Tetrahedral DNA nanostructures facilitate neural stem cell migration *via* activating RHOA/ROCK2 signalling pathway

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Abstract

Objectives: The main purpose of current study was to explore the effects of tetrahedral DNA nanostructures (TDNs) on neuroectodermal (NE-4C) stem cells migration and unveil the potential mechanisms.

Materials and methods: The successfully self-assembled TDNs were also determined by dynamic light scattering (DLS). A bidirectional wound-healing assay and transwell chamber assay were employed to test the migrating behaviour of NE-4C stem cells cultured under different conditions.

Results: Through an in vitro study, we found that stem cells could internalize TDNs quickly, and the cells' parallel and vertical migration was promoted effectively. Besides, the effects of TDNs were found being exerted by upregulating the gene and protein expression levels of *RhoA*, *Rock2* and *Vinculin*, indicating that the RHOA/ROCK2 pathway was activated by the TDNs during the cell migration.

Conclusions: In conclusion, TDNs could enter NSCs without the aid of other transfection reagents in large amounts, whereas only small amounts of ssDNA could enter the cells. TDNs taken up by NSCs activated the RHOA/ROCK2 signalling pathway, which had effects on the relevant genes and proteins expression, eventually promoting the migration of NE-4C stem cells. These findings suggested that TDNs have great potential in application for the repair and regeneration of neural tissue.

1 | INTRODUCTION

Neural stem cells (NSCs) orchestrate embryonic nervous system development and can equilibrate and repair injured nerve tissue when needed.¹⁻⁵ In the developing nervous system, pluripotent embryonic NSCs can proliferate, migrate and differentiate to form each component of the whole system.⁵⁻⁹ In recent years, because of their role in neural repair and regeneration, transplantation of NSCs has become increasingly attractive in cell transplantation for neurological diseases, such as cerebral palsy, spinal cord injury and motor neuron disease.^{1-5,9-11} While the proliferation, migration and differentiation of NSCs are promising, an effective intermediate is urgently needed to promote these biological behaviours of NSCs.¹⁰⁻¹⁷

DNA materials have been widely investigated with the aid of DNA nanotechnology.¹⁸⁻²⁶ DNA materials have shown potential in various

biomedical fields, such as anti-multidrug resistance and disease diagnostics/therapeutics.²⁰⁻²⁶ Different DNA nanostructures maintain various biological effects; among them, one of the most promising nanomaterials is tetrahedral DNA nanostructures (TDNs).²⁷⁻³⁷ This kind of DNA material has received increased research attention because of its biological safety and simplicity of synthesis.³⁴⁻³⁶ Self-assembled TDNs comprise 4 single-stranded DNAs (ssDNAs) that are designed based on the principle of base pairing. Therefore, TDNs possess excellent structural stability, mechanical rigidity and modification versatility.^{30,33,38} Previous studies of TDNs have shown that different cells could internalize TDNs, and then TDNs exerted influence on the cells' biological behaviours under certain conditions.³⁹⁻⁴⁴ For instance, TDNs could maintain the chondrocyte phenotype, promote chondrocyte proliferation and accelerate dental pulp stem cells osteo/odontogenic differentiation.^{35,44} Meanwhile, TDNs could

be modified by aptamers to detect tumour cells and are considered as potential carriers for drug delivery to reverse tumour resistance.^{30,42} In our recent work, we observed that TDNs promoted the proliferation of NSCs and accelerated the process of NSCs neuronal differentiation *in vitro*.⁴¹ However, whether TDNs could also exhibit effects in promoting the migration of NSCs remains unclear, and the potential underlying mechanism also remains elusive.

Physiologically, cellular migration is an intricate biological behaviour which is a pivotal ingredient of biological processes, including development of embryonic, immune responses and tissue regeneration.^{6-8,36,45} Migration is also considered a vital pathological component of diseases such as metastatic cancer and chronic inflammatory diseases.⁴⁶⁻⁵⁰ In the nervous system, after injury, NSCs migrate to the damaged area where they secrete certain growth factors to further accelerate NSCs migration, and then differentiate into neurocytes.⁵¹ This is a key process in the repair and regeneration of nerves and their accessory cells. However, the proliferation, migration and differentiation abilities of NSCs are poor, making it hard to repair and regenerate injured neural tissue.^{10,11,52,53} Therefore, studies exploring biomaterials that could promote the migration of NSCs safely and effectively will be of great significance in searching cures of neural system diseases. To find out whether TDNs could promote NSCs migration, together with our previous study,⁴¹ might provide systematic evidence of effects of TDNs on the biological behaviour of NSCs *in vitro* and of the possible use in future nerve tissue repair and regeneration.

In this study, using *in vitro* experiments, we investigated the performance of TDNs in the migration of neuroectodermal (NE-4C) stem cells; then, the potential mechanism was also explored. NE-4C stem cells are an *in vitro* model of NSCs and possess the abilities of proliferation and differentiation into the neuronal lineage. We hypothesized that TDNs could facilitate the migration of NSCs safely and effectively, thus representing a promising material for nerve tissue repair and regeneration in curing neurological diseases.

2 | MATERIALS AND METHODS

2.1 | Cell culture

NE-4C stem cells of mouse were bought from ATCC (CRL-2925, VA, USA). Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, USA) was used to culture the cells which containing 1% (v/v) Penicillin-streptomycin solution (Gibco, Grand Island, USA), and 10% (v/v) foetal bovine serum (FBS, Corning, New York, USA). The cells were cultivated in a humidified incubator at the temperature of 37°C, 5% CO₂ in the atmosphere. The growth medium was changed thrice every week.

2.2 | TDNs synthesis and characterization

The same concentrations of each ssDNAs (TaKaRa, Otsu, Japan) were added to TM buffer (Tris-HCl = 10 mmol/L; MgCl₂ = 50 mmol/L, pH = 8.0; Bio-Rad, Hercules, USA). The mixtures were heated for 10 minutes at 95°C and then cooled at 4°C for 40 minutes. TDNs'

concentration used in our experiment is 250 nmol/L, which was proven to be the optimal concentration in our previous studies.^{40,41,43}

To ensure the synthesis of self-assembled TDNs, we tested the size of the TDNs and the 4 ssDNA by Polyacrylamide gel electrophoresis (8% PAGE, Beyotime, Nanjing, China). The successfully self-assembled TDNs were also determined by dynamic light scattering (DLS) in ddH₂O at 250 nmol/L using a ZETAPals analyser (Brookhaven Instruments, Holtsville, NY, USA).

2.3 | Cellular uptake of TDNs

To ensure that the cells could take in TDNs, we treated the cells with TDNs or ssDNA, which were both modified with Cyanine-5 (Cy5). First, cells were seeded onto confocal dishes. The growth medium was changed to a medium with Cy5-TDNs or Cy5-ssDNA for 12 hours. Then, we washed the cell samples using phosphate-buffered saline (PBS, Gibco, Grand Island, USA) for 3 times, and fixed them with paraformaldehyde solution (4% w/v, Boster, Wuhan, China). After all samples were rinsed 3 times, FITC-labelled phalloidin (Sigma, St Louis, USA), together with DAPI (Sigma, St Louis, USA), was applied to stain each of cytoskeleton and nucleus. All samples were washed by PBS 3 times before being imaged under a confocal laser microscope (Nikon N-SIM, Japan).

Cellular uptake of TDNs was also tested by flow cytometry. Briefly, cells were firstly plated to 6-well plate (2×10^5 /well); then, we changed the medium with that containing Cy5-TDNs or Cy5-ssDNA. After 12 hours, the cells were collected and tested by the flow cytometer (FC500 Beckman, Brea, USA).

2.4 | Parallel cell migration (wound healing assay)

A bidirectional wound-healing assay was employed to test the migrating behaviour of NE-4C stem cells cultured under different conditions. Briefly, cells were first plated to 6-well plates and then incubated at 5% CO₂ and 37°C. Sterilized pipette tips were applied to form a bidirectional wound by scratching the single-layer cells. PBS was applied to wash the cell debris away. Cells were treated with medium without FBS (the control groups: without TDNs) and the same medium containing TDNs (the experimental groups: 250 nmol/L TDNs). Wound closure of cells was imaged after incubation for 0, 12 and 24 hours.

2.5 | Vertical cell migration (Transwell chamber assay)

Transwell insert (pore diameter: 8 µm; Corning) was applied to analyse the vertical cell migration.⁵⁴ Transwell inserts were attached to 6-well culture plate, and cells (1×10^5 /well) were seeded on the upper half of the insets. The growth medium was changed to the medium without FBS (the control groups: without TDNs; the experimental groups: 250 nmol/L TDNs). After incubated for 24 hours, cells in the upper insets were fixed in paraformaldehyde, staining with DAPI. The amount of cells presented in the lower part was determined by manual counting, and the results of migration were presented by using the mean number of cells.

TABLE 1 The primer sequences of relevant genes designed for qPCR

mRNA	Primer pairs	Product length (bp)
<i>GAPDH</i>	Forward 5'-AGAGGGATGCTGCCCTTACC-3' Reverse 5'-ATCCGTTACACCGACCTTC-3'	109
<i>RhoA</i>	Forward 5'-GCTCTTTTATAGCCCCGGTGT-3' Reverse 5'-CCACGATTGCTCAAGAACGC-3'	116
<i>Rock2</i>	Forward 5'-CTGTGATCCCAAGGGAAGGCT-3' Reverse 5'-ACTGACAGCAGCAGTATGCC-3'	147
<i>Vinculin</i>	Forward 5'-TGGTCTAGCAAGGGCAATGA-3' Reverse 5'-CTCGTCACCTCATCAGAGGC-3'	155

FIGURE 1 Successful synthesis and characterization of TDNs. A, Schematic diagram of TDNs. B, Confirmation of the successful synthesis of TDNs by 8% PAGE (polyacrylamide gel electrophoresis; TDNs: red circle). Lane 1 is S1. Lane 2 is S2. Lane 3 is S3. Lane 4 is S4. Lane 5 is S1+S2. Lane 6 is S1+S2+S3. Lane 7 is S1+S2+S3+S4, representing the successful synthesis of TDNs. C, Typical size distribution graph of TDN

2.6 | Semi-quantitative PCR and quantitative PCR

Total RNA of cells treating with or without TDNs was extracted by an RNeasy Plus Mini Kit and treated with a genomic DNA eliminator. The RNA samples were reverse transcribed into cDNA using a synthesis kit (TaKaRa, Ostu, Japan). The mRNAs (Table 1) expressions of each group were normalized to the housekeeper genes of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and evaluated. Semi-quantitative PCR was conducted with a PCR kit (Mbi, TaKaRa, Ostu, Japan), while quantitative PCR (qPCR) was conducted using SYBR Green II PCR master mix (TaKaRa, Ostu, Japan) and a Bio-Rad CFX96™ thermal cycler (Bio-Rad). The semi-quantitative PCR procedure included a 30-second denaturation at 94°C, 30-second annealing cycle at 55-65°C and a 30-second elongation cycle at 72°C, for 28-35 amplification cycles. Products were detected by 2% agarose gel electrophoresis in TAE buffer and stained using Gel-Red (Sigma, St Louis, USA). The procedure for qPCR comprised 2 minutes of denaturation at 95°C, 5 seconds annealing at 95°C and 30 seconds elongation at 60°C for 40 cycles.

2.7 | Western blotting

After 24 hours treated with 250 nmol/L TDNs or vehicle control, NE-4C stem cells were washed with PBS after thrice loop operations, and a Whole Cell Lysis Assay (KeyGEN Institute of Biotechnology, Jiangsu, China) was applied to harvest all the proteins. The protein samples and 5× loading buffer (Beyotime, Nanjing, China) were mixed together at a ratio of 4:1, and then the mixed samples were heated for 3 minutes at 100°C. The mixed samples were subjected to SDS-PAGE (Beyotime, Nanjing, China) at different gel concentrations to separate the proteins, and then transferred to Polyvinylidene fluoride membranes (PVDF, TaKaRa, Ostu, Japan). After incubating with 5% skim milk powder solution

TABLE 2 Base sequence of each ss DNA

ss DAN	Base sequence	Direction
Cy5-S1	Cy5-ATTTATCACCCGCCATAGTAGACGTATCACCAGGCAGTTGAGACGAACATTCTAAGTCTGAA	5'→3'
S1	ATTTATCACCCGCCATAGTAGACGTATCACCAGGCAGTTGAGACGAACATTCTAAGTCTGAA	5'→3'
S2	ACATGCGAGGGTCCAATACCGACGATTACAGCTTGCTACACGATTACAGACTTAGGAATGTTCCG	5'→3'
S3	ACTACTATGGCGGGTGATAAAACGTGTAGCAAGCTGTAATCGACGGGAAGAGCATGCCCCATCC	5'→3'
S4	ACGGTATTGGACCCTCGCATGACTCAACTGCCTGGTGATACGAGGATGGGCATGCTCTTCCCCG	5'→3'

FIGURE 2 Cellular uptake of TDNs. A, Interaction of NE-4C stem cells with 250 nmol/L Cy5-ssDNA (the control group) or 250 nM Cy5-TDNs for 12 h (Cy5: red, cytoskeleton: green, nucleus: blue). Scale bars are 25 μ m. B, Semi-quantitative analysis of fluorescence of Cy5-ssDNA and Cy5-TDNs. Data are presented as mean $\pm$ SD ($n = 4$). Statistical analysis: *** $P < .001$. C, After treatment with 250 nmol/L Cy5-ssDNA or 250 nmol/L Cy5-TDNs for 12 h, flow cytometry examination and the analysis of cellular uptake of Cy5-ssDNA and Cy5-TDNs (negative control: treated with nothing; positive control: treated with Cy5-ssDNA; TDNs: treated with Cy5-TDNs). D, Semi-quantitative analysis of the cellular uptake in flow cytometry. Data are presented as mean $\pm$ SD ($n = 4$). Statistical analysis: ** $P < .01$, *** $P < .001$

for 1 hour, all membranes were incubated with primary antibodies (anti-GAPDH, anti-RhoA, anti-Rock2, and anti-Vinculin; Abcam, Cambridge, U.K) overnight at 4°C. All membranes were rinsed with TBST thrice next day and incubated with secondary antibodies for 1 hour. All membranes were washed thrice with TBST and tested by an enhanced chemiluminescence detection system (Bio-Rad).

2.8 | Immunofluorescence staining

Cells in confocal dishes treated with or without TDNs for 24 hours were fixed in paraformaldehyde solution. All samples were then treated with 0.5% Triton X-100 and then blocked with 5% sheep serum. The samples were treated overnight with primary antibodies solution (anti-RhoA, anti-Rock2 and anti-Vinculin; Abcam, Cambridge, U.K) at 4°C. The next day, the cells were incubated with secondary antibodies for 1 hour. The cytoskeleton was stained with phalloidine at 37°C for 1 hour and the nuclei with DAPI for 10 minutes. Every step in this experiment was followed by 3 washes with PBS for 10 minutes. Finally, a confocal laser microscope (Nikon N-SIM, Japan) was applied to capture images.

2.9 | Statistical analysis

SPSS (version 23.0) was applied for data analysis. Intergroup differences were compared using analysis of variance (ANOVA) and t test, and $P < .05$ indicated that the test results were statistically significant. All experiments and analyses were conducted in triplicate and replicated more than 3 times.

3 | RESULTS

3.1 | TDN characterizations

TDNs comprised 4 different ssDNA (Figure 1A), and each ssDNA was designed with a specific sequence based on the base complementation pairing rules (Table 2). To evaluate the synthesis and characterization of the TDNs, 8% PAGE was performed to assess the size and size distribution of the TDNs. Figure 1B shows that the TDNs were successfully synthesized. Figure 1C shows that the average size of TDNs was 16.801 ± 0.237 nm. These results indicated that the TDNs were successfully constructed.

FIGURE 3 A, Facilitated effects of TDNs on NE-4C stem cells horizontal migration tested by a bidirectional wound-healing assay at 0, 12 and 24 h. B, Histogram representation of the percentage of NE-4C stem cells migration area at 0, 12 and 24 h on treatment without (the control group) or with TDNs. Data are presented as mean $\pm$ SD ($n = 4$). Statistical analysis: * $P < .05$, *** $P < .001$

3.2 | Cellular uptake of TDNs

To ensure that NE-4C stem cells could take in abundant TDNs, but not ssDNA, fluorescence staining and flow cytometry were used to track TDNs and ssDNA. As shown in Figure 2A,B, the fluorescence signal of Cy5 in the cells treated with Cy5-loaded TDNs was stronger than that from cells treated with Cy5-loaded ssDNA. Flow cytometry showed that more cells took in Cy5-loaded TDNs than Cy5-loaded ssDNA (Figure 2C-D). These results indicated that the

cells could take in TDNs specifically, which is a prerequisite of TDNs to affect cellular biological behaviour.

3.3 | Changes in NE-4C stem cell migration

To observe TDNs' actions of horizontal migration on cells, we used 250 nmol/L TDNs to treat cells and analysed the migration by a wound-healing assay, which has been applied to detect the cell migration in vitro widely. Cells migrated towards the wound areas for 24 hours, and

FIGURE 4 A, Schematic diagram of the vertical cell migration tested by Transwell chamber assay. B, Fluorescence images showing the facilitated effects of TDNs on NE-4C stem cell migration. The transmigrated NE-4C stem cells' nuclei were stained with DAPI (nucleus: blue). Scale bars are 100 μ m. C, Cell number of transmigrated NE-4C stem cells was presented by histogram. Data are presented as mean $\pm$ SD ($n = 3$). Statistical analysis: *** $P < .001$

FIGURE 5 A, Semi-quantitative PCR of *RhoA* upon exposure to 250 nmol/L TDNs for 24 h. B, Semi-quantitative analysis of semi-quantitative PCR of about *RhoA* gene expression level. Data are presented as mean $\pm$ SD (n = 4). Statistical analysis: *** P < .001. C, Quantitative PCR of *RhoA* upon exposure to 250 nmol/L TDNs for 24 h. Data are presented as mean $\pm$ SD (n = 4). Statistical analysis: * P < .05. D, Western blotting analysis of *RhoA* protein expression level after treatment with 250 nmol/L TDNs for 24 h. E, Semi-quantitative analysis of western blotting about *RhoA* protein expression level. Data are presented as mean $\pm$ SD (n = 4). Statistical analysis: *** P < .001. F, After treated with 250 nmol/L TDNs for 24 h, *RhoA* showed higher expression level in cells. Immunofluorescent images of cells treated with or without TDNs (*RhoA*: red, cytoskeleton: green, nucleus: blue). Scale bars are 25 μ m. G, Semi-quantitative analysis of average optical density of Figure 5F. Data are presented as mean $\pm$ SD (n = 4). Statistical analysis: *** P < .001

FIGURE 6 A, Semi-quantitative PCR of *Rock2* upon exposure to 250 nmol/L TDNs for 24 h. B, Semi-quantitative analysis of semi-quantitative PCR of about *Rock2* gene expression level. Data are presented as mean $\pm$ SD (n = 4). Statistical analysis: ** P < .01. C, Quantitative PCR of *Rock2* upon exposure to 250 nmol/L TDNs for 24 h. Data are presented as mean $\pm$ SD (n = 4). Statistical analysis: *** P < .001. D, Western blotting analysis of *Rock2* protein expression level after treatment with 250 nmol/L TDNs for 24 h. E, Semi-quantitative analysis of western blotting about *Rock2* protein expression level. Data are presented as mean $\pm$ SD (n = 4). Statistical analysis: *** P < .001. F, After treated with 250 nmol/L TDNs for 24 h, *Rock2* showed higher expression level in cells. Immunofluorescent images of cells treated with or without TDNs (*Rock2*: red, cytoskeleton: green, nucleus: blue). Scale bars are 25 μ m. G, Semi-quantitative analysis of average optical density of Figure 6F. Data are presented as mean $\pm$ SD (n = 4). Statistical analysis: *** P < .001

FIGURE 7 A, Semi-quantitative PCR of *Vinculin* upon exposure to 250 nmol/L TDNs for 24 h. B, Semi-quantitative analysis of semi-quantitative PCR of about *Vinculin* gene expression level. Data are presented as mean $\pm$ SD ($n = 4$). Statistical analysis: $**P < .01$. C, Quantitative PCR of *Vinculin* upon exposure to 250 nmol/L TDNs for 24 h. Data are presented as mean $\pm$ SD ($n = 4$). Statistical analysis: $***P < .001$. D, Western blotting analysis of *Vinculin* protein expression level after treatment with 250 nmol/L TDNs for 24 h. E, Semi-quantitative analysis of western blotting about *Vinculin* protein expression level. Data are presented as mean $\pm$ SD ($n = 4$). Statistical analysis: $***P < .001$. F, After treated with 250 nmol/L TDNs for 24 h, *Vinculin* showed higher expression level in cells. Immunofluorescent images of cells treated with or without TDNs (*Vinculin*: red, cytoskeleton: green, nucleus: blue). Scale bars are 25 μ m. G, Semi-quantitative analysis of average optical density of Figure 7F. Data are presented as mean $\pm$ SD ($n = 4$). Statistical analysis: $***P < .001$

FIGURE 8 Schematic diagram showing the underlying mechanism of TDNs promoted the NE-4C stem cells migration via activating RHOA/ROCK2 pathway

the control group cells migrated markedly slower than the groups cells treated with 250 nmol/L TDNs (Figure 3A). Statistical analysis of the area of wound healing showed that NE-4C stem cells migration was promoted significantly after treatment with TDNs (Figure 3B).

To further confirm that TDNs could affect cell vertical migration, Transwell chamber assay was used. According to Figure 4A,B, the stem cells were plated onto the upper chamber of Transwell and treated with or without 250 nmol/L TDNs for 24 hours. Compared with the control groups, more cells of the experimental groups incubated with 250 nmol/L TDNs, migrated onto the lower surface of the Transwell. Moreover, the quantitative analysis shown in Figure 4C indicated that TDNs could enhance the migration of NE-4C stem cells significantly. These results showed that TDNs could facilitate the stem cell migration, which is a prerequisite for the regeneration of the injured nervous system.

3.4 | TDNs promote cell migration via the RHOA/ROCK2 pathway

Previous studies demonstrated that the RHOA/ROCK2 pathway is closely related to cell migration; therefore, changes in the expressing levels of ras homologue family member A (*RhoA*), rho associated coiled-coil containing protein kinase 2 (*Rock2*) and *Vinculin* were tested in this study. From the results of semi-quantitative PCR (Figures 5A-B, 6A-B, and 7A-B) and quantitative PCR (Figures 5C, 6C and 7C), the expressions of *RhoA*, *Rock2* and *Vinculin* in cells with the treatment of TDNs for 24 hours were activated by comparison with the control group.

To further explore the mechanism by which TDNs activate the RHOA/ROCK2 pathway, we used both western blotting and immunofluorescence staining to analyse the protein levels of *RhoA*, *Rock2*

and *Vinculin*. From the results of western blotting, the group with TDNs had higher levels of *RhoA*, *Rock2* and *Vinculin* than the control group (Figures 5D-E, 6D-E and 7D-E); meanwhile, the immunofluorescence staining intensities of the 3 proteins in cells treated with TDNs were much stronger than those in the cells of the control group (Figures 5F,G, 6F,G and 7F,G). The gene expression and protein analysis results were consistent, thus indicated that TDNs could accelerate cells migration by upregulating RHOA/ROCK2 signalling pathway (Figure 8).

4 | DISCUSSION

NSC transplantation is an emerging therapy for various neurological and neuro-traumatic diseases with great potential, due to its role in nervous tissue repair and regeneration.^{1-5,9,55-57} However, some challenges remain to be addressed before NSC transplantation could be eventually used in clinical application.¹²⁻¹⁷ One of the biggest challenges is that transplanted NSCs cannot self-renewal, migrate or differentiate with high efficiency.^{10,11,52,53} In previous studies, when NSCs were transplanted into a specific nervous tissue site to promote NSCs proliferation, migration and differentiation, many biological agents were tested through co-transplantation with NSCs, such as scaffolds, neurotrophic growth factors and neurotrophic drugs.^{12-17,51} However, they all have shown either low efficacy or resistance to degradation.¹²⁻¹⁷ Thus, there still lack an effective biomaterial which could promote NSCs proliferation, migration and differentiation, and new types of materials or drugs with lower toxicity, lower cost and better biosecurity are urgently required.

Recently, TDNs, a tough and flexible nanomaterial, have been shown with great potential in different biological functions, such as cell proliferation, differentiation and anti-inflammation.^{39-44,58} Recently, we demonstrated that TDNs could enhance the proliferation and differentiation of NSCs in vitro.⁴¹ In present study, we further investigated the effects of TDNs and found that they could promote NSCs migration via upregulating the related genes and proteins of RHOA/ROCK2 signalling pathway in vitro.⁵⁹ While the migration of NSCs from the transplantation site to the injured areas play a critical part in neurogenesis,^{6-8,60} findings of current study, together with those from the recent one,⁴¹ systematically showed that TDNs could enhance NSCs proliferation, migration and differentiation. Despite the lack of animal experimental data in our study, we strongly believe that TDNs possess potential uses for nervous tissue repair and regeneration of cells in neurological diseases. Therefore, further studies in vivo are necessary.

In addition to their innate biological functions, TDNs are ideal biological carriers. According to previous studies, TDNs could be modified by aptamers to detect tumours and could deliver drugs to treat tumours.^{29,30,42} Therefore, we postulated that TDNs could serve a dual purpose: on one hand, TDNs could help NSCs proliferate, migrate and differentiate in nervous tissue regeneration; on the other hand, TDNs might act as drug carriers for medicines that could increase neurogenesis, such as neurotrophic

growth factors. Taken together, the data in our studies have shown that treatment with TDNs would enhance neurological recovery by increasing neurogenesis including cell proliferation, migration, and differentiation. Thus, TDNs have the potential to be further researched and developed as a therapeutic for neuroregeneration, and a kind of carriers for deliver drugs to treating neurological diseases.

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CONFLICT OF INTEREST

All authors declare no competing financial interest.

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