Rapid differentiation of hiPSCs into functional oligodendrocytes using an OLIG2 synthetic modified messenger RNA

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Transcription factors (TFs) have been introduced to drive the highly efficient differentiation of human-induced pluripotent stem cells (hiPSCs) into lineage-specific oligodendrocytes (OLs). However, effective strategies currently rely mainly on genome-integrating viruses. Here we show that a synthetic modified messenger RNA (smRNA)-based reprogramming method that leads to the generation of transgene-free OLs has been developed. An smRNA encoding a modified form of OLIG2, in which the serine 147 phosphorylation site is replaced with alanine, OLIG2S147A, is designed to reprogram hiPSCs into OLs. We demonstrate that repeated administration of the smRNA encoding OLIG2 S147A lead to higher and more stable protein expression. Using the single-mutant OLIG2 smRNA morphogen, we establish a 6-day smRNA transfection protocol, and glial induction lead to rapid NG2+ OL progenitor cell (OPC) generation (>70% purity) from hiPSC. The smRNA-induced NG2+ OPCs can mature into functional OLs in vitro and promote remyelination in vivo. Taken together, we present a safe and efficient smRNA-driven strategy for hiPSC differentiation into OLs, which may be utilized for therapeutic OPC/OL transplantation in patients with neurodegenerative disease.
Oligodendrocytes (OLs), which are myelinating cells of the central nervous system (CNS), are extremely promising therapeutic targets for cell replacement-based therapies for myelin loss or dysfunction, such as in patients with multiple sclerosis and white matter ischemic injury. Reprogramming human-induced pluripotent stem cells (hiPSCs) to OLs is a profoundly promising approach for disease modeling, drug development, and OL transplantation based therapeutic approaches. Research advances have made it possible to generate surface antigen O4 (O4)-positive and myelin basic protein (MBP)-positive OLs from hiPSCs within only ~20 days by overexpressing key transcription factors (TFs). However, the traditional approach to achieving TF overexpression generally involves ectopic virus-mediated gene delivery, and viral integration into the genome initially is an obstacle to the therapeutic use of OLs. Therefore, synthetic modified messenger RNAs (smRNAs) were developed in vitro to diminish the innate immune response and improve the delivery of genetic material that can be efficiently translated into specific functional proteins into mammalian cells. In contrast to DNA-based gene manipulation, the introduction of smRNA carries no risk of genomic integration, as smRNAs are translated in the cytoplasm without being delivered into the nucleus, indicating that smRNA delivery is a safer and more efficient method for inducing protein expression.

Instability and a small window for inducing protein expression are the major obstacles when using smRNAs for cellular reprogramming. For mRNAs to be effectively translated in vitro, the 5'-terminal m7GpppG cap and the 3'-terminal poly(A) sequence need to be incorporated into the mRNAs structure for in vitro transcription (IVT). 5-Methyl-CTP, pseudo-Ψ-UTP, and other modified nucleotides have also been incorporated into mRNA to reduce immunogenicity and increase stability. An smRNA was used to directly reprogram the fate of human somatic cells into hiPSCs. Warren et al. employed smRNAs to drive the expression of “reprogramming” factors and successfully developed a method for hiPSC generation. In particular, this method of inducing protein expression mediated by smRNAs has the potential to become a very useful technology for cell-based therapies and regenerative medicine. Moreover, smRNAs have been used to direct the fate of reprogrammed hiPSCs into tissue-specific cell types; however, smRNA-driven differentiation hiPSCs into OLs has been largely unreported. We and other researchers have sought to develop protocols to achieve uniform and reproducible cultures of hiPSC-derived OL progenitor cells (OPCs). We identified factors that facilitate the differentiation of many OPCs derived from hiPSCs, optimized methods, defined conditions, and tested the survival and differentiation of the cells in animal models. Transplantation of iPSC-derived OPCs that have been better “instructed” to follow the OL lineage may facilitate the recovery of patients with CNS diseases. Regulating the expression of the TF OLIG2 affects a major regulatory transcription pathway in OL genesis. Overexpression of OLIG2 in primary neural stem cells (NSCs) has been reported to facilitate myelinating OL generation and contribute to remyelination of the corpus callosum in mice with experimental allergic encephalomyelitis (EAE). Although previous studies have shown that the expression of oligodendroglial lineage marker genes, such as nerve/glial antigen 2 (NG2, also known as CSPG4) and platelet-derived growth factor receptor alpha (PDGFRα, also known as CD140a), was not induced following overexpression of the TF OLIG2 alone in neural progenitor cells (NPCs), our published data have shown that GANT61, a small Shh inhibitor, can abolish the development of motor neurons (MNs) and improve OL specification by regulating the posttranslational phosphorylation of the OLIG2 protein. Therefore, it seems that the significance of OLIG2 overexpression in OL specification should be further emphasized. Indeed, post-translational modifications (PTM) of the OLIG2 protein has been shown to influence cell fate transitions. Li et al. reported that a specific serine residue (serine 147) of OLIG2 is essential for MN-OL switching. Replacing serine 147 with an alanine residue (S147A) was found to abolish MN production without preventing OL formation. We expect that we will establish better protocols for inducing hiPSC differentiation into OLs through the forced expression of an smRNA coding mutant OLIG2 (OLIG2S147A) and that this OLIG2 smRNA may be used to develop more efficient protocols for inducing mRNA-driven differentiation of hiPSCs into OLs.

In this study, we established a novel approach for inducing efficient and rapid hiPSC differentiation into OL lineage-specific cells through repeated administration of an smRNA and found that phosphosite modification of OLIG2 effectively promoted the efficiency of this smRNA-driven hiPSC differentiation process. This novel single virus-free smRNA-driven method will broaden the application of OL replacement therapy in various diseases involving myelin injury.

Results

An smRNA encoding OLIG2 with a phosphorylation site modification drives highly efficient oligodendroglial lineage cell differentiation of hiPSC-derived NPCs. We delivered smRNA to induce the expression of a mutant OLIG2 protein that recapitulates the induction of oligodendroglial TFs during OL development. Two vectors carrying mRNAs encoding wild-type OLIG2 and mutant OLIG2 with a specific serine-to-alanine modification were synthesized for T7 promoter-driven IVT (Figs. 1a and S1). Before testing these OLIG2 smRNAs, we first determined the efficiency of smRNA delivery into NPCs derived from hiPSCs. Using a cationic liposome-based delivery system, we introduced eGFP smRNA into NPCs as an in vitro tracer. As shown in Fig. 1b, transfection of 300 ng of eGFP smRNA along into NPCs resulted in a transfection efficiency greater than 95%. Moreover, immunofluorescence staining for Flag revealed Flag-tagged OLIG2WT and Flag-tagged OLIG2S147A expression in NPCs at 24 h posttransfection (Fig. 1c). Based on these results, these smRNAs were successfully delivered into NPCs and translated into proteins. Next, smRNA-induced cytotoxicity, which would decrease the efficiency of cellular reprogramming, was determined using the CCK-8 assay. No significant cytotoxicity was observed in smRNA-transfected cells compared to untransfected cells (Fig. S2). These results supported our strategy of utilizing mutant OLIG2 smRNA to drive hiPSC-derived NPC differentiation into OLs.

Blocking OLIG2 phosphorylation at serine 147 has been shown to abolish MN production without preventing OL production and to trigger the switch from an MN fate to an OL fate. Using our optimized strategy for smRNA synthesis and transfection, we used smRNAs encoding wild-type and phosphorylation site-modified OLIG2 to determine which protein induces greater protein expression and OL specification. The smRNA encoding OLIG2 with the S147A phosphorylation site modification resulted in 1.2-fold higher protein expression than the smRNA encoding wild-type OLIG2 at 16 h posttransfection, and this difference peaked 24 h after smRNA transfection (Fig. 1d). We then asked whether OLIG2WT smRNA or OLIG2S147A smRNA more strongly induced the expression of validated downstream targets of OLIG2, such as SOX10, which is required for OL differentiation and maturation. As shown in Fig. 1e, cells underwent three or six daily transfections of smRNAs to determine the most suitable and efficient differentiation strategy. NPCs derived from the three different hiPSC lines were plated in 12-well plates precoated with...
the reduced growth factor Matrigel/laminin, and SOX10 mRNA expression was measured. The qPCR results suggested that six daily transfections of OLIG2S147A induced 9.8-fold higher SOX10 mRNA expression than three daily transfections in hiPSC-derived NPCs. Similarly, we found that OLIG2S147A smRNA drove higher expression of a OL lineage marker gene (SOX10) than OLIG2WT smRNA in hiPSC2- and hiPSC3-derived NPCs (Fig. 1f). Type I interferon (IFN) is considered a marker for antiviral defense responses mediated by interferon-γ- and NF-κB-dependent pathways in mammalian cells. The expression of the signaling
marker IFN was transient, as evidenced by significant down-regulation of IFN 48 h after six daily smRNA transfections (Fig. S3). Thus, the smRNA encoding OLIG2 with a phosphorylation site modification was a better driver of OL differentiation from hiPSC-derived NPCs than its respective wild-type counterpart.

**smRNA-induced hiPSC differentiation into OPCs.** Since six daily smRNA transfections resulted in higher OL differentiation potential, we next established 6-day smRNA transfection schemes for mRNA-induced differentiation of hiPSCs into OLs (Fig. 2a). First, NANOG- and SSEA4-positive cells of the hiPSC1 line (Fig. 2b) were differentiated into NPCs by culture in NIM for 7 days, and the cultured cells expressed PAX6 and NESTIN (Fig. 2c). One day after smRNA transfection, the culture medium was replaced with GIM containing different morphogens (T3, SAG and PDGFR-AA). After 6 days, the GIM was changed to DM lacking PDGF-AA and SAG to promote OL generation and maturation. Six days after smRNA treatment and GIM transfection, smRNA-induced NG2+ OPCs were identified. In contrast, NPCs transfected with or without eGFP smRNA failed to attach or survive in culture with GIM; however, NPCs transfected with OLIG2S147A smRNA promoted NG2+/SOX10+/PDGFRA+ OPC generation to a greater degree than NPCs transfected with OLIG2WT smRNA (Fig. 2d, e). To verify the reproducibility of this phenomenon in a variety of hiPSC lines, the two other hiPSC lines also underwent smRNA-mediated OL differentiation. Similarly, there were more NG2-, SOX10-, and PDGFRA-positive cells among hiPSC2- or hiPSC3-derived NPCs transfected with OLIG2S147A smRNA than those transfected with OLIG2WT smRNA (Fig. 2e). Additionally, cell death was not increased in hiPSC-derived NPCs transfected with OLIG2WT or OLIG2S147A smRNA compared with eGFP smRNA-transfected and untransfected cells after 6 daily transfections (Fig. S4). We analyzed the cultured cells after 6 daily transfections using flow cytometry to further characterize OPC generation induced by OLIG2S147A smRNA. The OLIG2S147A smRNA-treated cells were largely NG2+ cells (74.94 ± 2.68%), unlike the OLIG2WT smRNA-transfected cells (27.72 ± 1.25%, Fig. 2f, g). PDGFRA, another marker, was utilized for OPC identification. A total of 32.82% ± 2.68% of OLIG2S147A smRNA-treated cells were PDGFRA+ OPCs; however, only 6.68 ± 1.25% of OLIG2WT smRNA-treated cells were PDGFRA+ OPCs (Fig. 2f, g). These results were consistent with those for the other two hiPSC lines showing that OLIG2S147A smRNA induced hiPSC differentiation into OPCs. Blocking OLIG2 phosphorylation downregulates the expression of the MN-specific marker NKX2.2, which results in the initiation of OL production25. We thus determined NGN2 mRNA expression levels in OLIG2WT smRNA and OLIG2S147A smRNA-treated cultures and found that OLIG2S147A smRNA downregulated NGN2 mRNA expression to a significantly greater extent than OLIG2WT smRNA but upregulated the mRNA expression of NKX2.2, which is required for OPC/OL differentiation (Fig. 2h). qPCR further showed that the mRNA expression level of HB9, a TF downstream of NGN2, was also decreased in OLIG2S147A smRNA-induced OPCs (Fig. 2i). Taken together, these results suggested that the smRNA encoding OLIG2 with the phosphorylation site modification facilitated highly efficient OPC generation.

**Maturation of OLIG2 smRNA-induced OPCs into functional OLs.** Differentiation of OPCs into mature OLs upon stimulation with both extrinsic and intrinsic factors is a key event required for axon myelination in the CNS. We next investigated whether smRNA-induced OPCs subsequently matured into functional OLs. The O4 epitope, which is a mature OPC or pre-OL marker, was utilized to assess the terminal differentiation of OPCs28. To determine the kinetics, efficiency, and yield of mRNA-mediated OL lineage specification, we performed weekly flow cytometry assays to evaluate O4+ OPC/OL generation in our culture system. As shown in Fig. 3a, b, the percentage of OLIG2S147A smRNA-induced O4+ cells was 40.49 ± 2.54% on day 14 and increased to 77.16 ± 6.24% by day 21; however, only 0.09 ± 1.25% O4+ cells were observed on day 7, and 11.97 ± 2.24% O4+ cells were observed on day 21 in the OLIG2WT smRNA-treated cultures. The expression of MBP, a membrane actin-binding protein in CNS myelin, is a critical marker of OLs and multilayered compact myelin29. Immunofluorescence staining for MBP confirmed that unlike OLIG2WT smRNA transfection, OLIG2S147A smRNA transfection led to rapid differentiation and maturation of OPCs (Fig. 3c, d) from three different hiPSC lines. Consistent with our previous data, on day 21 of differentiation, 3.4-fold (p < 0.001) and 2.6-fold (p < 0.003) increases in the mRNA expression levels of MBP and myelin oligodendrocyte glycoprotein (MOG), respectively, were observed in OLs transfected with OLIG2S147A smRNA compared to those transfected with OLIG2WT smRNA (qPCR, n = 3, Fig. 3e). This outcome was associated with a concurrent increase in the expression of other myelin-related genes, i.e., myelin-associated glycoprotein (MAG) (3.5-fold, p = 0.005, n = 3) and proteolipid protein 1 (PLP1) (3.2-fold, p = 0.039, n = 3, Fig. 3e). Together, these results indicated that OLIG2WT smRNA-induced OPCs showed limited maturation, whereas OLIG2S147A smRNA-induced OPCs were capable of maturing toward the terminal stage because critical myelin-related genes were expressed at high levels.

To further characterize MN generation during OL differentiation induced by smRNA, immunofluorescence staining of two MN-specific marker genes (β III-tubulin (TU1) and HB9) was performed in cells from the hiPSC1 line. As shown in Fig. 3f, g, a smaller proportion of hiPSC1-derived NPCs transfected with OLIG2S147A smRNA were induced to differentiate into TU1- or HB9-positive MNs than those transfected with OLIG2WT smRNA. Our results demonstrated that the OLIG2S147A smRNA inhibited MN production and might have triggered the MN-OL fate switch of hiPSC-derived NPCs.

**OLIG2 smRNA-induced OLs generate a multilayer myelin sheath in the brains of mice with cuprizone-induced demyelination.** To assess myelination in vivo, FACS-purified OLIG2S147A smRNA-induced O4+ cells (purity > 92%, Fig. S5a) frozen in liquid nitrogen were resuscitated and cultured until they reached a suitable state for transplantation. The cells were stained with an antibody against O4 and displayed a distinct, typical branched morphology of mature OPCs or pre-OLs (Fig. S5b). They were injected into the corpus callosum of mice with cuprizone-induced demyelination, and mouse brain slices were obtained (Fig. 4a, b). Immunohistochemistry revealed efficient engraftment, as indicated by the presence of human hN+ cells coexpressing MBP in brain tissue 6 weeks after transplantation (Fig. 4c). We next sought to investigate the formation of a compact myelin ultrastructure around mouse axons by OLIG2S147A smRNA-induced OLs in vivo. TEM of corpus callosum samples from mice with cuprizone-induced demyelination that had received vehicle or OLIG2S147A smRNA-induced O4+ OPCs was performed (n = 5). We found that significant demyelination and disintegration occurred in mice from the vehicle group (Fig. 4d); however, the corpus callosum of the cell-transplanted mice showed dense mature compact myelin, which was characterized by major concentrically organized dense lines (Fig. 4e) and interlaminar tight junctions. The density of
Fig. 2 A highly efficient strategy for generating smRNA-induced oligodendroglial lineage cells from hiPSC-derived NPCs. 

**a** Diagram of the six daily smRNA differentiation protocols for hiPSCs. 

**b** Representative images of immunofluorescence staining for the human NANOG and SSEA4 proteins in cultured hiPSC1 cells (scale bar, 200 μm).

**c** Representative images of immunofluorescence staining for the human PAX6 and NESTIN proteins in hiPSC1-derived NPCs (scale bar, 20 μm).

**d** Representative images of immunofluorescence staining for NG2, SOX10, and PDGFRα in smRNA-transfected cells of the hiPSC1 line on day 6 (scale bar, 50 μm).

**e** Quantification of the percentage of NG2, SOX10, and PDGFRα in smRNA-transfected cells of the hiPSC1 line on day 6 after smRNA transfection (n = 3; *p < 0.05, **p < 0.01, ***p < 0.001 using two-tailed Student’s t test). OLIG2WT mRNA induced greater NG2+/SOX10+/PDGFRα+= OPC production than OLIG2S147A smRNA.

**f** Representative flow cytometry analyses of the expression of NG2 and PDGFRα in smRNA-transfected cells of the hiPSC1 line on day 6.

**g** Quantification of NG2+ and PDGFRα+ cells among OLIG2WT and OLIG2S147A smRNA-transfected cells of the three different hiPSC lines on day 6 (n = 3, **p < 0.01, ***p < 0.001 using two-tailed Student’s t test).

**h** The mRNA expression levels of a proneural marker gene (NGN2) and oligodendroglial lineage marker gene (NKX2.2) involved in OL differentiation on day 6 after smRNA transfection (n = 3; ***p < 0.001 using two-tailed Student’s t test).

**i** The mRNA expression levels of the MN-specific marker gene HB9 on day 6 after smRNA transfection (n = 3; **p < 0.01 using two-tailed Student’s t test).
myelinated axons in the OPC group was significantly higher than that in the vehicle group and was similar to that in the sham group (Fig. 4f, g). Remyelination by engrafted OPCs was further analyzed and quantified by calculating the g-ratio, which is the ratio of the axon diameter to the total diameter of a myelinated fiber\textsuperscript{30}. Compared to the vehicle group, the OPC group showed significantly lower g-ratio values (Fig. 4h). LFB staining showed spontaneous remyelination in the OPC group (Fig. 4i). These results indicated that OLIG2 smRNA-induced OLs matured into myelin-forming cells and enhanced the remyelination process in the demyelinated mouse brain.

The HSP70 complex binds to and promotes the function of OLIG2 in driving OL differentiation. We performed proteomic
analysis to identify proteins that bind OLIG2<sup>S147A</sup> and compare them with those that bind OLIG2<sup>WT</sup>, confirm the underlying mechanism of OL differentiation mediated by OLIG2 smRNA, and determine the potential partners of OLIG2 in promoting OPC/OL generation. As shown in Fig. 5a, we performed Flag IP by pulling down Flag-tagged OLIG2<sup>WT</sup> and OLIG2<sup>S147A</sup> and their binding proteins from NPCs 24 h after transfection with either OLIG2<sup>WT</sup> or OLIG2<sup>S147A</sup> smRNA. (Fig. 5b). Proteomic analysis led to the identification of 67 differentially expressed proteins in NPCs transfected with either OLIG2<sup>WT</sup> or OLIG2<sup>S147A</sup> smRNA (Supplementary Data 1). These differentially binding proteins were subjected to pathway enrichment analysis using the DAVID bioinformatics database (Supplementary Data 2). They were found to be enriched in the myelin sheath (Fig. 5c) and to be possible positive modulators of OLIG2 transcriptional activity that drive more efficient OL differentiation. Among these elevated proteins that were enriched in the myelin sheath, proteins associated with OLIG2<sup>S147A</sup> were found to include multiple...
Fig. 4 smRNA-induced OPCs give rise to functional myelin following transplantation in the brains of mice with cuprizone-induced demyelination. 

(a) Diagram showing the transplantation site in the corpus callosum. 
(b) schematic of the mouse demyelination model and cell transplantation. Female C57BL/6 mice were provided a diet containing 0.2% cuprizone for 5 weeks. After demyelination was induced for 2 weeks, purified OLIG2S147A smRNA-transfected O4+ OPCs (10⁵) were injected into the demyelinated mouse brain. 
(c) Representative images of immunofluorescence staining for hN and MBP. 
(d) Electron micrographs showing that cuprizone induced myelin disintegration and shedding in the mouse brain; scale bar, 200 nm. 
(e) The density of myelinated axons in each group (n = 6; *p < 0.05 using one-way ANOVA test). 
(f) Western blot analysis of the OLIG2 binding ratio of HSP70 complex components (HSPA5, HSPA8 and HSPA9) involved in the myelin sheath pathway.

Fig. 5 Proteomic analysis identified proteins that differentially bound to OLIG2S147A and OLIG2WT smRNA. 
(a) Schematic of the proteomic analysis protocol using hiPSC-derived NPCs transfected with OLIG2S147A or OLIG2WT smRNA. 
(b) Western blot analysis of the OLIG2 binding ratio of HSP70 complex components (HSPA5, HSPA8 and HSPA9) involved in the myelin sheath pathway.

Chaperones, particularly three main members of the HSP70 complex (HSPA5, HSPA8, and HSPA9) (Fig. 5d), indicating a potential role for the HSP70 complex in regulating OLIG2-driven OL differentiation.

Total protein was extracted from NPCs 24 h after transfection of either OLIG2WT or OLIG2S147A smRNA to validate the identities of the TF OLIG2-binding proteins. IP and immunoblot analyses were performed to validate the binding of the two candidate proteins HSPA8 and HSPA9, which are mainly located in the nucleus and cytosol, to OLIG2. Indeed, based on our working model (Fig. 6a), HSPA8 and HSPA9 were consistently more strongly associated with OLIG2S147A than with OLIG2WT (Fig. 6b). Next, the HSP70 complex agonist ML346 (ML) and antagonist VER-155508 (VE) were utilized to determine whether the efficiency of OL generation was regulated by HSP70 activity. ML acts as an activator of HSP70 expression by increasing HSF-1 activity. ML treatment increased the yield of MBP-positive OLs significantly after VE treatment, and VE also attenuated the effect of ML in promoting OL production. Furthermore, we determined the transcriptional activity of OLIG2 by measuring the mRNA expression level of the OLIG2 target gene SOX10 in response to OLIG2S147A smRNA transfection and ML and/or VE treatment (Fig. 6e). ML treatment significantly upregulated SOX10 expression following transfection of OLIG2S147A smRNA, while VE treatment attenuated this induction of SOX10 expression and the effect of ML in promoting SOX10 upregulation. We validated these findings by investigating the effect of genetic elimination of HSP70 factors on OL differentiation. To this end, three HSP70 siRNAs were used to knockdown HSP70 expression in hiPSC-derived NPCs (Fig. 6f).

We found that the siRNA targeting HSP70 decreased MBP-positive OL generation after OLIG2S147A smRNA transfection (Fig. 6g). Additionally, HSP70 siRNA significantly upregulated
Expression (Fig. 6h). Taken together, these results indicated that these two HSP70-modulating compounds may have regulated OL generation by affecting the transcriptional activity of OLIG2.

Discussion
smRNA-driven differentiation strategies for generating hiPSCs or hiPSC-derived specific cell types have been well documented in recent decades.10,11,35. In this study, an smRNA encoding a TF was introduced into cells through nongenome-integrating viruses. This approach not only increased the expression level of the TF but also prevented the risk of tumorigenicity caused by genome integration. Here, we report for the first time that an smRNA-based differentiation strategy induced highly efficient generation of human OL cells from hiPSC-derived NPCs, while OLIG2 smRNA failed to directly reprogram hiPSCs to OLs. We show that repeated transfection of OLIG2 smRNA containing a phosphorylation site modification significantly enhanced the efficiency of OL generation from NPCs derived from hiPSCs.

Proteins are important functional molecules that maintain normal life activities. PTM of proteins is an important way to regulate the structure, localization, stability, and degradation rate of proteins. Previous studies have reported that protein phosphorylation promotes protein degradation in most cases.36,37. Protein expression after OLIG2 smRNA transfection was much higher than that after OLIG2 WT smRNA transfection, as shown in Fig. 3d. We speculate that inhibiting the phosphorylation of OLIG2 at serine 147 may delay the degradation of OLIG2.
and improve the stability of OLIG2 expression. To the best of our knowledge, although some TFs or TF cocktails have been identified as key factors in OL development that depend on the integration of retroviral vectors,6,14,16-18, OL fate conversion through OLIG2 activation only has not been achieved. smRNA-based gene delivery has been shown to be highly efficient and safe for cell fate reprogramming.38-41. A major advantage of the single OLIG2 smRNA-based approach described in this article is the ability to design and modify the mRNA translation sequence to promote protein expression and transcriptional activity. This strategy is crucial to regulate the developmental fate switch of NPCs from MNs to OLs and to generate large numbers of functional OLs from hiPSCs. Li et al. reported that the TF OLIG2 participates in MN-OL fate switching and is dephosphorylated at the onset of OL genesis. Replacing serine 147 with an alanine residue (S147A) has been shown to abolish MN production without preventing OL production in transgenic mice and chicks and cultured P19 cells.42. Apparently, by controlling PTM of the OLIG2 protein, such as phosphorylation, the function of OLIG2 in promoting MN generation in the early neurogenesis stage can be eliminated.21. Indeed, our results confirmed that OLIG2 smRNA with a phosphorylation site modification downregulated the expression of the MN-related genes NGN2 and HB9 and enhanced the expression of OLIG2 and functional O4+ OL generation. These findings are consistent with those reported in other publications. For example, Xue et al. reported that a phosphorylation site modification in pronuclear Atoh1 and NgN2 effectively enhanced lineage-specific neuron generation from hiPSCs.35. Proper PTM results in more precise localization and higher translational activity. Therefore, smRNA-induced OLIG2 expression is essential for MN-OL fate switching, and OLIG2 smRNA delivery is a practical strategy for inducing OL differentiation from hiPSCs.

Our understanding of demyelinating diseases and the development of new therapeutic options are hampered by the limited ability to obtain stable human OLs. As demonstrated here, OLIG2 smRNA-driven OL differentiation from hiPSCs is very robust, resulting in the production of up to 70% O4+ OPCs. Similar to OLs generated by other protocols, these smRNA-induced OPCs mature into OLs in vitro, indicating that they may promote remyelination in the CNS after demyelination. Although we observed that transplanted smRNA-induced OPCs survived in the mouse brain and eventually differentiated into myelinating OLs, remyelination might not have resulted exclusively from the direct effect of donor-derived OPCs. Indeed, OPCs have been shown to directly regulate myelin sheath formation in the CNS. In addition, exosome-mediated molecular signals from OPCs may also play an important role in remyelination after myelin injury, but corresponding in-depth studies are lacking. Exosomes are membranous vesicles of nanometer size (30-100 nm in diameter) that are secreted by many types of cells, such as glial cells and immune cells.42. Exosomes contain mRNAs, microRNAs (miRs), various proteins (such as Alix and HSP70), and signaling molecules (CD9 and CD63) that are critical mediators of intercellular signal transduction. For example, exosomes secreted from bone marrow mesenchymal stromal cells (MSCs) exert a significant therapeutic effect on enhancing remyelination and reducing neuroinflammation in the demyelinated CNS.43. Therefore, the bystander effect of remyelination induced by infused OPCs in mice with cuprizone-induced demyelination should not be ignored. Herein, we propose that exosome-derived infused OPCs may act on resident OPCs, indirectly and partially promoting remyelination upon OPC transplantation.

Reprogramming of hiPSCs into OPCs/OLs largely relies on regulators that modulate the stability and/or transcriptional activity of the OLIG2 protein. Our proteomic analysis identified OLIG2-binding proteins in hiPSC-derived NPCs for the first time. OLIG2S147A and OLIG2WT showed differential binding affinity for a set of binding partners. Poly (ADP-ribosyl) polymerase-1 (PARP-1) has been identified as a positive modulator of OL differentiation and remyelination.44-47. Previous studies performed in our laboratory also revealed that PARP-1 acts as an intrinsic driver of OL development and myelination by stabilizing myelin mRNA translation. Specific inhibition of PARP1-mediated PARylation activity in OLIG2-expressing cells further compromises OL differentiation and CNS remyelination.32. Indeed, consistent with our proteomic data, the level of PARP-1 bound to OLIG2S147A was ~15-fold higher than that bound to OLIG2WT (Fig. S6). Therefore, PARP-1 might be a crucial factor that mediates OLIG2 PTM involved in OL differentiation. Moreover, the increase in PARP-1 binding to OLIG2S147A was consistent with the elevated expression of downstream myelin-associated genes, such as MBP, MOG, MAG and PLP1, as shown in Fig. 3e. Higher binding affinity of OLIG2S147A for PARP-1 than OLIG2WT indicated a potential mechanism by which OLIG2S147A promotes differentiation ability, but these molecular events remain to be further studied in detail. Most importantly, in the present study, we found that the HSP70 complex might stabilize OLIG2 and lead to more robust oligodendroglia lineage-specific gene induction (particularly at the bulk level). We show, for the first time, that the HSP70 complex is a functional OLIG2-binding partner and that the HSP70 agonist ML promotes OL generation. The HSP70 complex is widely known to function as a chaperone that supports the folding of diverse target proteins.48,49. Several studies have shown that constitutively expressed HSP70 is required for the optimal expression of MBP during OL differentiation.50. Our study revealed a novel function for the HSP70 complex in promoting the transcriptional activity of OLIG2 and its function in OL lineage specification. Our findings provide an entirely new perception and understanding of the structural basis of the OLIG2/HSP70 interaction to optimize OL production from NPC-derived hiPSCs.

Next, several important limitations of this smRNA-driven strategy are discussed. First, it was unlikely to directly induce OPC differentiation from uncommitted hiPSCs. Indeed, according to our data, forced expression of OLIG2 smRNA alone did not result in the generation of NG2- or PDGFRα-positive cells; in contrast, only neural-committed NPCs were observed. This outcome was consistent with previous findings showing that the expression of the single TF SOX10 alone does not directly induce an OL fate of hiPSCs.51. Recently, Chanoumoud et al. published a rapid protocol for the generation of O4+ cells from human fibroblasts induced by ectopic expression of a TF cocktail including SOX10, OLIG2, and NKX6.2 and showed that the fibroblasts further differentiated into MBP+ mature OL-like cells within 16 days.52. TF cocktails show a powerful propensity for inducing OL lineage specification. OLIG2WT smRNA showed limited ability to achieve OL specification of hiPSCs, partially due to the lack of certain partner proteins. It is logical to assume that other essential TF smRNAs that can directly generate OLs from hiPSCs or even human fibroblasts will be identified in future studies. Another limitation of this study involves the transplantation experiment; it is unclear whether hiPSC-derived O4+ cells also possess the ability to promote myelin regeneration in vivo. Although we validated the effectiveness of our smRNA-driven OL differentiation strategy in a variety of hiPSC lines, the presented animal data could be hiPSC1 line-specific, as the findings were not proven for any other hiPSC lines. Furthermore, it is insufficient to focus only on the effect of OPCs in demyelinating diseases. hiPSC-derived OPC transplantation has also shown significant therapeutic potential in other human brain diseases, such as ischemic stroke. Therefore, it is very meaningful to
broaden the application scope of smRNA-induced OPCs and further confirm the efficacy of OPC transplantation in other disease models.

In summary, highly efficient hiPSC differentiation strategies are critical for the application of hiPSC technology in regenerative medicine. Traditional methods based on chemical compounds and viruses are relatively slow, variable, or unsafe. In this study, a highly efficient strategy was established based on using a smRNA encoding the single transcription factor OLIG2 with dephosphorylation modification to drive rapid hiPSC differentiation into functional OLs. These discoveries will facilitate the applications of hiPSC-derived OLs in demyelinating disease modeling and therapy and guide the development of robust methods for generating various lineage-specific progeny from hiPSCs.

**Methods**

**smRNA synthesis and cell transfection.** The coding sequences of human OLIG2WT and OLIG2S107A were cloned into a vector containing the T7 promoter and poly(A) tail for IVT. The open reading frames (ORFs) of OLIG2 were cloned into the PC2R-UTR-R1R2 vector according to the LR reaction protocol (Thermo Fisher Scientific, Waltham, MA, USA). The PC2R-UTR-R1R2 vector was linearized with a restriction enzyme that cuts outside the ORF. The linearized vector was used as the template for T7 polymerase promoter sequence and a 3’ UTR-directed primer with a long poly T tail. The PCR product was gel purified and used as a template for a protocol that was performed essentially as described by Mandal and Rossi. The IVT reaction included an anti-reverse cap analog (ARCA, B8175, APEXBio, Houston, USA) and the modified nucleotides 5′-methylcytidine-5′-triphosphate and pseudouridine-5′-triphosphate, to reduce cytotoxicity due to the activation of innate immune responses. The purified smRNA was verified and stored as aliquots at −80 °C until use (Table S1). In each well of a 12-well plate, we incubated smRNAs with 1.5 μL of ScreenFect transfection reagent in 75 μL of transfection buffer for 15 min before adding the mixture to cultured cells. Cell proliferation and death were analyzed using the Cell Counting Kit-8 (CCK-8) assay kit (CK101-91, Data Inventory Biotechnology, Hong Kong, China) according to the manufacturer’s instructions.

**hiPSC culture and neural differentiation.** hiPSC lines (hiPSC-UC17P3-C3P9, hiPSC-UC3F2P2-C3P3, and hiPSC-UC5F3P3-C5P4; referred to as hiPSC1, hiPSC2, and hiPSC3, respectively) were obtained from the Guangzhou Institute of Bio-medicine and Health Chinese Academy of Science (Guangzhou, China). All three hiPSC lines in this study were derived from normal human urine epithelial cells, and cytogenetic analysis of all hiPSC lines showed a normal karyotype (Fig. S7). For growth under feeder-free conditions, hiPSCs were cultured in Matrigel-coated 12-well plates or 60-mm dishes using Stem Cell Technologies, Stem Cell Media (Stemline, NJ, USA) medium. The cells were incubated at 37 °C in 5% CO2, and were mechanically split every 3 days at 1/8 ratios using ReLeSR ( Stem Cell Technologies, Canada) according to the methods described by the manufacturer.

For the differentiation of hiPSCs into NPCs. Briefly, passages 20 to 30 hiPSCs were collected and mechanically dissociated. The differentiated colonies were replated in a six-well plate. Once the confluence of hiPSCs reached about 20%, Culture medium was then switched to Neural Induction Medium 1 for 2 days (NIM1: 50% DMEM/F12, 50% Neurobasal, 1X B27, 1X N2, 1X GlutaMAX, 10 ng/ml hLIF, 25 mM ammonium bicarbonate by centrifugation at 12,000 × g). The sample was incubated in the dark for 15 min at room temperature. The ultratrafaction tube was washed with 200 μL of 8 M urea 3 times and 200 μL of 8 M urea 3 times. The reduction reaction was performed for 2 h at 37 °C. The solution was removed by centrifugation, and 200 μL of an 8 M urea solution supplemented with 50 mM iodoacetamide (IAA) was added. The sample was incubated in the dark for 15 min at room temperature. The ultratrafaction tube was washed with 200 μL of 8 M urea three times and 200 μL of 250 mM ammonium bicarbonate three times by centrifugation at 12,000 × g. The Pierce Protein A/G magnetic beads were washed three times using Pierce IP lysis/wash buffer before LC-MS/MS analysis.

**OPC differentiation.** For OL lineage differentiation, a two-step differentiation protocol was utilized. hiPSC-derived NPCs were seeded at a density of 1 × 10^5 cells per well in a 12-well plate coated with poly-L-ornithine/laminin (Sigma-Aldrich, MO, USA). NPCs were transduced with OLIG2WT or OLIG2S107A smRNA using ScreenFect transfection reagent (293-75991, Wako, Japan) for 6 days, and the culture medium was changed to glial induction medium (GIM) for 4 days. Then, the medium was replaced with differentiation medium (DM). A detailed description of the GIM and DM is provided in Table S2. The cells were also cryopreserved in a medium containing 40% Neurobasal medium with B27 supplement, 50% fetal bovine serum (DIB-12B-10X50ML, Data Inventory Biotech, China) and 10% DMSO.

**Immunofluorescence staining and immunohistochemical staining.** Cells on coverslips were fixed with 4% paraformaldehyde for 15 min at room temperature, and covered with PBS three times. After fixation, cells were then blocked with 4% bovine serum albumin (BSA, A8010, Solarbio, China) for 30 min at 37 °C and then incubated with an appropriate primary antibody overnight. The next day, the coverslips were incubated with fluorescent dye-conjugated secondary antibodies (Invitrogen, San Diego, CA, USA) for 1 h at room temperature in the dark. The coverslips were washed three times with PBS counterstained with 2-5 μM 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlington, CA, USA) for at least 10 min, washed with PBS three times, and mounted with mounting medium (Invitrogen). For immunohistochemical staining of mouse brain samples, after the paraffin sections were dehydrated, they were rehydrated at 0.1 mol/L sodium citrate (pH 6.0) for antigen retrieval. Then, they were permeabilized with 0.2% Triton and blocked with 3% BSA solution at 37 °C for 30 min. The sections were incubated with primary antibody overnight at 4 °C and then with secondary antibody for 1 h the next day. After that, tissue autofluorescence was eliminated with a kit (Vectorlabs, Vector True View Autofluorescence Quenching Kit), and then the membranes were incubated with secondary antibodies labeled with HisP (Solarbio, China), and protein expression was normalized to the expression of the housekeeping protein tubulin in the same sample.

**Quantitative real-time PCR (qPCR)**. Total RNA was extracted using an EZ-Prep RNA Purification Kit (B0004D, EZBioScience, Roseville, USA), and the RNA concentration was measured by a Nanodrop spectrophotometer (Thermo Scientific, USA). cDNA was subsequently synthesized using an EZscript All-in-one Reverse Transcription Kit (RT3P, EZBioScience). qPCR was performed using Real Time PCR EasyTM-SYBR Green I (QP-01014, Foregene, China). The primer sequences used for qPCR are provided in Table S4 (Tsuchiue, China).

**Immunoprecipitation (IP)**. Twenty-four hours after smRNA transfection, whole-cell lysates were extracted, and the protein concentration was determined with a BCA protein assay kit (Thermo Fisher Scientific, USA). Fifty micrograms of total protein were loaded into each well of a 10% SDS-PAGE gel and separated by electrophoresis. The primary antibodies were listed in Table S3. After the overnight incubation with the membranes were incubated with secondary antibodies labeled with HisP (Solarbio, China), and protein expression was normalized to the expression of the housekeeping protein tubulin in the same sample.

**LC-MS/MS**. Aliquots of proteins were mixed with 200 μL of 8 M urea in Nanopore centrifugal DEVICES ( Pall). The devices were centrifuged at 12,000 × g for 20 min at 20 °C. All subsequent centrifugation steps were performed under the same conditions to allow maximal concentration. Then, 200 μL of 8 M urea solution supplemented with 10 mM DTT was added, and the reduction reaction was performed for 2 h at 37 °C. The solution was removed by centrifugation, and 200 μL of an 8 M urea solution supplemented with 50 mM iodoacetamide (IAA) was added. The sample was incubated in the dark for 15 min at room temperature. The ultratrafaction tube was washed with 200 μL of 8 M urea three times and 200 μL of 250 mM ammonium bicarbonate three times by centrifugation at 12,000 × g. The Pierce Protein A/G magnetic beads were washed three times using Pierce IP lysis/wash buffer before LC-MS/MS analysis.

**OPC differentiation.** For OL lineage differentiation, a two-step transfection protocol was utilized. hiPSC-derived NPCs were seeded at a density of 1.5 × 10^5 cells per well in a 12-well plate coated with poly-L-ornithine/laminin (Sigma-Aldrich, MO, USA). NPCs were transduced with OLIG2WT or OLIG2S107A smRNA using ScreenFect transfection reagent (293-75991, Wako, Japan) for 6 days, and the culture medium was changed to glial induction medium (GIM) for 4 days. Then, the medium was replaced with differentiation medium (DM). A detailed description of the GIM and DM is provided in Table S2. The cells were also cryopreserved in a medium containing 40% Neurobasal medium with B27 supplement, 50% fetal bovine serum (DIB-12B-10X50ML, Data Inventory Biotech, China) and 10% DMSO.
Flow cytometry analysis. Cells were enzymatically harvested using Accutase, centrifuged and resuspended in 100 μL of FACS buffer (1X PBS, 2% fetal bovine serum and 0.02% sodium azide). To determine NG2, PDGFRα, and O4 expression, OLIG2 smRNA-induced progenies were incubated or not (as a control) with 1/20 dilutions of NG2-PE (C60635P, Signalway antibody, USA), PDGFRα-APC (2X3512, Biolog, USA), and O4-APC (130-114-978, Miltenyi Biotec, Germany) antibodies or for 15 min at 4 °C, washed and resuspended in 150 μL of FACS buffer. The cells were analyzed using a BD flow cytometer (Beckman Coulter, CytoFLEXS). All flow cytometry data were further analyzed using BD CytoExpert software (BD Biosciences, USA).

Transplantation of smRNA-induced OPCs in a cuprizone-induced demyelination mouse model. Eight- to ten-week-old female C57BL/6 mice (n = 5) were fed an AIN-93G purified rodent diet containing 0.2% cuprizone (Dyets, D200218) for 5 weeks; thereafter, the cuprizone-infused food was removed, and the mice were given a normal diet. Mice with demyelination induced by cuprizone serve as a well-proven model to test the functional capacity of transplanted OPCs to form MBP−positive compact myelin sheaths around axons. The demyelinated mice were injected with 100,000 OLIG2σ1475 smRNA-induced O4+ OPCs into the corpus callosum using a Hamilton syringe (KW-ZSB, NIKKEWBO, China) at the following coordinates according to our previously published protocols52: 0.2 mm anterior to the interaural line and 1.1 mm medial to the bregma and 2 mm dorsoventral from the skull surface. The control group included mice fed normal food for 5 weeks and underwent sham operation. The mice were intraperitoneally injected with 10 mg/kg cyclosporine daily for immune suppression beginning 2 days before cell transplantation. Postoperative care was provided according to Institutional Animal Care and Use Committee (IACUC) protocols approved by Sun Yat-sen University. The mice were sacrificed after 8 weeks, and corpus callosum sections (2–3 mm) containing the injection site were microdissected and processed for imaging under an electron microscope or for confocal imaging. The animal study protocol was approved by the Sun Yat-sen University Animal Use and Care Committee (SYSU-IACUC-2021-00076).

Transmission electron microscopy. Brain samples were isolated from mice perfused with 2% paraformaldehyde and then the corpus callosum was isolated and fixed with PBS containing 2.5% glutaraldehyde for 2 h. Next, the corpus callosum was washed, fixed with 1% osmium tetroxide, dehydrated in acetone, and embedded in epon. Afterward, 70–80 nm thin sagittal sections cut with a diamond knife were mounted on copper slot grids coated with Formvar. After staining with uranyl acetate and lead citrate, they were scanned with a JEM-1230 transmission electron microscope. Approximately 100 myelinated axons were measured for each group, and the g-ratios were calculated using ImagePro software (NIH, USA).

Construction of siRNAs and cell transfection. Three heat shock protein 70 (HSP70)-specific siRNAs targeting the human HSP70 sequence were synthesized as double-stranded oligonucleotides (MHBO, China). hiPSC-derived NPCs were seeded 24 h prior to transfection in 12-well plates. Cell transfection was conducted using Lipofectamine 2000 transfection reagent (DIB034, Data invention Biotech, China). Briefly, cells were incubated with HSP70 siRNAs (0.5 nM) or NC siRNA control in NSMM at 37 °C and 5% CO2 for 24 h. The culture medium was replaced with normal medium at the end of the incubation period. The efficacy of HSP70 knockdown was assessed after 2 days by western blotting. The sequences of the siRNAs designed in this study are provided in Table S5.

Statistics and reproducibility. Prism 8.0.2 for Windows (GraphPad) was used for the statistical analysis. All least three independent replicates were performed for each experiment. The number of biological replicates “n” define the number of individual samples used per group for each experiment as indicated in the legends. The significance of differences between two measurements was determined by two-tailed unpaired Student’s t test, and the significance of differences among groups of three or more was determined by one-way analysis of variance (ANOVA) test. The data are presented as the mean ± standard error of the mean. Differences in mean values between different treatment groups were considered significant at p < 0.05 (presented as “***”), p < 0.01 (presented as “**”), and p < 0.001 (presented as “***”).

Data availability. The datasets used and/or analyzed during the current study are available from the corresponding authors upon reasonable request. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD036975 and 10.6019/PXD036975. Source data underlying the graphs is presented in Supplementary Data 3. Original and uncropped immunoblots are presented in Fig. S8.

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Author contributions

J.X., W.B.D. and Y.C.N. designed the experiments and interpreted the data. J.X., Z.H.Y. and R.W. carried out most of the experiments with technical assistance from Z.H.Y, R.Y., J.X., W.B.D. and Y.C.N. designed the experiments and interpreted the data. J.X., Z.H.Y., W.B.D. and Y.C.N. provided critical input into the overall research direction. J.X. and Y.C.N. wrote the paper with input from all coauthors. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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