Generation of homogeneous midbrain organoids with *in vivo*–like cellular composition facilitates neurotoxin-based Parkinson's disease modeling

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Abstract

Recent studies have demonstrated the generation of midbrain-like organoids (MOs) from human pluripotent stem cells. However, the low efficiency of MO generation and the relatively immature and heterogeneous structures of the MOs hinder the translation of these organoids from the bench to the clinic. Here we describe the robust generation of MOs with homogeneous distribution of midbrain dopaminergic (mDA) neurons. Our MOs contain not only mDA neurons but also other neuronal subtypes as well as functional glial cells including astrocytes and oligodendrocytes. Furthermore, our MOs exhibit mDA neuron–specific cell death upon treatment with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), indicating that MOs could be a proper human model system for studying the *in vivo* pathology of Parkinson's disease (PD). Our optimized conditions for producing homogeneous and mature MOs might provide an advanced patient-specific platform for *in vitro* disease modeling as well as for drug screening for PD.

Significance Statement

Although recent studies have demonstrated the generation of human PSC-derived MOs which display structural and functional features of midbrain, a few issues including the low generation efficiency and reproducibility of MOs, heterogeneous and immature structures of MOs, and physiologically irrelevant cellular composition of MOs hinder the clinical translation of organoid technology. This study described the novel strategy for the robust generation of homogeneous MOs using specific combination of dual SMAD inhibitors and *in vitro* WNT gradient. MOs generated by our optimized protocol with *in vivo*-like cellular composition are as structurally and functionally mature as the developing midbrain. DAC3.0 MOs with functional glial cells facilitate MPTP-based *in vitro* disease modeling of PD.

Introduction

Induced pluripotent stem cell (iPSC) technology [1,2] represents a novel approach to patient-specific disease modeling and drug screening [3]. Many previous studies have employed patient-specific iPSCs for reproducing *in vivo* pathophysiology of various diseases in a dish, including neurodevelopmental disorders [4], neurodegenerative diseases [5], cardiovascular diseases [6], liver diseases [7], and others [8]. However, current two-dimensional (2D) differentiation protocols typically produce singular cell types, but not multiple cell types, from pluripotent stem cells (PSCs) [9,10]. Previous studies on *in vitro* disease modeling were based on 2D differentiation systems and could not fully recapitulate *in vivo* disease pathology [10,11], as organs consist of a highly orchestrated three-dimensional (3D) structure comprising multiple cell types, each of which has a unique function. Indeed, previous iPSC-based *in vitro* modeling studies of Parkinson's disease (PD) using 2D differentiation technology described either no PD-associated pathophysiology [12,13] (no phenotype and non-PD phenotype) or only early-stage symptoms of PD [14-16] such as a neuronal differentiation defect, abnormal autophagy, and accumulation of α-synuclein, without recapitulating the end-stage pathophysiology of PD such as massive mDA neuron–selective cell death [17].

Recent advances in the stem cell field have successfully demonstrated the generation of miniaturized 3D organ-like structures, so-called organoids, from human PSCs. By taking advantage of the self-organization capacity of PSCs, together with specific differentiation signals, organoids representing distinct organs [18], such as lung [19], liver [20], kidney [21], stomach [22], pancreas [23], intestine [24], and colon [25], could be generated. The generation of brain organoids that correspond to different parts of brain, such as cerebral cortex [26-30], optic cups [31], hippocampus [32], pituitary gland [33], and cerebellum [34], has been also described. Moreover, recent studies have also demonstrated the generation of midbrain-like organoids (MOs) from either human PSCs [35,36] or pre-differentiated neuroepithelial stem cells from human iPSCs [37]. In contrast to cerebral organoids (COs) [28,35], MOs express markers typical for

human midbrain [35] and produce neuromelanin-like granules [36,37], which display similar structures to those from human substantia nigra [38], suggesting that MOs have the potential for application in disease modeling and drug screening for midbrain-specific neurodegenerative diseases such as PD.

However, a few issues need clear resolution before translating organoid technology to the clinic. Firstly, the efficiency of organoid generation is typically very low [39], resulting in organoid technology of low reproducibility. Secondly, nearly all kinds of organoids, including MOs, exhibit heterogeneous structures [10,40], potentially compromising the reliability of organoid-based drug screening and disease modeling data. Thirdly, not all cellular components could be reproduced in organoids even after long-term maturation, yielding organoids with physiologically irrelevant cellular composition [10]. For example, MOs generated from PSCs contain no or a paucity of glial cells [35,36]. Finally, yet most importantly, organoids representing distinct organs typically display relatively immature structures compared with their *in vivo* counterparts [40], although they conserve some key structural characteristics.

In the current study, we developed a robust protocol for the generation of MOs. For this, we first screened small molecules for dual SMAD inhibition in order to achieve efficient specification into mesencephalon and, subsequently, into midbrain. Among the distinct small molecule combinations, the combination of dorsomorphin, A83-01, and CHIR99021 facilitated the robust production of MOs, with global enrichment of mDA neurons. Moreover, we also showed that the WNT signal is a key determinant of the regional identity of MOs, and by fine mapping the WNT gradient, we generated functionally and electrophysiologically mature MOs that produce dopamine and neuromelanin-like granules. Our MOs contain not only mDA neurons but also multiple neuronal subtypes, depicting a neuronal organization that is similar to the *in vivo* scenario. More importantly, we showed that these MOs contain functional glial cells, a finding that has not been described previously. Finally, the MOs containing functional astrocytes underwent massive cell death upon treatment with the astrocyte-mediated dopaminergic neurotoxin 1-methyl-4-

phenyl-1,2,3,6-tetrahydropyridine (MPTP). Our novel and robust protocol for generating MOs might hold potential usefulness for understanding the early development of the midbrain *in vivo* and for modeling PD *in vitro*.

Materials and Methods

Generation of brain organoids

To generate brain organoids, $1x10^4$ single cell–dissociated hESCs were plated on each well of ultra low–attachment U-bottom 96-well plates (Corning). At 24hrs after plating, BGM (see supplementary methods) was added to EBs. The medium was replaced every other day. Brain organoids were embedded into growth factor–reduced matrigel (Corning) droplets. Matrigel-embedded brain organoids were transferred to 6-cm petri dishes containing BGM with proper supplements for MOs and COs for inducing basal-apical lamination. For maturation, brain organoids were cultured on an orbital shaker (Stuart) with proper maturation medium (see supplementary methods).

Results

1. Robust mesencephalon specification by specific dual SMAD inhibitors

Although previous studies [35,36] have successfully demonstrated the generation of MOs, the efficiency of MO generation and issues regarding the structural and functional heterogeneity of these MOs have not been addressed. The utility of MO technology for *in vitro* disease modeling and drug screening for midbrain-specific neurodegenerative diseases depends on an optimized protocol for robustly generating a structurally and functionally homogeneous population of MOs. First, we sought to optimize mesencephalon specification, the first step in the generation of MOs (Fig. 1A). For this, first we treated human embryonic stem cells (hESCs) with distinct combinations of dual SMAD inhibitors for inducing early neuroectodermal commitment [41] and CHIR99021 for regional specification into mesencephalon [42,43]. $1x10^4$ single-cell dissociated hESCs were plated on u-bottom 96-well plates with 0.8 μM CHIR99021 [36] and distinct combinations of dual SMAD inhibitors, which had been used in previous organoid studies: i.e., 200 ng/ml Noggin + 10 μM SB431542 (**NS**) [36,44], 2 μM dorsomorphin + 2 μM A83-01 (**DA**) [35], 100 nM LDN + 10 μM SB431542 (**LS**) [35], and 10 μM dorsomorphin + 10 μM SB431542 (**DS**) [30] for achieving efficient mesencephalon specification (Fig. 1A).

The embryoid bodies (EBs) generated under all conditions, except for DS, were uniformly of high quality; few EBs were produced with DS and they were of relatively poor quality (Fig. 1B and Fig. S1A). As expected, all dual SMAD inhibition conditions robustly activated neuroectodermal markers, with complete suppression of pluripotency and mesendodermal markers (Fig. 1C). Unexpectedly, under the DS condition, mesendodermal markers were also highly activated (Fig. 1C). After the mesencephalon specification step, we induced the ventralization of mesencephalon-specified early EBs (day 4) to achieve further specification into the mesencephalic floor plate. This was done by treating EBs with both FGF8, a midbrain-hindbrain boundary-derived morphogen, and SAG, a sonic hedgehog agonist, for 5 days (Fig. 1A). Then,

we embedded midbrain-specified organoids (hereafter referred to as MOs) into matrigel droplets to induce basal-apical lamination, followed by MO maturation (Fig. 1A).

After 1 week of midbrain maturation (1WM), we observed MOs of similar number and size for all dual SMAD inhibition conditions, except for DS (Fig. S1B and C). As there were practically no viable MOs with DS (Fig. S1D), we compared only the NS, DA, and LS conditions for the remainder of the analysis. After 2WM, we observed MOs of similar size expressing both tyrosine hydroxylase (TH), a marker for mDA neurons, and ASCL1, a marker for their progenitor population, under all conditions (Fig. 1D, E and Fig. S1E). Our qPCR analysis showed that midbrain markers were gradually increased in MOs generated under both the DA and LS conditions (hereafter DA-MOs and LS-MOs, respectively) in a time-dependent manner, compared with MOs generated with NS (NS-MOs) (Fig. 1F). Taking these results together, we were able to optimize the conditions for mesencephalon specification of brain organoids, by comparing multiple conditions for dual SMAD inhibition for the robust generation of MOs that contain TH-positive mDA neurons.

2. Generation of homogeneous midbrain organoids by specific dual SMAD inhibition

Distinct combinations of dual SMAD inhibitors robustly led brain organoids to undergo early neuroectodermal commitment as well as mesencephalon specification (Fig. 1C and F). However, after 1WM, we observed that MOs from different conditions exhibited distinct cellular responses, i.e., highly specific activation of midbrain markers in DA-MOs and LS-MOs but residual expression of forebrain markers in NS-MOs (Fig. 1F). These different responses in gene expression pattern prompted us to investigate the effect of dual SMAD inhibition on the structural heterogeneity of MOs. For this, we examined the numbers and distribution patterns of TH-positive cells in MOs produced under distinct conditions (NS-, DA-, and LS-MOs) after 4WM.

As a result, NS-MOs and LS-MOs exhibited heterogeneous distribution of TH-positive mDA neurons (Fig. 2A). In contrast, strong expression of TH was evenly observed in DA-MOs (Fig.

2A). Notably, more than 85.7% ±15.8% of MAP2-positive neurons in DA-MOs were TH-positive, while MOs under the other conditions showed polarized distributions of fewer TH-positive neurons (47.5% ±14.0% in NS-MOs and 31.5% ±11.3% in LS-MOs) (Fig. 2B), indicating that more homogeneous MOs with more TH-positive mDA neurons were produced with DA. We also observed the relatively more abundant and homogeneous distribution of LMX1A, a marker for midbrain-specific progenitors and ASCL1, a marker for mDA neuronal progenitors in DA-MOs compared with NS- and LS-MOs (Fig. S2). Similarly, both LMX1A and TH were homogeneously expressed in DA-MOs but weakly and heterogeneously expressed in both NS-MOs and LS-MOs (Fig. 2C). Surprisingly, TBR1, a marker for cerebral cortex, was still highly expressed in both NS-MOs and LS-MOs but not expressed in DA-MOs (Fig. 2D). Similar gene expression patterns were also observed in our RNA-sequencing (RNA-seq) data using day-7 EBs. In general, MOs produced with each combination showed activation of neuronal genes (Fig. S1F and G). However, both NS- and LS-MOs exhibited high activation of cerebral cortex markers as well as non-neuronal genes (Fig. 2E and S1H). In contrast, DA-MOs exhibited strong suppression of those markers (Fig. 2E and S1G). Finally, a whole mount imaging of MOs produced under these different conditions again confirms our finding that MOs containing a high number and homogeneous distribution of TH-positive mDA neurons were produced with DA (Fig. 2F). Taken together, our data indicates that dual SMAD inhibition by DA during the early phase of MO generation not only induces robust specification into mesencephalic floor plate but also produces structurally homogeneous MOs.

3. WNT gradient is a key determinant for the regional identity of midbrain organoids

The WNT signaling pathway is critical for inducing distinct regional identities of the developing brain [42,43]. Previous trials for generating MOs had employed specific concentrations of CHIR99021, either 0.8 μM [36] or 3 μM [35]. However, the WNT gradient had not been properly recapitulated *in vitro* for generating MOs, probably resulting in structurally and functionally

heterogeneous brain organoids. Thus, as the second step in the generation of MOs, we sought to further fine-tune the regional identity of DA-MOs by screening for the ability of specific concentrations of CHIR99021 to generate homogeneous and mature MOs. For this, we tried to generate the *in vitro* WNT gradient by using different concentrations of CHIR99021 (0, 0.8, 1.5, 2, 2.5, or 3 μM) together with IWP2, a WNT inhibitor [45,46] that eliminates endogenous WNT (Fig. 3A). We found that there was a positive correlation between the concentration of CHIR99021 used and the size of DA-MOs generated (Fig. S3A and B), with the higher concentrations of CHIR99021 leading to gradually lower levels of pro-apoptotic genes and hence larger MOs (Fig. S3C). In general, a higher concentration of CHIR99021 led to the gradual increase in the expression of a subset of genes related to neuronal processes (Fig. 3B, 3C and S3D). The expression levels of cortex markers were decreased in a concentration-dependent manner (Fig. 3D). In contrast, at higher concentrations of CHIR99021, midbrain markers were highly expressed, with maximum levels reached at 3 μM CHIR99021 (Fig. 3E). Our RNA-seq analysis yielded similar changes at the transcriptional level. Compared with DA-MOs at lower concentration of CHIR99021, DA-MOs at higher concentrations (2 and 3 μM) exhibited increased expression of midbrain markers and suppression of cerebral cortex markers (Fig. 3F and G). We next performed whole mount imaging and found that DA-MOs treated with higher concentrations of CHIR99021 displayed homogeneous distribution of mDA neurons with stronger expression of TH (Fig. 3H). Notably, both 5 and 10 μM of CHIR99021 caused regional specification into more caudalized regions with decreased numbers of mDA neurons (Fig. S3E, F, and G). Collectively, our data indicates that the WNT gradient is a key determinant in establishing the regional identity of brain organoids and that a specific concentration of WNT (3 μM) facilitates the generation of homogeneous MOs.

4. DAC3.0 MOs are structurally and functionally mature

Throughout our optimization steps for the generation of MOs, we were able to generate MOs that

homogeneously contain TH-positive mDA neurons using DA and 3 μM CHIR99021 (hereafter DAC3.0 MOs). To address the structural homogeneity of DAC3.0 MOs, we next analyzed the structure of these MOs by immunohistochemistry. DAC3.0 MOs after 1WM showed early-stage progenitors expressing either ASCL1 or LRTM1 (Fig. 4A). DAC3.0 MOs after 4WM displayed a well-defined laminated structure containing a $FOXA2^+$ (Fig. 4A) ventricular zone, an $ASCL1^+$ (Fig. 4A)/$LMX1A^+$ (Fig. 4B) intermediate zone, and a TH^+/$MAP2^+$/DAT^+ (Fig. 4B) marginal zone, indicating that DAC3.0 MOs are structurally similar to the developing midbrain (Fig. 4C and S4A, Supplementary video1). Gene expression profiling by RNA-seq showed enhanced expression levels of maturation-related genes, indicating the gradual maturation of DAC3.0 MOs (Fig. S4B and C). DAC3.0 MOs were found to homogeneously contain TH-positive mDA neurons but not to express cerebral cortex markers FOXG1 and TBR1 (Fig. S4D). Moreover, DAC3.0 MOs (8WM) were found to contain mature mDA neurons expressing NURR1, GIRK2, and CALB (Fig. 4D and S4E). Transmission electron microscopy (TEM) analysis showed a typical apical-basal differentiation pattern with abundant neuronal bundles in the marginal zone and cells tightly connected in the ventricular zone (Fig. 4E). Taken together, our structural analysis indicates that DAC3.0 MOs are structurally mature and similar to the developing midbrain.

To investigate the functionality of DAC3.0 MOs, we assessed the production of dopamine. DAC3.0 MOs began producing dopamine at 12WM, with the highest dopamine level found at 20WM (Fig. 4F). Moreover, DAC3.0 MOs were found to be electrically active, as evidenced by the action potential elicited by neurons in DAC3.0 MOs (Fig. 4G and S4F, G). Notably, neuromelanin-like granules, which accumulate in the substantia nigra pars compacta in humans, were found to accumulate homogeneously in DAC3.0 MOs (Fig. 4H). Fontana-Masson staining showed that neuromelanin-like granules accumulate in the outer layer of DAC3.0 MOs, an area enriched for functional mDA neurons, but not in cerebral organoids that had maturated during the same periods (Fig. 4I), suggesting that mDA neurons from DAC3.0 MOs might produce

neuromelanin-like granules. TEM analysis revealed the presence of neuromelanin-like granules in DAC3.0 MOs (Fig. 4J and S4H). Collectively, our data indicates that DAC3.0 MOs are as structurally and functionally mature as the developing midbrain.

5. DAC3.0 MOs display cell type composition similar to that of midbrain

Midbrain contains not only mDA neurons, a functional cell type representative of the midbrain [47], but also several other cell types, such as different neuronal subtypes, astrocytes, and oligodendrocytes [48], that exert roles in maintaining the functional homeostasis of the midbrain [49-51]. Thus, we next investigated the cellular composition of DAC3.0 MOs. We found that both GABAergic and mDA neurons are intimately co-located in most of the rosettes (Fig. 5A), like in midbrain, where GABAergic neurons closely interact with mDA neurons in the substantia nigra, ventral tegmental area, and rostromedial tegmental nucleus [50]. In contrast, DAC0 MOs (0 μM CHIR99021) and NS-MOs (0.8 μM CHIR99021) exhibited heterogeneous distribution of GABAergic neurons. Indeed, most of the rosettes in NS-MOs produced exclusively either TH- or GAD67-positive neurons (Fig. S5A) and most of the rosettes in DAC0 MOs produced mainly GABAergic neurons (Fig. S5B). Moreover, we found glutamatergic neurons in DAC3.0 MOs (Fig. 5B and S5C).

Although a previous study had described the generation of functionally mature MOs, as shown by the MOs' mature electrophysiological ability and production of neuromelanin-like granules [36], the presence of mature glial cell types such as astrocytes and oligodendrocytes in MOs has been poorly described [52]. First, we performed RNA-seq analysis and found that both astrocyte and oligodendrocyte markers were highly enriched in mature DAC3.0 MOs (Fig. 5C and S5E, F). Notably, neuronal genes were activated earlier in DAC3.0 MOs than were markers for both astrocytes and oligodendrocytes (Fig. 5C and S5D), as in the developing midbrain, where neurogenesis precedes gliogenesis [53]. Moreover, we observed numerous glial cells in our mature DAC 3.0 MOs. After 8WM, we detected both astrocytes (Fig. 5D and E) and

oligodendrocytes (Fig. 5F and G) expressing multiple cell type–specific markers (Fig. S6A and B). DAC 3.0 MOs also exhibited morphology typical of both astrocytes (Fig. 5H and S6C) and oligodendrocytes (Fig. 5J and S6E). Our TEM analysis clearly showed functional astrocytes containing glycogen granules in contact with neuronal axons (Fig. 5I and S6D). Moreover, we found oligodendrocytes that show myelination, like their *in vivo* counterparts (Fig. 5K and S6F). Taken together, our data indicates that DAC3.0 MOs have a similar cellular composition to the midbrain, with multiple functional cells types.

6. DAC3.0 MOs with functional astrocytes facilitate dopaminergic neurotoxin–based *in vitro* modeling of Parkinson's disease

Parkinson's disease (PD), a prevalent neurodegenerative disease [54], is caused primarily by the death of mDA neurons [55]. However, the mechanisms underlying this selective cell death in the brain of patients with PD remain largely unknown. To replicate the neuropathology of PD, many previous studies had used animal models with a variety of dopaminergic toxins [56]. Among them, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is known to be the most reliable and frequently used toxin due to its ability to stably induce clinical symptoms that are indistinguishable from PD [57,58]. After crossing the blood-brain barrier, MPTP is converted first into 1-methyl-4-phenyl-2,3-dihydropyridinium (MPDP) by monoamine oxidase B (MAO-B) and then into 1-methyl-4-phenylpyridinium (MPP^+) in astrocytes [58]. The MPP^+ released into the extracellular space could bind to the dopamine transporter (DAT) and gain entry into mDA neurons [59]. As the concentration of MPP^+ in the mitochondria increases [60], oxygen free radicals are produced and ATP synthesis decreases [61,62]. Since astrocytes play a key role in the mode of action of MPTP [58,63], MPTP, a representative and reliable dopaminergic neurotoxin, has not been properly used in previous iPSC-based *in vitro* modeling studies of PD. Instead, previous studies [64-66] have treated iPSC-derived mDA neurons with MPP^+, the activated form of MPTP, to evaluate the toxic effect of MPTP, as neuron-to-glial cell interactions cannot be

replicated by 2D differentiation technology.

As DAC3.0 MOs contain a large number of glial cells, such as astrocytes, we next investigated whether DAC3.0 MOs could replicate the neuropathology of PD upon treatment with MPTP. To this end, we treated DAC3.0 MOs (8WM) with four different concentrations of MPTP (0, 10, 50, and 100 μM) for 48 hrs and assessed MPTP-mediated cell death (Fig. 6A). MPTP-treated DAC3.0 MOs exhibited massive cell death, as evidenced by the increased numbers of both Caspase-3– and TUNEL-positive cells (Fig. 6B and C). Moreover, the number of apoptotic cells increased in a dose-dependent manner (Fig. 6D and E), indicating that the cell death induced in DAC3.0 MOs is mediated solely by MPTP treatment. Notably, MPTP-mediated cell death is observed largely in TH-positive mDA neurons (Fig. 6F and S7A) but rarely in other cellular components, such as GABAergic neurons, astrocytes, and oligodendrocytes (Fig. 6F and S7B). Taken together, our data indicates that DAC3.0 MOs produced by our optimized protocol contain functional glial cells that facilitate the action of MPTP, a representative dopaminergic neurotoxin, which could be used for the *in vitro* modeling of PD.

Discussion

Our understanding of human diseases depends mainly on pathological outputs of internal organs, without having a fundamental understanding of the underlying etiology. Emphasis has been placed on establishing *in vitro* disease modeling systems that could effectively reproduce the disease pathophysiology. iPSC technology has provided a novel concept for patient-specific disease modeling. However, current 2D differentiation protocols are insufficient for closely replicating *in vivo* situations because 2D conditions typically produce a singular differentiated target cell type without forming the *in vivo*–like tissue architecture composed of multiple cellular components.

Recent organoid technology has opened up a new avenue for *in vitro* modeling of various diseases and understanding early development even at the organ level. As organoids theoretically contain multiple cell types with a similar structure to their *in vivo* counterparts, the potential exists to achieve more precise and reliable *in vitro* disease modeling for various diseases. Indeed, in many cases, the cause and progression of neurodegenerative diseases are mediated by multiple cellular components in organs [67,68]. However, it has not been described whether organoids from patients could sufficiently mimic the *in vivo* disease pathology, which is caused by tight crosstalk between distinct cellular components. In the current study, we describe the generation of DAC3.0 MOs that exhibit structural and functional similarities to the midbrain, *in vivo*, as evidenced by their laminated structures (Fig. 4A and B), homogeneous distribution of mature mDA neurons (Fig. 3H and 4D), production of dopamine (Fig. 4F), electrophysiological activity (Fig. 4G), and production of neuromelanin-like granules (Fig. 4H, I, and J). Most importantly, DAC3.0 MOs display midbrain-like cellular makeup with multiple functional cells types (Fig. 5, S5, and S6), such as astrocytes and oligodendrocytes. Therefore, to address whether DAC3.0 MOs can recapitulate the phenotype of PD mediated by crosstalk between distinct cellular components, we treated these organoids with MPTP, a dopaminergic neurotoxin that causes the selective cell death of mDA neurons via functional astrocytes [58,63]. Upon treatment of DAC3.0 MOs with MPTP,

we observed that mDA neurons underwent cell death, indicating that brain organoid technology could even recapitulate *in vivo* pathophysiology mediated by cell-to-cell interactions—i.e., between astrocytes and mDA neurons.

Organoid technology presents an advanced concept of disease modeling and drug screening, as it could replicate the *in vivo* scenario at higher resolution compared to conventional 2D differentiation methods. However, the low generation efficiency, heterogeneous structures, and biased or limited cellular compositions of the resultant organoids have been critical hurdles for the clinical translation of organoid technology. To overcome these issues, we first tried to optimize the early mesencephalon specification step by comparing distinct combinations of dual SMAD inhibitors. Moreover, by creating an *in vitro* WNT gradient, we fine mapped specific concentrations of WNT that facilitate the generation of structurally and functionally homogeneous MOs (DAC3.0 MOs). Further efforts for generating DAC3.0 MOs in a high-throughput drug screening platform are urgently required for the clinical translation of organoid technology. Our optimized protocol for robustly producing homogeneous MOs might be useful for the precise *in vitro* modeling of PD and the efficient screening of drug candidates for PD.

Conclusion

In the current study, we described the novel strategy for the robust generation of homogeneous MOs, namely DAC3.0 MOs using specific combination of dual SMAD inhibitors and *in vitro* WNT gradient. DAC3.0 MOs exhibit structural and functional similarities to the midbrain, *in vivo*, as shown by their laminated structures, homogeneous distribution of mature mDA neurons, robust production of neuromelanin-like granules, and midbrain-like cellular makeup with functional glial cells. Moreover, DAC3.0 MOs can recapitulate *in vivo* pathophysiology of PD mediated by crosstalk between distinct cellular components.

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Author Contributions

T.H.K. and D.W.H.: conception and design; J.H.K., S.H., J.K., C.P., J.L., H.K.R., J.E.N, J.K.: performed the experiments; K.K., J.J., H.S.J., H.N., N.K., K.H.H., W.S., C.H.C., I.J.R.: data analysis; D.W.H.: manuscript writing, final approval of manuscript.

Disclosure of Potential Conflicts of Interests

The authors indicated no potential conflicts of interests.

Figure legends

Figure 1. Robust specification into mesencephalon by specific dual SMAD inhibitors

(**A**) Schematic illustration of the procedure for generating MOs. The generation of MOs can be achieved by a sequential protocol that includes mesencephalon specification, mesencephalic floor plate induction, basal-apical lamination, and maturation.

(**B**) The number and quality of EBs generated from distinct combinations of dual SMAD inhibitors. Data are presented as mean ± SD from three independent experiments.

(**C**) Expression of pluripotency, neuroectoderm, mesendoderm, and apoptosis markers was analyzed by qPCR using EBs grown under distinct dual SMAD inhibition conditions (7 days). Expression levels are normalized to those of undifferentiated hESCs. *$p < 0.05$.

(**D**) Morphology and average diameter of MOs (2WM) generated with distinct combinations of dual SMAD inhibitors. Data are presented as mean ± SD from three independent experiments. Scale bar, 500 μm.

(**E**) Representative confocal images of NS-, DA-, and LS-MOs (2WM) expressing TH, MAP2, and ASCL1. Scale bar, 50 μm.

(**F**) Expression of forebrain- and midbrain-specific marker genes was analyzed by qPCR in a time-course manner, on days 7, 14, and 21 after differentiation. Expression levels are normalized to those of undifferentiated hESCs. Data are presented as mean ± SD of triplicate values. *$p < 0.05$.

Figure 2. Structural homogeneity of DA-MOs.

(**A**) Confocal images showing distribution of mDA neurons expressing both MAP2 and TH in MOs grown under distinct conditions (NS-, DA-, and LS-MOs) after 4WM. Scale bar, 100 μm.

(**B**) Percentage of TH-positive mDA neurons. Data are presented as mean ± SD from three

independent experiments. ***$p < 0.001$.

(**C** and **D**) Confocal images showing expression pattern of the midbrain markers LMX1A and TH (**C**) and cerebral cortex markers TBR1 and TUJ1 (**D**) in NS-, DA-, and LS-MOs (4WM). Scale bar, 100 μm.

(**E**) The Integrative Genomics Viewer (IGV), a basic RNA-seq processing tool displaying expression patterns of the cerebral cortex marker FOXG1 and the midbrain marker LMX1A in day-7 EBs.

(**F**) Multiple section images of whole mount confocal microscopy showing the distribution of TH-positive mDA neurons in MOs grown under distinct conditions (4WM). Scale bar, 100 μm.

Figure 3. WNT gradient is a key determinant of homogeneous MO generation.

(**A**) Schematic illustration depicting the procedure of generating homogeneous MOs by using an *in vitro* WNT gradient.

(**B**) Heatmap representing the global gene expression profile of MOs (3WM) generated by the *in vitro* WNT gradient. The red-blue color scale is the normalized expression value, denoted as the row Z-score. Red and blue colors indicate increased expression and decreased expression, respectively. Differentially expressed genes among each sample are categorized into five groups (class 1 to 5).

(**C**) GO enrichment analysis based on the gene set highly enriched in class 1.

(**D** and **E**) Expression of forebrain- (**D**) and midbrain-specific marker genes (**E**) was analyzed by qPCR in DA-MOs with different concentrations of CHIR99021 (2WM). Expression levels are normalized to those of non-treated DA-MOs. Data are presented as mean ± SD of triplicate values. *$p < 0.05$.

(**F** and **G**) Heatmaps representing the expression patterns of markers related to the midbrain (**F**) and forebrain (**G**) using DA-MOs produced by the *in vitro* WNT gradient (3WM). Color bar at the bottom indicates gene expression in $\log_2$ scale. Red and green colors represent higher and

lower expression levels, respectively.

(**H**) The representative section images of whole mount confocal microscopy displaying global enrichment of TH-positive mDA neurons in DA-MOs with the *in vitro* WNT gradient (3WM). Scale bar, 200 μm.

Figure 4. Structural and functional maturity of DAC3.0 MOs.

(**A** and **B**) Confocal images showing micro-anatomical structures of early- (**A**) (1WM) and late-stage (**B**) (4WM) DAC3.0 MOs. Scale bar, 50 μm.

(**C**) Illustration describing the layer structure of developing *in vivo* midbrain.

(**D**) Confocal images displaying markers of the mature mDA neurons NURR1, GIRK2, and Calbindin. Scale bar, 25 μm.

(**E**) TEM images of cross-sectional and longitudinal-sectional views of an axon bundle in the marginal zone and adherent junctions (arrows) in the ventricular zone of DAC3.0 MOs (2WM). Scale bar, 2 μm.

(**F**) Levels of dopamine produced by DAC3.0 MOs were measured by high performance liquid chromatography (HPLC) 7 days after differentiation (12WM, and 20WM). *$p < 0.05$.

(**G**) Representative traces of multiple action potentials (Aps) recorded from neurons in DAC3.0 MOs (8WM), evoked by current injection.

(**H**) Accumulation of neuromelanin-like granules in DAC3.0 MOs were observed at 10WM. Pigmented cytoplasm of neurons (arrows) and neuromelanin-like aggregates (arrowheads) are shown in DAC3.0 MOs. Scale bar, 20 μm.

(**I**) Fontana-Masson staining of DAC3.0 MOs. Cerebral organoid was used as a negative control. Scale bar, 50 μm.

(**J**) TEM images displaying the fine structure of neuromelanin-like granules (arrowheads) in DAC3.0 MOs. Scale bar, 1 μm.

Figure 5. *In vivo*–like cell type composition of DAC3.0 MOs.

(**A**) Confocal images representing neural lobes containing TH-positive mDA neurons and GAD67-positive GABAergic neurons in DAC3.0 MOs (8WM). Scale Bar, 100 μm.

(**B**) A magnified view of GABA-positive GABAergic neurons and GLUT-positive glutamatergic neurons in DAC3.0 MOs (8WM). Scale bar, 25 μm.

(**C**) Heatmaps representing the expression patterns of markers related to astrocytes and oligodendrocytes in DAC3.0 MOs (3WM, 8WM, and 20WM). Color bar at the left bottom indicates gene expression in $\log_2$ scale. Red and green colors represent higher and lower expression levels, respectively.

(**D**) Confocal images showing the global distribution of astrocytes expressing GFAP in DAC3.0 MOs (8WM). Scale bar, 100 μm.

(**E**) Percentage of GFAP-positive astrocytes in DAC3.0 MOs. Data are presented as mean ± SD of triplicate values.

(**F**) Global distribution of oligodendrocytes expressing MBP in DAC3.0 MOs (8WM). Scale bar, 100 μm.

(**G**) Percentage of MBP-positive oligodendrocytes in DAC3.0 MOs. Data are presented as mean ± SD of triplicate values.

(**H**) Representative image of astrocytes expressing GFAP/S100β (left panel) and morphology of single astrocyte (right panel). Scale bar, 25 μm.

(**I**) TEM images displaying direct connection between astrocyte containing glycogen granules and neuronal axon (Ax). Scale bar, 2 μm.

(**J**) Morphology of oligodendrocytes expressing MBP located close to TH-positive mDA neurons. Scale bar, 25 μm.

(**K**) TEM images showing myelinating oligodendrocytes in DAC3.0 MOs. Scale bar, 2 μm.

Figure 6. MPTP-based *in vitro* modeling of Parkinson's disease using DAC3.0 MOs.

(**A**) Schematic illustration of MPTP assay using DAC3.0 MOs.

(**B** and **C**) Confocal images showing Caspase-3 expression (**B**) and the presence of TUNEL-labeled mDA neurons (**C**) in MPTP-treated DAC3.0 MOs (0, 10, 50, and 100 μM). Scale bar, 100 μm.

(**D** and **E**) Percentage of Caspase-3–positive (**D**) and TUNEL-positive (**E**) mDA neurons in MPTP-treated DAC3.0 MOs. Data are presented as mean ± SD of triplicate values. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

(**F**) mDA neuron–specific cell death by MPTP treatment. TUNEL-positive cells were barely observed in GABAergic neurons (GABA), astrocytes (AQP4), and oligodendrocytes (PLP). Scale bar, 25 μm.

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Legend for graphical abstract

This study describes the novel strategy for the robust generation of homogeneous MOs using specific combination of dual SMAD inhibitors and *in vitro* WNT gradient. DAC3.0 MOs generated by our optimized protocol with *in vivo*-like cellular composition are as structurally and functionally mature as the developing midbrain. DAC3.0 MOs with functional glial cells including astrocytes facilitate MPTP-based *in vitro* disease modeling of PD, suggesting its potential usefulness for an advanced patient-specific platform for *in vitro* disease modeling as well as for drug screening for PD.