MicroRNAs and their potential therapeutic applications in neural tissue engineering

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2015

Nguyen, L. H., Diao, H. J., & Chew, S. Y. (2015). MicroRNAs and their potential therapeutic applications in neural tissue engineering. *Advanced Drug Delivery Reviews*, 88, 53-66.

<https://hdl.handle.net/10356/80905>

<https://doi.org/10.1016/j.addr.2015.05.007>

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Manuscript Draft**

Manuscript Number: ADDR-D-15-00027R2

Title: microRNAs and their potential therapeutic applications in neural tissue engineering

Article Type: MicroRNAs in tissue engineering_Chew

Keywords: RNA interference; central nervous system; spinal cord injury; neurons; gene silencing; oligodendrocytes; nerve regeneration; astrocytes; neural transdifferentiation; neural differentiation.

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Abstract: The inherent poor regeneration capacity of nerve tissues, especially in the central nervous system, poses a grand challenge for neural tissue engineering. After injuries, the local microenvironment often contains potent inhibitory molecules and glial scars, which do not actively support axonal regrowth. microRNAs can direct fate of neural cells and are tightly controlled during nerve development. Thus, RNA interference using microRNAs is a promising method to enhance nerve regeneration. Although the physiological roles of microRNAs expression levels in various cellular activities or disease conditions have been extensively investigated, the translational use of these understanding for neural tissue engineering remains limited. This review aims to highlight essential microRNAs that participate in cellular behaviors within the adult nervous system and their potential therapeutic applications. In addition, possible delivery methods are also suggested for effective gene silencing in neural tissue engineering.

microRNAs and their potential therapeutic applications in neural tissue engineering

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Abstract. *The inherent poor regeneration capacity of nerve tissues, especially in the central nervous system, poses a grand challenge for neural tissue engineering. After injuries, the local microenvironment often contains potent inhibitory molecules and glial scars, which do not actively support axonal regrowth. microRNAs can direct fate of neural cells and are tightly controlled during nerve development. Thus, RNA interference using microRNAs is a promising method to enhance nerve regeneration. Although the physiological roles of microRNAs expression levels in various cellular activities or disease conditions have been extensively investigated, the translational use of these understanding for neural tissue engineering remains limited. This review aims to highlight essential microRNAs that participate in cellular behaviors within the adult nervous system and their potential therapeutic applications. In addition, possible delivery methods are also suggested for effective gene silencing in neural tissue engineering.*

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Key words: RNA interference, central nervous system, spinal cord injury, neurons, gene silencing, oligodendrocytes, nerve regeneration, astrocytes, neural transdifferentiation, neural differentiation.

INTRODUCTION

The importance of neural tissue engineering has been recognized for decades due to the limited regeneration capacity of nerve tissues after injuries, especially within the central nervous system (CNS). Unlike the peripheral nervous system (PNS), where damaged axons can regenerate depending on the extent of the injuries, damaged CNS axons often do not regenerate in their native environment [1, 2]. This poor regeneration is an outcome of the existence of a hostile microenvironment that is formed by a complex series of events after nerve injuries [3]. This series of events has been studied comprehensively and reviewed in many articles [1-3]. Briefly, in the CNS, injuries trigger the activation of astrocytes. In particular, reactive astrocytes are recruited to the site of injury and form a barrier, termed the glial scar, to cover the lesion. Besides protecting the adjacent tissues from further damaged, the glial scar also physically blocks regenerating axons from entering the lesion, thereby inhibiting the regeneration of neuronal connections [2]. Reactive astrocytes also produce inhibitory molecules, such as chondroitin sulfate proteoglycans, which further prevent neuronal growth [2]. In addition, myelin of the CNS contains potent growth inhibitory molecules, such as Nogo, myelin-associated glycoprotein, and oligodendrocyte-myelin glycoprotein, which also deter neurite outgrowth [4-6]. Therefore, an important approach in neural tissue engineering is to manipulate and neutralize the local environment to make it more permissive for nerve regeneration [1, 3]. However, recreation of the growth-promoting environment after nerve injuries remains challenging.

Currently, neural tissue engineering focuses on physical therapy using nerve grafts and biomaterial scaffolds, or on biochemical therapy using cells and

neurotrophic factors [1, 3]. However, utilization of a single therapy is not always effective. Synergistic combination of these therapies could better mimic the complex milieu of signals in the nerve tissue, thereby resulting in improved recovery. Yet challenges remain to implement this treatment clinically. Alternative treatments for nerve injuries are, therefore, subjects of further investigation.

Recent studies demonstrated that the cellular responses triggered after nerve injuries are tightly regulated by various signaling pathways involving microRNAs (miRNAs) [7]. Since their discovery, miRNAs together with their biogenesis and functions have been extensively studied and documented [8, 9]. Specifically, miRNAs do not encode proteins but function as post-transcriptional regulators to down-regulate protein expression by preventing translation or triggering mRNA degradation [8, 9]. As miRNAs are often only partially complementary to their target mRNAs, a single miRNA can concurrently regulate a broad network of related genes, giving rise to robust and widespread chains of responses. Hence, RNA interference using miRNAs is a promising method to repair the injured nervous system. Important miRNAs that participate in cellular activities within the adult nervous system will be reviewed in this paper. They have been summarized in Table 1 and will be divided into two groups: (i) potential miRNA candidates for cell transplantation, and (ii) miRNAs that can manipulate the local cell populations for tissue repair. Furthermore, an overview of delivery methods suitable for gene silencing in neural tissue engineering will be presented.

A. miRNAs for directing cell fate in cell transplantation

Cell transplantation is extensively attempted in neural tissue engineering to restore functional loss due to damage or disease in the nervous system. Animal studies have shown that neuronal replacement and partial reconstruction of neuronal circuit are possible [10]. In cell replacement therapy, it is critical that the balance between proliferation and differentiation of transplanted stem/precursor cells is timely regulated. Ideally, these transplanted cells can first proliferate to reach a therapeutically appropriate number and then differentiate into desired neural cell types. These neural cell types, including neurons, astrocytes, and oligodendrocytes (OLs), are the main cell types of the nervous system. Since these cells are generally damaged and lost after nerve injury, they need to be replenished for structure and function restoration. miRNAs have the ability to modulate this balance in a complex molecular network, thus RNA interference using miRNAs holds great promise as a useful tool to control cell proliferation and cell fate commitment of transplanted cells. In most cases, cell fate commitment is analyzed through the expression of specific markers at different developmental stages. Some of these frequently used markers are summarized in Table 1. In this section, various cell sources together with potential miRNA candidates for cell replacement therapy in neural tissue engineering will be reviewed.

1. Directing commitment of neural lineage stem/progenitor cells

a. Neural stem/progenitor cells (NSPCs)

NSPCs have been widely used in neural tissue engineering due to their capacity to self-renew and terminally differentiate into different mature neural cell types, such as neurons, astrocytes, and oligodendrocytes [11-14]. NSPCs can be

harvested from many sources, such as enriched or cultured fetal human NSCs and embryonic stem cell-derived NSCs, for therapeutic uses [15]. However, it is difficult to effectively direct NSPCs differentiation into a desired cell type. Many studies showed that besides differentiating into neurons, NSPCs also favorably differentiated into glial cells or remained as precursor cells when transplanted into the CNS [16-19]. Hence, the ability to control self-renewal and differentiation of transplanted NSPCs is critical for successful cell replacement therapy.

Enhancing NSPCs proliferation

The ability to control proliferation allows the derivation of sufficient cell numbers to restore tissue structure and functionality. In this regard, miR-137, miR-184, and miR-195 may be potential candidates for enhancing NSPC proliferation. On the other hand, the opposite effect can be achieved by down-regulating these miRNAs via anti-miR treatment.

miR-137 is an intrinsic modulator of adult neurogenesis. This miRNA exerts its effects by targeting EZH2, a critical regulator of NSCs survival, self-renewal, and differentiation [20, 21]. *In vitro*, as compared to the scrambled miRNA treatment, overexpression of miR-137 by lentiviral vectors in mouse adult NSCs (aNSCs) increased cell proliferation by 90% [20]. Correspondingly, decreased neuronal and astrocytic differentiations were observed (65% and 51%, respectively) [20]. The potency of miR-137 in controlling cell proliferation was further demonstrated in miR-137-overexpressing mice. Specifically, a 58% enhancement in cell proliferation and a 30% decrease in neuronal differentiation were reported [20]. In contrast, transfection of anti-miR-137 into mouse aNSCs increased the number of Tuj1⁺ neurons and

GFAP⁺ astrocytes by 2.1-fold and 2.4 fold, respectively, compared to transfection of nonspecific anti-miRNAs [20].

Comparatively, miR-184 appears to be less potent in regulating the proliferation and differentiation of NSCs *in vitro*. Specifically, the overexpression of miR-184 in mouse aNSCs *in vitro* resulted in only 50% and 22% increases in cell proliferation when they were transfected with miRNA mimics and lentiviral vectors, respectively [22]. However, the effect of miR-184 seems to be more potent than miR-137 *in vivo* since miR-184-overexpressed aNSCs in the dentate gyrus proliferated 5.0-fold faster than the negative control [22]. Meanwhile, mouse aNSCs transfected with anti-miR-184 gave rise to 2.1-fold more Tuj1⁺ neurons and 52% more GFAP⁺ astrocytes *in vitro* [22]. Numbl, a known regulator of brain development, has been suggested as a physiological downstream target of miR-184 [22].

The third miRNA identified for its effects on enhancing NSPC self-renewal is miR-195. However, the potency of miR-195 appears to be the weakest as compared to the two aforementioned miRNAs both *in vitro* and *in vivo*. In particular, relative to negative miRNA treatment, the *in vitro* overexpression of miR-195 by miRNA mimics in mouse aNSCs only resulted in 18% increased cell proliferation [23]. The effect of miR-195 on NSC differentiation was also less pronounced. Overall, 32% and 38% reductions in the number of Tuj1⁺ neurons and GFAP⁺ astrocytes were obtained, respectively [23]. Moreover, the overexpression of miR-195 did not lead to any significant effect on mouse aNSCs proliferation and neuronal differentiation *in vivo* [23]. Correspondingly, mouse aNSCs transfected with anti-miR-195 exhibited only 17% more Tuj1⁺ neurons and 26% more GFAP⁺ astrocytes [23]. The negative

feedback loop that exists between miR-195 and its downstream target, MBD1, may be the possible reason behind the less effective outcomes of miR-195 treatment. Hence, it is likely that interference of miR-195 expression alone may be insufficient to impart significant alteration in the balance between NSC proliferation and differentiation.

Being able to control the time window for the expression of these miRNAs in NSPCs is crucial. At first, these miRNAs need to be up-regulated to produce therapeutically relevant numbers of cells and then down-regulated to enhance differentiation efficiency. To date, some scaffold designs have been developed to provide temporal controlled release of multiple drugs. In particular, poly(lactide-co-glycolide) (PLG) scaffold can deliver both vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF), one after the other, to promote angiogenesis [24]. This scaffold was fabricated using a mixture of polymer particles, which were pre-mixed with VEGF, and polymer microspheres, which encapsulated PDGF. As a result, VEGF was mainly associated with the surface of the polymer and released rapidly. Comparatively, the release of PDGF was slower as it was associated with the microsphere degradation rate. Similarly, Alexander *et al.* recently reported that the temporal releases of interleukin 10 (IL-10) and endothelial nitric oxide synthase (eNOS) polyplexes were achieved *in vitro* by loading them into collagen and collagen microspheres, respectively [25]. Thus, extending from these previously established platforms, it is possible to impart control over the temporal availability of multiple miRNAs/anti-miRs. Such scaffolding constructs will provide a powerful tool for directing NSPC commitment process.

Enhancing neuronal commitment

For NSPC transplantation, after acquiring a therapeutically relevant number of cells, it is critical that these NSPCs can differentiate into the desired mature cell types. The ability to direct neuronal commitment of NSPCs has been an area of intense research due to the need for neuronal replacement in neurological pathologies ranging from traumatic injuries to degenerative diseases. To date, many miRNAs are shown to regulate neuronal differentiation of these cells.

Among these miRNAs, miR-124 supports neurogenesis of NPCs *in vivo* by suppressing SCP1 expression [26]. In particular, electroporation of miR-124 mimics in chick neural tube triggered the concomitant increase in the expression level of neuronal marker, neurofilament (NF), and a 20% decrease in NPC proliferation [26]. Furthermore, miR-124a, a member of the miR-124 family, promotes NSPC neuronal differentiation at the expense of their proliferation. Overexpression of miR-124a in subventricular zone (SVZ)-derived NPCs *in vitro* led to a 4.5-fold increase in the expression of DCX, a marker of migrating neuroblasts and immature neurons, and a 39% reduction in cell proliferation [27]. miR-124a regulates this cellular balance by targeting the Notch signaling pathway, a highly conserved pathway that is important in controlling the survival and cell fate determination of NSCs [27, 28]. Appeared to be less potent than miR-124a *in vitro*, the miR-200 family also regulates neuronal differentiation of NPCs by targeting the expression of Sox2 and E2F3 proteins. In the presence of B27 supplement, overexpression of the miR-200c/141 cluster in ventral midbrain/hindbrain (vMH) NPC culture led to an 18% increase of Tuj1⁺ neurons at three days post transfection [29]. Hence, the overexpression of miR-124 or miR-200 family in transplanted NSPCs may help increase the efficiency of neuronal differentiation.

In comparison to the aforementioned miRNAs, miR-25 could promote both proliferation and neuronal differentiation of NSPCs when it is overexpressed in synergy with other miRNAs. On its own, *in vitro* ectopic expression of miR-25 in mouse adult NSPCs promoted their proliferation by 17% through TGF- β and insulin/IGF-FoxO related pathways [30]. However, once overexpressed in culture together with miR-106b and miR-93 in the miR-106b~25 cluster, the proliferation and neuronal differentiation of mouse adult NSPCs increased by 22% and 2.6 fold, respectively [30]. These findings suggest a possible approach to improve the potency of NSPC transplantation treatment by first up-regulating miR-25 to expand the cell pool, follow by overexpressing the miR-106b~25 cluster to promote neuronal differentiation. However, the detailed *in vivo* functions of miR-25 and miR-106b~25 cluster await further examination.

Enhancing neuronal and astrocytic commitment

Alongside neuron replacement, astrocyte replacement after nerve injury is also important due to their active regulatory roles in the adult CNS. Astrocytes in the adult hippocampus promote neurogenesis by enhancing NSC proliferation and instructing them to adopt neuronal fate [31]. In addition, astrocytes regulate synapse formation, synaptic transmission, and functional synaptic plasticity [32]. Therefore, enhancing both neuronal and astrocytic commitment of transplanted NSPCs via miRNA treatment may be beneficial for the recovery of neural circuits after nerve injury.

Along this line, miR-9 is a potential candidate as it facilitates neuronal and astrocytic differentiation of aNSCs, depending on microenvironmental factors, by

targeting TLX. Specifically, in the presence of retinoic acid, a neuronal differentiation factor, transfection of miR-9 increased the number of Tuj1⁺ neurons by 32% [33]. However, when coupled with glial differentiation medium that contained forskolin, a 78% increase in GFAP⁺ astrocytes was observed [33]. Correspondingly, a reduction in cell proliferation occurred in both cases [33]. Furthermore, the effects of miR-9 on NSC differentiation are also dependent on the maturation state of the target cells. At 16-20 days *in vitro* (DIV), miR-9 promotes proliferation of human embryonic stem cell-derived NPCs by targeting stathmin [34]. As such, anti-miR-9 treatment decreased cell proliferation by 20% after three days of transfection [34]. Altogether, these findings indicate the importance of microenvironment and temporal considerations in miR-9 related therapeutics. Therefore, in order to effectively monitor neurogenesis using miR-9, the synergistic effects of neuronal inductive factors are required. Additionally, depending on the desired outcome, either cellular proliferation or differentiation, miR-9 can be introduced at different developmental stages of NSCs.

Besides miR-9, let-7d is an alternative miRNA that acts in synergy with neuronal and astrocytic inductive factors to direct NSC differentiation. In fact, being an upstream regulator of miR-9 and TLX signal cascades, let-7d promotes NSC differentiation more effectively. Under neuronal differentiation condition, let-7d transfection in mouse NSCs increased number of Tuj1⁺ neurons by 1.6-fold (versus 1.3-fold increase with miR-9 treatment) [35]. When cultured under the glial differentiation medium, a 2.4-fold increase in GFAP⁺ astrocytes was obtained (versus 1.8-fold increase with miR-9 overexpression) [35]. Similarly, let-7b, another member of the let-7 family, also accelerates neuronal and astrocytic commitment of NSCs by

targeting TLX. Under the same aforementioned differentiation media, the overexpression of let-7b in mouse aNSCs led to 2.0-fold and 2.4-fold more Tuj1⁺ neurons and GFAP⁺ astrocytes, respectively [11].

On the other hand, there is an auto-regulated feedback loop between let-7, Lin-28, and miR-125 during NSC development. Specifically, the expression level of let-7 is negatively controlled by Lin-28, while the expression of Lin-28 in turn is repressed by let-7 and miR-125 [36]. Thus, to facilitate NSC differentiation, one can either up-regulate let-7 and/or miR-125 or down-regulate Lin-28. However, future works are required to compare efficiency of these approaches *in vivo*.

Enhancing neuronal commitment and maturation of NSPCs-derived neurons

In addition to deriving early immature neurons, it is critical to ensure subsequent maturation of differentiated neurons to achieve signal transduction and functionalities. In this regard, miR-34a is a potential candidate since it modulates neural differentiation of NSCs by targeting SIRT1 [37]. The overexpression of miR-34a by pre-miR-34a treatment in NSCs *in vitro* gave rise to 93% more postmitotic NeuN⁺ neurons at 72 hours post transfection [37]. Moreover, the neurite length of derived Tuj1⁺ cells was 22% longer than the negative miRNA-treated cells at 48 hours post transfection [37]. Thus by targeting SIRT1, miR-34a enhances neuronal maturation and neurite elongation of NSC-derived neurons *in vitro*. Similarly, miR-7 also plays an important role in neurite outgrowth and synapse formation by regulating the expression of synapsin gene. In particular, the overexpression of miR-7 in hNSCs *in vitro* by lentiviral miR-7 precursor transfection resulted in a 2.6-fold increase in synapsin expression in the derived neurons [13].

To date, many miRNAs have been identified for their effects on promoting neuronal differentiation of NSPCs. However, the differentiation potencies of these miRNAs were most likely determined from the presence of neuronal markers, such as Tuj1 and NF, rather than the maturation and biological functions of the resulting neurons. Since only matured and functional neurons are clinically useful for cell replacement therapy, future works that evaluate these cellular behaviors are required for fruitful translational applications.

Enhancing oligodendrocyte commitment

OLs are the myelin-forming cells of the CNS [38, 39]. They produce myelin sheaths that wrap around axons to physically protect them, electrically insulate them, and induce a clustering of sodium channels along them for saltatory nerve conduction to support high-speed transmission of nerve impulses over long distances [38, 40]. The importance of myelination is highlighted by the presence of a board range of neurological diseases following myelin failure or loss, such as leukodystrophies and multiple sclerosis [38, 41]. After demyelination, axons can be spontaneously remyelinated by new mature OLs to restore the myelin sheaths [39, 42]. During adulthood, these new mature OLs can be generated via the differentiation process starting from NSPCs to oligodendrocyte precursor cells (OPCs) and then to OLs [43].

In comparison with neuronal differentiation, less is known about regulating OL differentiation of NSPCs. However, the disruption of Dicer function has led to the failure of OL development and myelin formation, suggesting that miRNA is required in this process [44]. Indeed, the number of NG2⁺, PDGFR α ⁺, and Olig2⁺ OPCs

derived from miR-7a-mimic-transfected mouse NPCs increased by 2.0-fold each after three days of culture in the presence of low concentrations of growth factors (EGF and bFGF) [44]. In addition, electroporation of miR-7a-expressing vectors into developing mouse cortex resulted in 8.2-fold and 13.5-fold increases in the population of PDGFR α ⁺ and Olig2⁺ OPCs, respectively [44]. Two proneuronal differentiation factors, Pax6 and NeuroD4, have been identified as possible targets of miR-7a for its function to induce OL commitment of NPCs [44]. After this oligodendroglial lineage specification of NPCs, the multiple-stages process to derive functional myelinating OLs from OPCs is regulated by other miRNAs, which will be reviewed in the next section.

Taken together, it is possible that by fining-tune the expression profiles of multiple miRNAs in NSPCs, these cells could be either proliferated or differentiated efficiently into different mature cell types of interest.

b. Oligodendrocyte precursor cells (OPCs)

OPCs are progenitor cells in the CNS that are capable of self-renewal and OL differentiation [45]. Unfortunately, about 50% of local OPC pool would be lost after spinal cord injury [46]. The loss of these cells may account for the delay in recovery of the number of mature OLs after injury [45]. Hence, OPC replacement would be a useful strategy to regenerate injured nerve tissues. In this regard, miR-7a would be of interest as it maintains OPCs at the proliferation stage by inhibiting the expression of myelin genes [44]. As such, the overexpression of miR-7a in OPCs would lead to an increase in the amount of BrdU incorporation, an indicator of proliferating cells. Correspondingly, the percentage of BrdU incorporation by miR-7a-overexpressed rat

OPCs was 4.9-fold higher than that of the control OPCs at two days after transfection [44]. This finding suggests that overexpression of miR-7a in proliferating OPCs would help to generate sufficient number of OPCs for cell replacement therapy.

After acquiring a therapeutically relevant number of OPCs, it is critical that these cells can be terminally differentiated into myelinating OLs. miR-219 and miR-338 are critical regulators of OL differentiation and myelination in the vertebrate CNS that share many common targets, including Sox6, Hes5, and ZFP238 [47, 48]. The overexpression of each of these miRNAs alone in OPCs was sufficient to elicit OL differentiation even without the need of specific differentiation condition. In particular, the transfection of miR-219 mimics into mouse OPCs resulted in 2.1-fold more RIP⁺ OLs and 3.0-fold more MBP⁺ OLs at four days post transfection in OL growth medium [47]. Similarly, the overexpression of miR-338 in mouse OPCs led to 3.4-fold and 4.6-fold increases in the number of RIP⁺ and MBP⁺ OLs under the same culture conditions [47]. Moreover, the overexpression of these two miRNAs is effective to promote OL differentiation not only *in vitro* but also *in vivo*. Specifically, utero electroporation of miR-219-expressing vectors into the mouse-developing cortex at E14.5 led to 47% more Olig2⁺ OPCs and 25% more PDGFR α ⁺ OPCs in comparison with the negative plasmid control [47]. Similarly, utero electroporation of miR-338-expressing vectors increased the number of Olig2⁺ and PDGFR α ⁺ OPCs by 47% and 56% [47]. Hence, given the potency of miR-219 and miR-338 in OL differentiation under non-specific *in vitro* and *in vivo* conditions, treatment of these miRNAs would be useful to promote OPC maturation and myelination for nerve regeneration in the CNS.

Besides miR-219 and miR-338, miR-23a also supports OL differentiation of OPCs. By targeting LMNB1, the overexpression of miR-23a in mouse primary glial culture led to a 2.9-fold increase in the number of CNP⁺ OLs compared to the negative control [49]. Additionally, by targeting PTEN and 2700046G09Rik, miR-23a-overexpressed OPCs from transgenic mice resulted in 5.1-fold more MBP⁺ OLs and 3.8-fold more MAG⁺ OLs after 4 DIV in the differentiation medium containing T3 [50].

On the other hand, miR-138 promotes the early phase (specified by CNP and MBP expressions) but postpones the later phase (specified by MOG expression) of OL differentiation (Table 1). Under proliferative condition, overexpression of miR-138 in OPCs led to 2.4-fold more CNP⁺ OLs and 1.9-fold more MBP⁺ OLs compared to the control miRNA mimic after 7 DIV [48]. However, overexpression of miR-138 in differentiation medium supplemented with combination of PDGF and T3 for 7 DIV resulted in 31% more CNP⁺ OLs, 54% more MBP⁺ OLs, but 54% fewer MOG⁺ OLs compared to the negative control [48]. Consequently, by promoting the early phase and delaying the later phase, miR-138 could elongate the immature phase of OL differentiation, thereby extending the time frame in which a terminally differentiating OL can select and properly myelinate nearby axons [48]. Cumulatively, by manipulating the level of these specific miRNAs, one could possibly promote OL maturation and myelination after nerve injury.

2. Directing neural transdifferentiation of other cell lineages

Cell replacement strategy for the nervous system employs not only stem cells and precursor cells of the neural lineages but also cells from other lineages, such as

MSCs and fibroblasts. While most transdifferentiation studies have employed different cocktails of transcription factors, recent studies show that miRNAs can also induce cell fate transition. Moreover, miRNAs can be introduced into cells in their mature form without initiating innate immune responses; whereas transcription factors need to be introduced mostly as DNAs, which hold the risk of integrating into host genome, thereby eliciting permanent effects [51]. However, directing transdifferentiation via miRNA treatment has only been shown for derivation of neurons although using transcription factors, other neuronal cells, such as OPCs, have been derived [52, 53].

a. Mesenchymal stem cells (MSCs)

MSCs can be easily isolated from many adult tissue sources, further expanded, and differentiated into a variety of cell types under specific conditions [54-57]. In particular, ectopic enforced expression of miR-9 or miR-124 is able to induce bone marrow MSCs (BMSCs) transdifferentiation into neurons. The overexpression of miR-9 in BMSC culture increased the number of MAP-2⁺ neurons and NSE⁺ neurons by 4.4% and 8.6%, respectively, after six days induction by β -mercaptoethanol [58]. Similarly, the transfection of miR-124-expressing vectors into BMSCs resulted in the remarkable elevation of neuronal markers, Tuj1 and MAP-2, after six days of *in vitro* differentiation [59]. However, detailed culture conditions and the transfection efficiency were not reported in this paper. In addition, these miR-124-overexpressed BMSCs also developed neuron-like morphology with dendrite and neurite structures [59]. More importantly, the expression of synaptophysin, a marker of synapse formation, was also increased in these transfected cells, thus indicating that miR-124 promotes neuronal differentiation of BMSCs toward mature functional neurons [59].

Altogether, by overexpressing these miRNAs, functional neurons can be effectively derived from MSCs for cell replacement therapy.

In contrast to miR-9 and miR-124, miR-128 negatively regulates the differentiation of BMSCs into neuron-like cells through the modulation of Wnt3a, a component of the Wnt signaling pathway [60]. Specifically, miR-128 mimic-transfected BMSCs possessed significant reductions in the expression levels of six nerve cell markers, including Tuj1, MAP-2, NF-M, GFAP, Nestin, and NSE, after five days of culture in medium containing bFGF and EGF as compared to the non-transfected cells [60]. On the contrary, differentiation of BMSCs that were transfected with miR-128 inhibitor resulted in 72% increased Tuj1, 2.6-fold increased MAP-2, 67% increased NF-M, 2.3-fold increased GFAP, 78% increased Nestin, and 50% increased NSE relative to non-transfected BMSCs [60]. These findings suggest that neurons can be efficiently derived from MSCs for treatment of nerve injury by suppressing miR-128, an inhibitor of this transdifferentiation process. Yet, this study only evaluated the expression levels of nerve cell markers and the change in cell morphology but not the functionalities of the derived neurons. Therefore, further work is required to confirm the translational applications of anti-miR-128 treatment.

b. Fibroblasts

Similar to MSCs, fibroblasts is an attractive alternative cell source for transplantation because they are easily isolated from autologous donors, expanded in cultures, and genetically modified *in vitro* for transdifferentiation purposes [61]. Indeed, the direct neuronal transdifferentiation of fibroblasts have been demonstrated using miRNAs. Specifically, the treatment of miR-9/9* and miR-124 cocktail

successfully reprogrammed human neonatal fibroblasts to induced neurons as compared to failed outcomes with either of these miRNAs alone. In particular, when these two miRNAs were overexpressed together in fibroblasts, less than 5% MAP-2⁺ cells were obtained at 30 days after lentiviral transfection [62]. However, when coupled with the overexpression of transcription factor NEUROD2, miR-9/miR-124 transfection resulted in a 10-fold increase in neuronal transdifferentiation. Specifically, 50% of the cell population expressed MAP-2 at 30 days after transfection [62]. Taken together, while the synergistic effect of miR-9/miR-124 induced neuronal transdifferentiation of fibroblasts, the inclusion of additional factors, such as NEUROD2, is required in order to enhance transdifferentiation efficiency.

On the other hand, when coupled with the culture media containing Noggin for 4-7 days and forskolin for subsequent 10-14 days, the overexpression of miR-124 in postnatal fibroblasts gave rise to a Tuj1⁺ cell population [63]. Correspondingly, these cells possessed multiple small processes but no mature neuronal morphology [63]. Whereas under the same culture conditions, treatment of postnatal fibroblasts with the combination of miR-124 and two transcription factors, BRN2 and MYT1L, gave rise to 55% MAP-2⁺ and 46% NeuN⁺ cells with typical neuronal morphology and functions [63]. Thus, to effectively induce reprogramming of fibroblasts into mature functional neurons, the synergism of miRNAs and transcription factors might be required.

B. Directing cellular reaction of local mature cells in the injured sites

Besides stem/progenitor cells, different populations of mature cells surround the sites of injury within the nervous system, ranging from neurons, astrocytes, OLs

and Schwann cells, to microglia, and macrophages. Their responses towards the injury via changes in gene expression influence neural tissue regeneration outcomes. Post-transcriptional regulation by miRNAs is one of the effective gene expression regulation mechanisms. Accordingly, by manipulating the expression levels of key miRNAs in these cells, it might be possible to modulate their responses towards the injury, thereby facilitating tissue regeneration and functional recovery.

1. Axonal regeneration & dendrite formation

The first signal of nerve injury is axonal swelling followed by its disintegration [64]. This process, known as Wallerian degeneration, leads to breakdown of the axonal cytoskeleton and then fragmentation of the axons [64, 65]. Moreover, together with the loss of axons, nerve injury also causes damage to dendrites resulting in decreased dendritic complexity and loss of the synaptic networks [66]. Hence, one of the traditional strategies in neural tissue engineering is to enhance neurite regrowth to form new axons and dendrites.

Along these lines, neurite outgrowth may also be promoted by regulating miRNA expression. Indeed, the growth promoting effect of overexpressing miRNAs was demonstrated with miR-124, a miRNA that is down-regulated in injured motor neurons [67, 68]. Specifically, the transfection of miR-124-expressing vectors into primary cortical neurons *in vitro* significantly increased the number of protruded primary neurites at 43 hours after electroporation [68]. In contrast, depending on the down-stream effects of a miRNA, one may also achieve enhanced neurite outgrowth effect by decreasing the expression of the target miRNA through the delivery of anti-miRs. Accordingly, by transfecting primary hippocampal neurons with anti-miR-137,

total dendritic length was increased by 25% as compared to the control anti-miR treatment at 48 hours post transfection [69]. Thus, enhanced axonal regeneration and dendrite formation may be achieved by either up-regulating miR-124 expression or down-regulating miR-137 expression in local neurons after nerve injury.

Besides focusing on enhancing neurite regrowth, another consideration of neural tissue engineering is the formation of synaptic reconnections. This can be achieved by modulating dendritic structures, synaptic strength, and synaptic plasticity using miRNAs. Indeed, miR-125b and miR-132 oppositely regulate dendritic spine structures and synaptic functions. By targeting NR2A, the overexpression of miR-125b in hippocampal neurons *in vitro* led to 22% increased dendritic protrusion length and 11% decreased dendritic protrusion width at three days post transfection [70]. These long and thin dendritic protrusions were correlated with the weakening of synaptic transmission as miR-125b-overexpressed neurons exhibited 25% drop in the amplitude of mean miniature excitatory post-synaptic current (mEPSC) [70]. On the other hand, miR-132-overexpressed hippocampal neurons acquired 9% increased protrusion width and 15% decreased protrusion density at three days post transfection *in vitro* [70]. These induced wider spines were associated with 29% improved mean mEPSC amplitude [70]. Therefore, changes in spine morphology that resulted from alterations in expression levels of miRNAs would affect synaptic strength. This indicates the importance of miRNAs in directing the formation of regenerated dendrites and remodeling of synaptic networks after nerve injury.

2. Remyelination

OLs synthesize myelin membranes that ensheath axons of the CNS to protect

them and accelerate their information conduction speed. Consequently, damage of myelin membranes and failure of remyelination after nerve injury can disrupt neural signals, leading to nerve degeneration [69]. miR-23 is abundantly expressed in OLs and involved in myelin formation, maintenance, and proper myelin folding [49, 50]. The overexpression of miR-23a in transgene (TG) mice led to 50%, 80%, and 35% increase in MBP, CNP, and MAG levels, respectively, in the corpus callosum as compared to wildtype (WT) mice [50]. The increase in expression levels of myelin genes was correlated with the increase in myelin thickness as a corresponding 27% reduction in G-ratio was also observed in the homogenous TG mice [50]. Hence, miR-23 treatment could be employed to elevate the expression levels of these myelin genes in the local OLs and thus facilitating their remyelination process after nerve injury in the CNS.

On the other hand, Schwann cells are responsible for synthesizing myelin sheath in the PNS [2]. They also play a critical role in generating a supportive environment within the PNS, as they produce cell adhesion molecules, integrins, and neurotrophins [1]. Moreover, Schwann cells are involved in synapse formation [1]. For these reasons, the ability of Schwann cells to promote nerve regeneration has been extensively studied. Unlike OLs, Schwann cells are able to dedifferentiate back to an immature-like state following axonal damage [71]. After dedifferentiation, Schwann cells proliferate to increase cell number for the repair process to begin [2]. Therefore, enhancing the proliferation efficiency of Schwann cells during this preparation phase may speed up nerve regeneration. Along this line, miRNA profiling has identified miR-34a as an inhibitor of this proliferation step by acting on two downstream targets, Notch1 and Ccnd1. In particular, at day 3 in the coculture of

Schwann cells and dorsal root ganglions (DRGs), the overexpression of miR-34a led to a 50% reduction in Schwann cell proliferation [71]. Correspondingly, this result suggests that anti-miR-34a treatment can potentially provide the opposite effect of enhancing Schwann cell proliferation for an efficient repair process. Hence, future works that investigate this application of anti-miR-34a would be beneficial.

After the transient phase of proliferation, dedifferentiated Schwann cells migrate into the site of injury to form interconnected tubes that act as conduits to direct axonal regrowth and provide a supportive extracellular milieu for tissue regeneration [2, 71, 72]. Thus, manipulating the expression of miRNAs that regulate this migration process could be a useful strategy to promote tissue regrowth. Specifically, miR-9 has been identified as a negative regulator of Schwann cell migration. The disruption of miR-9 activity in Schwann cells in turn enhanced their migration ability. As indicated by transwell assays, anti-miR-9 transfection increased Schwann cell migration by 78% versus control cells [73]. In addition, cell migration was enhanced by 50% with anti-miR-9 treatment in wound healing assays [73]. Overall, the results provide evidences that suppressing miR-9 expression would significantly improve the migration ability of Schwann cells and may serve as a potential therapeutic approach to support axonal regrowth.

Given the primary role of Schwann cells in PNS regeneration, the ability to control their proliferation and migration processes together via miRNA treatment would be attractive. In this regard, miR-221 and miR-222 are potential candidates. Particularly, miR-221 mimic transfection into Schwann cells increased cell proliferation and migration by 2.4-fold and 4.3-fold, respectively [74]. On the other

hand, Schwann cells transfected with miR-222 had a 2.7-fold increase in cell proliferation and a 2.6-fold increase in cell migration [74]. In contrast to miR-221 and miR-222, miR-182 suppresses Schwann cell proliferation and migration by targeting FGF9 and NTM [75]. Consequently, when treated with anti-miR-182, Schwann cell proliferation and migration were improved by 1.9-fold and 2.3-fold, respectively, as compared to control cells [75]. Thus, a potential therapeutic approach for nerve regeneration may be to overexpress miR-221, miR-222, and/or anti-miR-182 so as to enhance the proliferation rate and migration ability of Schwann cells after PNS injury.

As the peripheral axons will regenerate along the interconnected tubes assembled by Schwann cells, these Schwann cells need to re-differentiate to form new myelin sheath [71]. Recently, miR-140 has been found to hinder this redifferentiation process and prevent Schwann cells to form myelin *in vitro*. In particular, when cocultured with DRGs, the overexpression of miR-140 in Schwann cells resulted in a 76% decrease in the number of myelinated segments [71]. This finding suggests another approach to promote the regenerative process of peripheral nerve after injury - by suppressing the expression of miR-140, a negative regulator of Schwann cell redifferentiation. Yet, the potential of anti-miR-140 treatment in this regard must be further evaluated.

3. Glial scar formation

Astrocyte is the most abundant cell type in the CNS comprising approximately 50% of total glial cell number [76-78]. In response to pathological conditions, astrocytes leave their quiescent state and become activated by undergoing proliferation (astrocytosis) and hypertrophy (astrogliosis), which are associated with

the elevated expression levels of GFAP and vimentin [76, 77, 79]. The ultimate result is the formation of glial scars, which may be essential for wound repair but also inhibit axonal regrowth [80]. Hence, a potential treatment to injuries in the CNS could be to control glial scar formation by modulating the proliferation and hypertrophy extents of astrocytes surrounding the injured site.

miR-145 attenuates both astrogliosis and astrogliosis of astrocytes. In particular, the overexpression of miR-145 in astrocytes *in vitro* led to 49% less BrdU incorporated cells, 49% reduced cell size, and 42% reduced number of cellular processes compared to the negative control [81]. In agreement with the *in vitro* results, a reduction of astrogliosis was observed in rat spinal cords, which were transfected with lentiviral miR-145, at seven days post-injury. Specifically, the size of astrocytes and the area of GFAP⁺ processes were decreased by 62% and 66%, respectively [81]. Altogether, this data demonstrates that the overexpression of miR-145 in astrocytes could result in a thinner glial scar by inhibiting both astrogliosis and astrogliosis processes. This reduction in scar thickness would potentially explain for the observation of 2.8-fold more Iba1⁺ amoeboid microglial/macrophages infiltrating at the lesion border 1-week post injury [81]. As compared to the PNS, there is a limited recruitment of inflammatory cells into the CNS injury [2, 82]. Consequently, the availability of myelin and cell debris at the injured site was prolonged and inhibits axonal regrowth [2, 83, 84]. Thus, an enhanced infiltration of inflammatory cells into the injury site may be beneficial to the axonal regeneration as debris is removed more efficiently.

Besides miR-145, miR-21 is also able to attenuate astrogliosis. Specifically, in the transgenic mouse line that overexpressed miR-21, GFAP and vimentin levels at the lesion border were decreased by 47% and 33%, respectively, at 2-weeks post spinal cord injury [85]. However, this reduction in astrocytic hypertrophy was unexpectedly correlated with an 80% increase in the lesion size [85]. As such, the weakening of astrogliosis after spinal cord injury did not result in a better wound closure. This excessive tissue damage could be due to the continuous invasion of destructive immune cells into the lesion since the blood-brain barrier could not be repaired under attenuated astrogliosis condition. Indeed, the inhibition of miR-21 in the injured mice spinal cords sustained astrocytic hypertrophy, which was associated with an 80% increase in axonal neurofilament expression through the lesion site [85]. Hence, the timing and extend of astrocytosis and astrogliosis as modulated by miRNAs must be tightly regulated post-injury to first facilitate debris clearance and then aid blood-brain barrier reformation to ultimately enhance tissue regeneration.

4. The inflammatory response

The inflammatory response is one of the key steps in the wound healing process following nerve injuries [2]. Signaling molecules released from the injured site attract inflammatory cells to the lesion [2]. There are two notable differences between the PNS and CNS in terms of the recruitment of inflammatory cells and their subsequent responses towards nerve injuries. First, in the PNS, numerous monocytes are recruited from the blood and differentiate into macrophages in the first 3-5 days post-injury [2, 86]. In contrast, due to the presence of the blood-brain barrier, macrophage infiltration into the CNS tissue is remarkably limited [2, 86]. Hence, debris removal by phagocytosis takes months in the CNS as opposed to days in the

PNS [2]. Second, the reduction in the level of inflammatory cell recruitment in the CNS is substituted by resident microglia. Microglia, which make up 10-20% of the CNS glia cells, act like macrophages in inflammatory response [2, 87]. They are normally maintained in a quiescent state and only become activated once necrotic debris is present or the blood-brain barrier is compromised after nerve injury [2, 88-90]. Upon activation, microglia, together with recruited macrophages, govern the immune response by phagocytosis and release of inflammatory mediators.

Depending on external stimulus and environment, activation of macrophages and microglia could result in either M1 (classical activation) or M2 phenotype (alternative activation) [87, 91, 92]. *In vitro*, lipopolysaccharides (LPS) or interferon- γ (IFN- γ) is commonly used to induce M1 phenotype as characterized by enhanced expression levels of M1-associated molecules, such as TNF- α , IL-6, IL-12, and iNOS [87, 93, 94]. On the other hand, IL-4, IL-10, or TGF- β is used to yield the M2 phenotype with the elevation of TGF- β 1, Arg-1, and FIZZ1 expression [87, 94]. Besides expressing distinct molecular markers, these different activated phenotypes also exert different functions in the nerve tissues. M1 cells produce high levels of nitric oxide and proinflammatory cytokines that are essential for host defense [95]. However, these molecules also cause damage to healthy neighboring tissues, thereby initiating secondary injuries [95]. In contrast, M2 cells promote tissue repair while suppressing destructive immunity [95]. Particularly, in the case of injured mouse spinal cord, M1 macrophages induced neuronal toxicity while M2 macrophages promoted axonal regeneration [95]. In addition, M2 polarization is essential for efficient remyelination in the CNS as there is an M1-to-M2 switch at the beginning of remyelination in rodent models [96]. Thus, harnessing the beneficial properties of

macrophages and microglia by modulating their polarization towards the M2 phenotype provides great potential for the treatment of nerve injuries.

a. Macrophages

As discussed previously, polarizing macrophages towards the M2 phenotype might aid in nerve regeneration. This desired polarization could be achieved via miRNA treatment, which either down-regulates M1-associated markers and/or up-regulates M2-associated markers. In the case of down-regulating M1-associated markers, miR-92a is a potential candidate. In particular, miR-92a inhibits M1 activation by repressing the expression of MKK4 [97]. Under LPS exposure, miR-92a-overexpressed macrophages exhibited 43% decreased TNF- α level and 44% decreased IL-6 level at 24 hours post-transfection [97]. Meanwhile, miR-124 promotes macrophage polarization towards the M2 phenotype by targeting the transcription factor C/EBP- α [98]. Specifically, the overexpression of miR-124 in bone marrow-derived macrophages resulted in the down-regulation of M1-associated markers (98% decreased TNF- α level and 72% decreased iNOS level) and the up-regulation of M2-associated proteins (3.2-fold increased TGF- β 1 level, 2.7-fold increased Arg-1 level, and 2.8-fold increased FIZZ1 level) [98].

Furthermore, secondary injuries in the neighboring tissues can also be limited by eliminating the already-polarized M1 macrophages via miRNA treatment. For example, the overexpression of let-7c in M1 macrophages can diminish the expression of their M1 phenotype. Indeed, M1 macrophages transfected with let-7c mimic possessed 55% decreased CCR7 level, a typical marker of M1 macrophage [93]. In addition, despite being exposed to LPS, let-7c overexpression also reduced

IL-12 and iNOS expression levels by 70% and 36%, respectively [93]. Apart from inhibiting the expression of M1-associated markers in M1 macrophages, let-7c also participates in promoting the transition of M1 to M2 phenotype. Specifically, as compared to control cells, let-7c mimic transfection into M1 macrophages enhanced FR- β expression (M2 marker) by 50% [93]. Moreover, under the exposure of IL-4, these cells exhibited 2.7-fold increased Arg-1 level and 3.8-fold increased FIZZ1 level [93]. Taken together, the overexpression of either of these miRNAs in macrophages could be employed to promote M2 phenotype, thereby limiting neurotoxic responses and supporting tissue repair in the nervous system.

b. Microglia

miR-124, a brain-specific miRNA, is highly expressed in quiescent microglia but down-regulated in activated microglia [98]. The effect of miR-124 overexpression *in vivo* was recently studied on the experimental autoimmune encephalomyelitis (EAE) mouse model, which is characterized by autoimmune inflammation of the CNS associated with microglia activation. In particular, systemic administration of miR-124 into these mice resulted in 27% more resting microglia and 73% less activated microglia and peripheral macrophages relative to the control group [98]. Moreover, the inflammation lesions were reported in the control group but undetectable in the miR-124 injected mice [98]. These results reveal that the inflammatory response towards nerve injury could be attenuated to prevent secondary injury by overexpressing miR-124 in microglia.

Contrarily to macrophages, little is known about the mechanisms responsible for regulating microglia phenotype in the CNS [94]. To date, only miR-155 has been

extensively studied for its function to regulate the M1/M2 phenotype ratio in microglia. Specifically, miR-155 is associated with the M1 phenotype and has a strong pro-inflammatory role in microglia [99]. Hence, by inhibiting the expression level of miR-155, M1 activation of microglia can potentially be suppressed to limit microglia-mediated neurotoxic response and enhance axonal regeneration. Indeed, the transfection of anti-miR-155 into microglia decreased the expression levels of TNF- α , IL-6, and iNOS by 55%, 75%, and 25%, respectively, under the LPS exposure [99].

c. Astrocytes

In the CNS, beside microglia, activated astrocytes also function like macrophages as they are capable of phagocytosis and antigen presentation [76]. In the case of myelinated axons, axonal degeneration and fragmentation after injury result in the accumulation of myelin debris, which contains a variety of axonal growth inhibitors. Removal of these growth inhibitors via astrocyte and microglia phagocytosis facilitates axon regeneration [2, 83, 84]. Activated astrocytes also participate in immune response by mediating different signaling pathways. In particular, miR-146a is a regulator of astrocyte-mediated immune response. It participates in IL-1 β mediated release of inflammatory mediators. *In vitro*, the transfection of miR-146a mimics into the astrocytoma cell line, U373, resulted in the reductions of IL-6, IL-8, G-CSF, INF- γ , TNF- α expression levels by 97%, 83%, 55%, 67%, and 80%, respectively, after 24 hours exposed to IL-1 β [100]. These results reveal the anti-inflammatory role of miR-146a in astrocytes. Correspondingly, miR-146a treatment has potential to prevent secondary injuries and promote tissue repair.

Taken together, the aforementioned examples highlight the significant roles of miRNAs in the regulation of cellular behaviors in the nervous system. A complete understanding of the miRNA regulatory network will ultimately provide more useful approaches and precise targets for therapeutic applications in nerve regeneration.

C. miRNA and tumors

As miRNAs participate in various cellular activities, such as cell growth, proliferation, migration, differentiation, and apoptosis, it is not surprising that various miRNAs are also involved in human cancer [27, 101]. Many miRNAs function as oncogenes by repressing the expression of multiple genes in different signaling pathways [101]. For example, while miR-221 and miR-222 support Schwann cell proliferation and migration *in vitro*, they also promote the growth of glioblastoma, the most common and aggressive primary brain tumor in adults, by suppressing p27^{Kip1}, a key negative regulator of the cell cycle [102, 103]. Therefore, since miRNAs have a wide impact on different cellular processes, RNA interference using miRNAs must be tightly controlled in many aspects, such as the cell types that overexpress miRNAs, the expression level of miRNAs, and the transience and localization of the miRNA effects, in order to support tissue regeneration without causing undesirable cancer formation. Taking these points into considerations, various delivery methods have been designed to date. Some notable delivery methods will be discussed in the next part of this review.

D. miRNA delivery methods

Although miRNAs hold many potential applications in nerve regeneration, practical considerations must be made in terms of their delivery. Apart from the

oncogenicity effects (outlined in section C), miRNAs are also labile molecules. Specifically, the ribose sugar group of RNA molecule is chemically reactive; thereby RNAs are unstable and more prone to degradation [104, 105]. Moreover, besides the instability nature of RNAs, cellular uptake and endosomal escape are two main barriers for efficient miRNA delivery. First, the negative charge of miRNA molecules prevents them from crossing biological membranes, hence deterring their cellular uptake [106, 107]. Second, after taken up by cells, miRNAs have to leave the endosome and enter the cytoplasm to avoid lysosomal degradation [106]. Therefore, miRNA treatment for nerve regeneration requires effective delivery methods for transporting miRNAs into the cytoplasm of the targeted cells. Since each method has its own advantages and drawbacks, direct comparison remains elusive. Thus, this section will focus on the effectiveness of some frequently used methods in neural tissue engineering based on available works.

1. Viral transduction

Due to the high transfection efficiency, viral transduction has frequently been the first choice for delivering miRNAs and studying their functions. In this approach, retrovirus and lentivirus are extensively used. Retroviruses can infect various types of dividing cell, including NSPCs. Specifically, miR-25 was overexpressed by 8-fold in NSPCs using retroviral vectors containing miR-25 precursor [30]. Similarly, an 8-fold increase in miR-124 expression also reported in neurospheres transduced by retroviral miR-124 vectors [108]. However, the drawback of using retroviral vectors is their inability to transduce quiescence cells [109]. On the contrary, lentiviral vectors has the added advantage of being able to transduce non-dividing cells [109], resulting in its enhanced usage popularity in transfection of cells for neural tissue engineering. In

particular, 80% of GFAP⁺ astrocytes in the rat spinal cords were transduced by lentiviral vectors encoding miR-145 at 24 hours after infection [81]. Similarly, Szulwach *et al.* reported nearly 100% transfection efficiency of lentiviral vectors expressing miR-137 in aNSCs [20]. Furthermore, the efficiency of lentiviral transduction was also illustrated by the overexpression of delivered miRNAs inside host cells. In this regard, the transfection of lentiviral vectors encoding miR-9 into MSCs *in vitro* resulted in a 2.8-fold increase in the miR-9 expression level as compared to the untransfected cells [58].

Besides high transfection efficiency, viral transduction offers sustained expression of miRNAs in the transfected cells due to the integration of viral genome into host genome [110]. Although such sustained expressions of miRNAs are desirable for basic cell biological studies, the genomic integration can lead to uncontrolled insertional mutagenesis [110, 111], therefore, limiting the use of viral-mediated miRNA delivery in translational therapeutic applications.

2. Non-viral transfection

As compared to viral transduction, non-viral delivery methods emerged with their own advantages, such as the lack of mutagenesis, low immunogenicity, and the capacity of delivering a large number of therapeutic agents. Hence, for translational applications, non-viral approaches are preferred.

a. Electroporation

Electroporation is a classic method for gene delivery by permeabilizing cell membrane using electric forces. This method is appropriate for both transient and

stable transfection [112]. As compared to commercialized liposomes and synthesized cationic polymers, electroporation can transport miRNAs more efficiently into virtually any cell types, even hard-to-transfect cells like NPCs [110, 113]. As such, the delivery of miR-18a and miR-19a mimics by the means of electroporation dramatically elevated the expression levels of these two miRNAs in NPCs. Particularly, these two miRNAs were overexpressed by 389-fold and 236-fold, respectively, relative to control cells [114]. Likewise, the delivery of miR-18a and miR-19a inhibitors by electroporation into NPCs nearly abolished expression levels of these two miRNAs [114]. However, there remains a significant trade-off between transfection efficiency and cell viability [112]. The resulting damages on cellular membranes due to electroporation often leads to the imbalanced ion gradient across cellular membrane, hence causing non-specific materials transport and ultimately cell death [110]. Therefore, the majority of electroporation protocols were developed based on the balance between acceptable transfection efficiency and cell viability. Under optimal electroporation conditions for many mammalian cell types, 50-75% of viable cells expressing the transgene were obtained [112]. However, several approaches have been introduced recently to enhance the transfection performance of electroporation, for example the use of patterning electrode pairs [115-117] or the addition of gold nanoparticles in electroporation solution [118].

b. Bolus/systemic delivery

Lipid-based transfection reagents

Like electroporation, the use of lipid-based reagents results in transient transfection [119]. The main advantages of this system are its biocompatibility, reproducibility, and ease of large-scale production [120]. As such, many conventional

reagents are available to date, for example Lipofectamine® 2000, Lipofectamine® RNAiMAX, siPORT™, HiPerFect, and siLentFect™. Despite their variation in structure, these reagents share some common features. In particular, they contain positively charged groups, which interact with the negatively charged sugar-phosphate backbone of RNA molecules [107]. This interaction aids the contact between RNA/reagent complex and cell membrane, thereby facilitating subsequent cellular uptake. However, the major shortcoming of lipid-based transfection reagents is their low transfection efficiency as compared to viral transduction and electroporation [119]. In particular, the transfection efficiency of miR-219 and miR-338 mimics in mouse primary OPCs using Lipofectamine® 2000 is only > 50% [47]. Meanwhile, the competence of Lipofectamine® in primary astrocytes was slightly higher as 80% of transfection efficiency was reported [100]. In addition, the silencing efficiency of a transfection reagent also varied depending on the target genes. Specifically, mRNA levels of Sox6 and Hes5 were down-regulated by 25% and 90%, respectively, by miR-219 mimic transfection using Lipofectamine® 2000 [47]. Apart from the poor transfection efficiency, another drawback of lipid-based transfection reagents is their potential cytotoxicity by lipofection. This cytotoxicity, accompanied by gene changes, could in turn hinder the desired outcome of gene-based therapy [121].

Cationic polymer

Cationic polymer is another class of non-viral vectors. In general, the positively charged amine groups in cationic polymers interact with the negatively charged phosphate groups of RNA molecules [111, 122]. Like lipid-based reagents, transfection efficiency and toxicity of cationic polymer are major issues that must be

considered for translational applications of cationic polymers [122, 123]. Currently, polyethylenimine (PEI) is the most promising cationic polymer due to its high transfection efficiency [122]. Specifically, it effectively protects oligonucleotides from lysosomal degradation and delivers them into the cytoplasm [111, 124]. In particular, the use of disulfide-crosslinked PEI (SSPEI), which was conjugated with rabies virus glycoprotein (RVG), to deliver miR-124a into Neuro2a neuroblastoma cells *in vitro* resulted in a 90% decrease in luciferase reporter activity [125]. Moreover, RVG-SSPEI showed significantly low cytotoxicity compared to PEI alone [125]. Besides PEI, chitosan is also extensively studied for gene delivery since it is biocompatible and able to effectively bind and compact oligonucleotides [126]. However, its transfection efficiency is generally limited in many cell types [111]. Thus, future works are required to modify these cationic polymers in order to overcome their drawbacks for clinical applications.

c. Scaffold-mediated delivery

Compared to the aforementioned methods, scaffold-mediated delivery is more advantageous for RNA interference in neural tissue engineering. The incorporation of RNAs within a scaffold protects these labile molecules from biodegradation. In addition, scaffold-mediated delivery allows the localized and sustained availability of gene silencing molecules, thus enhancing cellular uptake and potentially minimizing systemic off-target side effects [127, 128].

Currently, scaffold-mediated delivery of small non-coding RNAs remains at a nascent stage, with most reports being restricted to siRNA delivery. However, being chemically similar in nature (double stranded RNA molecules), the scaffold

incorporation and delivery mechanisms are generally similar and applicable between siRNAs and miRNA mimics. In this regard, the encapsulation of siRNAs within poly (ϵ -caprolactone-*co*-ethyl ethylene phosphate) (PCLEEP) nanofibers significantly enhanced the *in vitro* cellular uptake of gene silencing molecules into human dermal fibroblasts (HDFs) as compared to bolus delivery [129]. In addition, a sustained release of these encapsulated siRNAs for up to one month *in vitro* was reported, which prolonged *in vitro* silencing of collagen type I (COL1A1) by at least 2-3 times as compared to conventional bolus delivery [129].

Recently, collagen-nanohydroxyapatite scaffold has been introduced as an efficient reservoir for miRNA delivery to human MSCs *in vitro*. Specifically, by using this scaffold system to provide anti-GAPDH miRNA mimics, a 20% reduction in GAPDH expression was achieved over seven days of culture [130]. Meanwhile, miR-29B mimics were delivered by collagen scaffold *in vivo* to modulate extracellular matrix remodeling after injury. In particular, a 15% reduction in wound contraction was observed in rats treated with mir-29B-functionalized collagen scaffolds as compared to the untreated group [131].

Besides acting as a reservoir for sustained delivery of gene silencing molecules, topographical features of scaffolds may also serve as additional physical signals to modulate cellular behavior and gene uptake and silencing efficiencies. Using MSC as a model stem cell type, cells cultured on aligned fiber scaffolds demonstrated increased gene knockdown efficiencies when RE-1 silencing transcription factor (REST) and green fluorescent protein (GFP) were targeted (1.6 and 1.5-folds increase respectively, as compared to random fibers [132]. On the other

hand, using HDF as a model adult somatic cell type, gene-silencing efficiency was found to be dependent on fiber diameter. Specifically, smaller nanofiber diameters promoted the silencing of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and Collagen-I by 3.8 and 4.4-folds respectively as compared to scaffolds with an average diameter of 1.3 μm [132].

Apart from affecting gene-silencing extents, scaffold architecture also modulates cell phenotype. For example, fiber diameter affects NSPC differentiation [133] and oligodendrocyte myelination [134]. In terms of PNS myelination, fiber scaffolds, especially aligned fibers, promoted human Schwann cell maturation [135]. Moreover, three-dimensional nature of nanofibers affected differentiation outcomes of both mouse NPCs and rat OPCs. Specifically, the silencing of REST together with the use of nanofibers significantly enhanced neuronal commitment of mouse NPCs by 32% as compared to 2D film [136]. Meanwhile, higher proportion of OPCs was differentiated on nanofiber than on 2D conventional culture either with or without the presence of miR-219/miR-338 (3-fold versus 2-fold increase in RIP⁺ cells) [137]. Taken together, by providing mechanical support and developmental guidance for axonal regrowth, neotissue deposition [110, 138], sustained gene silencing and topographical features to direct cell fate, scaffold-mediated gene silencing approach is a promising strategy for RNA interference in neural tissue engineering.

CONCLUSIONS

The regeneration capacity of nerve tissues after injuries, especially within the CNS, is limited. This poor regeneration is mainly contributed by the hostile microenvironment at the injured sites. Thus, an important mission of neural tissue

engineering is to manipulate the environment, making it more permissive for tissue repair. Along this line, RNA interference using miRNAs appeared as a promising method. This review highlighted important miRNA candidates, which participate in cellular activities within the adult nervous system, and their potential therapeutic applications.

In addition, miRNA treatment for nerve regeneration requires effective delivery methods for transporting miRNAs safely into the cytoplasm of the targeted cells. Although various delivery methods have been designed to date, their translational applications remain limited. In this review, key advantages and drawbacks of some major delivery methods, which have been employed in neural tissue engineering so far, were discussed. Yet, future works to improve transfection and silencing efficiencies are required for more dramatic successes in nerve regeneration.

Acknowledgements:

This work was supported by the National Medical Research Council (NMRC) CBRG Grant (NMRC/CBRG/0002/2012).

Table 1: Frequently used markers for the evaluation of cell fate commitment

Cell type	Developmental stage	Markers	Reference
Neuron	Immature	Tuj-1, DCX	[139-141]
	Mature	NSE, NeuN, MAP-2	[141, 142]
Astrocyte		GFAP	[143, 144]
OPC		NG2, PDGFR α , Olig2	[44, 48, 145-147]
OL	Immature	RIP, CNP, MBP	[48, 148]
	Mature	RIP, MOG	[38, 48, 148]
M1		TNF- α , IL-6, IL-12, iNOS	[87, 93, 94]
M2		TGF- β 1, Arg-1, FIZZ1	[87, 94]

Table 2: miRNA involvement in neural cell fate commitment

Cell type	Function	miRNA/anti-miR	Silencing target	<i>In vitro</i> / <i>In vivo</i>	Form of miRNA/anti-miR	Delivery method	Overcome of miRNA/anti-miR treatment	Reference
NSPCs	Enhancing proliferation	miR-137	EZH2	<i>In vitro</i>	Lentivirus	Infection	90% increase in proliferation	[20]
				<i>In vivo</i>	Retrovirus	Injection	58% increase in proliferation	
		miR-184	Numbl	<i>In vitro</i>	miRNA mimic	FuGENE® HD	50% increase in cell proliferation	[22]
					Lentivirus	Infection	22% increase in cell proliferation	
				<i>In vivo</i>	Retrovirus	Injection	5.0-fold increase in cell proliferation	
		miR-195	MBD1	<i>In vitro</i>	miRNA mimic	FuGENE® HD	18% increase in cell proliferation	[23]
				<i>In vivo</i>	Retrovirus	Injection	<u>No</u> significant effect on proliferation	
		miR-25	TGF- β and insulin/IGF-FoxO related pathways	<i>In vitro</i>	Retrovirus	Infection	17% increase in cell proliferation	[30]
	Enhancing neuronal commitment	miR-124a	Notch signaling pathway	<i>In vitro</i>	miRNA mimic	Nanoparticles	4.5-fold increase in the expression of DCX	[27]
		miR-124	SCP1	<i>In vivo</i>	miRNA	Electroporation	An increase in the expression of NF	[26]

					mimic			
		miR-200c/141 cluster	Sox2 and E2F3	<i>In vitro</i>	Plasmid vector	<u>Lipofectamine® LTX with Plus™ reagent</u>	18% more Tuj1+ neurons	[29]
		miR-106b~25 cluster	TGF-β and insulin/IGF-FoxO related pathways	<i>In vitro</i>	Retrovirus	Infection	2.6-fold more Tuj1+ neurons	[30]
	Enhancing neuronal & astrocytic commitment	miR-9	TLX	<i>In vitro</i>	miRNA mimic	<u>siLentFect™</u>	32% more Tuj1+ neurons, 78% more GFAP+ astrocytes	[33]
		let-7d	miR-9 and TLX cascade	<i>In vitro</i>	miRNA mimic	<u>TransFectin™</u>	61% more Tuj1+ neurons, 2.4-fold more GFAP+ astrocytes	[35]
		let-7b	TLX	<i>In vitro</i>	miRNA mimic	<u>TransFectin™</u>	2.0-fold more Tuj1+ neurons, 2.4-fold more GFAP+ astrocytes	[11]
		Anti-miR-137	miR-137	<i>In vitro</i>	Single-stranded RNA	Electroporation	2.1-fold more Tuj1+ neurons, 2.4-fold more GFAP+ astrocytes	[20]
		Anti-miR-184	miR-184	<i>In vitro</i>	Single-stranded RNA	<u>FuGENE® HD</u>	2.1-fold more Tuj1+ neurons, 52% more GFAP+ astrocytes	[22]
		Anti-miR-195	miR-195	<i>In vitro</i>	Single-stranded RNA	<u>FuGENE® HD</u>	17% more Tuj1+ neurons, 26% more GFAP+ astrocytes	[23]

	Enhancing neuronal commitment & maturation	miR-34a	SIRT1	<i>In vitro</i>	pre-miR-34a	<u>Lipofectamine® 2000</u>	93% more postmitotic NeuN+ neurons, 22% increase in the length of Tuj1+ cell neurites	[37]
		miR-7	Many targets	<i>In vitro</i>	Lentivirus	Infection	2.6-fold increase in the expression of synapsin	[13]
	Enhancing oligodendrocyte commitment	miR-7a	Pax6, NeuroD4	<i>In vitro</i>	miRNA mimic	<u>Lipofectamine® 2000</u>	2.0-fold more NG2+ OPCs, 2.0-fold more PDGFRα+ OPCs, 2.0-fold more Olig2+ OPCs	[44]
				<i>In vivo</i>	Plasmid vector	Electroporation	8.2-fold more PDGFRα+ OPCs, 13.5-fold more Olig2+ OPCs	
OPCs	Enhancing proliferation	miR-7a	Myelin genes	<i>In vitro</i>	miRNA mimic	<u>Lipofectamine® 2000</u>	4.9-fold increase in cell proliferation	[44]
	Enhancing oligodendrocyte commitment	miR-219	Sox6, Hes5, ZFP238	<i>In vitro</i>	miRNA mimic	<u>Lipofectamine® 2000</u>	Under proliferative condition: 2.1-fold more RIP+ OLs, 3.0-fold more MBP+ OLs	[47]
				<i>In vivo</i>	Plasmid vector	Electroporation	47% more Olig2+ OPCs, 25% more PDGFRα+ OPCs	
		miR-338		<i>In vitro</i>	miRNA mimic	<u>Lipofectamine® 2000</u>	Under proliferative condition: 3.4-fold more RIP+ OLs, 4.6-fold more MBP+ OLs	[47]
				<i>In vivo</i>	Plasmid vector	Electroporation	47% more Olig2+ OPCs, 56% more PDGFRα+ OPCs	

		miR-23a	LMNB1	<i>In vitro</i>	Plasmid vector	<u>FuGENE® HD</u>	2.9-fold more CNP+ OLs	[49]
			PTEN, 2700046G09 Rik	<i>In vitro</i>	Transgene	Transgene	5.1-fold more MBP+ OLs, 3.8-fold more MAG+ OLs	[50]
		miR-138		<i>In vitro</i>	miRNA mimic	<u>Lonza/Amara</u> <u>Nucleofector™</u>	- Under proliferative condition: 2.4-fold more CNP+ OLs, 1.9-fold more MBP+ OLs - Under differentiation condition: 31% more CNP+ OLs, 54% more MBP+ OLs, 54% fewer MOG+ OLs	[48]
MSCs	Promoting neuronal differentiation	miR-9	Zfp521	<i>In vitro</i>	Lentivirus	Infection	4.4% more MAP-2+ neurons, 8.6% more NSE+ neurons	[58]
		miR-124		<i>In vitro</i>	Lentivirus	Infection	- Increase in the expression of Tuj1, MAP-2, and synaptophysin - miR-124-transfected BMSCs developed neuron-like morphology	[59]
		Anti-miR-128	miR-128	<i>In vitro</i>	RNA	<u>Lipofectamine®</u> <u>2000</u>	72% increased Tuj1 expression, 2.6-fold increased MAP-2 expression, 67% increased NF-M expression, 2.3-fold increased GFAP expression, 78% increased Nestin expression, and 50% increased NSE expression	[60]

Fibroblasts	Promoting neuronal differentiation	miR-9/9*		<i>In vitro</i>	Lentivirus	Infection	- Alone: no MAP+ cells - With miR-124: less than 5% MAP2+ cells - With miR-124 and NEUROD2: 50% MAP2+ cells	[62]
		miR-124		<i>In vitro</i>	Lentivirus	Infection	- Alone: no MAP+ cells - With miR-9/9*: less than 5% MAP2+ cells - With miR-9/9* and NEUROD2: 50% MAP2+ cells	[62]
				<i>In vitro</i>	Lentivirus	Infection	- Alone: few Tuj1+ cells with immature neuronal morphology - With BRN2 and MYT1L: 55% MAP2+ cells, 46% NeuN+ cells	[63]
Neurons	Aiding in axonal regeneration & dendrite formation	miR-124	Targets in cellular cytoskeleton	<i>In vitro</i>	Plasmid vector	<u>FuGENE® 6</u>	Increase in the number of protruded primary neurites	[68]
		Anti-miR-137	miR-137	<i>In vitro</i>	RNA	<u>Lipofectamine® 2000</u>	35% increase in total dendritic length	[69]
		miR-125b	NR2A	<i>In vitro</i>	Plasmid vector	<u>Lipofectamine® 2000</u>	22% increase in dendritic protrusion length, 11% decrease in dendritic protrusion width, 25% drop in mean mEPSC amplitude	[70]

		miR-132	p250GAP	<i>In vitro</i>	Plasmid vector	<u>Lipofectamine® 2000</u>	9% increase in dendritic protrusion width, 15% decrease in dendritic protrusion density, 29% increase in mean mEPSC amplitude, 84% increase in mean mEPSC frequency	[70]
OLs	Aiding in myelin formation and maintenance	miR-23a	PTEN, 2700046G09 Rik	<i>In vivo</i>	Transgene	Transgene	50% increase in the expression of MBP, 80% increase in the expression of CNP, 35% increase in the expression of MAG, 27% reduction in g-ratio	[50]
Schwann cells	Inhibiting proliferation	miR-34a	Notch1, Ccnd1	<i>In vitro</i>	Lentivirus	Infection	50% reduction in cell proliferation	[71]
	Enhancing migration	Anti-miR-9	miR-9	<i>In vitro</i>	RNA	<u>Lipofectamine® RNAiMAX</u>	- Transwell assay: 78% increase in cell migration - Wound healing assay: 50% increase in cell migration	[73]
	Enhancing proliferation & migration	miR-221	LASS2	<i>In vitro</i>	miRNA mimic	<u>Lipofectamine® RNAiMAX</u>	2.4-fold increase in cell proliferation, 4.3-fold increase in cell migration	[74]
		miR-222	LASS2	<i>In vitro</i>	miRNA mimic	<u>Lipofectamine® RNAiMAX</u>	2.7-fold increase in cell proliferation, 2.6-fold increase in cell migration	[74]
		Anti-miR-182	miR-182	<i>In vitro</i>	RNA	<u>Lipofectamine® RNAiMAX</u>	89% increase in cell proliferation, 2.3-fold increase in cell migration	[75]

	Inhibiting redifferentiation	miR-140	Egr2	<i>In vitro</i>	Lentivirus	Infection	76% decrease in the number of myelinated segments	[71]
Astrocytes	Inhibiting astrocytosis & astrogliosis	miR-145	GFAP, c-myc	<i>In vitro</i>	Lentivirus	Infection	49% reduction in cell proliferation, 49% reduction in cell size, 42% decrease in the number of cell processes	[81]
				<i>In vivo</i>	Lentivirus	Infection	62% reduction in cell size, 66% decrease in the area of GFAP signal	
	Inhibiting astrogliosis	miR-21		<i>In vivo</i>	Transgene	Transgene	47% decrease in GFAP expression, 33% decrease in vimentin expression	[85]
	Attenuating inflammation	miR-146a	IL-1 β mediated release	<i>In vitro</i>	miRNA mimic	<u>Lipofectamine®</u>	97% reduction in IL-6 expression, 83% reduction in IL-8 expression, 55% reduction in G-CSF expression, 67% reduction in INF- γ expression, 80% reduction in TNF- α expression	[100]
Macrophages	Promoting M2 activation	miR-92a	MKK4	<i>In vitro</i>	miRNA mimic	<u>INTERFERin®</u>	43% reduction in TNF- α expression, 44% reduction in IL-6 expression	[97]
		miR-124	C/EBP- α	<i>In vitro</i>	miRNA mimic	<u>Lipofectamine®</u> 2000	98% decrease in TNF- α expression, 72% decrease in iNOS expression, 3.2-fold increase in TGF- β 1 expression, 2.7-fold increase in Arg-1 expression, 2.8-fold increase in FIZZ1 expression	[98]

	Suppressing M1 phenotype	let-7c	C/EBP- δ	<i>In vitro</i>	miRNA mimic	HiPerFect	<ul style="list-style-type: none"> - Alone: 55% decrease in CCR7 expression, 50% increase in FR-β expression - With LPS: 70% decrease in IL-12 expression, 36% decrease in iNOS expression - With IL-4: 2.7-fold increase in Arg1 expression, 3.8-fold increase in FIZZ1 expression 	[93]
Microglia	Attenuating microglial activation	miR-124	C/EBP- α	<i>In vivo</i>	miRNA mimic	<u>Lipofectamine®</u> 2000	27% more resting microglia, 73% less peripheral macrophages and activated microglia	[98]
	Inhibiting M1 activation	Anti-miR-155	miR-155	<i>In vitro</i>	RNA	Lipoplexes	With LPS: 55% decrease in TNF- α expression, 75% decrease in IL-6 expression, 25% decrease in iNOS expression	[99]

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