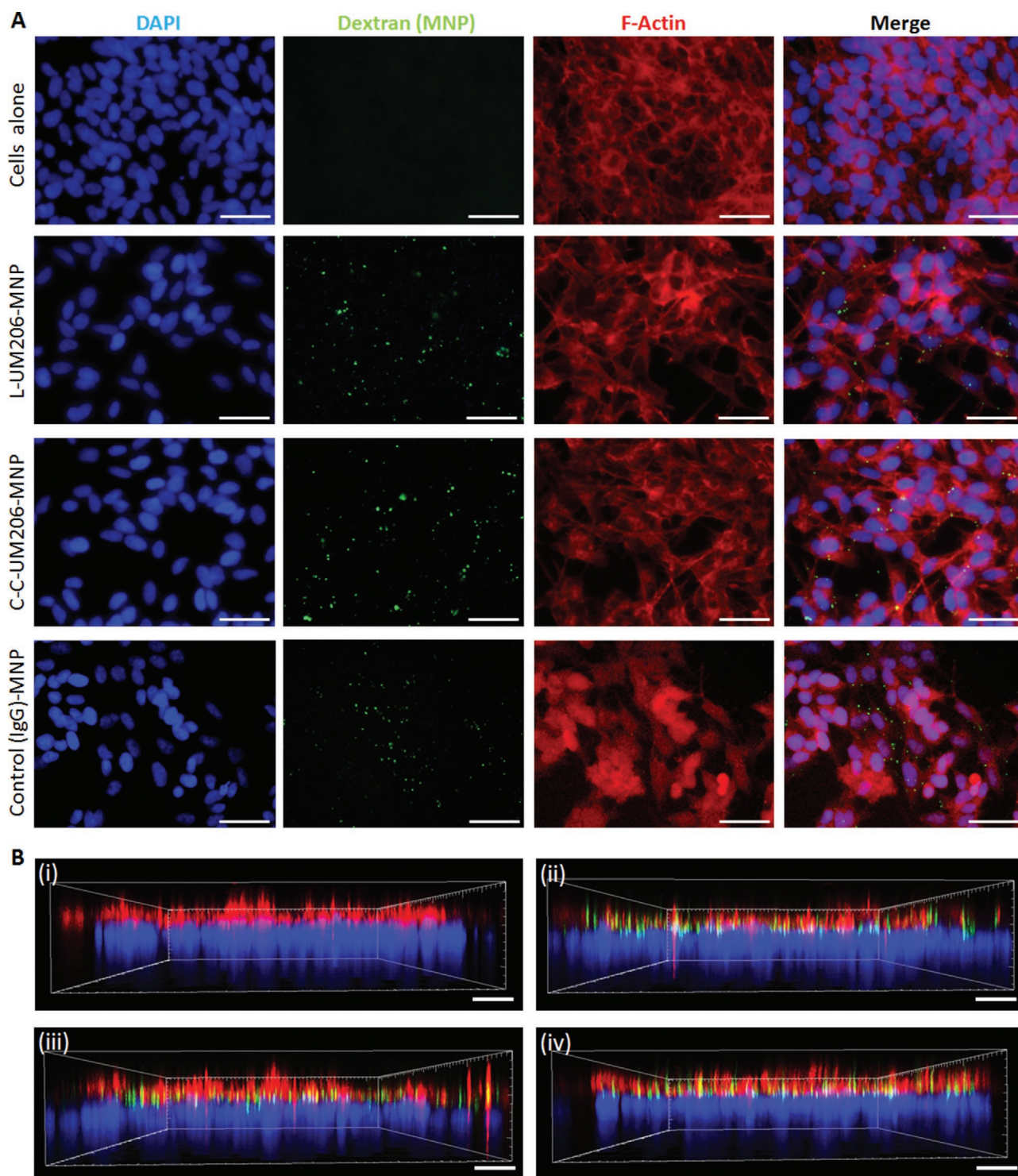
Figure 3. Labeling of SH-SY5Y with functionalized MNP. A) Immunofluorescence images showing unlabeled cells (top row) and cells labeled with L-UM206-MNP (second row), C-C-UM206-MNP (third row), and Control (IgG)-MNP (bottom row). Nuclei are shown by DAPI staining (blue); MNP are shown by anti-dextran staining (green); and cell cytoskeleton by Actin staining (red). Representative images of $n = 3$; scale bar represents 25 µm. B) Confocal microscopy confirmed that most MNP are bound at the cell membrane 3 h post-labeling. Cells alone are shown in (i), L-UM206-MNP (ii), C-C-UM206-MNP (iii), and Control (IgG)-MNP (iv). Nuclei are shown by DAPI staining (blue), MNP by anti-dextran staining (green), and cell membrane by CM-Dil staining (red). Representative images of $n = 3$; scale bar represents 30 µm.
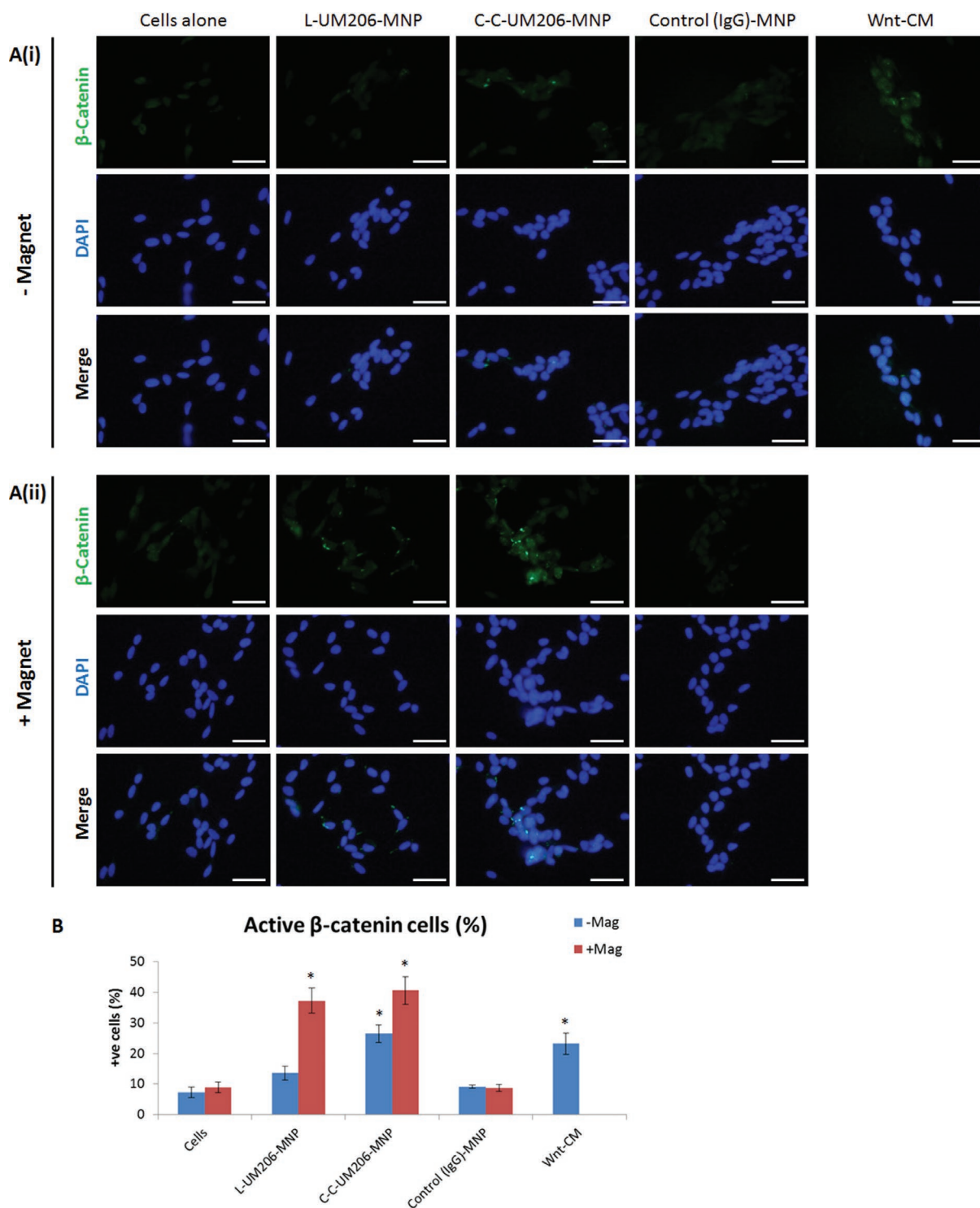
Figure 4. UM206-MNP initiates β-catenin activation. Active β-catenin mobilization was visualized by immunofluorescence 24 h after 3 h (1 Hz) magnetic stimulation. Untreated cells and cells treated with L-UM206-MNP or Control (IgG)-MNP showed negligible levels of β-catenin activation (A(i)), while addition of Wnt3A-conditioned media (Wnt-CM) or C-C-UM206-MNP resulted in minor increases in nuclear translocation over baseline levels. Treatment with magnetic field stimulation alone or with Control (IgG)-MNP had no observable effect on β-catenin translocation over basal levels, while an increase over the magnetic field–only control was observed when cells were treated with L-UM206-MNP or C-C-UM206-MNP with magnetic field stimulation (A(ii)). Cell nuclei are shown by DAPI staining (middle row). Representative images of $n = 3$; scale bar represents 25 µm. Quantification of active β-catenin cell numbers confirmed this trend with significant increases in active β-catenin positive cells after treatment with L-UM206-MNP (with magnetic field only), C-C-UM206-MNP (with or without magnetic field), or Wnt-CM. In contrast, control groups treated with magnetic field alone or Control (IgG)-MNP had no overall effect on active β-catenin cell numbers (B). Error bars represent SEM; $n = 3$; * denotes $p < 0.05$.
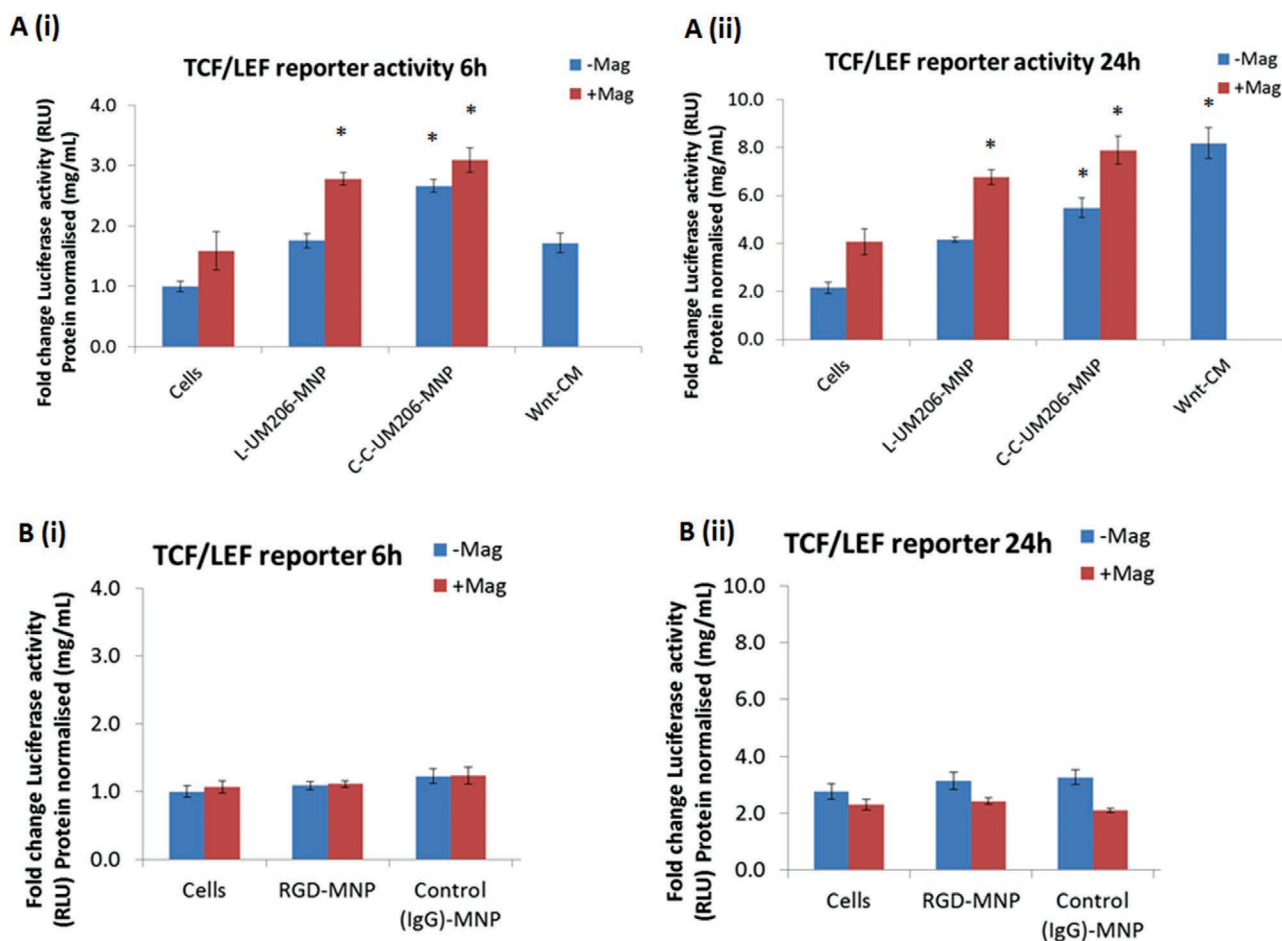
Figure 5. UM206-MNP induces TCF/LEF reporter activation. A TCF/LEF responsive luciferase reporter was used to assess end point Wnt signaling after 3 h (1 Hz) treatment with MNP and magnetic stimulation. After 6 h, treatment with L-UM206-MNP or Wnt-CM resulted in marginal increases in reporter activity, while treatment with C-C-UM206-MNP resulted in significant reporter activation. Treatment with magnetic field alone had no significant effect on reporter activity. In contrast, treatment with either L-UM206-MNP or C-C-UM206-MNP with added magnetic field stimulation resulted in significant reporter activation (A(i)). The same trend continued up to 24 h with significant reporter activity observed in response to L-UM206-MNP (with magnetic field) and C-C-UM206-MNP (with or without magnetic field). Wnt-CM also significantly increased reporter activity at this time point (A(ii)). Control particles functionalized with either RGD peptide (RGD-MNP) or IgG (Control IgG-MNP) caused no significant increases in reporter activity after at 6 h (B(i)) or 24 h (B(ii)). Error bars represent SEM; $n = 4$. * denotes $p < 0.05$.

or C-C-UM206-MNP treatment alone (trend only). Addition of magnetic field stimulation augmented this effect with a significant increase in TH/DAT-positive cells observed after treatment with C-C-UM206-MNP with magnetic field stimulation. In contrast, TH/DAT-positive cell numbers remained comparable to the non-treated control after treatment with either magnetic field alone, Wnt-CM, or Control (IgG)-MNP with or without magnetic stimulation (Figure 6B).

2.7. MNP Maintain TH Expression in Injected Cells Cultured in Rat Brain Slices

Due to the increased signaling response to C-C-UM206-MNP, these MNPs were then used in subsequent ex vivo experiments. Both C-C-UM206 and Control (IgG)-MNP-labeled cells were delivered into the striatum of postnatal rat brain slices and intermittently treated with magnetic field stimulation over

7 days. Slices were cultured in RA-supplemented media for 3 days followed by PMA-supplemented media for further 4 days to support dopaminergic differentiation of injected cells. Fluorescent microscopy confirmed that cells were still present at the striatum injection site after 7 days, as shown by Pkh26 staining (**Figure 7**A). Furthermore, some populations of injected cells were found to express TH at levels which were broadly consistent across all groups (Figure 7B), indicating a dopaminergic-like phenotype.

3. Discussion

In this study, we examined the role of Wnt signaling in neuronal differentiation of SH-SY5Y cells and probed the neurogenic effects of remote mechanoactivation of Wnt signaling using magnetic nanoparticles and magnetic fields. We first examined basal Frizzled receptor expression levels in SH-SY5Y.
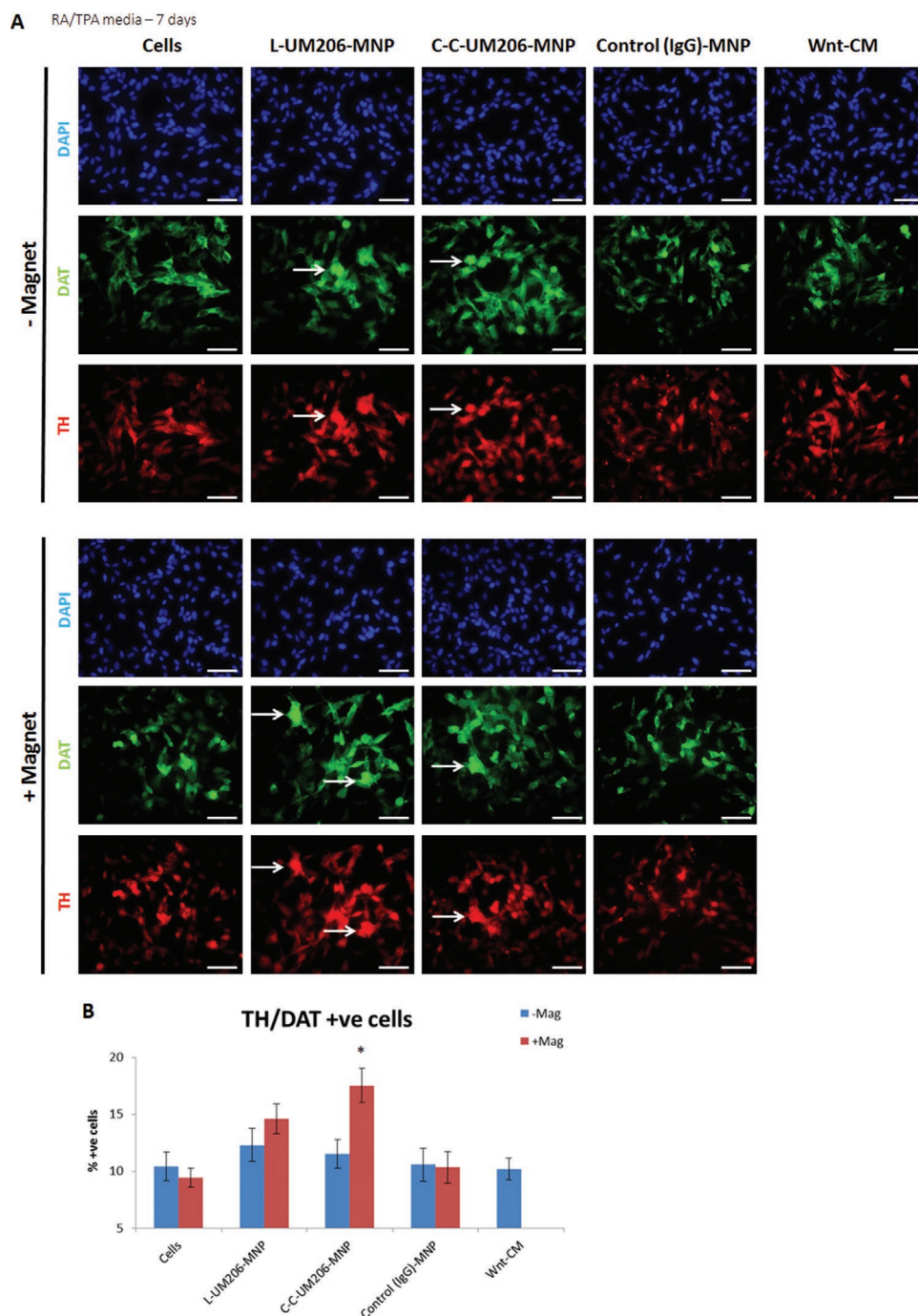
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Figure 6. UM206-MNP enhances dopaminergic marker expression in dopaminergic media in vitro. Fluorescent images showing DAT (green) and TH (red) after 7 days of intermittent magnetic stimulation (3 h, 1 Hz). DAPI was used to visualize cell nuclei (blue). Non-treated cells and Control-MNP treated cells both showed a base level of DAT and TH expression, while treatment with L-UM206-MNP or C-C-UM206-MNP (without magnetic field) or Wnt-CM over 7 days resulted in marginal changes in DAT and TH expression. Both markers were largely unaffected by magnetic field stimulation alone. In contrast, treatment with L-UM206-MNP or C-C-UM206-MNP with magnetic field resulted in increases in DAT and TH expression over magnetic field–only control. Control (IgG)-MNP with magnetic field treatment reduced marker expression to control levels (A). Representative images of $n = 3$; scale bar = 100 µm. Quantification of TH/DAT-positive cell numbers confirmed this trend with increases in overall TH/DAT-positive cell numbers in response to either L-UM206-MNP or C-C-UM206-MNP alone (trend only). Addition of magnetic field stimulation augmented this effect with significant increases in TH/DAT-positive cells observed after treatment with C-C-UM206-MNP with magnetic field stimulation (B). Error bars represent SEM; $n = 3$; * denotes $p < 0.05$.
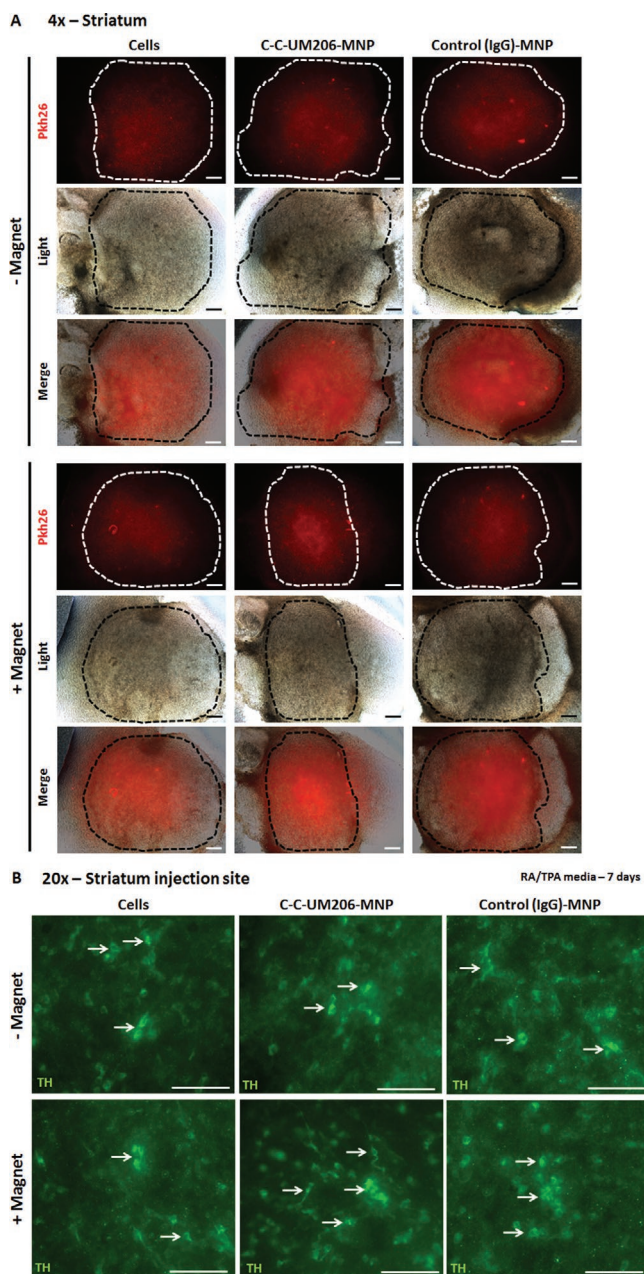
Figure 7. MNP maintains TH expression in injected cells cultured in ex vivo rat brain slices. Fluorescent and light microscopy images showing location of Pkh26-labeled cells 7 days after injection into the striatum of rat brain slices of the nigrostriatal pathway (A). Cells are shown by Pkh26 staining (red). Rat brain tissue is shown in the light channel. The approximate striatum boundary is denoted by the dotted line. Representative images of $n = 3$; scale bar = 200 µm. TH expression was detected in some populations of injected cells in the striatum (indicated by arrows) after 7 days of intermittent magnetic stimulation (3 h, 1 Hz), with expression levels broadly consistent across all groups (B). Representative images of $n = 3$; scale bar = 100 µm.

We confirmed that both Frizzled 1 and 2 are expressed in SH-SY5Y, consistent with Wei et al.,[24] who also showed Frizzled 1 is expressed in SH-SY5Y (Frizzled 2 was not studied). The increasing expression of Frizzled receptors during RA- and BDNF-induced neuronal differentiation also suggests a role for Wnt signaling, particularly during the early stages of neuronal differentiation. Furthermore, our observation that differentiated neuronal cells also co-expressed Frizzled receptors is further evidence that Wnt signaling is important in mature neuronal function. These results are to be expected given the well-established link between Wnt signaling and neuronal development. Other groups have also alluded to the role of Wnt signaling during neuronal stem and pre-cursor cell differentiation.[14,16–18] Our results also suggest that cross-talk between the Wnt pathway and RA and BDNF signaling is important for regulation of neuronal differentiation. Previous work has demonstrated the link between RA, BNF, and Wnt signaling. For example, Yang et al. showed that BDNF overexpression promoted the growth of human embryonic spinal cord neurons and upregulated Wnt, Frizzled, and Dsh expression.[41] Uemura et al. showed that retinoic acid (RA) causes increases in nuclear β-catenin levels with subsequent increases in cyclin D1 expression.[42] Furthermore, RA has also been shown to activate WNT1 signaling, while repressing expression of Wnt antagonists.[43]

We then confirmed that it was possible to remotely activate the Wnt pathway in SH-SY5Y using mechano-stimulation of Frizzled receptors mediated by magnetic fields and peptide-functionalized MNP. In this study, dextran-coated particles were functionalized with UM206 peptides allowing specific binding to Frizzled receptors expressed at the cell membrane. This allowed remote stimulation and activation of the Wnt signaling cascade by applying an external oscillating magnetic field which generates a torque on the MNP–receptor complex with forces in the pN range, as described previously.[29,44] Mechanoactivation of Wnt signaling has been previously reported through multiple mechanisms. Subjecting human mesenchymal stem cells (hMSCs) or osteoblasts to oscillatory shear stress or dynamic mechanical strain has been shown to promote β-catenin mobilization and TCF/LEF-responsive gene transcription.[45–47] While further work is required to ascertain the precise mechanism of MNP-mediated activation of Frizzled receptors, our previous work suggests that Frizzled clustering may be part of this initial activation mechanism, an effect which may be enhanced upon magnetic oscillation of the receptor-bound MNP.[30] However, endocytosis of the Wnt-receptor signalosome is also known to play a role in Wnt signal transduction.[48,49] Due to the fact that MNPs are also commonly endocytosed by target cells,[50] internalization of the receptor-bound MNPs may in fact also play a role in MNP-mediated signaling. The long-term uptake and ultimate fate of receptor-bound MNP was not investigated in the current study, and these remain intriguing questions for future studies to explore.

Downstream indicators of Wnt pathway activity in response to UM206-MNP were investigated by assessing the hallmarks of Wnt pathway activation: β-catenin localization and end-point activation of TCF/LEF-responsive signaling.[51] Our results demonstrate that both L-UM206-MNP and C-C-UM206-MNP were capable of initiating nuclear translocation of β-catenin and activating TCF/LEF reporter expression in SH-SY5Y cells, especially when combined with magnetic field stimulation. These results are in agreement with our previous study which showed remote UM206-MNP-mediated activation of Wnt signaling in

hMSCs.[30] Interestingly, in contrast to our previous work in hMSCs, in SH-SY5Y cells, we observed broadly comparable pathway activation between L-UM206 and C-C-UM206-MNP, with somewhat increased pathway activity observed after C-C-UM206-MNP treatment. The activity of C-C-UM206-MNP may be expected given that the cyclic peptide has previously been shown to induce TCF/LEF activity.[36] One explanation for the cell-type dependent effects of these peptides could be differences in receptor expression levels between the two cell types; this could conceivably alter the binding kinetics of the functionalized MNP and therefore alter the degree of pathway activation between the two peptide conformations.

We then sought to elucidate the effects of MNP-mediated Wnt pathway activation on SH-SY5Y cell fate. Wnt activation using UM206-MNP was shown to augment dopaminergic differentiation of SH-SY5Y in conjunction with the Phorbol ester PMA. This demonstrates that mechanoactivated Wnt signaling can be a beneficial and synergistic stimulus for regulating dopaminergic differentiation of neuronal precursor cells. In this study, mechanoactivation was combined with the phorbol ester PMA, which has been previously shown to induce neurite extension and up-regulate BDNF, NeuN, and neuron-specific enolase expression,[52–55] while promoting tyrosine hydroxylase expression possibly through protein kinase C activation.[56] In the context of this work, our results could be expected given the neurogenic and dopaminergic phenotype inducing role of both PMA and Wnt signaling.

As dopaminergic differentiation is tightly controlled in vivo and relies on a number of pathways, it is therefore important to acknowledge the reciprocal role of Wnt signaling as part of a network of signaling pathways, such as PMA-induced signaling, which are required for properly controlled dopaminergic neuron development and function. Future work should investigate MNP-mediated control over differentiation of neuronal stem cells as well as tracking the long-term phenotype of MNP-differentiated cells. Our results clearly highlight the complex and contrasting role of Wnt signaling in regulating dopaminergic differentiation. In our study, in contrast to transient MNP-mediated Wnt activation, combining PMA with a constant Wnt3A signal provided by Wnt-conditioned media led to negligible changes on dopaminergic marker expression. This indicates that a constant canonical Wnt signal may be restricting terminal differentiation of some cells. This effect has also been observed in ventral midbrain (VM) cells treated with Wnt3a, which expanded the cell population but restricted their dopaminergic differentiation.[14] Incidentally, this effect was also mirrored in our previous work which showed that hMSC differentiation was reduced in Wnt-conditioned media cultured cells.[30] Given the complex role of Wnt signaling in regulating dopaminergic differentiation, this result may also be expected. In the context of dopaminergic differentiation, Castelo-Branco et al. elegantly demonstrated the opposing effects that Wnt signaling can have on dopaminergic differentiation of precursor cells. In their study, canonical Wnt3a was shown to enhance Nurr1+ dopaminergic precursor cells while restricting TH+ cell numbers; in contrast, non-canonical Wnt5a led to enhancement of TH+ cells from Nurr1+ populations.[14] This suggests that canonical Wnt signaling may be beneficial in progenitor populations while withdrawal of Wnt or a switch to non-canonical

signaling may be required for terminal dopaminergic differentiation. In summary, our results highlight the beneficial role that transient and controlled activation of Wnt signaling with MNP can have on neuronal differentiation as opposed to a constant Wnt stimulus provided by exogenous Wnt protein.

We then sought to translate our approach into an ex vivo model of the nigrostriatal pathway. We demonstrated delivery of MNP-labeled cells into postnatal rat brain slices and showed that ex vivo stimulation of MNP-labeled cells had a similar, if somewhat restricted, effect on dopaminergic marker expression in this model. This suggests that paracrine factors in the host tissue may also be regulating implanted cell behavior. Indeed, other groups have shown that fibroblast growth factors, Shh, as well as both canonical and non-canonical Wnts are expressed in the ventral midbrain during development.[10,14,15,17,57] It is therefore feasible that the complex spatial and signaling cues that are replicated in ex vivo slice models may directly influence the fate of delivered cells by regulating multiple pathways, including Wnt Signaling. This serves to show the importance of controlling external spatial and biochemical cues for optimal differentiation of implanted cells and highlights one of the major challenges that must be overcome for successful adoption of cell therapies in neurodegenerative disease.

4. Conclusion

In conclusion, there is considerable interest in developing Wnt modulators to therapeutically regulate Wnt signaling. Easily synthesized ligands conjugated to magnetic nanoparticles therefore present an attractive route for targeting cell signaling pathways. The use of conjugated MNP to activate Wnt signaling may have useful applications as a research tool or in drug discovery in an approach that is amenable to translation to the clinic. Furthermore, the ability to tune signaling activity by inducing MNP torque using external magnetic fields affords flexible and temporal control over the translational forces applied to target receptors and subsequent activation of differentiation pathways. Our results demonstrate that remote activation of cell signaling pathways using targeted magnetic particles and alternating magnetic fields can be used to influence neuronal cell signaling and precursor cell differentiation toward a dopaminergic phenotype. This unique approach opens a potential avenue for novel therapeutic treatments, in the form of injectable cell therapies, with potential applications for treating neurodegenerative diseases such as Parkinson's disease.

5. Experimental Section

Cell Culture: SH-SY5Y cells (PHE, UK) were grown to confluency in basal media consisting of DMEM:F12 (Corning) supplemented with 1% antibiotics and 10% fetal bovine serum (FBS) (Gibco) and incubated at 37 °C and 5% CO_2. Culture medium was replaced twice per week, and cells were passaged once they reached approximately 80% confluency. Cells of passage 18–28 were used in all experiments. For neuronal differentiation, after seeding on pre-coated laminin (Sigma) plates, cells were cultured in basal media for 24 h to allow cell attachment. Cells were differentiated in neuronal induction media as described in Figure S1, Supporting Information. Briefly, media was changed to neuronal

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differentiation media consisting of basal media with the addition of 10 mM RA (Sigma) for 4 days after which media was switched to serum-free media with RA and 25 ng mL^{-1} BDNF (R&D systems) for a minimum of 8 days. Neuronal differentiation was confirmed microscopically by observing morphology changes and by staining for betaIII-tubulin (Figure S2, Supporting Information). Alternatively, cells were differentiated in modified neuronal media to support dopaminergic differentiation according to Knaryan et al.[21] Cells were cultured in reduced serum (3% FBS) media containing 10 mM RA for 3 days followed by 80 nM PMA (Sigma) for further 4 days. For Wnt media experiments, differentiation media was supplemented with 100 ng mL^{-1} recombinant human WNT3a (R&D systems), or diluted (1:5) Wnt-conditioned media (collected from Wnt3a overexpressing L-M (TK-) cells (ATCC).

MNP Functionalization: 250 nm of SPIO carboxyl–functionalized magnetic nanoparticles (Micromod, Germany) were covalently coated with UM206 peptide/RGD or rabbit-IgG (Abcam) by carbodiimide activation method, as described previously.[30] Briefly, 2 mg of particle suspension (for each ligand) was activated with EDAC/NHS in 0.5 M MES buffer (Sigma) for 1 h at room temperature. After washing, particles were coated overnight at 4 °C with 20 µg of either Linear-UM206 peptide, Cyclic-UM206 peptide (Pepceuticals, UK), RGD (Sigma), or 40 µg of anti-rabbit-IgG (Abcam) in 0.1 M MES buffer (Sigma). For Control (IgG)-MNP, the suspension was washed in 0.1 M MES buffer, then re-suspended in 0.1 M MES containing 20 µg of rabbit-IgG (Abcam) and mixed at room temperature for 3 h. All particle suspensions were blocked with 25 mM glycine (Sigma), washed in PBS (Lonza), and re-suspended in 1 mL PBS (Fisher). MNP functionalization was confirmed using dynamic light scattering and zeta potential (see Supporting Information). The peptide/protein coating on the MNP surface was quantified and number of ligands per MNP estimated using a total protein assay as described in Supporting Information. MNP morphology was also characterized by transmission electron microscopy (Figure S3, Supporting Information).

Cell MNP Labeling: Cells were cultured in reduced serum basal media for 3 h prior to addition of 25 µg MNP/2 × 10^5 cells. Cells were incubated with MNP for 1.5 h and then washed with PBS to remove unbound particles before addition of fresh media.

Magnetic Stimulation: Magnetically stimulated groups were treated with ≥25 mT magnetic fields provided by arrays of NdFeB magnets in 1–3 h sessions at 1 Hz using a vertically oscillating magnetic force bioreactor (MICA Biosystems, UK). The bioreactor was housed in a cell culture incubator maintained at 37 °C, 5% CO$_2$.[29,30] Non-stimulated control groups were kept in identical external conditions (without magnetic field).

Transient Transfections: Cells were transiently transfected with a Gaussia luciferase reporter under control of an 8× TCF/LEF promoter, as described previously.[29,30] Briefly, cells were grown to approximately 70% confluency in antibiotic-free media in 24-well plates. Cells were then transfected with 0.5 µg of TCF/LEF reporter in reduced serum Opti-MEM using Lipofectamine LTX reagent (Invitrogen) according to the manufacturer's instructions. Transfection media was aspirated after 4 h and replaced with fresh basal media. Luciferase activity was assessed 48–72 h after transfection using Biotek Synergy 2 plate reader.

Luciferase Reporter Assays: Experiments were performed in reduced serum basal media in 24-well plates. At each time point, media samples were taken and analyzed for secreted Gaussia luciferase activity using a luciferase flash assay kit (Thermo Pierce) on a Biotek Synergy 2 plate reader operating Gen5 software. Luciferase activity was normalized to the total protein content of the respective cell lysates obtained at the experimental end-point and quantified using a BCA assay kit (Fisher).

Polymerase Chain Reaction: Total RNA was extracted using an RNAeasy extraction kit (Qiagen) according to the manufacturer's instructions. Reverse transcription was performed on 1 µg RNA using a high-capacity reverse transcription kit (Applied Biosystems). Polymerase chain reaction (PCR) mixes were prepared using diluted cDNA mixed with PCR master mix (Applied Biosystems) and commercially available primers for Frizzled 1, Frizzled 2, DRD2, DRD3, TH, DAT, 18S, and GAPDH (Qiagen). Thermocycling was performed on a Stratagene MX3000P system. Gene expression was normalized to GAPDH or 18S as

stated. Fold change in gene of interest expression was calculated using the delta-delta CT method. PCR products were resolved on a 2% agarose gel and imaged using a GelDoc-It2 imager system (UvP).

Immunocytochemistry: For establishment of neuronal differentiation and receptor expression targets, cells were stained with β3-tubulin (Abcam) diluted 1:1000 and either anti-Frizzled 1 (Santa Cruz) diluted 1:200 or anti-Frizzled 2 (Santa Cruz) diluted 1:200. All primary antibodies were diluted in 1% BSA/PBS and incubated overnight at 4 °C. After washing with PBS, cells were stained with goat anti-mouse-FITC (Sigma) diluted 1:1000 and rabbit anti-goat-555 (Life technologies) diluted 1:2000 for Frizzled 1/2. All secondary antibodies were diluted in 1% BSA/PBS and incubated for 2 h at room temperature. Cells were washed with PBS X3 cells, then counterstained with DAPI (Sigma) for 15 min and stored in PBS.

MNP labeling of cells was determined using immunofluorescence to identify the dextran matrix of the MNP. After labeling with MNP, cells were fixed with 4% PFA in PBS (Fisher) for 10 min, permeabilized with 0.1% Triton-X in PBS for 10 min, washed with PBS, then blocked with 2% BSA/PBS for 2 h. Samples were then stained with an anti-dextran antibody (Stem Cell Technologies) diluted 1:1000 in 1% BSA in PBS overnight at 4 °C. Cells were then washed with PBS and stained with anti-mouse-FITC secondary antibody (Sigma) diluted 1:1000 in 1% BSA in PBS for 1 h at room temperature. Cells were then washed with PBS and counterstained with DAPI to visualize cell nuclei and either Phalloidin–Atto 565 (Sigma) to visualize F-actin or CM-Dil (Fisher) to visualize the cell membrane. For β-catenin staining, 24 h after treatment, cells were fixed with 90% methanol (Fisher) for 10 min. Cells were permeabilized with 0.1% Triton-X in PBS (Sigma) for 10 min and then then blocked in 2% BSA (Fisher) in PBS for 1 h at room temp. Cells were then incubated with anti-active β-catenin antibody (Millipore) diluted 1:1000 in 1% BSA in PBS overnight at 4 °C. Afterward, cells were washed, then incubated with anti-Mouse-FITC secondary antibody (Sigma) in 0.1% BSA in PBS for 1 h at room temp. Cells were then washed with PBS and counterstained with DAPI (Sigma). Active β-catenin-positive cells were quantified by cell counting using ImageJ. A minimum of 100 cells were counted in each group. For dopaminergic marker studies, cells were PBS washed, then fixed with 4% PFA in PBS for 10 min. Cells were then permeabilized with 0.1% Triton-X100 (Sigma) in PBS for 10 min. Cells were washed with PBS, then blocked using 2% BSA (Fisher) in PBS for 2 h. The blocking solution was aspirated, and cells were stained with primary antibodies as described below. Cells were stained with rabbit anti-tyrosine hydroxylase (TH) antibodies (Abcam) diluted 1:200 in 1% BSA/PBS and incubated overnight at 4 °C. Cells were washed with PBS X3 followed by incubation with Alexa Flour goat anti-rabbit IgG-594 secondary antibodies (Life Technologies) diluted 1:500 in 1% BSA/PBS for 2 h at room temperature. Samples were then re-blocked with BSA as above, then stained with rat anti-dopamine transporter (DAT) antibodies diluted 1:500 in 1% BSA/PBS (Santa Cruz Biotech.) overnight at 4 °C. Cells were washed with PBS × 3 followed by incubation with Rabbit anti-Rat-488 secondary antibodies (Life Technologies) diluted 1:2000 in 1% BSA/PBS for 2 h at room temperature. Cells were washed with PBS × 3; cells then counterstained with DAPI (Sigma) for 15 min and stored in PBS before imaging. TH and/or DAT-positive cells were quantified by cell counting using ImageJ. A minimum of 150 cells were counted in each group. Fluorescence microscopy was performed on an Eclipse Ti-S microscope (Nikon) or a Fluoview FV1200 laser scanning confocal microscope (Olympus) operating FV10-ASW (v4.2) imaging software.

Rat Brain Organotypic Slice Culture: Organotypic slice cultures were prepared from postnatal day 5–8 rat pups according to the interface method.[58] Pups were terminally anesthetized by intraperitoneal injection of pentobarbital in accordance with UK Animals (Scientific Procedures) Act 1986. Following decapitation, the brain was removed and placed in sterile ice-cold dissection medium composed of Gey's balanced salt solution (GBSS, Sigma), 25 mM HEPES (Gibco), 2 mM GlutaMAX (Gibco), 36 mM D-glucose (Fisher), and antibiotic–antimycotic (Gibco, 100 U mL^{-1} penicillin, 100 µg mL^{-1}, 0.25 µg mL^{-1} amphotericin B). The hemispheres were bisected and 300 µm sagittal sections were cut at a 10° angle using a vibratome (Leica). Following removal of the cortex and hippocampus, slices were placed in dissection medium for 1 h at 4 °C.

Three to four slices per hemisphere were transferred to 30 mm 0.4 μm pore membrane inserts (Millipore, PICM03050) in six-well plates and cultured for 7 days in slice culture medium composed of 50% Minimum Essential Medium (MEM, Gibco), 25% heat-inactivated horse serum (Gibco), 25% Hank's Balanced Salt Solution (HBSS, Gibco), 25 mM HEPES, 2 mM GlutaMAX, 36 mM D-glucose, and antimitotic–antimycotic. The medium was changed every 2–3 days. After 7 days, the medium was changed to serum-free medium composed of Neurobasal A medium (Gibco), B27 (Gibco), 25 mM HEPES, 2 mM GlutaMAX, 36 mM D-glucose, and antimitotic-antimycotic.

Delivery of SH-SY5Y Cells into Slices: SH-SY5Y cells were first labeled with the membrane tracker Pkh26 (Sigma), and then tagged with MNPs as described above. Approximately, 4.10^5 cells were delivered into the striatum of triplicate slices by pipette with the aid of a dissection microscope. Serum-free medium was supplemented with 10 mM RA for 3 days followed by serum-free medium supplemented with 80 nM PMA for further 4 days. Magnetic stimulation was performed in 3×1-h sessions over 7 days; control slices were incubated under identical conditions but without loading. After 7 days, slices were washed with PBS and fixed with 4% PFA (Sigma) for 30 min . Slices were washed and stored in PBS prior to staining.

Organotypic Slice Immunohistochemistry: Slices were blocked in PBS containing 10% normal goat serum (Vector Labs), 1% triton X-100 (Sigma) for 2 h at room temperature, followed by incubation with tyrosine hydroxylase antibody (1:1000, Millipore) in antibody solution (PBS containing 10% normal goat serum, 0.3% triton X-100) for 3 days at 4 °C. Slices were washed 3 times with PBS followed by incubation with Alexa Fluor goat anti-rabbit 488 antibody (1:500, Life Technologies) in antibody solution for 3 h at room temperature. Slices were washed three times with PBS, counterstained with DAPI for 15 min, mounted onto slides, and coverslipped.

Statistical Analysis: All data are presented as means +/− SEM or SD as stated. Statistical significance at 95% confidence level was determined using 1-way ANOVA with post hoc Tukey's tests using Mini-tab (v16). Data were tested for normality and equal variance before analysis using Mini-tab.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

A.E.H. is a director and co-founder of MICA Biosystems Ltd. She receives no salary and holds 50% of the shareholding in MICA Biosystems Ltd. MICA Biosystems Ltd. had no involvement in the research and/or preparation of this article.

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