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Scaffold Mediated Gene Knockdown for Neuronal Differentiation of Human Neural Progenitor Cells

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The use of human induced pluripotent stem cell-derived neural progenitor cells (hiPSC-NPCs) is an attractive therapeutic option for damaged nerve tissues. To direct neuronal differentiation of stem cells, we have previously developed an electrospun polycaprolactone nanofiber scaffold that was functionalized with siRNA targeting Re-1 silencing transcription factor (REST), by mussel-inspired bioadhesive coating. However, the efficacy of nanofiber-mediated RNA interference on hiPSC-NPCs differentiation remains unknown. Furthermore, interaction between such cell-seeded scaffolds with injured tissues has not been tested. In this study, scaffolds were optimized for REST knockdown in hiPSC-NPCs to enhance neuronal differentiation. Specifically, the effects of two different mussel-inspired bioadhesives and transfection reagents were analyzed. Scaffolds functionalized with RNAiMAX Lipofectamine-siREST complexes enhanced the differentiation of hiPSC-NPCs into TUJ1⁺ cells (60 % as compared to 22 % in controls with scrambled siNEG after 9 days) without inducing high cytotoxicity. When cell-seeded scaffolds were transplanted to transected spinal cord organotypic slices, similar efficiency in neuronal differentiation was observed. The scaffolds also supported the migration of cells and neurite outgrowth from the spinal cord slices. Taken together, the results suggest that this scaffold can be effective in enhancing hiPSC-NPC neuronal commitment by gene-silencing for the treatment of injured spinal cords.

Introduction

Injuries and diseases to the nervous system often result in debilitating conditions. Hence, in search for methods to repair damaged nerve tissues, the use of neural progenitor cells (NPCs) has emerged as an attractive option for cell transplantation^{1,2}. In particular, induced pluripotent stem cell (iPSC)-derived NPCs hold great potential since patient-specific neurons may be generated³. To direct cell fate, RNA interference (RNAi) via siRNA delivery is an effective approach without depending on protein and biochemical cocktails⁴. By targeting repressor element-1 silencing transcription factor (REST), which controls pathways that are repressive against neuronal commitment, NPC differentiation into neurons can be enhanced⁵⁻¹².

Previous studies showed that generating neurons using scaffolds, such as electrospun fibers, is advantageous by providing structural support for cell transplantation and a better mimic of the *in vivo* microenvironment^{13,14}. However, conventional ways of incorporating siRNA into scaffolds through bulk encapsulation often involve harsh fabrication processes that may alter the integrity of siRNAs^{9,15}. In contrast, surface modification of electrospun scaffolds with mussel-inspired

bioadhesive coatings is a simple and effective method to immobilize siRNA complexes due to the facile reactivity of such coatings towards biomolecules¹⁶⁻²⁰. Previously, we showed that neuronal differentiation was enhanced in mouse NPCs and human mesenchymal stem cells (MSCs)^{19,20} by the synergistic integration of topographical cues from electrospun nanofibers and REST knockdown. However, the efficacy of such an approach in enhancing neuronal differentiation from hiPSC-NPCs remains unknown. Further optimization is also needed to extend the platform for hiPSC-NPCs, as different mussel-inspired bioadhesives and transfection reagents used in previous studies suggest that a unique optimal combination may be needed for different cell types^{19,20}.

Besides evaluating the response of hiPSC-NPCs towards scaffolds, it is also crucial to understand the interactions between such cell-seeded substrates and injured tissues to ensure good host-implant integration. To achieve this, we introduce a novel approach of utilizing an organotypic spinal cord slice culture. Due to the preserved cyto-architecture of spinal cord tissues^{21,22}, such organotypic slice cultures serve as an excellent bridge between isolated cell cultures and *in vivo* experiments without sacrificing large number of animals. Specifically, we evaluated the interactions between hiPSC-NPC-seeded scaffolds and the injured spinal cord tissues.

In this study, we hypothesized that a combination of aligned fiber topography and sustained RNAi will synergistically enhance neuronal commitment of hiPSC-NPCs. By demonstrating the effectiveness of the scaffold in promoting

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neuronal differentiation of hiPSC-NPC, this platform will be advanced a step closer towards *in vivo* applications. In particular, we compared two mussel-inspired bioadhesives (polydopamine and polyDOPA-melanin) and two transfection reagents (Lipofectamine RNAiMAX and *TransIt*-TKO) to achieve the highest differentiation efficiency with minimal cytotoxicity (Fig 1). Thereafter, using the optimized scaffold, we investigated the efficacy of transplanting the hiPSC-NPC-seeded substrate for spinal cord injury treatment, using an *in vitro* organotypic slice culture. The capability of the scaffolds in supporting the migration of neural cells and neurite outgrowth from the spinal cord tissues was also evaluated.

Results

Scaffold characterization

As shown in Fig 2A, the morphology of the scaffolds remained unchanged even after functionalization with different surface coatings and siRNA complexes. Importantly, the coating and siRNA complexes did not mask the underlying fiber morphology. Besides that, the average fiber diameter did not increase after siRNA adsorption (Fig 2B). Specifically, the average fiber diameter of the plain PCL scaffolds was $1.3 \pm 0.5 \mu\text{m}$, which has been demonstrated to be effective in inducing neuronal differentiation from NPCs²³

To visualize the distribution of the siRNA complexes on the scaffolds, fluorescently-tagged RNA, Cy5-RNA, were incorporated onto the scaffolds. As shown in Fig 2C, the distributions of the Cy5-RNA complexes on the scaffolds were fairly even regardless of the treatment. However, larger aggregates of Cy5-RNA were observed with TKO complexes, especially on DOPA-coated scaffolds. The average loading efficiency of siRNA complexes on the scaffolds were also similar, which was about 90 % in all groups (Fig 2D). These siRNA complexes were then released slowly from the scaffolds (Fig 2E). Although siRNA complexed with RNAiMAX on DOPAMINE scaffolds appeared to be released at a faster rate as compared to the other groups, a cumulative of only 2 % release was observed after 28 days. This indicated that majority of the siRNA complexes were retained on the scaffolds.

Cellular uptake and viability

As illustrated in Fig 3A, hiPSC-NPCs cultured on Cy5-RNA-incorporated scaffolds took up Cy5-RNA on Day 1 as well as Day 9 in all groups. However, there were fewer cells on scaffolds with TKO complexes at Day 9. In addition, the siRNA-TKO complexes were also larger on the scaffolds and large aggregates were observed in the cells on these scaffolds.

Cell viability was assessed using the Live/Dead Viability/Cytotoxicity Kit. There was no significant difference in cell viability between plain DOPAMINE, plain DOPA, DOPAMINE-RNAiMAX, and DOPA-RNAiMAX scaffolds at Day 9 (Fig 3B). However, significant decrease in cell viability was observed in scaffolds with siRNA-TKO complexes on both

DOPAMINE and DOPA coatings. These observations suggested that TKO complexes induced more significant cytotoxicity in hiPSC-NPCs.

Neuronal differentiation

Neuronal differentiation of hiPSC-NPCs that were cultured on the scaffolds was assessed by immunostaining of TUJ1, a neuronal marker. As indicated in Fig 4A and 4B, regardless of the type of transfection reagents and bioadhesive coatings used, there were significantly higher numbers and percentages of TUJ1⁺ cells in the presence of siREST as compared to treatment with negative, scrambled siNEG. Furthermore, scaffolds with DOPAMINE and RNAiMAX combination had higher percentage of TUJ1⁺ cells than the DOPA-RNAiMAX scaffolds. Overall, DOPAMINE-RNAiMAX scaffolds had the highest percentage (59.9%) and number of TUJ⁺ cells (64 cells per ROI). Therefore, DOPAMINE-RNAiMAX scaffolds were chosen for subsequent experiments.

Using DOPAMINE-RNAiMAX scaffolds, the expression of *REST* was studied to verify target gene knockdown (Fig 4C). Correspondingly, *REST* expression was decreased significantly by scaffolds with *REST* siRNA at all experimental time points. This correlated with the upregulation of *TUJ1* expression. CNP and GFAP were also stained to assess oligodendroglia and astroglia differentiation respectively (Fig S1A). There were no CNP⁺ and GFAP⁺ cells observed on the scaffolds. However, Nestin⁺ cells were present on the scaffolds, but the difference between siREST-incorporated scaffolds and the siNEG-incorporated controls was not statistically significant (Fig S1B).

Organotypic slice culture: Efficacy of scaffold in supporting and enhancing neuronal differentiation

The objective of using the organotypic slice culture was to evaluate the efficacy of the scaffolds in enhancing neuronal differentiation from hiPSC-NPCs after transplantation into injured spinal cord tissues. In addition, this also allowed us to assess the response of the injured host tissue towards the transplanted scaffold. Fig 5A shows the integration of a scaffold with the transected spinal cord tissues after being cultured for 7 days. The tissues, along with the scaffolds, were stained with neural cell markers and an antibody for human nuclei (HuNu) to differentiate the transplanted hiPSC-NPCs from cells that have migrated from the rat spinal cord tissues. Fig 5B shows the distribution of TUJ1⁺ hiPSC-NPCs on the scaffolds. There was no significant difference in the number of HuNu⁺ human cells between siNEG and siREST (Fig 5C). However, the percentage of HuNu⁺TUJ1⁺ cells was significantly higher on siREST-incorporated scaffolds (53%, Fig 5D). In addition, we did not observe any HuNu⁺CNP⁺ and HuNu⁺GFAP⁺ cells on the scaffolds (Fig S2). These findings were similar to the *in vitro* results, and showed that the siREST-incorporated substrates supported neuronal differentiation even after transplantation, in the presence of the injured spinal cord tissues.

Organotypic slice culture: Efficacy of scaffold in supporting and enhancing neuron adhesion and neurite extension

Besides allowing easy transplantation of hiPSC-NPCs and supporting neuronal differentiation of these progenitor cells, HuNu-TUJ⁺ rat neurons from the spinal cord tissue were also observed on the scaffolds (Fig 6A). However, the number and percentage of HuNu-TUJ⁺ neurons on the substrates were not affected by the presence of siREST (Fig 6B). One possible reason might be due to the fact that the siREST sequence was designed to target human cells. Similarly, neurites from the tissues also extended onto the scaffolds and aligned along the fiber direction (Fig 6A). As the neurites were touching each other, it was difficult to track individual neurite length. Therefore, we counted the number of neurites that crossed the distances of 500 μ m and 1000 μ m away from the scaffold-tissue interface as well as the number of neurites that crossed the 2 mm injury gap. As depicted in Fig 6C–6F, the number of neurites at different distances from the scaffold-tissue interfaces was not significantly different between the treatment groups. Although the number of neurites on the scaffolds was highly variable between animals and spinal cord slices, it was not significantly affected by the different slice locations across the coronal and sagittal planes (Fig S3).

Organotypic slice culture: Efficacy of scaffold in supporting glial cell migration

Besides neurons, glial cells such as HuNu-CNP⁺ oligodendrocytes, HuNu-GFAP⁺ astrocytes and HuNu-OX42⁺ microglia also migrated onto the scaffolds (Fig 7A). However, most of the migrated cells were mainly near the scaffold-tissue interface. When quantified, the number and percentage of each type of glial cells were not significantly different between siREST and siNEG (Fig 7B). The number and percentage of glial cells were also highly variable between animals and spinal cord slices.

Discussion

Currently, there is a lack of effective treatment options for neural diseases and injuries. Although cell replacement therapy has been proposed as a promising solution, the two-fold objectives of directing cell fate effectively and allowing cell transplantation have not been achieved. An alternative treatment approach may be to control cell fate by RNAi and topographical cues from scaffolds, which also allows subsequent cell transplantation. Correspondingly, the effectiveness of such an approach was demonstrated with mouse NPCs and human MSCs, where siREST-functionalized electrospun scaffolds enhanced neuronal differentiation from the respective stem cells^{19, 20}. Here, we expanded the application of the platform to enhance the differentiation of hiPSC-NPCs into TUJ1⁺ neurons, and evaluated the interactions between these cell-seeded scaffolds and injured spinal cord tissues. This will advance the approach closer towards *in vivo* applications.

Non-viral delivery of siRNA from scaffold surfaces was demonstrated to be effective using different mussel-inspired bioadhesives: polydopamine and polyDOPA-melanin^{19, 20}. Furthermore, different transfection reagents, both liposomal RNAiMAX and non-liposomal TKO were used in these studies. However, the efficacy of these protocols in transfecting hiPSC-NPCs was unknown. In this study, these protocols were compared and optimized for hiPSC-NPCs transfection. Surprisingly, scaffolds with TKO were found to be cytotoxic to hiPSC-NPCs, which did not happen in hMSCs under the same drug dosage¹⁹. The reason is unclear but it appears to be a cell type-dependent response. It is likely that the cytotoxicity towards hiPSC-NPCs was due to high dosage and aggregates formed on the scaffolds as bolus delivery of the same complexes at a lower dosage (50 nM siRNA with 0.5 μ l TKO) did not kill the cells (Fig S4). Therefore, the results suggest that optimization should be done when using similar approach of scaffold-mediated delivery for other cell types.

Although the release of siRNA from the scaffolds was slow, cellular uptake of Cy5-RNA and REST knockdown were observed even at 9 days after cell seeding. These observations suggest that the siRNAs might have been delivered through reverse transfection, where cells internalized the siRNA complexes directly from the fibers. Similar mechanism has also been suggested in other studies that delivered siRNA by mussel-inspired bioadhesives-coated surfaces^{20, 24}.

Both polydopamine and polyDOPA-melanin were formed by similar oxidative polymerizations under alkaline condition, from dopamine and DOPA monomers respectively. Between polydopamine and polyDOPA-melanin, the latter is more negatively charged and believed to have a higher adhesive strength towards positively-charged siRNA complexes due to its extra carboxyl group²⁵. This was supported by the observation of a slightly lower siRNA release from our polyDOPA-melanin scaffolds (Fig 2D) despite similar siRNA loading efficiency.

Immunostaining with pan-neuronal marker, TUJ1, was used as a quick and simple method to assess neuronal differentiation on different scaffolds²⁶. Previously, NPCs generated from hiPSCs using the same protocol differentiated into TUJ1⁺ neurons²⁷. Here, we attempted to accelerate the neuronal differentiation with REST knockdown and fiber scaffolds. We found that siREST-RNAiMAX complexes enhanced neuronal differentiation on polydopamine-coated fibers. As compared to previous results, the neuronal differentiation efficiency of hiPSC-NPCs on siREST-functionalized scaffolds (60 % TUJ1⁺) was slightly higher than mNPCs on similar scaffolds (~40 % TUJ1⁺)²⁰. The effect of siREST was also stronger in this study (60 % TUJ1⁺ on siREST vs. 22 % TUJ1⁺ on siNEG), suggesting that siREST enhanced neuronal differentiation of hiPSC-NPCs more efficiently than mNPCs. However, we did not observe any MAP2⁺ cells within the study duration, which is relatively short as compared to previous study that used the same differentiation protocol and cell source (60 days)^{27,28}. The optimal parameters for REST knockdown as obtained here, can be used for long-term

differentiation of hiPSC-NPCs into mature neurons in future studies. Based on results from previous related studies^{19,20}, we expect REST knockdown with nanofibers to enhance neuronal maturation as well.

The interaction between injured spinal cord and siREST-loaded scaffolds were then evaluated using the organotypic slice culture, which acts as a bridge between isolated cell cultures and *in vivo* experiments. Specifically, the cyto-architecture and neuron-glial interactions are preserved in organotypic slice cultures, thus allowing features of spinal cord injuries such as glial scar formation and microglial activation to be observed^{21,22}. The organotypic slice culture also helps in reducing the cost and number of animals that are required, as compared to *in vivo* experiments²¹. Comparatively, we observed similar extents of neuronal differentiation in the hiPSC-NPCs when they were cultured on siREST-loaded scaffolds even in the presence of the transected spinal cord tissues (53 % TUJ1⁺ in Fig 5 vs. 60% TUJ1⁺ in Fig 4). In contrast, neuronal differentiation in the presence of siNEG showed a trend of decreased neuronal differentiation after transplantation to the injured spinal cord tissues (12% in Fig 5 vs. 22% in Fig 4). Hence, the results suggest the efficacy of the siREST-incorporated substrates in providing effective differentiation signals to direct cell fate even in less optimal post-injury situations.

The extension of neurites and migration of neural cells from the rat spinal cord tissues were also observed on the scaffolds. However, siREST did not alter the response of the host tissue, as the sequence of siREST was designed to target human cells instead of rat cells. Furthermore, it is also possible that the effects of siREST is smaller as compared to the variation across different spinal cord slices and animals.

Differences in location of the slices in the spinal cord and the location within the slices may have contributed to the variations in tissue response. This may happen due to differences in the types of neural cell that exist at the different locations within the spinal cord. For example, interneurons at the dorsal horn and motor neurons at the ventral horn may display varying extents of neurite outgrowth after spinal cord transection. These potential differences were not evaluated in the literature²⁹ but we did not observe obvious differences in the number of neurite extensions due to slice location in this study (Fig S1).

Axonal outgrowth and extension have been evaluated and quantified in both cross-sectional and transected longitudinal slice cultures^{21,30,31}. In these studies, axonal outgrowth was quantified by the number of neurite processes per area or neurite length. As a comparison, motor neurons at the ventral portion of spinal cord cross-sectional slices extended axons up to distances of $1456 \pm 70.76 \mu\text{m}$ and $1229 \pm 80.09 \mu\text{m}$ along fibrin and collagen hydrogels respectively³¹. Here, we showed that functionalized electrospun fibers enabled some neurites to extend across the 2 mm injury gap. Besides that, we did not observe any difference in glial cell response due to the presence of siRNA on the scaffolds. Since dexamethasone was shown to

elicit similar effects on microglia in organotypic slice cultures and *in vivo*³², the lack of excessive glial response in our study suggests that there is high potential that our scaffolds would not aggravate spinal cord injuries after transplantation *in vivo*.

In this study, cells were seeded on the scaffolds with dense PCL fibers, which provide topographical cues to the cells. For *in vivo* transplantation, modification to the scaffolds may be needed. Firstly, PCL has relatively high stiffness (~20 kPa) as compared to softer substrates that favour neural differentiation (<1 kPa)^{33,34}. In the literature, PCL fibers are commonly used as a scaffold for neuronal differentiation by providing topographical cues and it is also one of the softest among the commonly used polymers (as compared to PLGA³⁵ and PEEK³³, for example). Despite current knowledge that soft substrates favour neural differentiation that is mediated through the RhoA/ROCK pathway³⁶⁻³⁸, these studies were done on 2D and hydrogel systems. Potentially, a softer fibrous scaffold than PCL may induce better neuronal differentiation and the interplay between stiffness and topography on neuronal differentiation may be further explored. Secondly, the PCL fibrous scaffold, which was used in this study did not support cell penetration. To overcome this, cells may be seeded on suspended fibers that are less dense and then embedded within a three-dimensional hydrogel. The feasibility of cell seeding on sparse suspended fibers and combining fibers with hydrogel for *in vivo* transplantation have been demonstrated previously³⁹⁻⁴¹. Results from this study can be applied as a reference for *in vivo* scaffold design to deliver hiPSC-NPCs and siRNA.

Altogether, this culture system could serve as a platform to select optimal scaffolds for implantation *in vivo* and predict scaffold-tissue interactions. Further investigations can be conducted in the future to evaluate the transplantation of other types of cells, integration of neurons with host tissues at longer time points, and the inflammatory response of the host tissue using such organotypic slice cultures.

Experimental

Materials

REST siRNA (siREST, s11932) and non-targeting negative control siRNA (siNEG, 4390844) were purchased from Ambion (Waltham, MA, USA). *TransIt*-TKO was purchased from Mirus Bio (Madison, WI, USA). Polycaprolactone (PCL), poly-L-ornithine, 2,2,2-trifluoroethanol (TFE), dopamine hydrochloride, 3,4-dihydroxy-L-phenylalanine (DOPA), NeuralQ medium, GS-21, and Triton-X 100 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fibroblast Growth Factor-2 was purchased from Peprotech. M-MLV Reverse Transcriptase was obtained from Promega (Madison, WI, USA). Rabbit anti-betaIII tubulin (TUJ1) was procured from Biolegend (San Diego, CA, USA). Mouse anti-2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNP) was purchased from DSHB (Iowa City, IA, USA). Rabbit anti-microtubule associated protein-2 (MAP2), chicken-anti glial fibrillary acid protein (GFAP), mouse anti-CD11b integrin αM

(OX42), and mouse anti-human nuclei antibody (HuNu) were obtained from Merck-Millipore (Burlington, MA, USA). All other cell culture reagents were purchased from ThermoFisher (Waltham, MA, USA).

Cell culture

Human NPCs were derived from hiPSCs using a Neuro EB protocol as described previously²⁷. Thereafter, hiPSC-NPCs were cultured on poly-L-ornithine and laminin coated culture dishes in maintenance medium. The maintenance medium consisted of DMEM/F12 supplemented with 1 % glutaMAX, 1 % N2 supplement, 2 % B27 supplement, 1 % penicillin-streptomycin and 20 ng/ml FGF2. Cells were maintained in a humidified incubator at 37 °C and 5 % CO₂. Thereafter, the cell culture medium was changed every 2 days. P7-P8 cells were used for seeding onto the scaffolds.

Scaffold preparation

Fig 1A shows the procedure for scaffold preparation. The polymer solution was prepared by dissolving PCL (MW: 70,000-90,000) in TFE (12.5% w/v). Thereafter, 2 ml of polymer solution was loaded into a 3 ml syringe with a 21G blunt needle. Following that, fibers were electrospun at a flow rate of 1.5 ml/h onto a rotating collector (1,200 rpm). Voltages of +10 kV and -4 kV were applied to the needle and rotating collector respectively. The distance between the rotating collector and the needle tip was set at 12 cm. The fiber mats were then cut into round pieces with a diameter of 15.6 mm to be fitted into wells of 24-wells plates.

The bioadhesive coatings on the scaffolds were prepared as described previously^{19, 20, 42, 43}. Briefly, for polydopamine deposition, dopamine hydrochloride was dissolved in 10 mM Tris buffer (pH 8.5) at 0.5 mg/ml. The scaffolds were then immersed into the dopamine solution and incubated on a shaker (100 rpm) for 4 h²⁰. For polyDOPA-melanin coating, scaffolds were immersed into 2 mg/ml DOPA that was dissolved in a buffer that consisted of 10 mM Bicine and 250 mM NaCl (pH 8.5) and incubated for 6 h on a shaker (100 rpm)^{19, 42, 43}. Following that, all scaffolds were washed and coated with 0.5 ml of poly-L-ornithine (0.1 mg/ml) for 1 h. The scaffolds were then washed and freeze dried overnight. Thereafter, all scaffolds were UV irradiated and coated with 0.5 ml of laminin (20 µg/ml) for 4 h.

siRNA complexes were formed with either RNAiMAX or TKO based on formulations as described previously^{19, 20}. For RNAiMAX, 2 µg siRNA and 6 µl RNAiMAX were diluted and mixed to form complexes for 5 min according to manufacturer's protocol²⁰. For TKO, 2 µg siRNA and 3 µl TKO were diluted and mixed to form complexes for 15 min¹⁸. Thereafter, siRNA complexes were added to the scaffolds respectively after laminin was removed and incubated at 37 °C for 1 h. Following that, scaffolds were washed once and hiPSC-NPCs were seeded at a density of 50,000 cells/cm² in maintenance medium. After 2 days, the medium was changed into differentiation medium.

The cells were then cultured for an additional 7 days with a change of medium every 2 days. The differentiation medium consisted of 50% DMEM/F12, 50% NeuralQ medium, 1 % N2 supplement, 2 % GS-21 supplement, 1 % glutaMAX and 1 % penicillin-streptomycin.

Scaffold characterization: surface morphology, siRNA distribution, loading efficiency, and release kinetics

The surface morphology of the scaffolds was observed under a scanning electron microscope (SEM, JOEL, JSM-6390LA) at a 10 kV accelerating voltage after freeze-drying overnight and sputter coated with platinum for 90 s at 20 mA. The average fiber diameters were measured by ImageJ with at least 100 fibers per sample. The distribution of siRNA on the scaffolds was observed by using fluorescently-tagged RNA oligonucleotides. Specifically, Cy5-tagged non-targeting RNA oligonucleotides (Cy5-RNA) were complexed with the respective transfection reagents and adsorbed onto the scaffolds as mentioned above. The scaffolds were then visualized under an epifluorescent microscope (Leica DMI8).

To determine siRNA loading efficiency, scaffolds with siRNA complexes were washed once with 500 µl PBS. The concentration of the unbound RNA in the supernatant was then determined by RiboGreen assay after being treated with 1 µg/ml heparin. The fluorescence intensity was measured using a microplate reader (Tecan, Infinite 200). The mass of siRNA in the supernatant was termed m_{washed} while the mass of initially loaded RNA was denoted as m_{loaded} . The initial RNA loading efficiency was then calculated as $(m_{\text{loaded}} - m_{\text{washed}}) / m_{\text{loaded}} \times 100\%$. Thereafter, scaffolds (diameter $\varnothing$ 15.6 mm, 9.3 ± 0.1 mg) were completely submerged into 500 µl PBS and incubated in a humidified incubator at 37 °C until the drug release profile plateaued at 28 days. At each time point, 250 µl of the supernatant was collected and replaced with the same amount of fresh PBS. The amount of siRNA that was released was then quantified by RiboGreen assay as mentioned and the cumulative release profile was plotted as percentage of actual mass loaded.

Cellular uptake

Cellular uptake of siRNA was observed by using Cy5-RNA-incorporated scaffolds. At 1 day and 9 days after cell seeding, scaffolds with cells were fixed and stained with phalloidin-AF488 and DAPI. Images were then taken at 400× magnification under a confocal microscope (Carl Zeiss LSM800) to observe the presence of Cy5-RNA in the cell cytoplasm.

Cell viability assay

Cell viability was assessed with Live/Dead Viability/Cytotoxicity Kit. Briefly, at the end of the culture after 9 days, scaffolds were incubated in calcein-AM and ethidium homodimer-1, which were diluted in PBS for 45 min. Thereafter, images were taken using an epifluorescent microscope (Leica DMI8) using a 10× objective. Images were taken at five random and non-overlapping areas for each scaffold. The experiment was

repeated for 3 times. Following that, all images were processed with ImageJ and the number of live cells and dead cells were counted. At least 1000 cells were counted for each sample group ($n = 3$).

Immunofluorescent staining of scaffolds

All scaffolds were fixed with 4% paraformaldehyde (PFA) at the end of the experiment. Scaffolds were then treated with 0.1 % Triton-X for 15 min before being blocked with 5 % BSA for 1 h. After that, the scaffolds were incubated in rabbit anti-TUJ1 (1:1000) overnight at 4 °C. The scaffolds were then stained with secondary antibody (1:500) and the nuclei were counterstained with DAPI. Next, the scaffolds were mounted and imaged using a 20× objective under an epifluorescence microscope (Leica DMI8). Images were taken at five random and non-overlapping areas for each scaffold. The experiment was repeated for 3 times. Finally, all images were processed with ImageJ and the number of TUJ1⁺ cells was counted. At least 150 cells were counted for each sample group ($n = 3$).

Gene expression

At Day 2, Day 5 and Day 9 after cell seeding, total RNA was extracted by TRIzol reagent and chloroform. RNA was then precipitated by isopropanol and washed with 75 % ethanol. Thereafter, the quality and quantity of RNA were checked by Nanodrop spectrophotometer. Reverse transcription of 500 ng of RNA to cDNA was then carried out using M-MLV Reverse Transcriptase. Following that, real time PCR was performed using SYBR Select Master Mix in ABI StepOne Plus. The PCR condition used was 10 min at 95 °C, followed by 15 s at 95 °C and 1 min at 60 °C for 40 cycles. The sequences of the primers were obtained from the literature and are presented in Table 1⁴⁴. Gene expression was analyzed using relative expression by $\Delta\Delta C_t$ method after confirming that all the primers had similar amplification efficiencies. Gene expressions were normalized relative to *GAPDH* for each time point.

Table 1. Primer sequences for real time PCR

Primers	Sequence (5' to 3')
<i>GAPDH</i>	Forward: AGCCACATCGCTCAGACAC
	Reverse: GCCCAATACGACCAATCC
<i>REST</i>	Forward: TGGAAAATGCAACTATTTTTCAGA
	Reverse: GAACTTGAGTAAGGACAAAGTTCACA
<i>TUJ1</i>	Forward: GCAACTACGTGGGCGACT
	Reverse: CGAGGCACGTACTTGTGAGA

Organotypic slice culture

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC), NTU. Fig 1B demonstrates the procedure for the organotypic slice culture. Briefly, spinal cords from the thoracolumbar region were isolated from postnatal rat pups of 5 to 7 days old. Spinal cords were then cut into 350 μ m longitudinal slices along the sagittal

plane with a tissue chopper. A full transection was made to the slices with microscissors to simulate a complete transection spinal cord injury. Thereafter, a 2 mm gap was created in between the transected spinal cords. A scaffold prepared as mentioned above was cut into 2 mm $\times$ 3 mm dimension and applied to the spinal cord to bridge the transected site. The tissues were then cultured on a tissue culture insert with 0.4 μ m pore size that provided an air-liquid interface for 7 days. The culture medium consisted of neurobasal medium, 2 % B27 supplement, 1 % glutaMAX, and 1 % penicillin-streptomycin and was changed every 2 days. At the end of 7 days, tissues were fixed in 4% PFA for 1 h.

Immunofluorescent staining of spinal cord organotypic slices

Tissue slices with scaffolds were incubated in block solution (3% horse serum, 2% BSA, 0.5% Triton-X in PBS) on a shaker for 1 h at room temperature. Following that, the tissues were incubated in primary antibodies that were diluted in block solution on a shaker for overnight at 4 °C. Primary antibodies used were: rabbit anti-TUJ1 (1:1000), mouse anti-HuNu (1:500), chicken anti-GFAP (1:500), mouse anti-CNP (1:25), mouse anti-*OX42* (1:200). Thereafter, the tissues were washed with block solution for 3 times and incubated in secondary antibodies (1:500) and DAPI diluted in block solution for 2 h at room temperature. Subsequently, the tissues were washed thrice with PBS and mounted for imaging. Images were taken at 200X with an epifluorescent microscope (Leica DMI8). Images were stitched to analyze the viability of hiPSC-NPCs and the number of neurite extensions. The number of hiPSC-NPCs on each scaffold was counted within a 1000 μ m $\times$ 500 μ m area in the center of scaffold. To analyze the number of neurite extensions, the number of neurites at 500 μ m and 1000 μ m from both scaffold-tissue interfaces as well as the number of neurites crossing the 2 mm injury gap were counted. To assess cell migration from the rat spinal cord slices, images were taken using a confocal microscope (Carl Zeiss LSM800) using a 40× oil objective. Three images were taken within a distance of 400 μ m from each scaffold-tissue interface and positively stained cells were counted using ImageJ. At least 110 cells were counted for each sample from at least 3 experiments.

Statistical analysis

Statistical analysis was carried out using SPSS. All the values were represented as mean $\pm$ standard error of mean. One-way ANOVA and the Tukey post-hoc tests were used for comparison of means after verifying for normality and equal variance. Otherwise, Mann-Whitney U test was used for pairwise analysis.

Conclusions

In conclusion, we optimized and demonstrated a scaffold-mediated siREST delivery system to enhance hiPSC-NPCs neuronal differentiation. We identified that siREST-RNAiMAX complexes and polydopamine-incorporated electrospun fiber scaffolds were able to knockdown REST expression and enhance

hiPSC-NPCs differentiation into TUJ1⁺ neurons with minimal cytotoxicity. Similar effectiveness of the siREST-loaded scaffolds in enhancing neuronal differentiation was also observed when the scaffolds were transplanted to transected spinal cord slices. Upon examination, the aligned fiber scaffolds guided the growth direction of neurites and supported cell migration from the spinal cord tissues. Taken together, the organotypic slice culture results suggest that this scaffold can be effective in delivering hiPSC-NPCs to injured spinal cords. Thus, this approach may be a potential strategy for neural regeneration by generating neurons from hiPSC-NPCs.

Conflicts of interest

There are no conflicts to declare.

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Figure 1. Experimental procedure. (A) Flow chart for scaffold preparation and *in vitro* optimization. (B) Flow chart for spinal cord organotypic slice culture.

Figure 2. Scaffold characterization. (A) SEM images for of PCL electrospun scaffolds with and without functionalization. **(B)** Average fiber diameter of the scaffolds with and without functionalization. **(C)** Distribution of cy5-RNA on the scaffolds, showing more diffused signals of cy5-RNA-RNAiMAX complexes. **(D)** Loading efficiency for different combinations of scaffolds and siRNA complexes. **(E)** siRNA release profile in terms of % of experimental loading. Mean $\pm$ S.E.M. $n = 3$.

Figure 3. Cellular uptake and viability. (A) Cellular uptake of cy5-RNA at Day 1 and Day 9. (B) Cell viability as measured using the LIVE/DEAD Viability/Cytotoxicity Kit.; Mean $\pm$ S.E.M.; * : $p < 0.05$ compared to plain; $n = 3$.

Figure 4. Neuronal differentiation and REST knockdown. (A) Representative images with TUJ1 staining (red) and nuclei counterstained by DAPI (blue) at Day 9 after seeding. **(B)** Percentage and number of TUJ1⁺ cells per ROI; ROI: images taken using a 20× objective; Mean ± S.E.M.; * : $p < 0.05$; $n = 3$. **(C)** Gene expression for DOPAMINE – RNAiMAX scaffolds relative to GAPDH and siNEG of each time point; mean ± S.E.M., * : $p < 0.05$; $n = 4$.

Figure 5. Scaffold with injured spinal cord tissues after culturing for 7 days on cell culture insert. (A) Representative image of scaffold integration with transected spinal cord tissues. (B) Representative images of HuNu⁺ hiPSC-NPCs in the middle of the scaffold stained for TUJ1. (C) Number of hiPSC-NPCs counted by the number of HuNu⁺ human nuclei stained; ROI: 1000 × 500 μm in the middle of scaffolds; mean ± S.E.M., n = 5. (D) Percentage of HuNu⁺TUJ1⁺ double positive cells per HuNu⁺ cells in the middle of scaffold; mean ± S.E.M., * : p < 0.05, n = 5.

Figure 6. Neuron migration and neurite extension from spinal cord tissue slices onto scaffolds. (A) Representative images of TUJ1 staining at the scaffold-tissue interface. Images are maximum projections of z-stack images (thickness: 27 μ m) taken using a 20 $\times$ objective. Arrow indicates aligned fiber direction. (B) Number per ROI and percentage of HuNu-TUJ1⁺ cells per DAPI within 400 μ m of the interface; ROI: sum of 3 images taken using a 40 $\times$ objective; mean $\pm$ S.E.M., $n = 5$. (C) Number of neurites at 500 μ m (green arrows) and 1000 μ m (white arrows) from both rostral and caudal scaffold-tissue interfaces. (D) Number of neurites that crossed the entire 2-mm injury gap; mean $\pm$ S.E.M.; $n = 8$. (E) Number of neurites that crossed the 500 μ m line; mean $\pm$ S.E.M.; $n = 8$. (F) Number of neurites that crossed the 1000 μ m line; mean $\pm$ S.E.M.; $n = 8$.

Figure 7. Glial cells migration on electrospun scaffolds from spinal cord slice tissues. (A) Representative images at the scaffold-tissue interface stained for CNP⁺ oligodendrocytes, GFAP⁺ astrocytes and OX42⁺ microglia. Images are maximum projection of z-stack images (thickness: 27 μ m) taken using a 20 $\times$ objective. (B) Number per ROI and percentage of each type of glial cells per DAPI within 400 μ m of both rostral and caudal scaffold-tissue interfaces; ROI: sum of 6 images taken on scaffold within 400 μ m of scaffold-tissue interfaces using a 40 $\times$ objective; mean $\pm$ S.E.M., n = 3.