Localised non-viral delivery of nucleic acids for nerve regeneration in injured nervous systems

Zhang, Na; Chin, Jiah Shin; Chew, Sing Yian

2018

Zhang, N., Chin, J. S., & Chew, S. Y. (2018). Localised non-viral delivery of nucleic acids for nerve regeneration in injured nervous systems. *Experimental Neurology*.
doi:10.1016/j.expneurol.2018.09.003

<https://hdl.handle.net/10356/89781>

<https://doi.org/10.1016/j.expneurol.2018.09.003>

© 2018 Elsevier. This is the author created version of a work that has been peer reviewed and accepted for publication by *Experimental Neurology*, Elsevier. It incorporates referee's comments but changes resulting from the publishing process, such as copyediting, structural formatting, may not be reflected in this document. The published version is available at: [<http://dx.doi.org/10.1016/j.expneurol.2018.09.003>].

Downloaded on 26 Apr 2023 19:37:01 SGT

Manuscript Number: EXNR-18-362R1

Title: Localized non-viral delivery of nucleic acids for nerve regeneration in the injured nervous systems

Article Type: SI: Neural regeneration

Keywords: Gene delivery; Scaffolds; RNA interference; Neural tissue engineering; Electrospinning; Gene silencing; siRNA; microRNA

Corresponding Author: Dr. Sing Yian Chew,

Corresponding Author's Institution: Nanyang Technological University

First Author: Na Zhang

Order of Authors: Na Zhang; Jiah Shin Chin; Sing Yian Chew

Abstract: Axons damaged by traumatic injuries are often unable to spontaneously regenerate in the adult central nervous system (CNS). Although the peripheral nervous system (PNS) has some regenerative capacity, its ability to regrow remains limited across large lesion gaps due to scar tissue formation. Nucleic acid therapy holds the potential of improving regeneration by enhancing the intrinsic growth ability of neurons and overcoming the inhibitory environment that prevents neurite outgrowth. Nucleic acids modulate gene expression by over-expression of neuronal growth factor or silencing growth-inhibitory molecules. Although in vitro outcomes appear promising, the lack of efficient non-viral nucleic acid delivery methods to the nervous system has limited the application of nucleic acid therapeutics to patients. Here, we review the recent development of efficient non-viral nucleic acid delivery platforms, as applied to the nervous system, including the transfection vectors and carriers used, as well as matrices and scaffolds that are currently used. Additionally, we will discuss possible improvements for localised nucleic acid delivery.

COVER LETTER

Sing Yian CHEW, Ph.D.
Associate Professor
Nanyang Technological University
School of Chemical and Biomedical Engineering
Lee Kong Chian School of Medicine
62 Nanyang Drive, Singapore 637459
E-mail: sychew@ntu.edu.sg
Tel: +65-6316-8812

August 30, 2018

Ahmet Hoke, M.D., Ph.D.
Editor-in-Chief
Experimental Neurology

Dear Dr. Hoke:

Attached, please find our revised submission, "**Localized non-viral delivery of nucleic acids for nerve regeneration in the injured nervous systems**" submitted for consideration of publication in Experimental Neurology, SI: Neural Regeneration.

This review has highlighted the important nucleic acid candidates, which participate in cellular activities within the nervous system, and their mechanisms of gene modulation. Following that, the available transfection methodologies of neuronal cells and the application of these methods for promoting nerve regeneration in the injured nervous systems were discussed. Lastly, we included design considerations for scaffold fabrication to enable efficient localized nucleic acids delivery. We thank all the reviewers for their critical review of this work. We have improved on our manuscript with their suggestions.

Thank you for your kind consideration.

Sincerely,

A handwritten signature in black ink, appearing to read "Sing Yian Chew", is located below the "Sincerely," text.

Sing Yian Chew, Ph.D.

Manuscript ID: EXNR-18-362

Title: Localized non-viral delivery of nucleic acids for nerve regeneration in the injured nervous systems

Journal: Experimental Neurology

We thank the reviewer for providing constructive comments. We have highlighted all changes in yellow and listed point-by-point responses below.

Reviewer #1: Major concerns

1. Phrasing is frequently difficult to understand, with considerable scattered grammatical errors. I recommend careful revision or hiring the services of an English proof-editor. E.g. Abstract - "gene expressions"; used. utilized; Introduction, page 4 - "various disorders", improperly used instead of applications, as skin wound healing/bone regeneration are not disorders. Closer attention should also be paid to the usage of proper verb tenses, e.g. "long-term gene expression, which brought about". Such errors abound throughout the manuscript and should be rectified before future considerations.

Response:

The manuscript has been carefully amended. All tenses have been corrected as well.

2. The manuscript reads more as a book chapter than a review, primarily due to its structure. Introductory subchapters are used to define certain notions and contain information that is not absolutely relevant for understanding further content. These definitions could be better refined and interspersed where appropriate to the content, instead of being grouped together.

Response:

We have reorganized the revised manuscript to make it more succinct.

Firstly, we agree that the definitions of nucleic acids in the Introduction section is not relevant for understanding further contents. Therefore, we have removed that part and only left two tables with schematics. The two comparison tables (Table 1A and 1B) serve to identify differences between nucleic acids and allow readers to make appropriate selections of delivery methods.

Since nerve regeneration originates from neuronal cells, delivering nucleic acids to individual neurons plays a significant role in modulating axon regeneration. Neurons are post-mitotic cells that are difficult to transfect. Hence, we have dedicated section 2.1 to look into various ways of achieving efficient nucleic acid transfection in neurons.

Section 2.2 is one of the main sections in this manuscript as it illustrates the recent works that involve the application of nucleic acids to promote nerve regeneration *in vivo*. Here, we explained the gap between *in vitro* and *in vivo* transfection for the nervous system. We believe this progression would help readers understand the review better.

3. In Chapter 2.2, information is presented as an enumeration of findings from various research articles with little intellectual input from the authors especially compared to subchapter 2.3.

Response:

We have now summarised this section and presented relevant comparisons between studies and transfection platforms, to reflect more intellectual input in the revised manuscript.

Besides that, we have now added additional comments on page 15 of the revised manuscript. Line 14 now reads, *'Taken together, lipofection is the most commonly used method for the transfection of neuronal cells due to its high transfection efficiency and low cytotoxicity. As compared to electrical and physical transfection methods, lipofection is more applicable for transfecting a large number of neurons in one go. On the other hand, for single cell studies, single-cell electroporation and microinjection are more appealing due to their transfection accuracy. However, these methods are recommended for the transfection of robust neurons (i.e. PC12 and invertebrate neurons) as the electrical and mechanical stimuli could jeopardize cell viability. Altogether, several factors such as neuronal cell types and their survival rates should be taken into consideration before deciding on the transfection approach.'*

4. A lot of space is allocated to chapter 2.2, regarding methods for in vitro delivery of nucleic acids. Considering the topic of this review, I do not find this subchapter justified in its current length, unless more arguments can be provided for the translation of the in vitro parameters to actual therapeutics.

Response:

As suggested, the length of the original chapter 2.2 (now currently listed as chapter 2.1) has been shortened. Only the crux of each *in vitro* transfection methods is presented in the main text.

5. The conclusion drawn at the end of this review is over-simplified and should be revised.

Response:

This point is acknowledged, and the conclusions have been amended accordingly.

Conclusions, Page 41, now reads, *'The injured nervous system holds limited regenerative capability, especially within the CNS. Although the PNS has some regenerative capacity, its ability to grow remains limited when crossing large lesion gaps. Although the delivery of nucleic acids via viral vehicles into the injured nervous system holds great potential in enhancing nerve regeneration in vivo, the lack of safe and efficient delivery systems has limited the application of these molecules in patients who suffer from traumatic nerve injuries. Therefore, the exploration of non-viral delivery approaches is of great necessity. Along this line, this review has highlighted the important nucleic acid candidates, which participate in cellular activities within the nervous system, and their mechanisms of gene modulation. Following that, we focused on the transfection of neuronal cells and summarised the non-viral vectors that have been used in vitro as well as their corresponding transfection efficiencies. More importantly, we reviewed recent animal studies on non-viral delivery of therapeutic nucleic acids for nerve regeneration. Specifically, the combinatorial approach of nucleic acid therapeutics with scaffolds provides a synergistic and promising treatment option for nerve regrowth. Besides that, scaffold-mediated non-viral nucleic acid delivery allows controlled modulation of gene expression while providing topographical cues to guide axonal regeneration.*

Although scaffold-mediated non-viral nucleic acid delivery approaches have been applied to nerve injury repair, many challenges remain in the development of these bio-functionalized scaffolds. These challenges include maintaining or enhancing the stability of nucleic acids against biodegradation, improving cellular uptake efficiencies as well as the temporal control of the expression of target gene. As such, these aspects should be taken

into consideration when designing scaffolds for more effective therapeutic treatment for nerve repair.'

Minor concerns

6. Introduction - The authors mention that significant safety issues and complications arise from using viral vectors for transfection but do not explain. Some examples should be briefly described or summarized in a table.

Response:

The safety issues and complications of using viral vectors for delivering of nucleic acids is now added into the revised manuscript. Page 4, Line 2 now reads, *'In particular, viral vectors often lead to unwanted immune responses, increased risk of insertional mutagenesis, and face difficulties in storage, which are critical problems that limit their clinical applications²³⁻²⁶.'*

7. Introduction - References that support the following statement "nucleic acids packaged in viral vectors such as adeno-associated virus or herpes simplex virus remain the leading candidate for neuron-targeted gene therapy as they have high transfection efficiencies and enable long-term gene expression" should be highlighted as summarized/ described" in the references quoted, since all 3 papers are reviews.

Response:

This point is acknowledged and the references that support this statement have been changed into experimental papers at Page 4, Line 1, reference No. 19-22.

8. Introduction - Concluding phrase for this chapter must be rephrased for clarity. "Finally, we will focus on localized nucleic acids delivery via scaffolds where some design considerations for better control over delivery and uptake of nucleic acids by injured neurons will be discussed as future strategies to enhance nerve regeneration by nucleic acid therapeutics."

Response:

This point has been amended accordingly. Page 4, Line 19 now reads, *'Finally, we will focus on the delivery of nucleic acids via scaffolds to achieve localized and sustained therapeutic outcomes. Design considerations for better control over the delivery and uptake of nucleic acids by injured neurons will be discussed as future strategies to enhance nerve regeneration by nucleic acid therapeutics.'*

9. Several references appear to be missing. Take for example the first paragraph of the Gene overexpression subchapter - an example of plasmids successfully used to overexpress growth factors should be included.

Response:

We have screened through the manuscript carefully and inserted the appropriate references accordingly.

10. The definitions of types of nucleic acid tools and delivery methods could be greatly improved by use of general schematics for each type, particularly as there is mention of their structures/principle of action.

Response:

We have now added in schematics to represent structures of each nucleic acid and the principle of action for nucleic acids that are involved in gene silencing, as shown in Table 1A and 1B.

11. Lipofectamine 2000 is mentioned, however its improved version Lipofectamine 3000 is not. Should it not be compared with the other delivery vectors?

Response:

We have added the information of Lipofectamine 3000 in the main text. Page 12, Line 6 now reads, *'As an improved version of Lipofectamine 2000, Lipofectamine 3000 has also been widely used for neuronal cell transfection⁸⁷⁻⁸⁹. However, these studies did not report their transfection efficiency. As such, it is difficult to directly compare with other delivery vectors.'*

12. There effect of GDNF on neuronal regeneration has long been established, and it can be administered directly to an injury site. Why GDNF-gene transfection is a preferable therapeutic strategy compared to the protein itself should be supported with evidence comparing the two within the same experiment in order to justify the authors statement.

Response:

This point is acknowledged. However, the direct comparison between the utilization of GDNF protein and GDNF-gene transfection has not been reported. Nonetheless, this point is not only applicable for GDNF but also for other therapeutic proteins. Thus, we have elaborated on this point in the beginning of the in vivo section. Page 19, Line 23 now reads, *'Comparatively, although proteins can also be administered to an injury site, one advantage of using nucleic acids is that multiple therapeutic genes can be delivered from the same*

delivery systems². In addition, the administration of proteins to the injured site via local injection is often transient due to the labile nature of proteins, especially under the injured microenvironment¹²⁰. Hence, by modifying the genome of the transfected cells, protein expression can be prolonged¹²¹. Besides that, when transfecting cells that can proliferate, protein expression may be passed on¹²², thereby enabling long term therapeutic effects. More importantly, protein treatment works through the recognition by protein receptors. For proteins which receptors are lacking on the neurons, protein treatment is not applicable¹²³. Nucleic acid delivery could also overexpress transcription factors¹²⁴, which cannot be achieved by protein delivery.'

13. What is the time-course of the nucleic acid therapeutics in the studies referenced?

Response:

We have included this point in the main text. Page 24, Line 8 now reads, 'Usually, the injected nucleic acid therapeutics can last for 1 month and the time-course of observing its expression/effects is around 3-7 days^{121,140,145,146}. However, prolonged expression of the transgenes (eg. several months) is often needed to achieve more prominent functional recovery¹⁴⁹.' Page 26, Line 21 now read, 'As compared to intraspinal injection, scaffold-mediated nucleic acid delivery can last for several months (eg. 3 months)¹⁴⁹ and the transgene expression was observed up to 3-4 weeks after treatment^{161,162}.'

14. What does a low rate of axon growth mean?

Response:

For clarification, the low rate of axon growth has been changed into 'low rate of nerve regeneration (i.e. 1 mm/ day¹⁶⁸)' in the main text, page 27, Line 14. This rate of nerve

regeneration dose not match the regeneration requirement when crossing large lesion gaps.

15. The title of Chapter 4 should be changed to Conclusion, as the content of the chapter does not represent a proper summary of the review.

Response:

This point is acknowledged, and the title of Chapter 4 has been changed into "Conclusions"

Localised non-viral delivery of nucleic acids for nerve regeneration in injured nervous systems

Na Zhang^{1, #}, Jiah Shin Chin^{1,2, #}, Sing Yian Chew^{1,3, *}

¹ School of Chemical and Biomedical Engineering, Nanyang Technological University, Singapore 637459;

² NTU Institute of Health Technologies, Interdisciplinary Graduate School, Nanyang Technological University, Singapore 639798;

³ Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore 308232;

*Corresponding author:

Tel.: +65 6316 8812; Fax: +65 6794 7553; E-mail: sychew@ntu.edu.sg

[#]: These authors contributed equally in this work

Abstract

Axons damaged by traumatic injuries are often unable to spontaneously regenerate in the adult central nervous system (CNS). Although the peripheral nervous system (PNS) has some regenerative capacity, its ability to regrow remains limited across large lesion gaps due to scar tissue formation. Nucleic acid therapy holds the potential of improving regeneration by enhancing the intrinsic growth ability of neurons and overcoming the inhibitory environment that prevents neurite outgrowth. Nucleic acids modulate gene expression by over-expression of neuronal growth factor or silencing growth-inhibitory molecules. Although *in vitro* outcomes appear promising, the lack of efficient non-viral nucleic acid delivery methods to the nervous system has limited the application of nucleic acid therapeutics to patients. Here, we review the recent development of efficient non-viral nucleic acid delivery platforms, as applied to the nervous system, including the transfection vectors and carriers used, as well as matrices and scaffolds that are currently used. Additionally, we will discuss possible improvements for localised nucleic acid delivery.

Key words: Gene delivery; Scaffolds; RNA interference; Neural tissue engineering; Electrospinning; Gene silencing; siRNA; microRNA

1. Introduction

Treatment options targeted at stimulating nerve regeneration after injuries remain limited. Hence, continuous elucidation of the molecular mechanisms that underlie such poor nerve regeneration has driven the development of treatment strategies that aim at reversing neuropathologies at the molecular level. Correspondingly, treatments with nucleic acid therapeutics have emerged as a promising approach since it addresses the molecular causes of hindered nerve regeneration by manipulating gene expression profiles in targeted cells within the nervous system. In general, there are two main nucleic acid-based therapeutic approaches – gene therapy and gene silencing^{1–3}. Gene therapy for nerve regeneration is typically accomplished by introducing genes that encode for neurotrophic growth factors or corrective enzymes to injured neurons. Both pathological and functional outcomes have been observed through the use of such strategies in animal models^{4–6}. On the other hand, the implementation of gene silencing methods, such as RNA interference (RNAi), has witnessed the reduction in toxic protein expression levels and the minimization of growth inhibitory signals at nerve injury sites^{7–9}.

Nucleic acid-based therapy has seen significant advancement in various tissue repair applications, ranging from wound healing in skin^{10–13}, to bone regeneration^{14,15}, muscle repair¹⁶ and optic nerve repair¹⁷. However, due to the extreme difficulty in transfecting mature post mitotic neurons with genetic materials¹⁸, it has become crucial to use highly efficient gene vectors and carriers for effective transfection to occur within the nervous system. Hence, nucleic acids packaged in viral vectors such as the adeno-associated virus or herpes simplex virus remain the leading candidate for neuron-targeted gene therapy as they have high transfection efficiencies and enable long-term gene expression, which brings

1 about functional recovery in various animal model^{19–22}. However, significant safety issues
2 and complications have also arisen out of such viral delivery strategies. In particular, viral
3 vectors often lead to unwanted immune responses, increased risk of insertional
4 mutagenesis, and face difficulties in storage, which are critical problems that limit their
5 clinical applications^{23–26}. Even though viral vectors can be altered to remove viral
6 components that trigger the immune response, the modified viruses are often difficult to
7 produce with substantial yield and efficacy^{27,28}. On the other hand, non-viral nucleic acid
8 delivery strategies offer improved safety profiles and are promising alternatives.
9 Unfortunately, the limited transfection efficiencies of non-viral delivery platforms must first
10 be addressed before achieving functional nerve regeneration outcomes.

11
12 In order to improve the transfection efficiency of non-viral nucleic acid delivery
13 platforms, it is crucial to understand the molecular structures of these nucleic acids while
14 dwelling into the recent strategies that have been employed by the neural tissue
15 engineering field in order to deliver these molecules non-virally. Therefore, this review will
16 begin by looking into various types of therapeutic nucleic acids that have been used in tissue
17 engineering. Following that, we will highlight the available delivery and transfection
18 methodologies that are specific to neurons. We will also discuss the application of these
19 methods to promote nerve regeneration in the injured nervous systems. Finally, we will
20 focus on the delivery of nucleic acids via scaffolds to achieve localized and sustained
21 therapeutic outcomes. Design considerations for better control over the delivery and uptake
22 of nucleic acids by injured neurons will be discussed as future strategies to enhance nerve
23 regeneration by nucleic acid therapeutics.

2. Platforms for non-viral delivery of nucleic acids and their applications in the nervous system

Nucleic acids have been used to enhance or inhibit gene expression at transcription and post-transcriptional levels to direct tissue regrowth²⁹. The use of these nucleic acids has been explored in many tissue regeneration approaches, such as bone^{30–32}, skin^{33–35}, ligaments and tendons³⁶, cartilage^{37,38}, cardiac³⁹ and hepatic tissues⁴⁰. On the contrary, such use of nucleic acid-based therapeutics is significantly less reported for nerve regeneration. This phenomenon may be attributed to the challenges in neuronal cell transfection. The central nervous system (CNS) is protected by a barrier system that is composed of tight vascular junctions and glial elements, which forms the blood-brain barrier that prevents the access of therapeutics^{41,42}. Besides that, non-viral nucleic acid delivery systems should be designed to target and transfect specific neuronal populations while ensuring that the nucleic acids bind to these cells before being washed out of the nervous system^{43–45}. Furthermore, the design of an efficient non-viral delivery platform is dependent on the type of nucleic acid used.

An extensive literature search revealed that therapeutic nucleic acids can be broadly categorized by either gene overexpression or gene silencing. Plasmids and messenger RNAs (mRNAs) are the two most commonly used nucleic acids for overexpressing genes^{46–49} while antisense oligonucleotides (AS ODNs)^{50,51}, short interfering RNAs (siRNAs)^{52,53} and microRNAs (miRs)^{54,55} are most commonly involved in gene silencing for neural tissue engineering. Notably, the mechanisms of how these nucleic acids modulate gene expression are different. Hence, delivery considerations will vary from one type of nucleic acid to another. An overview of these nucleic acids, along with some of the important properties

- 1 that should be considered when designing non-viral platforms for the delivery of
- 2 therapeutic nucleic acids to the nervous system are listed in Tables 1A and 1B.

Table 1A: A summary of therapeutic nucleic acids for gene overexpression and some design considerations for development of non-viral delivery systems

Gene overexpression		
<i>Properties</i>	<i>Plasmids</i>	<i>mRNAs</i>
Structure	<p>Several kilo base pairs Double stranded DNA constructs</p>	<p>Long single stranded RNA up to 130 nucleotides in length</p>
Charge	Negatively charged due to phosphate backbone	
Place of action	Nucleus	Cytoplasm
Duration of gene regulation	Long-term or permanent depending on site of integration within host genome	Transient
Transfection barriers	Cell membrane and nuclear membrane	Cell membrane
References	[56],[57]	[58–63]

Table 1B: A summary of therapeutic nucleic acids for gene silencing and some design considerations for development of non-viral delivery systems

Gene silencing			
Properties	AS ODNs	siRNAs	miRs
Structure	<p>15 to 20 nucleotides 6 to 10 kDa Single stranded DNA</p>	<p>21 to 23 nucleotides 12 to 13.3 kDa Duplex RNA strand with 3' overhangs</p>	<p>21 to 25 nucleotides 14 to 15 kDa Duplex RNA strand with interspersed mismatches and 3' overhangs</p>
Charge	Negatively charged due to phosphate backbone		
Place of action	Cytoplasm		
Mechanism of gene regulation	<p><u>Recruiting protein factors such as RNase H</u></p> <p>AS ODN</p> <p>RNase H</p> <p>mRNA</p> <p>Steric blocking of ribosomes and other factors</p> <p>AS ODN</p> <p>mRNA</p> <p>Ribosome</p>	<p><u>Binding of siRNA to RISC facilitates separation of duplex</u></p> <p>Guide strand</p> <p>RNA Interference Silencing Complex (RISC)</p> <p>Passenger strand</p> <p>Degradation of passenger strand</p> <p>RISC-mediated cleavage of mRNA</p> <p>mRNA</p>	<p><u>Binding of miR to RISC facilitates separation of duplex</u></p> <p>Guide strand</p> <p>RNA Interference Silencing Complex (RISC)</p> <p>Passenger strand discarded</p> <p>RISC-mediated cleavage of mRNA</p> <p>Translational repression</p> <p>mRNA</p>

	<p><u>Modulate mRNA splicing</u></p>
<i>Complementary to mRNA</i>	Completely complementary to mRNA	Completely complementary to mRNA	Partially complementary to mRNA, typically targeting the 3' untranslated region (UTR) of mRNA
<i>Number of mRNA targets</i>	One	One	Multiple
<i>Duration of gene regulation</i>	Transient		
<i>Transfection barriers</i>	Cell membrane		
<i>References</i>	[64–67]	[68–72]	[72–77]

2.1 *In vitro* studies on transfection of neurons

Stimulating the intrinsic growth ability of neurons is crucial to achieve the desired regeneration outcomes after injuries in the nervous system. Nucleic acid therapeutics have emerged as promising approaches since they can potentially be used to downregulate growth inhibitory molecules (eg. Nogo, OMgp and MAG) or upregulate growth promoting factors. However, the application of nucleic acid therapy, through non-viral delivery methods, on neurons requires rigorous optimisation since neurons are especially sensitive to physical stress, temperature alterations, pH shifts and changes in osmolarity¹⁸. Despite these constraints, numerous non-viral methods of gene delivery, such as chemical transfection, electrical transfection and physical transfection have been established to deliver nucleic acids to neurons *in vitro* with impressive outcomes^{78,79,80}. Table 2 provides an overview of studies that have been carried out on neuronal cell transfection, including the transfection approaches, vectors used and their respective transfection efficiencies.

Chemical transfection methods

Calcium-phosphate/DNA co-precipitation

Calcium phosphate transfection remains a convenient and economical method for transfecting foreign genes into neurons. Specifically, transfection is performed by mixing calcium chloride with recombinant DNA in a phosphate buffer and allowing the formation of DNA/calcium phosphate precipitates. These precipitates are then added into the cell culture medium and administered to the cells, where they are then endocytosed and shuttled into the nucleus¹⁸. Notably, this method can be applied to neurons at all stages of its cell cycle, including those that have already formed neuronal networks⁸¹. Generally, the transfection

1 efficiency obtained using calcium phosphate as the carrier ranged between 0.5-5%⁸².

2 However, with further optimisations, it is possible to reach 50% transfection efficiency⁸¹.

4 *Lipofection*

5 Lipid-mediated gene delivery platforms work through the effects of cationic lipid
6 molecules. These lipid molecules contain a positively charged head group, which can
7 interact with the negatively charged nucleic acids to form complexes. The lipid-nucleic acid
8 complexes can then fuse with the cell membrane⁸³, and deliver the nucleic acids into the
9 cells effectively. To further facilitate the fusion of the complexes with the cell membrane,
10 the cationic lipid molecules are often combined with a neutral co-lipid (helper lipid). Besides
11 being used for transfecting large nucleic acids (i.e. DNA and mRNA), lipid-based vectors have
12 also been utilized for the delivery of small oligonucleotides due to their high transfection
13 efficiencies.

15 An example of cationic lipofection reagent that works well in neuron cultures is
16 Lipofectamine® RNAiMAX⁸⁴. For this transfection reagent, the transfection efficiency was
17 found to be affected by the culture medium as well as the volume ratio between the
18 transfection reagent and the nucleic acids. By simply using Neurobasal-A instead of DMEM
19 for transfecting miR-21 into cortical neurons, higher amounts of miR-21 could be detected
20 within the cells. Furthermore, the transfection efficiency peaked when the volume ratio of
21 Lipofectamine® RNAiMAX:miR-21 was 3:5⁸⁴.

23 Similar to Lipofectamine® RNAiMAX that is commonly used to deliver siRNA and
24 miRNA, Lipofectamine® 2000 is another cationic lipid reagent that is widely utilized for the

1 delivery of nucleic acids with larger number of base pairs (i.e. DNA, mRNA)⁸⁵. When
2 Lipofectamine® 2000 was utilized for the delivery of mRNAs into DRG neurons, a
3 transfection efficiency as high as 25% was observed (based on EGFP mRNA transfection)⁸⁶.
4 Besides that, further analysis validating the expression of several heterologous proteins
5 namely, a cannabinoid receptor (CB1R), a G protein inwardly rectifying K⁺ channel (GIRK4)
6 and a dominant-negative G protein α -subunit mutant, suggested successful mRNA
7 transfection⁸⁶.

8 As an improved version of Lipofectamine 2000, Lipofectamine 3000 has also been widely
9 used for neuronal cell transfection^{87–89}. However, these studies did not report their
10 transfection efficiency. As such, it is difficult to directly compare with other delivery vectors.

11
12
13 Overall, attributing to the ease of use, lipid-based carriers have been widely utilized
14 for the delivery of both large (i.e. plasmid DNA, mRNA) and small nucleic acids (i.e. siRNA,
15 miRNA)^{90,91}, *in vitro*. Additionally, liposomes do not induce strong toxicity and are highly
16 reproducible when used for transfecting various neurons^{84,91}. Compared to viral delivery
17 methods, there is also a lower risk of mutation and immune-related issues⁹². Expanding
18 from the success of *in vitro* neuronal cell transfection, lipid-based vectors have also been
19 widely used for *in vivo* studies, as highlighted in the subsequent section.

21 ***Electrical transfection methods***

22
23 Electroporation is a technique that enables the cellular plasma membrane to be
24 transiently permeable to its surrounding materials and was shown to work well for both
25 embryos and dissociated neurons⁹³. By exposing neurons to a voltage pulse, the nucleic

acids can then enter the cytoplasm via the pores that were formed in the cell membrane⁹⁴. Generally, the transfection efficiency of neurons through electroporation is relatively low (0.5–3%)⁹³. However, higher transfection efficiency can be achieved by sacrificing cell viability⁹³.

Buchser *et al.* used electroporation to transfect primary mouse cerebellar granule neurons (CGNs) and rat hippocampus neurons⁹⁵. According to them, increasing voltages gave higher transfection efficiency while resulting in lower cell viability. Moreover, a calcium-free intracellular buffer⁹⁶ provided significantly better transfection efficiency than standard extracellular buffers or media. With the necessary optimizations, the average transfection efficiency of mouse CGNs and hippocampal neurons reached up to $\sim 26.8\% \pm 8.6\%$ and $\sim 17.3\% \pm 3.2\%$, respectively, as evaluated by GFP expression changes⁹⁵.

A novel micropipette electroporation technique was developed by Haas *et al.* for single cell transfection⁹⁷. Single-cell transfection enables the individual monitoring of genetic changes in a specific cell. This technique allows for genetic changes to be made in a specific single neuron, which is suitable for studying single cell behaviour during live cell imaging. Single-cell electroporation is applicable for delivering both large plasmid DNAs⁹⁷ and small oligonucleotides⁹⁸.

The transfection efficiency of single-cell electroporation was affected by various factors, including pulse shape, the number of pulses delivered and the voltage amplitude. However, the limitation of electroporation is the requirement of a large number of neurons to survive the electrical pulse. In general, high cell density facilitates the transfection

outcomes as the firm cell-cell attachment prevents cell death. On the contrary, insufficient cells for electroporation caused higher cell death rate and unhealthy surviving cells. As compared to lipofection, electroporation was more commonly reported for the delivery of large plasmid DNA.

Physical transfection methods

Microinjection

Although electroporation or chemical-mediated transfection have been widely utilized for neuronal cell transfection, the post-mitotic nature of primary neurons somehow prevents effective protein expression⁹⁹. Hence, intranuclear injections may serve as an alternative. In particular, nucleic acids can be injected into the cytoplasm or cell nucleus with fine glass capillaries, during which substantial pressure is applied to disrupt the cell plasma membrane. However, one of the main disadvantages of this approach is the low cell survival rate. Thus, this method may be more suitable for transfecting more robust neurons, such as invertebrate neurons¹⁸. In addition, in dividing neuronal cell lines, such as PC12, the injected material is often diluted during cell division, hence resulting in the loss of effects of the injected substance¹⁰⁰.

Despite the drawbacks, microinjection provides substantial advantages. In theory, the transfection efficiency is 100%. As compared to traditional transfection or infection, single-cell microinjection allows targeted transfection of pre-defined cells of interest within a mixed culture. Although microinjection is not as efficient as other transfection methods as it needs to be done cell by cell, the delivery dosage and delivery location can be precisely controlled.

Biolistics (Gene gun)

Biolistic transfection is based on the injection of subcellular-sized particles that are coated with DNA into the cells¹⁰¹. This method is applicable to tissues, cells and organelles, and can be used both *in vitro* and *in vivo*. In general, three major steps are needed to inject the DNA into cells/tissues: (i) coating the particle with DNA, (ii) transferring the coated particles into a cartridge, and (iii) firing the DNA-coated microcarriers into cells/tissues using a pulse of helium gas¹⁰². The transfection efficiency in brain slices using a gene gun can reach around 30%¹⁰². Up to now, only a few reports are available where successful biolistic gene has been transferred into neurons or neuronal tissues^{103,104}. Biolistic transfection can overcome physical barriers such as the stratum corneum of the epidermis. It also allows multiple transfection with more than one type of DNA within the same sample^{105,106}. However, the major drawbacks of biolistic transfection method are high cell death and high cost of equipment, although the consumable costs thereafter are relatively low¹⁰².

Taken together, lipofection is the most commonly used method for the transfection of neuronal cells due to its high transfection efficiency and low cytotoxicity. As compared to electrical and physical transfection methods, lipofection is more applicable for transfecting a large number of neurons in one go. On the other hand, for single cell studies, single-cell electroporation and microinjection are more appealing due to their transfection accuracy. However, these methods are recommended for the transfection of robust neurons (i.e. PC12 and invertebrate neurons) as the electrical and mechanical stimuli could jeopardize cell viability. Altogether, several factors such as neuronal cell types and their survival rates should be taken into consideration before deciding on the transfection approach.

1

2 **Although** a plethora of transfection methods have been established, efficient

3 transfection of post-mitotic cells, such as mammalian neurons, remains a challenging task.

4 While numerous studies **are** exploring efficient platforms for transfecting neurons, most of

5 these studies focused on the evaluation of the transfection efficiencies. Hence, the

6 biological outcomes that may be induced by functional nucleic acids remain to be evaluated.

7 Therefore, future evaluations of the functionalities **of** the transfected neurons are required.

Table 2: An overview of different transfection methods on neurons *in vitro*

Delivery Methods	Nucleic acids	Vectors	Target neurons	Max. transfection efficiency achieved	Amount of nucleic acids used	Reference
Calcium-phosphate/ DNA co-precipitation	Plasmid encoding EGFP	Calcium phosphate	Hippocampal neurons	50%	1-4 µg of plasmid DNA	[81]
	Plasmid encoding Bcl-xL	Calcium phosphate	Hippocampal neurons	1-5%	Not mentioned	[82]
	Plasmid encoding GFP	Calcium phosphate	Hippocampal neurons	13.4%	3-5 µg of plasmid DNA	[107]
Lipofection	Plasmid encoding GFP	Lipofectamine 2000	Cortical neurons	8%	400 ng of plasmid DNA	[108]
	Plasmid encoding NGF	Lipofectamine	PC12 cell	3-4%	1 µg plasmid encoding NGF gene	[109]
	Plasmid encoding β-gal	DOTAP	Hippocampal neurons	3%	1.5 µg of plasmid DNA	[90]
	Plasmid encoding β-gal	FuGene	Primary Mesencephalic Neurons	12.7%	255 ng of plasmid DNA	[78]
	pCMV-EGFP	M9-assisted lipofectamine	Embryonic rat hippocampal neurons	25%	1 µg of plasmid DNA	[110]
	mRNA	Lipofectamine 2000	Neurosphere	40-50%	50-100 ng	[111]
	mRNA	Lipofectamine 2000	DRG neurons	25%	50-1000 ng of mRNA	[86]
	siRNA	Cyclodextrins	Hypothalamic neurons	45%	50 nM of siRNA	[112]
	siRNA	Lipofectamine, Stearyl-R8 and AVPs	Hippocampal neurons	83%,73% and 75%, respectively	10 pmol of siRNA	[91]
	siRNA	Octaarginine-PEG-lipid	Hypothalamic neurons	20%	50-100nM of siRNA	[113]
	siRNA	PEG-PEI	Neural stem cells	86.05 ± 5.22%	80 pmol of siRNA	[114]
	miR-21	Lipofectamine RNAiMAX	Cortical neurons	38.70%	600-700 ng of miR-21	[84]
Electroporation	Plasmid encoding GFP	Electroporation	Mouse cerebellar granule neurons	26.8% ± 8.6%	1-5 µg of plasmid cDNA	[95]
	Plasmid encoding GFP	Electroporation	Hippocampus neurons	17.3% ± 3.2%	1-5 µg of plasmid cDNA	[95]
	plasmid encoding EGFP	Electroporation	Brain slices	30%	0.25-2 µg of plasmid DNA	[97]
	Plasmid encoding EGFP	Electroporation	DRG neurons	15-20%	1-2 µg of plasmid DNA	[93]
	mRNA	Electroporation	Neurosphere	60-70%	50-100 ng	[111]
	Dextran-fluorescein/siRNA	Electroporation	DRG neurons	59 ± 1.2%	2.4-2.8 µg of siRNA	[115]

	miR-124	Electroporation	Neural stem cells	50-60%	5 g miR mimic	[116]
Biolistic	Plasmid encoding EGFP	Gene gun	Brain slices	30%	Not mentioned	[102]
	Plasmid encoding BDNF/NT-4	Gene gun	Rat visual cortex	0.6–0.8%	30 µg of plasmid DNA	[103]
	DNA/gold particles	Gene gun	Cerebellar granule cells / Hippocampal neurons	10%	1 µg DNA/ mg of gold particles	[104]

2.2 *In vivo* studies

Although several *in vitro* transfection methods have been explored and optimised for neuronal transfection, not all approaches are applicable for *in vivo* utilization. Among the transfection methods discussed above, chemical transfection is the most commonly used approach for *in vivo* studies due to their high transfection efficiency, ease of modification and low cytotoxicity. Electrical and physical transfection methods, on the other hand, are much less used due to the risk of inducing secondary injuries. Studies related to the non-viral delivery of nucleic acids for *in vivo* nervous system repair, such as the vectors used, the delivery methods and the therapeutic outcomes were summarized in Table 3.

Non-viral delivery of nucleic acids for central nervous system (CNS) regeneration

Injuries to the CNS often lead to long-term disability, mortality and psychological symptoms¹¹⁷. Primary injuries often result in contusions and bleeding while secondary injuries can occur months after the initial damage and include axonal damage, demyelination and vascular injuries¹¹⁸. Generally, the injured axon may regenerate if the microenvironment is favourable for regrowth¹¹⁹. Hence, nucleic acid-based therapy has emerged as a promising strategy for treating different nervous system injuries by either up-regulating the growth promoting molecules or down-regulating the growth inhibitory factors².

Comparatively, although proteins can also be administered to an injury site, one advantage of using nucleic acids is that multiple therapeutic genes can be delivered from the same delivery systems². In addition, the administration of proteins to the injured site via

1 local injection is often transient due to the labile nature of proteins, especially under the
2 injured microenvironment¹²⁰. Hence, by modifying the genome of the transfected cells,
3 protein expression can be prolonged¹²¹. Besides that, when transfecting cells that can
4 proliferate, protein expression may be passed on¹²², thereby enabling long term therapeutic
5 effects. More importantly, protein treatment works through the recognition by protein
6 receptors. For proteins which receptors are lacking on the neurons, protein treatment is not
7 applicable¹²³. Nucleic acid delivery could also overexpress transcription factors¹²⁴, which
8 cannot be achieved by protein delivery.

10 *Bolus delivery of polymer-based carriers*

11
12 Polymers play critical roles in non-viral gene delivery by providing controlled release
13 of therapeutic nucleic acids over long durations. Due to the transient nature of nucleic acids,
14 such as their fast degradation rate, repeated administrations are typically needed to achieve
15 the long-term expression of therapeutic genes in the treatment of nervous system injuries.
16 As such, sustained delivery of nucleic acids by polymer-based carriers is of great necessity.

17
18 Polymer-based carriers have been used to deliver both large plasmid DNAs and small
19 oligonucleotides. Among the non-viral vectors used *in vivo*, the polycationic polymer,
20 polyethyleneimine (PEI, 50-kDa), exhibits one of the highest transfection efficiency¹²⁵. Shi *et*
21 *al.* studied the effects of PEI/DNA on the injured rat spinal cord by intrathecal
22 administration¹²⁶. In particular, the naked DNA that encoded luciferase or PEI/DNA
23 complexes were injected into the spinal cord lumbar levels. Thereafter, the transgene
24 expression was improved significantly in the presence of PEI. In particular, the expression
25 level induced by the PEI complexes containing 4 µg of DNA was 40-fold higher than that

1 induced by the same amount of naked plasmid DNA. The data showed that the luciferase
2 activity at the lumbar and thoracic levels accounted for 50% of the total activity in the whole
3 spinal cord. Positively stained cells were also observed to display typical morphologies of
4 astrocytes and neurons. In addition, long-term gene expression was achieved with repeated
5 injections of PEI/DNA complexes. However, when repeated dosages were administered, a
6 70% attenuation of gene expression was observed following reinjection at a 2-week interval
7 due to apoptotic cell death¹²⁶.

8
9 To circumvent the problem of cytotoxicity, other studies demonstrated that the
10 modification of PEI by polyethylene glycol (i.e. PEGylation) could improve
11 biocompatibility^{127,128,129}. Specifically, as compared to PEI alone, PEGylated PEI was
12 significantly less toxic to neuronal precursor cell lines such as PC12 and NT2/D1 cells¹²⁶.
13 Furthermore, by using PEG-grafted PEI for DNA complexation, the attenuation of gene
14 expression (which was observed due to the cytotoxicity of PEI) was not detected after
15 repeated intrathecal injections¹²⁶. Hence, PEGylated PEI could significantly reduce the cell
16 death which was caused by using PEI alone.

17
18 Overcoming mRNA instability is vital for effective mRNA delivery^{130,131}.
19 Correspondingly, polymer-based carriers have shown their potential in solving these
20 issues¹³². A polyplex nanomicelle system using the polycation, poly [N9-[N-(2-aminoethyl)-2-
21 aminoethyl] aspartamide] ([PAsp(DET)]), was reported recently¹³³. Due to its core-shell
22 architecture based on the self-assembly of block copolymers (which consisted of PEG and
23 polyamino acids), this polyplex nanomicelle served as an effective mRNA carrier. As the
24 mRNAs were entrapped within the micelle, both stability and immunogenicity issues could

1 be simultaneously resolved¹³². Besides that, this system could enhance endosomal escape
2 due to pH-responsive membrane destabilisation by [PAsp(DET)]¹³⁴. Furthermore, it also
3 rapidly degraded into non-toxic forms under physiological conditions, which further
4 facilitated endosomal escape and minimized cell damage and toxicity after
5 administration^{135,136}.

6
7 Since PEI induces strong cytotoxicity, chitosan (CS), a natural linear cationic
8 polysaccharide, was explored as an alternative for *in vivo* gene delivery. Chitosan has been
9 widely used as drug carriers, wound dressings, and scaffolds for tissue engineering due to its
10 biocompatibility, biodegradability and low toxicity^{38,137}. As such, chitosan or chitosan-
11 functionalized nanoparticles (CNPs) have been widely investigated for non-viral gene
12 delivery¹³⁸. However, the low transfection efficiency of chitosan has hindered its
13 applications. Recently, modifications, such as grafting PEI onto chitosan, or creating a
14 chitosan-PEI composite have been developed for gene delivery *in vivo*¹³⁹. Das *et al.* tested
15 chitosan and PEI-coated magnetic micelles (CPmag micelles or CPMMs) as gene delivery
16 carriers and the efficacy of this carrier was evaluated in a mild traumatic brain injury (mTBI)
17 model. CPMM-tomato plasmid (ptd) conjugates expressing a red fluorescent protein (RFP)
18 were instilled into the nose of sham-operated rats or rats subjected to mTBI. CPMM-ptd
19 conjugates were shown to successfully enter the brain, and the red fluorescent protein (RFP)
20 expression was identified in the cortex and hippocampus at 48 hours after mTBI without
21 evoking any inflammatory response. These observations indicated the possibility of using
22 intranasally administered CPmag as a theragnostic vehicle for mTBI¹⁴⁰.

Lipid-based carriers

Besides serving as effective gene carriers for *in vitro* studies, lipid-based carriers are also widely used in animal works due to their biocompatibility, biodegradability and low toxicity^{141,142}. Takahashi *et al.* used lipofectamine-plasmid complexes (Plasmids: Lipofectamine=2:1) to regulate the expression of B-cell lymphoma-2 (Bcl-2), which is a protein that has been shown to prevent apoptosis^{143,144}. Therefore, targeting the expression of Bcl-2 could potentially prevent neuronal cell death after CNS injuries. Hence, lipofectamine-complexed plasmids encoding $\alpha 22\beta$ -gal $\alpha 4$ bcl-2 gene were injected into the right side of the T8 segment after spinal cord hemi-incision. The transgene expression was then confirmed by observing the expression of the reporter gene, LacZ, three days after administration. Colocalization of LacZ expression and Clarke's Nucleus (CN) neurons were detected at the spinal cord L1 level. Correspondingly, this treatment significantly reduced atrophy and the loss of axotomized Clarke's Nucleus (CN) neurons¹⁴⁵. Besides that, the axotomized red nucleus (RN) neurons were also protected by this treatment. Results showed that 87% of RN neurons survived two months after C3/C4 subtotal hemi-incision, suggesting that intraspinal administration of Bcl-2 gene could prevent retrograde cell loss and reduce atrophy of damaged RN¹⁴⁶.

Glial-derived neurotrophic factor (GDNF) supports the survival of motor neurons and promotes axonal regeneration after axotomy^{147,148}. Lu *et al.* showed that the administration of complexes of liposome plasmids that encoded GDNF promoted axon regeneration after spinal cord injury¹²¹. The liposome plasmid complexes were injected directly into the grey matter of the rat spinal cord (T7-T8 level). Thereafter, the expression of GDNF mRNA was detected one week after injection. Moreover, the expression of EGFP-GDNF was observed in

the cells around the injection site 4 weeks after injection, indicating that these plasmids lasted for at least one month. Furthermore, anterograde tracing confirmed the regeneration of corticospinal tracts four weeks after treatment. Behaviour tests, such as the inclined plane test and Basso, Beattie, and Bresnahan (BBB) scores exhibited improved functional recovery of the rats' hindlimbs. These observations suggested that the delivery of plasmids encoding GDNF could promote nerve repair after SCI. However, the transfection efficiencies and the cell damage after lipoplexed plasmids injection were not assessed. Also, the exact cell types that were transfected by lipoplexes remains unknown.

Thus far, intraspinal injection is one of the most common administration routes of nucleic acids for treating traumatic injuries in the CNS. Usually, the injected nucleic acid therapeutics can last for 1 month and the time-course of observing its expression/effects is around 3-7 days^{121,140,145,146}. However, prolonged expression of the transgenes (eg. several months) is often needed to achieve more prominent functional recovery¹⁴⁹. While most studies have focused on evaluating the functional outcomes that are induced by the administration of nucleic acids, it is also crucial to understand the possible side effects of gene delivery, the extent of cellular uptake of transgenes, clearance durations as well as the transfection efficiencies.

Scaffold-mediated non-viral nuclei acids delivery for SCI treatment

Scaffolds serve a crucial role in tissue regeneration by providing a means to control the local extracellular environment. These substrates may present biochemical¹⁵⁰, topographical¹⁵¹ and mechanical¹⁵² cues to cells. Beyond that, scaffolds may also be employed as controlled release vehicles for bio-molecules and therapeutic drugs^{153,154}.

Specifically, drug encapsulation within scaffolds can help to protect nucleic acids from biodegradation by shielding them from immune attacks and retain nucleic acids locally, thereby preventing systemic clearance¹⁵⁵. Importantly, the sustained nucleic acid delivery via scaffolds also increases the opportunity for cellular internalisation and the likelihood of successful transfection due to local availability of drugs¹⁵⁶. Consequently, scaffolds and nucleic acid-incorporated substrates are employed to guide neuronal cell growth, direct neuronal differentiation^{157,158} and promote functional recovery for the treatment of traumatic nerve injuries^{159,160}.

In one study, lipoplexed plasmid DNA was encapsulated in multichannel poly[lactide-co-glycolide] (PLG) neural conduits¹⁶¹. Before implanting into the animals, different extracellular matrix (ECM) components (fibronectin, collagen I, laminin I) were coated onto PLG to immobilise DNA. *In vitro* studies revealed that fibronectin produced the highest immobilisation efficiencies as compared with the other two coatings. Thereafter, luciferase assay indicated that 25 or 50 µg of fibronectin coating elicited the highest levels of transgene expression. The plasmid DNA-encapsulated PLG conduits were subsequently implanted into spinal cord hemi-sectioned rats (T9-T10 level)¹⁶¹. Three weeks after implantation, the transgene expression level was 2-fold higher than that of naked plasmids. Additionally, the transgene expression persisted for three weeks and axon regeneration was observed inside the channels. However, the regenerated axons did not exit the conduits and the functional recovery after treatment remains unknown.

A follow-up study by the same group then applied the multichannel PLG conduits to deliver DNA plasmids to support and direct cellular processes and promote gene transfer

1 following spinal cord hemisection at T9-T10¹⁶². The expression of the transgene was shown
2 to last for 44 days *in vivo*. Furthermore, the implantation of multichannel conduits
3 supported cell infiltration and axon growth. Immunohistochemistry confirmed that the
4 transfected cells at the implant site were mainly Schwann cells, fibroblasts, and
5 macrophages. These observations suggested that the synergistic effects of functional gene
6 expression and topographical cues could significantly improve nerve regeneration¹⁶².
7 However, the transgene expression was mainly detected in glial or immune-related cells.
8 Therefore, future studies are needed to analyse how these transfected cells affect nerve
9 regeneration.

10
11 Although multiple groups have explored the delivery of large nucleic acids, the
12 delivery of small oligonucleotides and gene silencing is less explored. Nonetheless, a study
13 done by our group introduced a three-dimensional (3D) nanofiber hybrid scaffold that
14 directed and enhanced axonal regeneration after SCI¹⁶³. The fabrication of this 3D hybrid
15 scaffold involved the combination of electrospun aligned fibres and collagen matrix. Mir-
16 222, an inhibitor of the PI3K pathway that is important to central axon growth¹⁶⁴, was then
17 chosen as the additional biochemical signal to enhance nerve regeneration after SCI. As a
18 biofunctionalized platform, the 3D aligned nanofiber-hydrogel scaffold provided sustained
19 non-viral delivery of proteins (NT-3) and miR-222, along with synergistic contact guidance
20 for nerve repair. Correspondingly, aligned axon regeneration was observed as early as one-
21 week post-injury. Furthermore, no excessive inflammatory response and scar tissue
22 formation was triggered after scaffold implantation.

1 Taken together, studies thus far have indicated that functionalized scaffolds serve as
2 promising nucleic acid delivery platforms for SCI treatment. As compared to intraspinal
3 injection, scaffold-mediated nucleic acid delivery can last for several months (eg. 3
4 months)¹⁴⁹ and the transgene expression was observed up to 3-4 weeks after
5 treatment^{161,162}. However, in contrast to *in vitro* neuronal cell transfection, the transfection
6 efficiency and the side effects of gene delivery after CNS injuries have not been clearly
7 discussed in the above studies. One possible reason might be due to the lack of robust
8 experimental methods to evaluate cellular uptake, transgene expression and gene silencing
9 effects under the injured microenvironment. In general, as compared to protein
10 therapeutics such as NT-3, BDNF and GDNF^{165,166,167}, *in vivo* nucleic acids transfection is not
11 commonly used to treat CNS injuries. However, given the promising outcomes of these
12 studies and the lack of robust regeneration using conventional protein-based methods, it
13 may be highly worthwhile to continue to establish more robust non-viral nucleic acid
14 transfection methods for CNS injury treatment.

16 ***Non-viral delivery of nucleic acids for peripheral nervous system (PNS) regeneration***

17
18 The PNS has some regenerative capacity. However, its ability to grow remains
19 limited when crossing large lesion gaps. Hence, in patients with PNS injuries, nerve
20 reconnection is often incomplete over large lesions due to the low rate of nerve
21 regeneration (i.e. 1 mm/ day¹⁶⁸) and misrouting of the regenerated axons¹⁶⁹. Hence,
22 locomotor recovery remains limited¹⁷⁰ and more effective therapeutic strategies are
23 needed¹⁷¹. Gene therapy-based strategies aim to provide target-specific neurotrophic
24 support to enhance the survival and regeneration of both sensory and motor axons and
25 finally, the recovery of function^{172,173}. To achieve this, artificial nerve guidance conduits are

commonly utilized to bridge large nerve defect gaps. The synergistic effects of nucleic acids and topographical cues provided by the nerve conduits could further direct and enhance nerve regeneration and functional recovery after PNS injuries.

Vascular endothelial growth factor (VEGF) is a potent angiogenic factor which stimulates the function of new blood vessels and enhances vascular permeability¹⁷⁴. Lope *et al.* reported the use of plasmid vectors, which expressed human VEGF165 gene¹⁷⁵ for sciatic nerve injury treatment. Plasmid vectors carrying the VEGF gene were then injected into the thigh musculature below the sciatic nerve followed by electroporation. Ten minutes later, the sciatic nerve was transected and a 6 mm collagen nerve guide conduit was implanted to bridge the injury gap. Consequently, the number of regenerated myelinated axons and blood vessels were notably larger in VEGF-encoding plasmid (VEGF plasmid)-treated animals as compared to the control group (vectors alone). Moreover, the functional sciatic index and gastrocnemius muscle weight significantly increased in VEGF plasmid-treated animals versus vectors alone. While the results indicated that VEGF plasmid administration supported and enhanced nerve regeneration, this method of gene transfection before an injury may not be clinically relevant.

The granulocyte colony-stimulating factor (G-CSF), a cytokine that induces survival, proliferation and differentiation of hematopoietic lineage cells¹⁷⁶, was introduced and evaluated by the same group. The synergistic effects of G-CSF and VEGF were further investigated using the same delivery vectors in the treatment of sciatic nerve injuries¹⁷⁷. In particular, plasmids encoding VEGF and/or G-CSF genes were injected locally (below the sciatic nerve in adult mice) and transfected via electroporation. The sciatic nerves were then

transected followed by the implantation of a polycaprolactone (PCL) nerve guide. The administration of G-CSF alone and G-CSF-VEGF cocktail improved nerve regeneration, and the improvement was even more significant in the cocktail treated groups. G-CSF-VEGF cocktail-treated animals showed remarkably improved motor **function** recovery as compared with the control groups (vectors alone). In addition, the myelinated axons, blood vessels and gastrocnemius muscle weight were also significantly increased with VEGF and G-CSF treatment. These works **suggest** that the combined treatment acted synergistically in improving regeneration after sciatic nerve transection lesions¹⁷⁷.

Generally, electroporation was used in the above studies for the transfection of foreign genes *in vivo*, but these studies mainly focused on the effects of the transgenes on nerve regeneration outcomes with minimal attention spent on evaluating secondary damages due to such physical transfection methods. Importantly, the introduction of these nucleic acid therapeutics before an injury has low clinical relevance for unpredictable **traumatic nerve injuries**. Furthermore, the studies did not evaluate the transfection efficiencies and the expression of transgenes, which makes it difficult to make comparisons with *in vitro* electroporation outcomes.

Although non-viral gene delivery approaches have been used for the treatment of PNS injuries, existing studies regarding scaffold-mediated gene delivery via non-viral methods remain limited. **As axotomized nerve terminals are usually far from their cell bodies, therapeutic nucleic acids that modulate gene expression in the cell soma (i.e. at DRGs) might not exert their effects efficiently due to the long distance.** Hence, exploring therapeutic nucleic acids that target the injured axons, such as nucleic acids that facilitate

local protein synthesis or new growth cone formation, might serve as an alternative for the treatment of PNS injuries.

Gene therapy-based cell transplantation for nervous system injury treatment

To precisely monitor the transfection process and the transfection efficiencies of target cells, the implantation of gene-modified cells to the injured nervous system has also been explored as an alternative to enhance nerve regeneration. This method has been used in both CNS and PNS injury treatments.

Primary olfactory-ensheathing glial (OEG) were transfected with cationic liposome-complexed recombinant plasmids that encoded NT-3. *In vitro* transgene expression analysis demonstrated that higher amount of NT-3 was released from NT-3-transfected OEG as compared to cells that were transfected with transfection reagent only. Subsequently, the transfected cells were implanted into the rat spinal cord directly after a thoracic spinal cord (T9) contusive lesion. Seven days after transplantation, spinal cord tissues that were injected with transfected OEG expressed high levels of NT-3 mRNA. More importantly, robust nerve regeneration and hindlimb functional recovery were observed three months after implantation¹⁷⁸. Table 3 summarises various studies on gene-modified cell transplantation for promoting nerve regeneration after CNS injuries. The study mentioned above was highlighted due to their comprehensive work *in vitro* and *in vivo*. Besides that, their results strongly suggested that gene-modified cell transplantation is effective after SCI. However, the use of scaffolds for delivering non-viral gene-modified cells for CNS injury treatment, to our knowledge, has not been attempted. We speculated that this might be due to many impeding factors that could affect the regeneration outcomes. Some examples

1 include the viability of cells encapsulated within the scaffold, the level of transgene
2 expression, the migration rate of the transplanted cells along with the mass of functional
3 molecules that are ultimately released from the scaffolds.

4
5 The implantation of gene-modified cells is also applicable for PNS injuries. Schwann
6 cells (SCs) are regarded as the therapeutic targets of PNS due to their role in promoting
7 tissue regeneration by secreting growth-promoting molecules, guiding regenerating axons
8 towards a target region and myelinating regenerated axons^{179,180}. Therefore, restoring the
9 function of SCs is crucial for PNS regeneration. Specifically, the entrapment of the low
10 molecular weight (18-kDa) isoform of fibroblast growth factor-2 (FGF-2) in artificial nerve
11 guidance conduits significantly enhanced the growth of myelinated and unmyelinated axons
12 across large lesion gaps¹⁸¹. SCs transfection was carried by Haastert *et al.*¹²² using complexed
13 Metafectene™ and plasmids that encoded 18-kDa-FGF-2 isoform and 21/23-kDa-FGF-2
14 isoform. Thereafter, the transfected SCs were seeded into silicone tubes and the tubes were
15 implanted into the rat sciatic nerve to bridge a 15-mm rat ischiatic nerve defect.
16 Consequently, functional assessment indicated a more robust regeneration of sensory
17 function by grafted SCs that over-expressed different FGF-2 isoforms as compared to normal
18 untreated SCs. Furthermore, the over-expression of the high molecular weight 21/23-kDa-
19 FGF-2 isoforms by grafted Schwann cells resulted in earlier signs of sensory recovery as
20 compared to the over-expression of 18-kDa-FGF-2. In contrast, motor recovery was
21 detected after the over-expression of 18-kDa-FGF-2, as revealed by the recording of
22 compound muscle action potentials (CMAP)¹²².

Kempton *et al.* bridged a 10-mm gap using a collagen nerve guidance conduit with gene-modified mesenchymal stem cells (MSCs) that overexpressed VEGF¹⁸². These nerve guidance conduits were filled with saline, Matrigel with mesenchymal stem cells (MSCs) or Matrigel with gene-modified MSCs (transfected with complexed Lipofectamine 2000 and plasmids that encoded VEGF165 gene). The treatment with VEGF-transfected MSCs significantly promoted nerve regeneration and facilitated blood vessel formation three weeks after implantation. However, the differentiation and function of MSCs after VEGF transfection were not evaluated. Hence, it remains unknown if the MSCs participated directly in tissue function or provided biochemical support through paracrine signalling.

Modifying genes of cells before transfection allows better control over the transfection efficiency of target cells. However, there are also some concerns, such as the low survival rate of the transplanted cells and their ability to function in the injured microenvironment.

Gene therapy has shown its potential in enhancing nerve regeneration after injuries by overexpressing growth-promoting factors and preventing neurons from cell death. The incorporation of scaffolds provides additional topographical cues, which further facilitate and modulate nerve regeneration. However, some inevitable challenges should be taken into consideration, such as low transfection efficiency, uncertain cellular uptake and unpredictable side effects in the process of non-viral gene transfection *in vivo*.

Table 3: Non-viral delivery of nucleic acids for nerve system repair

Injuries	Delivery methods	Therapeutic nucleic acids	Delivery vectors	Therapeutic outcomes	Amount of nucleic acids used	Reference
CNS	Injection	Plasmid DNA	Polyethylenimine (PEI)	Increase transgene expression	2-40 µg of plasmid DNA	[126]
	Injection	Plasmid DNA	PEI PEGylation	Decrease apoptosis	4 µg of plasmid DNA	[126]
	Nose instillation	Plasmid encoding RFP	Chitosan and PEI-coated magnetic micelles	Vector enter the brain parenchyma	10 µg of plasmid DNA	[140]
	Injection	Plasmid encoding Bcl-2	Lipofectamine	Reduce atrophy and loss of neurons	3~75 µg/25 µg of plasmid DNA	[145][146]
	Injection	Plasmid encoding GDNF	DC-Chol-liposomes	CST regeneration and function recovery	Not mentioned	[121]
	Scaffold implantation	Plasmid DNA	Lipofectamine	Significant transgene expression	800 µg of plasmid DNA	[162]
	Scaffold implantation	Plasmid encoding NT-3	PEGylated DMAEMA	Promote robust axonal regeneration	Not mentioned	[119]
	Scaffold implantation	Plasmid DNA	Transfast	Transgene expression lasted for 3 weeks	3 µg of plasmid DNA	[161]
	Scaffold implantation	Plasmids encoding NT-3	PEI PEGylation	Improved axonal regeneration and functional recovery	1 µg of plasmid DNA	[183]
	Injection	mRNA	Polyplex nanomicelle	Decrease immune responses	2 µg of mRNA	[133]
	Injection	siRhoA	PgP	Promote axon regeneration and decrease apoptosis	20 µg of siRNA	[184]
	Injection	siRNA	Hiperfect	Promote axon regeneration	0.5 µg of siRNA	[185]
	Scaffold implantation	miR-222	TKO	Promote nerve regeneration	48 µg of microRNA	[163]
	Cell transplantation	NT-3 overexpressed OEGs	liposomes	Promote nerve regeneration and hindlimb functional recovery	Not mentioned	[178]
	Cell transplantation	E-cadherin overexpressed NSCs	SuperFect	Induce differentiation of NSCs into neurons	5 µg	[186]
	Cell transplantation	BDNF overexpressed MSCs	PAsp(DET)	Promote the recovery of motor function	12 µg	[187]
	Cell transplantation	NRG1 overexpressed SCs	FuGene6	Promote neuroprotective and anti-apoptotic effects	3 µg	[188]

PNS	Injection	Plasmid encoding VEGF	Up vector plus electroporation	Promote DRG neurons survival and nerve regeneration	50 µg of plasmid DNA	[175]
	Injection	Plasmid encoding VEGF and G-CSF	Up vector plus electroporation	Promote motor function, nerve regeneration and blood vessel reformation	50 µg of plasmid DNA	[177]
	Scaffold implantation	Plasmid encoding FGF-2	Metafectene™	The recovery of sensory and motor function	Not mentioned	[122]
	Scaffold implantation	Plasmid encoding VEGF	Lipofectamine 2000	Promote nerve regeneration and blood vessel reformation	Not mentioned	[182]
	Cell transplantation	Gene modified SCs	pLVTHM	Potential in stimulating nerve regeneration	Not mentioned	[189]

3. Design considerations for better control over the delivery and uptake of nucleic acids by injured neurons to enhance nerve regeneration

From the non-viral delivery systems reviewed thus far, bulk of these systems depend on physical transfection methods, polymer-based as well as lipid-based carriers. While physical transfection methods such as electroporation have achieved significant levels of successful nucleic acid transfection *in vitro*, the possibility of causing secondary nerve tissue damage has limited their *in vivo* applications. On the other hand, the systemic delivery of nucleic acids via non-viral carriers faces a variety of problems. Although improvements have been made to enhance transfection efficacy of these non-viral carriers, it is undeniable that these delivery systems are still exposed to systemic clearance and serum nucleases – both of which lead to transient modulation of gene expression, which is often insufficient in achieving desired therapeutic effects. Most importantly, these delivery systems cannot provide topographical cues which are essential for guiding neurite extension across an injury site.

Scaffolds serve as supporting structures and provide physical signals that may direct cell fate, aid cell infiltration, attachment, growth^{154,190,191} and modulate gene transfection¹⁹². Additionally, the delivery of nucleic acids via scaffolds provides protection against nucleases and allows localised and sustained delivery of nucleic acids at the injury site. While scaffold-mediated non-viral delivery of nucleic acids appears promising based on available works, numerous areas still require improvements for better functional outcomes. The following sections will look at some design alternatives to improve non-viral nucleic acids delivery platforms for nerve regeneration. Specifically, these improvements aim to enhance the

1 efficiency of delivery, transfection and uptake of nucleic acids by neurons at the site of
2 injury.

3 4 **3.1 Enhancing stability of nucleic acids**

5 Nucleic acids are susceptible to bio-degradation and clearance from the body due to
6 the presence of extracellular nucleases and the immune system ¹⁵⁵. The chronic
7 inflammatory environment and the presence of reactive oxidative species secreted by
8 activated inflammatory cells render nucleic acids vulnerable to degradation at the trauma
9 site within the nervous system ^{193–198}.

10
11 Bio-responsive delivery systems can be considered to minimise such undesirable nucleic
12 acid degradation. These systems change their properties in response to a biological trigger
13 such as changes in pH, temperature, light or presence of biomolecules such as enzymes ¹⁹⁹.
14 Their abilities to adapt to the environment provide novel modes of release, e.g. release of
15 nucleic acids only in a well-defined disease or injury state. Along these lines, cell-matrix
16 interactions represent an interesting trigger for releasing nucleic acids from scaffolds. For
17 example, matrix metalloproteinases (MMPs) are enzymes that degrade both matrix and
18 non-matrix proteins. They have great importance in remodelling the extracellular
19 environment of cells ²⁰⁰. Correspondingly, MMP-degradable hydrogels supported cell
20 growth and modulated cell migration ^{201–203}. Since MMP expression increases during spinal
21 cord injury ^{204–208}, such MMP-degradable scaffolds could serve as an additional protective
22 measure for nucleic acids that have been encapsulated within the matrix. As cells penetrate
23 the matrix, MMPs are released locally, and nucleic acids that have been encapsulated within

the matrix may then be released. Consequently, this limits the exposure of the encapsulated nucleic acids only to cells that are migrating into and residing within the matrix.

3.2 Modulating mechanical properties of scaffolds to increase cellular uptake of nucleic acids

The mechanical properties of scaffolds and matrices that cells adhere to are increasingly being recognised for their roles in regulating cellular response^{209,210}. Similarly, the efficiency of gene uptake and cellular transfection have also been investigated as a function of matrix compliance¹⁹². In particular, increasing substrate stiffness led to an increase in polyplex uptake, de-condensation and eventual delivery to the nucleus¹⁹². Besides that, stiffer substrates were identified to promote higher nucleic acid transfection efficiency due to the enhanced rate of cell proliferation^{192,211,212} (although it should be noted that in the context of neurons, these cells do not undergo proliferation²¹³). Interestingly, when neurons were cultured on soft substrates, they formed significantly more branches than on stiffer matrices^{214,215}. Hence, coupling the delivery of nucleic acids that enhance axonal growth cone advancement with softer substrates may potentially promote nerve regeneration^{185,216–218}.

On the contrary, glial cells seem to prefer stiffer substrates^{219,220}. The mechanical mismatch between nerve tissues and implanted electrodes led to glial cell activation²²¹. While glial scar formation is undesirable for nerve regeneration across spinal cord injuries, increasing evidence indicates that the components of glial scar are important in triggering the proliferation of neural progenitors and stem cells. Astrocytes, in particular, have been

1 identified as one of the key regulators of these processes^{222,223}. Therefore, while bio-
2 functionalised scaffolds should be mechanically soft to prevent mismatch in stiffness at the
3 host-implant interface, these scaffolds should also have the appropriate stiffness to allow
4 glial cells to infiltrate and attract the migration of stem cells into spinal cord injury sites.

5
6 The hybrid fibre-hydrogel system from our group¹⁶³ could serve as a potential
7 platform to amalgamate various matrices of different mechanical stiffness. In particular, the
8 hydrogel matrix provides a mechanically soft interface between the scaffold and the injured
9 tissue. Hence, this hybrid scaffold may help to reduce glial scar formation while promoting
10 neurite branching and ingrowth into the scaffold. Furthermore, the encapsulation of nucleic
11 acids into this hydrogel matrix allows nucleic acid transfection to occur at the growth cones
12 of neurite extensions. Concurrently, the fibres within this hybrid scaffold serve as the
13 relatively stiffer matrix. Hence, these fibres may promote glial cell infiltration since glial cells
14 prefer stiffer substrates. As glial cells reside within the hybrid scaffold, their presence could
15 bring about the eventual attraction of neuronal stem cells, which play important roles
16 during nerve regeneration especially across large lesion sites.

18 3.3 Increasing specificity of cellular binding and uptake

19 The direct administration of cationic polyplexes to the site of injury often result in non-
20 specific cell association through interactions with anionic membrane proteins such as
21 proteoglycans²²⁴. Despite these drawbacks, the delivery of nucleic acids via these cationic
22 polyplexes is still being employed for transfecting neurons *in vitro* and *in vivo* due to ease of
23 application^{125,225}. In addition, understanding and predicting the efficiency of neuronal
24 nucleic acid uptake is complicated by the fact that neurons are highly polarised cells with

soma, axonal and dendritic domains that possess distinct membrane compositions^{226,227}. Furthermore, the physicochemical characteristics of nucleic acid carriers, including the size, charge and surface composition, may strongly influence the nature of their interaction with the neuronal plasma membrane. Hence, the design of non-viral nucleic acid delivery platforms is crucial to ensure that cells within the nervous system can specifically bind and uptake these therapeutic nucleic acids.

Bio-functionalised scaffolds could be designed to attract neurite extensions within the injured nervous system such that nucleic acids can reach these targeted group of cells/regions of the neuron. The covalent coupling of bioactive laminin epitope, IKVAV, along with the incorporation of a full-length laminin chain provided a permissive environment that attracted neurite outgrowth²²⁸. This approach could be considered as a way to attract the extension of neurites from injured neurons into scaffolds that are loaded with nucleic acids, such that growth cones at the ends of these neurite extensions can preferentially uptake these molecules.

3.4 Improving temporal control

Tissue development and regeneration generally occur in sequential phases²²⁹. Therefore, regeneration strategies should incorporate precise spatial and temporal controls to engineer mature and functional tissues²³⁰. Since the release profile of nucleic acid from scaffolds does not directly correlate with transgenic expression, obtaining temporal control remains challenging²³¹. In this respect, modifications in biomaterial designs can allow sequential delivery to be established. In particular, one type of nucleic acid may be encapsulated in a rapidly degrading polymer while the other type of nucleic acid may be

1 held within a slow degrading polymer to achieve sequential delivery of multiple factors ²³².
2 Alternatively, the layer-by-layer strategy may be engaged, where nucleic acids are loaded in
3 between two materials. Correspondingly, the degradation of one material layer will be
4 accompanied by the release of nucleic acids at the closest proximity to the degraded
5 material layer ²³³. MMP-degradable scaffolds can also be adapted for such differential
6 delivery of nucleic acids ¹⁹⁹.

7

4. Conclusions

The injured nervous system holds limited regenerative capability, especially within the CNS. Although the PNS has some regenerative capacity, its ability to grow remains limited when crossing large lesion gaps. Although the delivery of nucleic acids via viral vehicles into the injured nervous system holds great potential in enhancing nerve regeneration *in vivo*, the lack of safe and efficient delivery systems has limited the application of these molecules in patients who suffer from traumatic nerve injuries. Therefore, the exploration of non-viral delivery approaches is of great necessity. Along this line, this review has highlighted the important nucleic acid candidates, which participate in cellular activities within the nervous system, and their mechanisms of gene modulation. Following that, we focused on the transfection of neuronal cells and summarised the non-viral vectors that have been used *in vitro* as well as their corresponding transfection efficiencies. More importantly, we reviewed recent animal studies on non-viral delivery of therapeutic nucleic acids for nerve regeneration. Specifically, the combinatorial approach of nucleic acid therapeutics with scaffolds provides a synergistic and promising treatment option for nerve regrowth. Besides that, scaffold-mediated non-viral nucleic acid delivery allows controlled modulation of gene expression while providing topographical cues to guide axonal regeneration.

Although scaffold-mediated non-viral nucleic acid delivery approaches have been applied to nerve injury repair, many challenges remain in the development of these bio-functionalized scaffolds. These challenges include maintaining or enhancing the stability of nucleic acids against biodegradation, improving cellular uptake efficiencies as well as the temporal control of the expression of targeted genes. As such, these aspects should be

1 taken into consideration when designing scaffolds for more effective therapeutic treatment
2 for nerve repair.

4 **Acknowledgement**

5 This work is partially supported by the A*Star BMRC International Joint Grant-
6 Singapore-China Joint Research Program (Project No. 1610500024); the SingHealth-NTU-
7 Research Collaborative Grant (SHS-NTU/038/206); and the Singapore National Research
8 Foundation under its NMRC-CBRG grant (NMRC/CBRG/0096/2015). Na Zhang would like to
9 acknowledge NTU for providing the Nanyang Research Scholarship to carry out these
10 research works. Jiah Shin Chin would like to acknowledge NTU for supporting her work
11 under the Interdisciplinary Graduate School's Graduate Research Officer Scheme.

References

1. Tinsley, R. & Eriksson, P. Use of gene therapy in central nervous system repair. *Acta Neurologica Scandinavica* **109**, 1–8 (2004).
2. Blits, B. & Bunge, M. B. Direct Gene Therapy for Repair of the Spinal Cord. *J. Neurotrauma* **23**, 508–520 (2006).
3. Bowers, W. J., Howard, D. F., & Federoff, H. J. Gene therapeutic strategies for neuroprotection: Implications for Parkinson's disease. *Exp. Neurol.* **144**, 58–68 (1997).
4. Hong, C. S., Goins, W. F., Goss, J. R., Burton, E. A., & Glorioso, J. C. Herpes simplex virus RNAi and neprilysin gene transfer vectors reduce accumulation of Alzheimer's disease-related amyloid- β peptide in vivo. *Gene Ther.* **13**, 1068–1079 (2006).
5. Choi-Lundberg, D. L., Lin, Q., Schallert, T., Crippens, D., Davidson, B. L., ... Bohn, M. C. Behavioral and cellular protection of rat dopaminergic neurons by an adenoviral vector encoding glial cell line-derived neurotrophic factor. *Exp. Neurol.* **154**, 261–275 (1998).
6. Franich, N. R., Fitzsimons, H. L., Fong, D. M., Klugmann, M., During, M. J., & Young, D. AAV vector-mediated RNAi of mutant Huntingtin expression is neuroprotective in a novel genetic rat model of Huntington's disease. *Mol. Ther.* **16**, 947–956 (2008).
7. Forte, A., Cipollaro, M., Cascino, A., & Galderisi, U. Small Interfering RNAs and Antisense Oligonucleotides for Treatment of Neurological Diseases. *Curr. Drug Targets* **6**, 21–29 (2005).
8. Davidson, B. L. & Paulson, H. L. Molecular medicine for the brain: Silencing of disease genes with RNA interference. *Lancet Neurology* **3**, 145–149 (2004).
9. Rodriguez-Lebron, E. & Gonzalez-Alegre, P. Silencing neurodegenerative disease: Bringing RNA interference to the clinic. *Expert Review of Neurotherapeutics* **6**, 223–233 (2006).
10. Chandler, L. a, Gu, D. L., Ma, C., Gonzalez, a M., Doukas, J., ... Phillips, M. L. Matrix-enabled gene transfer for cutaneous wound repair. *Wound Repair Regen.* **8**, 473–9 (2000).
11. Tyrone, J. W., Mogford, J. E., Xia, Y., Mustoe, T. A., Chandler, L. A., ... Pierce, G. F. Collagen-embedded platelet-derived growth factor DNA plasmid promotes wound healing in a dermal ulcer model. *J. Surg. Res.* **93**, 230–236 (2000).
12. Doukas, J., Chandler, L. a, Gonzalez, a M., Gu, D., Hoganson, D. K., ... Pierce, G. F. Matrix immobilization enhances the tissue repair activity of growth factor gene therapy vectors. *Hum. Gene Ther.* **12**, 783–98 (2001).
13. Chandler, L. A., Doukas, J., Gonzalez, A. M., Hoganson, D. K., Gu, D. L., ... Pierce, G. F. FGF2-targeted adenovirus encoding platelet-derived growth factor-B enhances de novo tissue formation. *Mol. Ther.* **2**, 153–160 (2000).
14. Fang, J., Zhu, Y. Y., Smiley, E., Bonadio, J., Rouleau, J. P., ... Roessler, B. J. Stimulation of new bone formation by direct transfer of osteogenic plasmid genes. *Proc. Natl. Acad. Sci. U. S. A.* **93**, 5753–8 (1996).
15. Bonadio, J., Smiley, E., Patil, P., & Goldstein, S. Localized, direct plasmid gene delivery in vivo: Prolonged therapy results in reproducible tissue regeneration. *Nat. Med.* **5**, 753–759 (1999).
16. Doukas, J., Blease, K., Craig, D., Ma, C., Chandler, L. A., ... Pierce, G. F. Delivery of FGF genes to wound repair cells enhances arteriogenesis and myogenesis in skeletal muscle. *Mol. Ther.* **5**, 517–527 (2002).

- 1 17. Berry, M., Gonzalez, A. M., Clarke, W., Greenlees, L., Barrett, L., ... Baird, A. Sustained
2 effects of gene-activated matrices after CNS injury. *Mol. Cell. Neurosci.* **17**, 706–716
3 (2001).
- 4 18. Karra, D. & Dahm, R. Transfection Techniques for Neuronal Cells. *J. Neurosci.* **30**,
5 6171–6177 (2010).
- 6 19. Costantini, L. C., Jacoby, D. R., Wang, S., Fraefel, C., Breakefield, X. O., & Isacson, O.
7 Gene Transfer to the Nigrostriatal System by Hybrid Herpes Simplex Virus/Adeno-
8 Associated Virus Amplicon Vectors. *Hum. Gene Ther.* **10**, 2481–2494 (1999).
- 9 20. Kay, M. A., Manno, C. S., Ragni, M. V., Larson, P. J., Couto, L. B., ... High, K. A. Evidence
10 for gene transfer and expression of factor IX in haemophilia B patients treated with
11 an AAV vector. *Nat. Genet.* **24**, 257–261 (2000).
- 12 21. Kordower, J. H., Emborg, M. E., Bloch, J., Ma, S. Y., Chu, Y., ... Aebischer, P.
13 Neurodegeneration prevented by lentiviral vector delivery of GDNF in primate
14 models of Parkinson's disease. *Science* **290**, 767–73 (2000).
- 15 22. Naldini, L., Blömer, U., Gage, F. H., Trono, D., & Verma, I. M. Efficient transfer,
16 integration, and sustained long-term expression of the transgene in adult rat brains
17 injected with a lentiviral vector. *Proc. Natl. Acad. Sci. U. S. A.* **93**, 11382–8 (1996).
- 18 23. Baum, C., Düllmann, J., Li, Z., Fehse, B., Meyer, J., ... Von Kalle, C. Side effects of
19 retroviral gene transfer into hematopoietic stem cells. *Blood* **101**, 2099–2114 (2003).
- 20 24. Li, Z., Düllmann, J., Schiedlmeier, B., Schmidt, M., Von Kalle, C., ... Baum, C. Murine
21 leukemia induced by retroviral gene marking. *Science (80-.)*. **296**, 497 (2002).
- 22 25. Kafri, T., Morgan, D., Krah, T., Sarvetnick, N., Sherman, L., & Verma, I. Cellular
23 immune response to adenoviral vector infected cells does not require de novo viral
24 gene expression: implications for gene therapy. *Proc Natl Acad Sci U S A* **95**, 11377–
25 11382 (1998).
- 26 26. Thomas, C. E., Schiedner, G., Kochanek, S., Castro, M. G., & Lowenstein, P. R.
27 Preexisting antiadenoviral immunity is not a barrier to efficient and stable
28 transduction of the brain, mediated by novel high-capacity adenovirus vectors. *Hum.*
29 *Gene Ther.* **12**, 839–846 (2001).
- 30 27. Daya, S. & Berns, K. I. Gene therapy using adeno-associated virus vectors. *Clinical*
31 *Microbiology Reviews* **21**, 583–593 (2008).
- 32 28. Samulski, R. J. & Muzyczka, N. AAV-Mediated Gene Therapy for Research and
33 Therapeutic Purposes. *Annu. Rev. Virol.* **1**, 427–451 (2014).
- 34 29. Yan, M. Nucleic acid nanotechnology. *Science* **306**, 2048–2049 (2004).
- 35 30. Kasper, F. K., Young, S., Tanahashi, K., Barry, M. A., Tabata, Y., ... Mikos, A. G.
36 Evaluation of bone regeneration by DNA release from composites of oligo (poly
37 (ethylene glycol) fumarate) and cationized gelatin microspheres in a critical-sized
38 calvarial defect. *J. Biomed. Mater. Res. Part B Appl. Biomater.* **78**, 335–342 (2006).
- 39 31. Itaka, K., Ohba, S., Miyata, K., Kawaguchi, H., Nakamura, K., ... Kataoka, K. Bone
40 regeneration by regulated in vivo gene transfer using biocompatible polyplex
41 nanomicelles. *Mol. Ther.* **15**, 1655–1662 (2007).
- 42 32. Elangovan, S., D'Mello, S. R., Hong, L., Ross, R. D., Allamargot, C., ... Salem, A. K. The
43 enhancement of bone regeneration by gene activated matrix encoding for platelet
44 derived growth factor. *Biomaterials* **35**, 737–747 (2014).
- 45 33. Thomas-Virnig, C. L., Centanni, J. M., Johnston, C. E., He, L. K., Schlosser, S. J., ... Allen-
46 Hoffmann, B. L. Inhibition of multidrug-resistant acinetobacter baumannii by nonviral
47 expression of hCAP-18 in a bioengineered human skin tissue. *Mol. Ther.* **17**, 562–569

- (2009).
34. He, S., Xia, T., Wang, H., Wei, L., Luo, X., & Li, X. Multiple release of polyplexes of plasmids VEGF and bFGF from electrospun fibrous scaffolds towards regeneration of mature blood vessels. *Acta Biomater.* **8**, 2659–2669 (2012).
35. Guo, R., Xu, S., Ma, L., Huang, A., & Gao, C. The healing of full-thickness burns treated by using plasmid DNA encoding VEGF-165 activated collagen-chitosan dermal equivalents. *Biomaterials* **32**, 1019–1031 (2011).
36. Goh, J. C., Ouyang, H., Teoh, S., Chan, C. K. C., Lee, E., ... Lee, E. Tissue-engineering approach to the repair and regeneration of tendons and ligaments. *Tissue Eng.* **9 Suppl 1**, S31–S44 (2003).
37. Odabas, S., Feichtinger, G. A., Korkusuz, P., Inci, I., Bilgic, E., ... Piskin, E. Auricular cartilage repair using cryogel scaffolds loaded with BMP-7-expressing primary chondrocytes. *J. Tissue Eng. Regen. Med.* **7**, 831–840 (2013).
38. Lu, H., Lv, L., Dai, Y., Wu, G., Zhao, H., & Zhang, F. Porous Chitosan Scaffolds with Embedded Hyaluronic Acid/Chitosan/Plasmid-DNA Nanoparticles Encoding TGF- β 1 Induce DNA Controlled Release, Transfected Chondrocytes, and Promoted Cell Proliferation. *PLoS One* **8**, e69950 (2013).
39. Marsano, A., Maidhof, R., Luo, J., Fujikara, K., Konofagou, E. E., ... Vunjak-Novakovic, G. The effect of controlled expression of VEGF by transduced myoblasts in a cardiac patch on vascularization in a mouse model of myocardial infarction. *Biomaterials* **34**, 393–401 (2013).
40. Chien, Y., Chang, Y. L., Li, H. Y., Larsson, M., Wu, W. W., ... Huang, P. I. Synergistic effects of carboxymethyl-hexanoyl chitosan, cationic polyurethane-short branch PEI in miR122 gene delivery: Accelerated differentiation of iPSCs into mature hepatocyte-like cells and improved stem cell therapy in a hepatic failure model. *Acta Biomater.* **13**, 228–244 (2015).
41. Barchet, T. M. & Amiji, M. M. Challenges and opportunities in CNS delivery of therapeutics for neurodegenerative diseases. *Expert Opin. Drug Deliv.* **6**, 211–225 (2009).
42. Banks, W. A. From blood-brain barrier to blood-brain interface: New opportunities for CNS drug delivery. *Nature Reviews Drug Discovery* **15**, 275–292 (2016).
43. Hanz, S. & Fainzilber, M. Integration of retrograde axonal and nuclear transport mechanisms in neurons: Implications for therapeutics. *Neuroscientist* **10**, 404–408 (2004).
44. Von Bartheld, C. S. Axonal Transport and Neuronal Transcytosis of Trophic Factors, Tracers, and Pathogens. *Journal of Neurobiology* **58**, 295–314 (2004).
45. Syková, E. & Nicholson, C. Diffusion in brain extracellular space. *Physiol. Rev.* **88**, 1277–340 (2008).
46. Morgan, J. R., Barrandon, Y., Green, H., & Mulligan, R. C. Expression of an exogenous growth hormone gene by transplantable human epidermal cells. *Science (80-)*. **237**, 1476–1479 (1987).
47. Deodato, B., Arsic, N., Zentilin, L., Galeano, M., Santoro, D., ... Giacca, M. Recombinant AAV vector encoding human VEGF165 enhances wound healing. *Gene Ther.* **9**, 777–785 (2002).
48. Liechty, K. W., Nesbit, M., Herlyn, M., Radu, A., Scott Adzick, N., & Crombleholme, T. M. Adenoviral-mediated overexpression of platelet-derived growth factor-b corrects ischemic impaired wound healing. *J. Invest. Dermatol.* **113**, 375–383 (1999).

49. Lin, M. P., Marti, G. P., Dieb, R., Wang, J., Ferguson, M., ... Harmon, J. W. Delivery of plasmid DNA expression vector for keratinocyte growth factor-1 using electroporation to improve cutaneous wound healing in a septic rat model. *Wound Repair Regen.* **14**, 618–624 (2006).
50. Liang, S. L. & Pan, J. T. Pretreatment with antisense oligodeoxynucleotide against D2 or D3 receptor mRNA diminished dopamine's inhibitory effect on dorsomedial arcuate neurons in brain slices of estrogen-treated ovariectomized rats. *Brain Res.* **926**, 156–164 (2002).
51. Shohami, E., Kaufer, D., Chen, Y., Seidman, S., Cohen, O., ... Soreq, H. Antisense prevention of neuronal damages following head injury in mice. *J. Mol. Med.* **78**, 228–236 (2000).
52. Low, W. C., Rujitanaroj, P.-O., Lee, D.-K., Kuang, J., Messersmith, P. B., ... Chew, S. Y. Mussel-Inspired Modification of Nanofibers for REST siRNA Delivery: Understanding the Effects of Gene-Silencing and Substrate Topography on Human Mesenchymal Stem Cell Neuronal Commitment. *Macromol. Biosci.* **15**, 1457–1468 (2015).
53. Rungta, R. L., Choi, H. B., Lin, P. J. C., Ko, R. W. Y., Ashby, D., ... MacVicar, B. A. Lipid nanoparticle delivery of sirna to silence neuronal gene expression in the brain. *Mol. Ther. - Nucleic Acids* **2**, (2013).
54. Kosik, K. S. The neuronal microRNA system. *Nature Reviews Neuroscience* **7**, 911–920 (2006).
55. Khudayberdiev, S., Fiore, R., & Schratt, G. MicroRNA as modulators of neuronal responses. *Communicative and Integrative Biology* **2**, 411–413 (2009).
56. Uherek, C. & Wels, W. DNA-carrier proteins for targeted gene delivery. *Adv. Drug Deliv. Rev.* **44**, 153–166 (2000).
57. Scholz, C. & Wagner, E. Therapeutic plasmid DNA versus siRNA delivery: Common and different tasks for synthetic carriers. *Journal of Controlled Release* **161**, 554–565 (2012).
58. Pichon, X., A. Wilson, L., Stoneley, M., Bastide, A., A King, H., ... E Willis, A. RNA Binding Protein/RNA Element Interactions and the Control of Translation. *Curr. Protein Pept. Sci.* **13**, 294–304 (2012).
59. Barrett, L. W., Fletcher, S., & Wilton, S. D. Regulation of eukaryotic gene expression by the untranslated gene regions and other non-coding elements. *Cellular and Molecular Life Sciences* **69**, 3613–3634 (2012).
60. Brunner, S., Sauer, T., Carotta, S., Cotten, M., Saltik, M., & Wagner, E. Cell cycle dependence of gene transfer by lipoplex, polyplex and recombinant adenovirus. *Gene Ther.* **7**, 401–407 (2000).
61. Wilke, M., Fortunati, E., van den Broek, M., Hoogeveen, A. T., & Scholte, B. J. Efficacy of a peptide-based gene delivery system depends on mitotic activity. *Gene Ther* **3**, 1133–1142 (1996).
62. Zabner, J., Fasbender, A. J., Moninger, T., Poellinger, K. A., & Welsh, M. J. Cellular and molecular barriers to gene transfer by a cationic lipid. *J. Biol. Chem.* **270**, 18997–19007 (1995).
63. Leonhardt, C., Schwake, G., Stögbauer, T. R., Rappl, S., Kuhr, J. T., ... Rädler, J. O. Single-cell mRNA transfection studies: Delivery, kinetics and statistics by numbers. *Nanomedicine Nanotechnology, Biol. Med.* **10**, 679–688 (2014).
64. Galderisi, U., Cascino, A., & Giordano, A. Antisense oligonucleotides as therapeutic agents. *J. Cell. Physiol.* **181**, 251–7 (1999).

- 1 65. Crooke, S. T. Progress in antisense therapeutics. *Medicinal Research Reviews* **16**, 319–
2 344 (1996).
- 3 66. Watts, J. K. & Corey, D. R. Silencing disease genes in the laboratory and the clinic.
4 *Journal of Pathology* **226**, 365–379 (2012).
- 5 67. Wittrup, A. & Lieberman, J. Knocking down disease: A progress report on siRNA
6 therapeutics. *Nature Reviews Genetics* **16**, 543–552 (2015).
- 7 68. McManus, M. T. & Sharp, P. A. Gene silencing in mammals by small interfering RNAs.
8 *Nature Reviews Genetics* **3**, 737–747 (2002).
- 9 69. Bertrand, J. R., Pottier, M., Vekris, A., Opolon, P., Maksimenko, A., & Malvy, C.
10 Comparison of antisense oligonucleotides and siRNAs in cell culture and in vivo.
11 *Biochem. Biophys. Res. Commun.* **296**, 1000–1004 (2002).
- 12 70. Scherr, M., Morgan, M. a, & Eder, M. Gene silencing mediated by small interfering
13 RNAs in mammalian cells. *Curr. Med. Chem.* **10**, 245–256 (2003).
- 14 71. Deng, Y., Wang, C. C., Choy, K. W., Du, Q., Chen, J., ... Tang, T. Therapeutic potentials
15 of gene silencing by RNA interference: Principles, challenges, and new strategies.
16 *Gene* **538**, 217–227 (2014).
- 17 72. Lim, L. P., Lau, N. C., Garrett-Engle, P., Grimson, A., Schelter, J. M., ... Johnson, J. M.
18 Microarray analysis shows that some microRNAs downregulate large numbers of
19 target mRNAs. *Nature* **433**, 769–773 (2005).
- 20 73. Lee, Y., Kim, M., Han, J., Yeom, K.-H., Lee, S., ... Kim, V. N. MicroRNA genes are
21 transcribed by RNA polymerase II. *EMBO J.* **23**, 4051–4060 (2004).
- 22 74. Muthiah, M., Park, I.-K., & Cho, C.-S. Nanoparticle-mediated delivery of therapeutic
23 genes: focus on miRNA therapeutics. *Expert Opin. Drug Deliv.* **10**, 1259–1273 (2013).
- 24 75. Ruberti, F., Barbato, C., & Cogoni, C. Targeting microRNAs in neurons: Tools and
25 perspectives. *Experimental Neurology* **235**, 419–426 (2012).
- 26 76. Zhang, Y., Wang, Z., & Gemeinhart, R. A. Progress in microRNA delivery. *Journal of*
27 *Controlled Release* **172**, 962–974 (2013).
- 28 77. Bartel, D. P. MicroRNAs: Target Recognition and Regulatory Functions. *Cell* **136**, 215–
29 233 (2009).
- 30 78. Wiesenhofer, B. & Humpel, C. Lipid-Mediated Gene Transfer into Primary Neurons
31 Using FuGene: Comparison to C6 Glioma Cells and Primary Glia. *Exp. Neurol.* **164**, 38–
32 44 (2000).
- 33 79. Malamas, A. S., Gujrati, M., Kummitha, C. M., Xu, R., & Lu, Z.-R. Design and evaluation
34 of new pH-sensitive amphiphilic cationic lipids for siRNA delivery. *J. Control. Release*
35 **171**, 296–307 (2013).
- 36 80. Jiang, M. & Chen, G. High Ca²⁺-phosphate transfection efficiency in low-density
37 neuronal cultures. *Nat. Protoc.* **1**, 695–700 (2006).
- 38 81. Sun, M., Bernard, L. P., Dibona, V. L., Wu, Q., & Zhang, H. Calcium phosphate
39 transfection of primary hippocampal neurons. *J. Vis. Exp.* e50808 (2013).
40 doi:10.3791/50808
- 41 82. Alavian, K. N., Li, H., Collis, L., Bonanni, L., Zeng, L., ... Jonas, E. A. Bcl-xL regulates
42 metabolic efficiency of neurons through interaction with the mitochondrial F1FO ATP
43 synthase. *Nat. Cell Biol.* **13**, 1224–33 (2011).
- 44 83. Advanced transfection with Lipofectamine 2000 reagent: primary neurons, siRNA,
45 and high-throughput applications. *Methods* **33**, 95–103 (2004).
- 46 84. Han, Z., Ge, X., Tan, J., Chen, F., Gao, H., ... Zhang, J. Establishment of Lipofection
47 Protocol for Efficient miR-21 Transfection into Cortical Neurons In Vitro. *DNA Cell Biol.*

- 1 **34**, 703–9 (2015).
- 2 85. Scientific, T. F. Lipofectamine[®] 2000 and Lipofectamine[®] rnaiMaX transfection
3 reagents. (2013).
- 4 86. Williams, D. J., Puhl, H. L., & Ikeda, S. R. A Simple, Highly Efficient Method for
5 Heterologous Expression in Mammalian Primary Neurons Using Cationic Lipid-
6 mediated mRNA Transfection. *Front. Neurosci.* **4**, 181 (2010).
- 7 87. Han, L., Dong, Z., Liu, N., Xie, F., & Wang, N. Maternally Expressed Gene 3 (MEG3)
8 Enhances PC12 Cell Hypoxia Injury by Targeting MiR-147. *Cell. Physiol. Biochem.* **43**,
9 2457–2469 (2017).
- 10 88. Chen, Q., Zhang, F., Wang, Y., Liu, Z., Sun, A., ... Zhang, Q. The Transcription Factor C-
11 Myc Suppresses MiR-23b and MiR-27b Transcription during Fetal Distress and
12 Increases the Sensitivity of Neurons to Hypoxia-Induced Apoptosis. *PLoS One* **10**,
13 e0120217 (2015).
- 14 89. Zhu, L., Gomez-Duran, A., Saretzki, G., Jin, S., Tilgner, K., ... Armstrong, L. The
15 mitochondrial protein CHCHD2 primes the differentiation potential of human induced
16 pluripotent stem cells to neuroectodermal lineages. *J. Cell Biol.* **215**, 187–202 (2016).
- 17 90. Kaech, S., Kim, J. B., Cariola, M., & Ralston, E. Improved lipid-mediated gene transfer
18 into primary cultures of hippocampal neurons. *Mol. Brain Res.* **35**, 344–348 (1996).
- 19 91. Tonges, L., Lingor, P., Egle, R., Dietz, G. P. H., Fahr, A., & Bähr, M. Stearylated
20 octaarginine and artificial virus-like particles for transfection of siRNA into primary rat
21 neurons. *RNA* **12**, 1431–1438 (2006).
- 22 92. Zhdanov, R. I., Kuvichkin, V. V., Shmyrina, A. S., Jdanov, A. R., & Tverdislov, V. A. Role
23 of lipid membrane-nucleic acid interactions, DNA-membrane contacts and metal (II)
24 cations in origination of initial cells and in evolution of prokaryotes to eukaryotes.
25 *Bioelectrochemistry* **58**, 41–6 (2002).
- 26 93. Dib-Hajj, S. D., Choi, J. S., Macala, L. J., Tyrrell, L., Black, J. A., ... Waxman, S. G.
27 Transfection of rat or mouse neurons by biolistics or electroporation. *Nat. Protoc.* **4**,
28 1118–1127 (2009).
- 29 94. Washbourne, P. & McAllister, A. K. Techniques for gene transfer into neurons. *Curr.*
30 *Opin. Neurobiol.* **12**, 566–73 (2002).
- 31 95. Buchser, W. J., Pardin, J. R., Shi, Y., Bixby, J. L., & Lemmon, V. P. 96-well
32 electroporation method for transfection of mammalian central neurons.
33 *Biotechniques* **41**, 619–24 (2006).
- 34 96. Majoul, I., Straub, M., Hell, S. W., Duden, R., & Söling, H. D. KDEL-cargo regulates
35 interactions between proteins involved in COPI vesicle traffic: measurements in living
36 cells using FRET. *Dev. Cell* **1**, 139–53 (2001).
- 37 97. Haas, K., Sin, W. C., Javaherian, A., Li, Z., & Cline, H. T. Single-cell electroporation for
38 gene transfer in vivo. *Neuron* **29**, 583–91 (2001).
- 39 98. Boudes, M., Pieraut, S., Valmier, J., Carroll, P., & Scamps, F. Single-cell electroporation
40 of adult sensory neurons for gene screening with RNA interference mechanism. *J.*
41 *Neurosci. Methods* **170**, 204–211 (2008).
- 42 99. Lu, V. B., Williams, D. J., Won, Y.-J., & Ikeda, S. R. Intranuclear Microinjection of DNA
43 into Dissociated Adult Mammalian Neurons. *J. Vis. Exp.* (2009). doi:10.3791/1614
- 44 100. Zhang, Y. & Yu, L.-C. Single-cell microinjection technology in cell biology. *BioEssays* **30**,
45 606–610 (2008).
- 46 101. Dunaevsky, A. The Gene-Gun Approach for Transfection and Labeling of Cells in Brain
47 Slices. in *Methods in molecular biology (Clifton, N.J.)* **1018**, 111–118 (2013).

102. O'Brien, J. A. & Lummis, S. C. R. Biolistic transfection of neuronal cultures using a hand-held gene gun. *Nat. Protoc.* **1**, 977–81 (2006).
103. Wirth, M. J. & Wahle, P. Biolistic transfection of organotypic cultures of rat visual cortex using a handheld device. *J. Neurosci. Methods* **125**, 45–54 (2003).
104. Wellmann, H., Kaltschmidt, B., & Kaltschmidt, C. Optimized protocol for biolistic transfection of brain slices and dissociated cultured neurons with a hand-held gene gun. *J. Neurosci. Methods* **92**, 55–64 (1999).
105. O'Brien, J. A., Holt, M., Whiteside, G., Lummis, S. C., & Hastings, M. H. Modifications to the hand-held Gene Gun: improvements for in vitro biolistic transfection of organotypic neuronal tissue. *J. Neurosci. Methods* **112**, 57–64 (2001).
106. Jiao, S., Cheng, L., Wolff, J. A., & Yang, N. S. Particle bombardment-mediated gene transfer and expression in rat brain tissues. *Biotechnology. (N. Y.)* **11**, 497–502 (1993).
107. Köhrmann, M., Haubensak, W., Hemraj, I., Kaether, C., Leßmann, V. J., & Kiebler, M. A. Fast, convenient, and effective method to transiently transfect primary hippocampal neurons. *J. Neurosci. Res.* **58**, 831–835 (1999).
108. Halterman, M. W., Giuliano, R., Dejesus, C., & Schor, N. F. In-tube transfection improves the efficiency of gene transfer in primary neuronal cultures. *J. Neurosci. Methods* **177**, 348–54 (2009).
109. Zou, L. L., Huang, L., Hayes, R. L., Black, C., Qiu, Y. H., ... Yang, K. Liposome-mediated NGF gene transfection following neuronal injury: potential therapeutic applications. *Gene Ther.* **6**, 994–1005 (1999).
110. Ma, H., Zhu, J., Maronski, M., Kotzbauer, P. T., Lee, V. M.-Y., ... Diamond, S. L. Non-classical nuclear localization signal peptides for high efficiency lipofection of primary neurons and neuronal cell lines. *Neuroscience* **112**, 1–5 (2002).
111. McLenachan, S., Zhang, D., Palomo, A. B. A., Edel, M. J., & Chen, F. K. mRNA Transfection of Mouse and Human Neural Stem Cell Cultures. *PLoS One* **8**, e83596 (2013).
112. O'Mahony, A. M., Doyle, D., Darcy, R., Cryan, J. F., & O'Driscoll, C. M. Characterisation of cationic amphiphilic cyclodextrins for neuronal delivery of siRNA: Effect of reversing primary and secondary face modifications. *Eur. J. Pharm. Sci.* **47**, 896–903 (2012).
113. O'Mahony, A. M., Desgranges, S., Ogier, J., Quinlan, A., Devocelle, M., ... O'Driscoll, C. M. In Vitro Investigations of the Efficacy of Cyclodextrin-siRNA Complexes Modified with Lipid-PEG-Octaarginine: Towards a Formulation Strategy for Non-viral Neuronal siRNA Delivery. *Pharm. Res.* **30**, 1086–1098 (2013).
114. Liang, Y., Liu, Z., Shuai, X., Wang, W., Liu, J., ... Tao, E. Delivery of cationic polymer-siRNA nanoparticles for gene therapies in neural regeneration. *Biochem. Biophys. Res. Commun.* **421**, 690–695 (2012).
115. Boudes, M., Pieraut, S., Valmier, J., Carroll, P., & Scamps, F. Single-cell electroporation of adult sensory neurons for gene screening with RNA interference mechanism. *J. Neurosci. Methods* **170**, 204–211 (2008).
116. Jiang, D., Du, J., Zhang, X., Zhou, W., Zong, L., ... Jiang, H. miR-124 promotes the neuronal differentiation of mouse inner ear neural stem cells. *Int. J. Mol. Med.* **38**, 1367–1376 (2016).
117. Shoichet, M. S., Tate, C. C., Baumann, M. D., & LaPlaca, M. C. *Strategies for Regeneration and Repair in the Injured Central Nervous System. Indwelling Neural Implants: Strategies for Contending with the In Vivo Environment* (CRC Press/Taylor &

- Francis, 2008).
118. Mckee, A. C. & Daneshvar, D. H. The neuropathology of traumatic brain injury. in *Handbook of clinical neurology* **127**, 45–66 (2015).
119. Yao, L., Yao, S., Daly, W., Hendry, W., Windebank, A., & Pandit, A. Non-viral gene therapy for spinal cord regeneration. *Drug Discov. Today* **17**, 998–1005 (2012).
120. Storer, P. D., Dolbeare, D., & Houle, J. D. Treatment of chronically injured spinal cord with neurotrophic factors stimulates β -tubulin and GAP-43 expression in rubrospinal tract neurons. *J. Neurosci. Res.* **74**, 502–511 (2003).
121. Lu, K.-W., Chen, Z.-Y., Jin, D.-D., Hou, T.-S., Cao, L., & Fu, Q. Cationic Liposome-Mediated GDNF Gene Transfer after Spinal Cord Injury. *J. Neurotrauma* **19**, 1081–1090 (2002).
122. Haastert, K., Lipokatic, E., Fischer, M., Timmer, M., & Grothe, C. Differentially promoted peripheral nerve regeneration by grafted Schwann cells over-expressing different FGF-2 isoforms. *Neurobiol. Dis.* **21**, 138–153 (2006).
123. Cameron, A. A., Smith, G. M., Randall, D. C., Brown, D. R., & Rabchevsky, A. G. Genetic Manipulation of Intraspinal Plasticity after Spinal Cord Injury Alters the Severity of Autonomic Dysreflexia. *J. Neurosci.* **26**, 2923–2932 (2006).
124. Nie, D., Chen, Z., Ebrahimi-Fakhari, D., Di Nardo, A., Julich, K., ... Sahin, M. The Stress-Induced Atf3-Gelsolin Cascade Underlies Dendritic Spine Deficits in Neuronal Models of Tuberous Sclerosis Complex. *J. Neurosci.* **35**, 10762–72 (2015).
125. Boussif, O., Lezoualc'h, F., Zanta, M. A., Mergny, M. D., Scherman, D., ... Behr, J. P. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proc. Natl. Acad. Sci. U. S. A.* **92**, 7297–301 (1995).
126. Shi, L., Tang, G. P., Gao, S. J., Ma, Y. X., Liu, B. H., ... Wang, S. Repeated intrathecal administration of plasmid DNA complexed with polyethylene glycol-grafted polyethylenimine led to prolonged transgene expression in the spinal cord. *Gene Ther.* **10**, 1179–1188 (2003).
127. Ogris, M., Brunner, S., Schüller, S., Kircheis, R., & Wagner, E. PEGylated DNA/transferrin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. *Gene Ther.* **6**, 595–605 (1999).
128. Nguyen, H.-K., Lemieux, P., Vinogradov, S. V., Gebhart, C. L., Guérin, N., ... Kabanov, A. V. Evaluation of polyether-polyethyleneimine graft copolymers as gene transfer agents. *Gene Ther.* **7**, 126–138 (2000).
129. Kichler, A., Chillon, M., Leborgne, C., Danos, O., & Frisch, B. Intranasal gene delivery with a polyethylenimine-PEG conjugate. *J. Control. Release* **81**, 379–88 (2002).
130. Heil, F., Hemmi, H., Hochrein, H., Ampenberger, F., Kirschning, C., ... Bauer, S. Species-Specific Recognition of Single-Stranded RNA via Toll-like Receptor 7 and 8. *Science* (80-.). **303**, 1526–1529 (2004).
131. Karikó, K., Ni, H., Capodici, J., Lamphier, M., & Weissman, D. mRNA Is an Endogenous Ligand for Toll-like Receptor 3. *J. Biol. Chem.* **279**, 12542–12550 (2004).
132. Itaka, K. & Kataoka, K. Recent development of nonviral gene delivery systems with virus-like structures and mechanisms. *Eur. J. Pharm. Biopharm.* **71**, 475–483 (2009).
133. Uchida, S., Itaka, K., Uchida, H., Hayakawa, K., Ogata, T., ... Kataoka, K. In Vivo Messenger RNA Introduction into the Central Nervous System Using Polyplex Nanomicelle. *PLoS One* **8**, e56220 (2013).
134. Miyata, K., Oba, M., Nakanishi, M., Fukushima, S., Yamasaki, Y., ... Kataoka, K.

- 1 Polyplexes from Poly(aspartamide) Bearing 1,2-Diaminoethane Side Chains Induce
2 pH-Selective, Endosomal Membrane Destabilization with Amplified Transfection and
3 Negligible Cytotoxicity. *J. Am. Chem. Soc.* **130**, 16287–16294 (2008).
- 4 135. Masago, K., Itaka, K., Nishiyama, N., Chung, U., & Kataoka, K. Gene delivery with
5 biocompatible cationic polymer: Pharmacogenomic analysis on cell bioactivity.
6 *Biomaterials* **28**, 5169–5175 (2007).
- 7 136. Itaka, K., Ishii, T., Hasegawa, Y., & Kataoka, K. Biodegradable polyamino acid-based
8 polycations as safe and effective gene carrier minimizing cumulative toxicity.
9 *Biomaterials* **31**, 3707–3714 (2010).
- 10 137. Malmo, J., Sandvig, A., Vårum, K. M., & Strand, S. P. Nanoparticle Mediated P-
11 Glycoprotein Silencing for Improved Drug Delivery across the Blood-Brain Barrier: A
12 siRNA-Chitosan Approach. *PLoS One* **8**, e54182 (2013).
- 13 138. Nice, J. Synthesis and chracterization of polymeric micells delivery system as a drug
14 and gene delivery carrier to treat traumatic brain injury. (Clemson University, 2004).
- 15 139. Lou, Y.-L., Peng, Y.-S., Chen, B.-H., Wang, L.-F., & Leong, K. W. Poly(ethylene imine)- g
16 -chitosan using EX-810 as a spacer for nonviral gene delivery vectors. *J. Biomed.*
17 *Mater. Res. Part A* **88A**, 1058–1068 (2009).
- 18 140. Das, M., Wang, C., Bedi, R., Mohapatra, S. S., & Mohapatra, S. Magnetic micelles for
19 DNA delivery to rat brains after mild traumatic brain injury. *Nanomedicine*
20 *Nanotechnology, Biol. Med.* **10**, 1539–1548 (2014).
- 21 141. Helm, F. & Fricker, G. Liposomal Conjugates for Drug Delivery to the Central Nervous
22 System. *Pharmaceutics* **7**, 27–42 (2015).
- 23 142. Immordino, M. L., Dosio, F., & Cattel, L. Stealth liposomes: review of the basic science,
24 rationale, and clinical applications, existing and potential. *Int. J. Nanomedicine* **1**,
25 297–315 (2006).
- 26 143. Adams, J. M. & Cory, S. The Bcl-2 protein family: arbiters of cell survival. *Science* **281**,
27 1322–6 (1998).
- 28 144. Beattie, M. S., Shuman, S. L., & Bresnahan, J. C. Review : Apoptosis and Spinal Cord
29 Injury. *Neurosci.* **4**, 163–171 (1998).
- 30 145. Takahashi, K., Schwarz, E., Ljubetic, C., Murray, M., Tessler, A., & Saavedra, R. A. DNA
31 plasmid that codes for human Bcl-2 gene preserves axotomized Clarke’s nucleus
32 neurons and reduces atrophy after spinal cord hemisection in adult rats. *J. Comp.*
33 *Neurol.* **404**, 159–71 (1999).
- 34 146. Shibata, M., Murray, M., Tessler, A., Ljubetic, C., Connors, T., & Saavedra, R. A. Single
35 Injections of a DNA Plasmid That Contains the Human Bcl-2 Gene Prevent Loss and
36 Atrophy of Distinct Neuronal Populations after Spinal Cord Injury in Adult Rats.
37 *Neurorehabil. Neural Repair* **14**, 319–330 (2000).
- 38 147. Henderson, C. E., Phillips, H. S., Pollock, R. A., Davies, A. M., Lemeulle, C., ... Rosenthal,
39 A. GDNF: a potent survival factor for motoneurons present in peripheral nerve and
40 muscle. *Science* **266**, 1062–4 (1994).
- 41 148. Watabe, K., Ohashi, T., Sakamoto, T., Kawazoe, Y., Takeshima, T., ... Kim, S. U. Rescue
42 of lesioned adult rat spinal motoneurons by adenoviral gene transfer of glial cell line-
43 derived neurotrophic factor. *J. Neurosci. Res.* **60**, 511–519 (2000).
- 44 149. Nguyen, L. H., Gao, M., Lin, J., Wu, W., Wang, J., & Chew, S. Y. Three-dimensional
45 aligned nanofibers-hydrogel scaffold for controlled non-viral drug/gene delivery to
46 direct axon regeneration in spinal cord injury treatment. *Sci. Rep.* **7**, 42212 (2017).
- 47 150. Diao, H. J., Low, W. C., Milbreta, U., Lu, Q. R., & Chew, S. Y. Nanofiber-mediated

- 1 microRNA delivery to enhance differentiation and maturation of oligodendroglial
2 precursor cells. *J. Control. Release* **208**, 85–92 (2015).
- 3 151. Jiang, X., Cao, H. Q., Shi, L. Y., Ng, S. Y., Stanton, L. W., & Chew, S. Y. Nanofiber
4 topography and sustained biochemical signaling enhance human mesenchymal stem
5 cell neural commitment. *Acta Biomater.* **8**, 1290–302 (2012).
- 6 152. Jiang, X., Mi, R., Hoke, A., & Chew, S. Y. Nanofibrous nerve conduit-enhanced
7 peripheral nerve regeneration. *J. Tissue Eng. Regen. Med.* **8**, 377–85 (2014).
- 8 153. Magnani, M. Drug delivery and targeting system. *Emerging Therapeutic Targets* **2**,
9 145–146 (1998).
- 10 154. Salvay, D. M. & Shea, L. D. Inductive tissue engineering with protein and DNA-
11 releasing scaffolds. *Mol. BioSyst.* **2**, 36–48 (2006).
- 12 155. Roy, K., Wang, D., Hedley, M. L., & Barman, S. P. Gene delivery with in-situ
13 crosslinking polymer networks generates long-term systemic protein expression. *Mol.*
14 *Ther.* **7**, 401–408 (2003).
- 15 156. Cao, H., Jiang, X., Chai, C., & Chew, S. Y. RNA interference by nanofiber-based siRNA
16 delivery system. *J. Control. Release* **144**, 203–212 (2010).
- 17 157. Cao, H., Liu, T., & Chew, S. Y. The application of nanofibrous scaffolds in neural tissue
18 engineering. *Advanced Drug Delivery Reviews* **61**, 1055–1064 (2009).
- 19 158. Mahairaki, V., Lim, S. H., Christopherson, G. T., Xu, L., Nasonkin, I., ... Koliatsos, V. E.
20 Nanofiber matrices promote the neuronal differentiation of human embryonic stem
21 cell-derived neural precursors in vitro. *Tissue Eng. Part A* **17**, 855–63 (2011).
- 22 159. De Laporte, L., Huang, A., Ducommun, M. M., Zelivyanska, M. L., Aviles, M. O., ... Shea,
23 L. D. Patterned transgene expression in multiple-channel bridges after spinal cord
24 injury. *Acta Biomater.* **6**, 2889–2897 (2010).
- 25 160. Houchin-Ray, T., Huang, A., West, E. R., Zelivyanskaya, M., & Shea, L. D. Spatially
26 patterned gene expression for guided neurite extension. *J. Neurosci. Res.* **87**, 844–856
27 (2009).
- 28 161. De Laporte, L., Yan, A. L., & Shea, L. D. Local gene delivery from ECM-coated
29 poly(lactide-co-glycolide) multiple channel bridges after spinal cord injury.
30 *Biomaterials* **30**, 2361–8 (2009).
- 31 162. Laporte, L. De, Yang, Y., Zelivyanskaya, M. L., Cummings, B. J., Anderson, A. J., & Shea,
32 L. D. Plasmid Releasing Multiple Channel Bridges for Transgene Expression After
33 Spinal Cord Injury. *Mol. Ther.* **17**, 318–326 (2009).
- 34 163. Nguyen, L. H., Gao, M., Lin, J., Wu, W., Wang, J., & Chew, S. Y. Three-dimensional
35 aligned nanofibers-hydrogel scaffold for controlled non-viral drug/gene delivery to
36 direct axon regeneration in spinal cord injury treatment. *Sci. Rep.* **7**, 42212 (2017).
- 37 164. Zhou, S., Shen, D., Wang, Y., Gong, L., Tang, X., ... Ding, F. microRNA-222 Targeting
38 PTEN Promotes Neurite Outgrowth from Adult Dorsal Root Ganglion Neurons
39 following Sciatic Nerve Transection. *PLoS One* **7**, e44768 (2012).
- 40 165. Elliott Donaghue, I., Tator, C. H., & Shoichet, M. S. Local Delivery of Neurotrophin-3
41 and Anti-NogoA Promotes Repair After Spinal Cord Injury. *Tissue Eng. Part A* **22**, 733–
42 741 (2016).
- 43 166. Ikeda, O., Murakami, M., Ino, H., Yamazaki, M., Koda, M., ... Moriya, H. Effects of
44 brain-derived neurotrophic factor (BDNF) on compression-induced spinal cord injury:
45 BDNF attenuates down-regulation of superoxide dismutase expression and promotes
46 up-regulation of myelin basic protein expression. *J. Neuropathol. Exp. Neurol.* **61**,
47 142–53 (2002).

- 1 167. Zhang, L., Ma, Z., Smith, G. M., Wen, X., Pressman, Y., ... Xu, X.-M. GDNF-enhanced
2 axonal regeneration and myelination following spinal cord injury is mediated by
3 primary effects on neurons. *Glia* **57**, 1178–1191 (2009).
- 4 168. Sulaiman, W. & Gordon, T. Neurobiology of peripheral nerve injury, regeneration, and
5 functional recovery: from bench top research to bedside application. *Ochsner J.* **13**,
6 100–8 (2013).
- 7 169. Allodi, I., Udina, E., & Navarro, X. Specificity of peripheral nerve regeneration:
8 Interactions at the axon level. *Prog. Neurobiol.* **98**, 16–37 (2012).
- 9 170. Guo, J., Wang, X., Wen, J., Wu, W., Pan, M., ... Liu, Z. A novel artificial nerve graft for
10 repairing long-distance sciatic nerve defects: a self-assembling peptide nanofiber
11 scaffold-containing poly(lactic-co-glycolic acid) conduit. *Neural Regen. Res.* **9**, 2132
12 (2014).
- 13 171. Hoyng, S. A., de Winter, F., Tannemaat, M. R., Blits, B., Malessy, M. J. A., & Verhaagen,
14 J. Gene therapy and peripheral nerve repair: a perspective. *Front. Mol. Neurosci.* **8**, 32
15 (2015).
- 16 172. Zacchigna, S. & Giacca, M. Chapter 20 Gene Therapy Perspectives for Nerve Repair. in
17 *International review of neurobiology* **87**, 381–392 (2009).
- 18 173. Lim, S. T., Airavaara, M., & Harvey, B. K. Viral vectors for neurotrophic factor delivery:
19 A gene therapy approach for neurodegenerative diseases of the CNS. *Pharmacol. Res.*
20 **61**, 14–26 (2010).
- 21 174. Leung, D. W., Cachianes, G., Kuang, W. J., Goeddel, D. V., & Ferrara, N. Vascular
22 endothelial growth factor is a secreted angiogenic mitogen. *Science* **246**, 1306–9
23 (1989).
- 24 175. Pereira Lopes, F. R., Lisboa, B. C. G., Frattini, F., Almeida, F. M., Tomaz, M. A., ...
25 Martinez, A. M. B. Enhancement of sciatic nerve regeneration after vascular
26 endothelial growth factor (VEGF) gene therapy. *Neuropathol. Appl. Neurobiol.* **37**,
27 600–612 (2011).
- 28 176. Metcalf, D. Hematopoietic cytokines. *Blood* **111**, 485–91 (2008).
- 29 177. Pereira Lopes, F. R., Martin, P. K. M., Frattini, F., Biancalana, A., Almeida, F. M., ...
30 Martinez, A. M. B. Double gene therapy with granulocyte colony-stimulating factor
31 and vascular endothelial growth factor acts synergistically to improve nerve
32 regeneration and functional outcome after sciatic nerve injury in mice. *Neuroscience*
33 **230**, 184–197 (2013).
- 34 178. Wu, J., Sun, T.-S., Ren, J.-X., & Wang, X.-Z. Ex vivo non-viral vector-mediated
35 neurotrophin-3 gene transfer to olfactory ensheathing glia: effects on axonal
36 regeneration and functional recovery after implantation in rats with spinal cord injury.
37 *Neurosci. Bull.* **24**, 57–65 (2008).
- 38 179. Jessen, K. R. & Mirsky, R. Schwann cell precursors and their development. *Glia* **4**,
39 185–194 (1991).
- 40 180. Lobsiger, C. S., Taylor, V., & Suter, U. The Early Life of a Schwann Cell. *Biol. Chem.* **383**,
41 245–53 (2002).
- 42 181. Aebischer, P., Salessiotis, A. N., & Winn, S. R. Basic fibroblast growth factor released
43 from synthetic guidance channels facilitates peripheral nerve regeneration across
44 long nerve gaps. *J. Neurosci. Res.* **23**, 282–289 (1989).
- 45 182. Kempton, L. B., Gonzalez, M. H., Leven, R. M., Hughes, W. F., Beddow, S., ... Kerns, J.
46 M. Assessment of axonal growth into collagen nerve guides containing VEGF-
47 transfected stem cells in matrigel. *Anat. Rec.* **292**, 214–224 (2009).

- 1 183. Yao, L., Daly, W., Newland, B., Yao, S., Wang, W., ... Pandit, A. Improved axonal
2 regeneration of transected spinal cord mediated by multichannel collagen conduits
3 functionalized with neurotrophin-3 gene. *Gene Ther.* **20**, 1149–1157 (2013).
- 4 184. Gwak, S.-J., Macks, C., Jeong, D. U., Kindy, M., Lynn, M., ... Lee, J. S. RhoA knockdown
5 by cationic amphiphilic copolymer/siRhoA polyplexes enhances axonal regeneration
6 in rat spinal cord injury model. *Biomaterials* **121**, 155–166 (2017).
- 7 185. Singh, B., Singh, V., Krishnan, A., Koshy, K., Martinez, J. A., ... Zochodne, D. W.
8 Regeneration of diabetic axons is enhanced by selective knockdown of the PTEN gene.
9 *Brain* **137**, 1051–1067 (2014).
- 10 186. Zhang, C., Tu, F., Zhang, J., & Shen, L. E-cadherin-transfected neural stem cells
11 transplantation for spinal cord injury in rats. *J. Huazhong Univ. Sci. Technol. [Medical*
12 *Sci.* **34**, 554–558 (2014).
- 13 187. Uchida, S., Hayakawa, K., Ogata, T., Tanaka, S., Kataoka, K., & Itaka, K. Treatment of
14 spinal cord injury by an advanced cell transplantation technology using brain-derived
15 neurotrophic factor-transfected mesenchymal stem cell spheroids. *Biomaterials* **109**,
16 1–11 (2016).
- 17 188. Zhang, J., Zhao, F., Wu, G., Li, Y., & Jin, X. Functional and Histological Improvement of
18 the Injured Spinal Cord Following Transplantation of Schwann Cells Transfected With
19 NRG1 Gene. *Anat. Rec. Adv. Integr. Anat. Evol. Biol.* **293**, 1933–1946 (2010).
- 20 189. Shakhbazov, A., Archibald, S. J., Shcharbin, D., Bryszewska, M., & Midha, R. Aligned
21 collagen–GAG matrix as a 3D substrate for Schwann cell migration and dendrimer-
22 based gene delivery. *J. Mater. Sci. Mater. Med.* **25**, 1979–1989 (2014).
- 23 190. Langer, R. Drug Delivery and Targeting. *Sci.* **392**, 5–10 (1998).
- 24 191. Langer, R. Drugs on target. *Science* **293**, 58–59 (2001).
- 25 192. Kong, H. J., Liu, J., Riddle, K., Matsumoto, T., Leach, K., & Mooney, D. J. Non-viral gene
26 delivery regulated by stiffness of cell adhesion substrates. *Nat. Mater.* **4**, 460–464
27 (2005).
- 28 193. Li, S., Wu, S. P., Whitmore, M., Loeffert, E. J., Wang, L., ... Huang, L. Effect of immune
29 response on gene transfer to the lung via systemic administration of cationic lipidic
30 vectors. *Am. J. Physiol.* **276**, L796-804 (1999).
- 31 194. Kao, W. J. Evaluation of protein-modulated macrophage behavior on biomaterials:
32 Designing biomimetic materials for cellular engineering. *Biomaterials* **20**, 2213–2221
33 (1999).
- 34 195. Krieg, A. M., Yi, A. K., Matson, S., Waldschmidt, T. J., Bishop, G. A., ... Klinman, D. M.
35 CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* **374**, 546–549
36 (1995).
- 37 196. DiProspero, N. A., Meiners, S., & Geller, H. M. Inflammatory cytokines interact to
38 modulate extracellular matrix and astrocytic support of neurite outgrowth. *Exp.*
39 *Neurol.* **148**, 628–639 (1997).
- 40 197. Brewer, K. L., Bethea, J. R., & Yezierski, R. P. Neuroprotective effects of interleukin-10
41 following excitotoxic spinal cord injury. *Exp. Neurol.* **159**, 484–493 (1999).
- 42 198. Shah, M., Foreman, D. M., & Ferguson, M. W. J. Control of scarring in adult wounds
43 by neutralising antibody to transforming growth factor β . *Lancet* **339**, 213–214 (1992).
- 44 199. You, J. O., Almeda, D., Ye, G. J. C., & Auguste, D. T. Bioresponsive matrices in drug
45 delivery. *Journal of Biological Engineering* **4**, (2010).
- 46 200. Nagase, H., Visse, R., & Murphy, G. Structure and function of matrix
47 metalloproteinases and TIMPs. *Cardiovascular Research* **69**, 562–573 (2006).

201. Raeber, G. P., Lutolf, M. P., & Hubbell, J. A. Molecularly engineered PEG hydrogels: A novel model system for proteolytically mediated cell migration. *Biophys. J.* **89**, 1374–1388 (2005).
202. Lee, S. H., Moon, J. J., Miller, J. S., & West, J. L. Poly(ethylene glycol) hydrogels conjugated with a collagenase-sensitive fluorogenic substrate to visualize collagenase activity during three-dimensional cell migration. *Biomaterials* **28**, 3163–3170 (2007).
203. Lutolf, M. P., Lauer-Fields, J. L., Schmoekel, H. G., Metters, A. T., Weber, F. E., ... Hubbell, J. A. Synthetic matrix metalloproteinase-sensitive hydrogels for the conduction of tissue regeneration: Engineering cell-invasion characteristics. *Proc. Natl. Acad. Sci.* **100**, 5413–5418 (2003).
204. Xu, J., Kim, G. M., Ahmed, S. H., Yan, P., Xu, X. M., & Hsu, C. Y. Glucocorticoid receptor-mediated suppression of activator protein-1 activation and matrix metalloproteinase expression after spinal cord injury. *J. Neurosci.* **21**, 92–97 (2001).
205. Noble, L. J., Donovan, F., Igarashi, T., Goussev, S., & Werb, Z. Matrix metalloproteinases limit functional recovery after spinal cord injury by modulation of early vascular events. *J. Neurosci.* **22**, 7526–35 (2002).
206. Birkedal-Hansen, H., Moore, W. G. I., Bodden, M. K., Windsor, L. J., Birkedal-Hansen, B., ... Engler, J. A. Matrix metalloproteinases: A review. *Critical Reviews in Oral Biology and Medicine* **4**, 197–250 (1993).
207. Davies, S. J. A. & Silver, J. Adult axon regeneration in adult CNS white matter. *Trends in Neurosciences* **21**, 515 (1998).
208. Mautes, a E., Weinzierl, M. R., Donovan, F., & Noble, L. J. Vascular events after spinal cord injury: contribution to secondary pathogenesis. *Phys. Ther.* **80**, 673–687 (2000).
209. Discher, D. E., Janmey, P., & Wang, Y. L. Tissue cells feel and respond to the stiffness of their substrate. *Science* **310**, 1139–1143 (2005).
210. Xu, M., West, E., Shea, L. D., & Woodruff, T. K. Identification of a Stage-Specific Permissive In Vitro Culture Environment for Follicle Growth and Oocyte Development1. *Biol. Reprod.* **75**, 916–923 (2006).
211. Escriou, V., Carrière, M., Bussone, F., Wils, P., & Scherman, D. Critical assessment of the nuclear import of plasmid during cationic lipid-mediated gene transfer. *J. Gene Med.* **3**, 179–187 (2001).
212. Tseng, W. C., Haselton, F. R., & Giorgio, T. D. Mitosis enhances transgene expression of plasmid delivered by cationic liposomes. *Biochim. Biophys. Acta - Gene Struct. Expr.* **1445**, 53–64 (1999).
213. Herrup, K. & Yang, Y. Cell cycle regulation in the postmitotic neuron: Oxymoron or new biology? *Nature Reviews Neuroscience* **8**, 368–378 (2007).
214. Flanagan, L. A., Ju, Y. El, Marg, B., Osterfield, M., & Janmey, P. A. Neurite branching on deformable substrates. *Neuroreport* **13**, 2411–2415 (2002).
215. Georges, P. C., Miller, W. J., Meaney, D. F., Sawyer, E. S., & Janmey, P. A. Matrices with compliance comparable to that of brain tissue select neuronal over glial growth in mixed cortical cultures. *Biophys. J.* **90**, 3012–3018 (2006).
216. Sengottuvel, V., Leibinger, M., Pfreimer, M., Andreadaki, A., & Fischer, D. Taxol Facilitates Axon Regeneration in the Mature CNS. *J. Neurosci.* **31**, 2688–2699 (2011).
217. Hellal, F., Hurtado, A., Ruschel, J., Flynn, K. C., Laskowski, C. J., ... Bradke, F. Microtubule stabilization reduces scarring and causes axon regeneration after spinal cord injury. *Science (80-.)*. **331**, 928–931 (2011).
218. Iyer, A. N., Bellon, A., & Baudet, M.-L. microRNAs in axon guidance. *Front. Cell.*

- Neurosci.* **8**, 78 (2014).
219. Pogoda, K. & Janmey, P. A. Glial Tissue Mechanics and Mechanosensing by Glial Cells. *Front. Cell. Neurosci.* **12**, 25 (2018).
 220. Moshayedi, P., Ng, G., Kwok, J. C. F., Yeo, G. S. H., Bryant, C. E., ... Guck, J. The relationship between glial cell mechanosensitivity and foreign body reactions in the central nervous system. *Biomaterials* **35**, 3919–3925 (2014).
 221. Moshayedi, P., Ng, G., Kwok, J. C. F., Yeo, G. S. H., Bryant, C. E., ... Guck, J. The relationship between glial cell mechanosensitivity and foreign body reactions in the central nervous system. *Biomaterials* **35**, 3919–25 (2014).
 222. Walton, N. M. Derivation and large-scale expansion of multipotent astroglial neural progenitors from adult human brain. *Development* **133**, 3671–3681 (2006).
 223. Laywell, E. D., Steindler, D. A., & Silver, D. J. Astrocytic Stem Cells in the Adult Brain. *Neurosurgery Clinics of North America* **18**, 21–30 (2007).
 224. Mislick, K. a & Baldeschwieler, J. D. Evidence for the role of proteoglycans in cation-mediated gene transfer. *Proc. Natl. Acad. Sci. U. S. A.* **93**, 12349–12354 (1996).
 225. Abdallah, B., Hassan, A., Benoist, C., Goula, D., Behr, J. P., & Demeneix, B. A Powerful Nonviral Vector for In Vivo Gene Transfer into the Adult Mammalian Brain: Polyethylenimine. *Hum. Gene Ther.* **7**, 1947–1954 (1996).
 226. Cameron, P. L., Südhof, T. C., Jahn, R., & De Camilli, P. Colocalization of synaptophysin with transferrin receptors: Implications for synaptic vesicle biogenesis. *J. Cell Biol.* **115**, 151–164 (1991).
 227. Dotti, C. G., Parton, R. G., & Simons, K. Polarized sorting of glypiated proteins in hippocampal neurons. *Nature* **349**, 158–161 (1991).
 228. Frick, C., Müller, M., Wank, U., Tropitzsch, A., Kramer, B., ... Löwenheim, H. Biofunctionalized peptide-based hydrogels provide permissive scaffolds to attract neurite outgrowth from spiral ganglion neurons. *Colloids Surfaces B Biointerfaces* **149**, 105–114 (2017).
 229. Shea, L. D., Wang, D., Franceschi, R. T., & Mooney, D. J. Engineered bone development from a pre-osteoblast cell line on three-dimensional scaffolds. *Tissue Eng.* **6**, 605–617 (2000).
 230. Nishimura, I., Garrell, R. L., Hedrick, M., Iida, K., Osher, S., & Wu, B. Precursor tissue analogs as a tissue-engineering strategy. *Tissue Eng* **9 Suppl 1**, S77–S89 (2003).
 231. Jang, J. H., Rives, C. B., & Shea, L. D. Plasmid delivery in vivo from porous tissue-engineering scaffolds: Transgene expression and cellular transfection. *Mol. Ther.* **12**, 475–483 (2005).
 232. Jang, J. H. & Shea, L. D. Controllable delivery of non-viral DNA from porous scaffolds. *J. Control. Release* **86**, 157–168 (2003).
 233. Zhang, J., Chua, L. S., & Lynn, D. M. Multilayered thin films that sustain the release of functional DNA under physiological conditions. *Langmuir* **20**, 8015–8021 (2004).