COMMENTARY

Traceable microRNA-124 loaded nanoparticles as a new promising therapeutic tool for Parkinson's disease

Cláudia Saraiva^a, Lino Ferreira^{b,c,d}, and Liliana Bernardino^a

^aHealth Sciences Research Center, Faculty of Health Sciences, University of Beira Interior, Covilhã, Portugal; ^bCNC-Center for Neuroscience and Cell Biology, Coimbra, Portugal; ^cBiocant - Center of Innovation in Biotechnology, Cantanhede, Portugal; ^dInstitute for Interdisciplinary Research, University of Coimbra (IIIUC), Coimbra, Portugal

ABSTRACT

Parkinson's disease (PD), a neurodegenerative disorder characterized by the selective degeneration of the nigrostriatal dopaminergic pathway, is a major socio-economic burden in modern society. While there is presently no cure for PD, enhancing the number of neural stem cells (NSCs) and/or stimulating their differentiation into new neurons are promising therapeutic strategies. Many proneurogenic factors have been implicated in controlling NSCs activity, including the microRNA (miR)-124. However, current strategies described for the intracellular delivery of miR involve mostly unspecific or inefficient platforms. In Saraiva et al. we developed miR-124 loaded nanoparticles (NPs) able to efficiently deliver miR-124 into neural stem/progenitor cells and boost neuronal differentiation and maturation *in vitro*. *In vivo*, the intracerebroventricular injection of miR-124 NPs increased the number of new neurons in the olfactory bulb of healthy and 6-hydroxydopamine (6-OHDA) lesioned mice, a model for PD. Importantly, miR-124 NPs enhanced the migration of new neurons into the 6-OHDA lesioned striatum, culminating in motor function improvement. Given the recent advent of clinical trials for miR-based therapies and the theranostic applications of our NPs, we expect to support the clinical translation of our delivery platform in the context of PD and other neurodegenerative diseases which may benefit from enhancing miR levels.

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Parkinson's disease (PD), the second most common neurodegenerative disorder, is characterized by the progressive degeneration of the nigrostriatal dopaminergic pathway, resulting in motor and non-motor symptoms.¹ Current treatment such as L-DOPA or deep brain stimulation only benefits a small number of patients and may cause side effects. Therefore, the development of new therapeutic strategies is urgently needed. Recently, neural stem cells (NSCs) have been widely recognized as capable of replacing dead or damaged neurons in several neurodegenerative diseases, including PD.^{2,3} One of the stem cell-based therapies being tested for PD focuses on the transplantation of exogenous stem cells, or stem cell-based progenies at various stages of maturation (e.g. neuroblasts), to replace lost dopaminergic (DA) neurons.^{4,5} Yet, this approach revealed several limitations including low number of cells available for therapy, low survival and integration, immune rejection, graft-induced dyskinesias, and ethical issues. The recruitment of endogenous NSCs sources may overcome these issues.² The

subventricular zone (SVZ), located between the lateral ventricles and the striatum, is the largest neurogenic niche in the adult mammalian brain, including humans.⁶ Under physiologic conditions SVZ-derived neuroblasts migrate toward the olfactory bulb (OB). However, neurogenesis rate may fluctuate in response to brain injury or degeneration. For that reason, the development of novel approaches to effectively enhance the regenerative process driven by endogenous SVZ NSCs is a promising research area with high clinical and economic impact. In Saraiva et al. we used a 6-hydroxydopamine (6-OHDA) animal model for PD. Inconsistent data have been reported over the last years showing decrease, maintenance or even increase of neurogenesis in PD models and PD patients (review at van den Berge et al., 2013⁷). The differential activation of dopaminergic receptors in NSCs⁸⁻¹⁰ together with a high diversity of PD models (transgenic or toxin based models, acute or chronic administrations, different dosages and different spatial administration of toxins¹) may explain distinct outcomes.

All these discrepancies have generated a high debate in the scientific community about the limitations of these experimental models, such as the sample size, the controls groups used, the type of analysis performed to name a few. Our mouse model for PD is in accordance with abovementioned reports showing impaired SVZ neurogenesis. In Saraiva et al., we observed a reduction in the number of BrdU⁺ and DCX⁺BrdU⁺ cells in the SVZ (in accordance with Baker et al., 2004 and Sui et al., 2012^{11,12}) whereas the number of DCX⁺ cells was not altered (in agreement with Fricke et al., 2016¹³). More studies are needed to hamper our knowledge regarding the neurogenesis process in PD to improve future therapeutic regenerative strategies. Notably, microRNA (miR)-124 loaded nanoparticles (miR-124 NPs) were able to boost endogenous SVZ neurogenesis, counteracting the functional impairments found in the 6-OHDA animal model of PD (Fig. 1).

MiRs are small non-coding RNAs able to regulate hundreds of genes at the post-transcriptional level.¹⁴ The miR-124 is the most abundant and one of the best studied miRs in the brain. Indeed, miR-124 has

been described as a pro-neurogenic miR since its overexpression results in neuronal differentiation of progenitor cells,^{15,16} HeLa cells¹⁴ and also (in combination with others factors) of human neonatal foreskin fibroblasts.^{17,18} MiR-124 pushes the balance toward a neuronal profile by targeting mainly non-neuronal related genes, including Sox9 (involved in the glial and undifferentiated state of stem cells), Jagged-1 (Notch-1 ligand), Dlx2 (marker of undifferentiated stem/progenitor cells), PTBP1 (Polypyrimidine-tract binding protein 1; global repressor of alternative pre-mRNA splicing in non-neuronal cells) and SCP-1 (Small C-terminal domain phosphatase-1; an anti-neural co-factor of the RE1-silencing transcription factor: REST), among others.^{14,16,19} Moreover, about 25% of the miR-124 validated targets are downregulated in PD,²⁰ while miR-124 seems to be down-regulated in the *substantia nigra* of a PD mouse model.^{21,22} In line with these evidences, in Saraiva et al. we delivered miR-124 in order to boost SVZ NSC neurogenesis *in vitro* and ultimately promote brain repair in a PD mouse model *in vivo*.²³ The

Figure 1. MiR-124 loaded nanoparticles (miR-124 NPs) boost neuronal differentiation of neural stem/progenitors cells (NSPCs) from the subventricular zone (SVZ) *in vitro* and *in vivo*, ultimately leading to motor symptoms amelioration in a Parkinson disease (PD) mice model. Left panel (*In vitro*): SVZ NSPCs cultures treated with miR-124 NPs showed a shift from glial (Ki67⁺GFAP⁺) to a neuronal fate (Ki67⁺DCX⁺) culminating in a robust enhancement in the number of mature neurons (NeuN⁺) and a slight decrease of astrocytes (GFAP⁺). This neurogenic shift seems to be related with the downregulation of two miR-124 targets Sox9 and Jagged1. Right panel (*In vivo*): MiR-124 NPs were injected in the lateral ventricles of healthy and 6-OHDA-challenged mice followed by intraperitoneal administration of 5-bromo-2'-deoxyuridine (BrdU, a thymine analog that intercalates in the DNA of dividing cells). The number of proliferating neuroblasts (DCX⁺BrdU⁺) in the olfactory bulb, the end-point of the SVZ-derived neuroblasts, was increased both in healthy and in 6-OHDA mice when compared with the saline counterparts. Moreover, levels of new neurons (NeuN⁺BrdU⁺) found in the 6-OHDA-lesioned striatum were significantly increased by miR-124 NPs. Importantly, this enhancement of neurogenesis is accompanied by an amelioration of PD motor symptoms. In particular, 6-OHDA mice treated with miR-124 NPs showed a reduction in the net contralateral rotations upon subcutaneous administration of apomorphine.

delivery of miRs into the cells can be done by chemical modification, liposomes/microvesicles, virus and electroporation or eventually without the delivering system by using naked nucleic acids. Nevertheless, these strategies are not efficient, cannot be controlled, can raise safety issues and do not allow an accurate non-invasive imaging of the reprogramming factors' spatial release within the cells. In Saraiva et al. we used polymeric NPs to deliver miR-124 due to their ability to protect and increase its stability, as well as for their capability to deliver intracellularly higher concentrations of miR-124. The use of NPs to induce neuronal differentiation of endogenous adult NSCs is relatively recent and we were the first to establish it.^{24,25} However, previous formulations could not be tracked in a temporal and spatial frame in living animals and were not used in a context of brain pathology. In Saraiva et al. we developed polymeric NPs that can be tracked non-invasively *in vivo*, by magnetic resonance imaging (MRI), due to the presence of perfluoro-1,5-crown ether (PFCE). These NPs have also in its composition poly(lactic acid-co-glycolic acid) (PLGA) and a cationic peptide, protamine sulfate, that allows an efficient complexation of negatively charged molecules, namely miRs.^{23,26} It is also important to notice that all NPs components were already approved by the Food and Drug Administration (FDA) in other applications. Previously, we showed that this NP formulation was effective in promoting the intracellular delivery of miR-132 into endothelial cells, which subsequently exerted pro-survival and pro-angiogenic effects in an ischemic limb *in vivo* model.²⁶ In Saraiva et al. we showed that miR-124 NPs promote a robust enrichment in the number of mature neurons in SVZ NSCs cultures *in vitro*, while it decreases the number of astrocytic cells. MiR-loaded NPs were efficiently internalized by cells able to differentiate in response to miR-124 overexpression such as stem/progenitor and immature cells. MiR-124 NPs shift the profile of SVZ NSCs cultures from an undifferentiated and/or glial fate into a neuronal commitment fate by targeting Jagged1 and Sox9^{19,27,28} (Fig. 1). It also enhanced axonogenesis a crucial process for maturation and integration of newborn neurons. Accordingly, previous evidences showed that miR-124 overexpression not only promotes a neuronal commitment of progenitor cells^{19,29} but also controls the choice among neuronal or astrocytic differentiation.³⁰ In addition, miR-124 enhance axonogenesis by regulating cytoskeleton

proteins levels.^{15,31} *In vivo*, a single intracerebroventricular administration of miR-124 resulted in increased levels of migrating neuroblasts reaching the OB (the endpoint of SVZ-derived neurons) of healthy and 6-OHDA-challenged mice, where they fully differentiated into mature neurons (Fig. 1). MiR-124-induced neurogenesis at the SVZ-OB axis was most probably due from the division of stem/progenitor cells into neuronal progenitors (DCX⁺/BrdU⁺), but also from neuronal commitment of NSCs that have not undergone mitosis, or from late dividing cells generated after the BrdU pulse (total DCX⁺ cells). Based on our *in vitro* and *in vivo* data, we may also speculate that miR-124 NPs triggered a shift from astrocytic-like cells to neurons without causing depletion in the NSCs pool, allowing the maintenance of the neurogenic niche and consequently, the continuous generation of new neurons. Nevertheless, others have shown that in physiological conditions miR-124 overexpression by viral vectors promotes neurogenesis in SVZ that does not interfere with migration nor OB integration, while it leads to a decrease in the levels of dividing precursors.^{19,29} Therefore, additional studies are needed to address NSCs dynamics upon miR-124 treatment. Recent studies also showed that miR-124 upregulation culminate in dopaminergic neurons protection by regulating apoptosis and autophagy processes.^{22,32} Notably, in 6-OHDA-challenged mice, miR-124 NPs administration substantially enhanced migration and maturation of SVZ-derived neurons into the lesioned striatum leading to the amelioration of motor deficits (Fig. 1). Nevertheless, it is essential to scrutinize in detail the mechanisms and/or cells involved in miR-124-induced motor recovery. Accordingly, besides neuronal differentiation, miR-124 seems to regulate cell death and inflammatory responses, resulting in neuroprotection in PD,^{21,22,32} alleviation of cell death in Alzheimer's disease³³ or reduction of infarct volume in stroke,^{34,35} to name a few. Therefore, broader applications of our formulation in other brain pathologies are also anticipated. For example, miR-124 levels were found decreased in the SVZ NSCs and ischemic core of rodent models for stroke as well as in the plasma of ischemic stroke patients.³⁵⁻³⁷ In rodent models for stroke the overexpression of miR-124 decreases infarct volume, reduces microglial activation and improves neurogenesis via a Usp14-dependent REST degradation.^{34,35} Moreover, miR-124 upregulation also tend to shift microglia/

macrophages activity from a pro-inflammatory (type 1) to an anti-inflammatory state (type 2).³⁸ Indeed, miR-124 fosters anti-inflammatory pathways in microglia by targeting the signal transducer and activator of transcription 3 (STAT3) and tumor necrosis factor- α -converting enzyme (TACE) reducing the release of interleukin (IL)-6 and tumor necrosis factor- α (TNF- α).³⁹ These studies prove that miR-124 can be used as a broad therapeutic molecule in the setting of neurodegenerative disorders, acting as neuroprotector, anti-inflammatory and also enhancer of endogenous brain repair mechanisms.

The combination of miR-124 with others factors can also improve the design of more efficient therapies for neurodegenerative disorders. Upregulation of miR-124 together with miR-9 by lentivirus infection converted low levels of human neonatal foreskin fibroblasts to mature neurons.¹⁷ Recently, it was shown that miR-9 and miR-124 act synergistically to promote neuronal differentiation and dendritic branching by repressing Ras-related protein Rap-2a, suggesting that both miRNAs can co-act to trigger neuronal differentiation. Other reports also suggest a role for miR-9 and miR-124 on the specification and survival of dopaminergic neurons,⁴⁰ suggesting that the combination of both miRs may be beneficial for PD treatment. Stroke patients seem to have decrease levels of miR-124 and miR-9 in the serum within the first 24h, with miR-9 reduction being correlated with larger lesions.³⁷ These reports seem to indicate that the combination of miR-124 with others factors, such as miR-9, can be of great value for the development of novel therapies for brain pathologies such as PD or stroke.

The ability of active molecules to cross the blood-brain barrier (BBB) into the brain parenchyma is a vital aspect for brain repair therapies. In Saraiva et al., miR-124 NPs were delivered into the lateral ventricles by an intracerebral injection. This procedure, although highly invasive, allowed us to study the effect of miR-124 NPs on SVZ neurogenesis bypassing issues such as immunogenicity, biodistribution, pharmacodynamics and failure in crossing the BBB. Nasal administration can be considered as an alternative non-invasive procedure for brain delivery of miR-124 NPs since it bypasses the BBB. Noteworthy, PD patients may present several alterations in nasal cycle, nasal mucosa pH and mucociliary clearance time⁴¹ that may account for reduced biodistribution and bioavailability of NPs. How mucosal alterations impact absorption of

nanomaterials should be further investigated before moving into clinical trials. The systemic delivery of miR-124 NPs by intravenous or intraperitoneal injections should be considered in future studies to circumvent the need of an invasive stereotaxic surgery. The physical-chemical characteristics of NPs make them versatile vehicles that can be easily modified in terms of size, charge, shape and surface ligands to better direct them across the BBB.⁴² NPs may be coated with ligands or antibodies that are recognized by receptors/transporters or epitopes on brain endothelial cells⁴² to facilitate its passage to the brain parenchyma. Decoration of NPs with lactoferrin⁴³ or transferrin antibodies⁴⁴ are examples of formulations that present higher brain accumulation in pathological situations. Blood clearance by reticuloendothelial system uptake may also limits the amount of NPs that reaches the brain. The use of poly(ethylene glycol) (PEG) increases NPs blood circulation time and consequently their brain accumulation.⁴² We anticipate that our polymeric NPs could be decorated with PEG and specific ligands, such as lactoferrin, allowing us to proceed to a less invasive procedure such as the intravenous administration.⁴² Indeed, increased expression of lactoferrin and its receptors in dopaminergic neurons and in BBB endothelial cells in the striatum and *substantia nigra* of PD patients could aid the uptake of our NPs at lesioned regions and reduce peripheral toxicity and the escape to other non-lesioned brain regions.⁴⁵⁻⁴⁸ The combination of the NP system with specific cues to increase the targeting to specific sub-populations of the SVZ niche could also be beneficial to improve the neurogenic response and decrease off-target effects. For example, the decoration of NPs with epidermal growth factor receptor (EGFR)-binding peptides or Notch-1 ligands could trigger an increased uptake by proliferative progenitor cells forcing cell cycle exit and promoting subsequent neuronal differentiation. Yet, pharmacokinetics and biodistribution studies, central and peripheral toxicity analysis, mechanisms of BBB transport studies and functional recovery evaluation *in vivo* are essential before moving into clinical studies.

The use of miR-based therapeutics in clinical trials highlights their beneficial potential.⁴⁹ For example, MRX34, from Mirna Therapeutics, was the first miR mimetic to enter clinical trials. MRX34 is a tumor suppressor and mimics miR-34 actions. Phase 1 clinical trials in patients with carcinoma and in stage 4

metastatic diseases showed convincing results. Although clinical translation of miR-based therapies is occurring, the protection and efficient delivery of these small molecules are still a concern.⁵⁰ Our formulation composed by PLGA, PFCE and protamine sulfate gave proofs of being able to protect miR and efficiently deliver them into SVZ cells.²³ Moreover, miR-124 NPs were able to significantly enhance SVZ NSCs neuronal differentiation through Sox9 and Jagged1 targeting as well as axonogenesis by c-Jun N-terminal kinase (JNK) activation. Moreover, miR-124 NPs were able to promote migration of new neurons into the lesioned striatum of 6-OHDA-challenged mice leading to an amelioration of behavioral symptoms. Although some mechanistic aspects need scrutiny before advance into the clinics we clearly proved that miR-124 NPs represent a novel therapeutic strategy to promote endogenous NSCs repair mechanisms in the setting of a neurodegenerative disorder.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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