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Generation of Dopaminergic Neurons from iPSCs for Parkinson's Cell-Based Therapies and Functional Assessment Methods Evaluation

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Abstract

Dopamine is a neurotransmitter or a chemical messenger that is primarily responsible for emotions, motivation, and most importantly, smooth, coordinated movement. In Parkinson's Disease, dopaminergic neurons are degraded, causing a shortage of dopamine within the human body. With current pharmacological treatments, their ability to alleviate symptoms temporarily is necessary for combating PD, but they do not reverse dopaminergic neuron loss. To further study its pathological mechanisms, stem-cell therapies are widely used to generate dopaminergic neurons in vivo using induced pluripotent stem cells. With their pluripotency and self-renewal capabilities obtained using Oct3/4, Sox2, c-Myc, and Klf4, these cells are manipulated to a dopaminergic neuronal fate using transcription factors such as Lmx1a, FoxA2, Nurr1, and Tyrosine Hydroxylase (TH). Other procedures, such as utilizing dual SMAD inhibition or using 3D organoids for their maturation into neuron progenitor cells, are also widely used as other alternatives, with each method displaying key differences or protocols. This study aims to

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analyse in vitro validation techniques and in vivo functional assessments, such as microdialysis and the rotation of 6-OHDA rodents, assessing their utility and limitations with safety and efficacy, and ethical complexity. Finally, future research should continue to discover more about methods to underscore its therapeutic potential and continue to address potential inaccuracies with each variable implemented.

Keywords: dopamine, Parkinson's disease, neuron, pluripotent stem cells, therapeutic potential

Introduction

Parkinson's Disease is a progressive neurological disorder that affects approximately 10 million people worldwide, with a prevalence rate of about 1% in individuals 60 years and above (Bjørn Tysnes & Storstein, 2017). Its incurable and degenerative nature, actively dissociating neurons within patients' central nervous system, positions itself as a major focus within the medical research community. Although predominant in a certain age group, it is characterized by being insufferable; its devastating effects on motor functions, inability to conduct automatic reflexes (bradykinesia), and abnormal activity will continue to follow modern society's changes, remaining one of many non-communicable diseases (NCDs) directly targeting our brain.

The main explanation for the development of Parkinson's Disease lies within the dopaminergic neurons near the base of the brain in the substantia nigra. These neurons are responsible for the synthesis of dopamine, a crucial neurotransmitter that influences motor function and cognitive ability as it releases and transmits signals in the brain to conduct smooth, resolved movements. Although the cause of PD remains unknown, neurodegeneration is not

limited to dopaminergic neurons but also intracellular inclusions in the substantia nigra -dorsal to the cerebral peduncles (crus cerebri) within the midbrain. Scientists hypothesize that α-synuclein-containing Lewy bodies disrupt cellular functions within neurons and are associated with the malfunction of the Nigrostriatal pathway that is critical for facilitating movement, leading to Parkinson's. These abnormal aggregations of proteins catalyze the decomposition of neurons and are also the main initiators of Alzheimer's.

Additionally, the death of dopaminergic neurons activates glial cells such as microglia, which are hypothesized to take up cellular debris, instinctively triggering cytokine responses. Neighbouring microglia and astrocytes have been shown to injure neurons, leading to further neurodegeneration within the substantia nigra.

Within the current treatments for PD, it constitutes temporal management of motion symptoms. However, it does not entirely resolve dopaminergic deterioration due to external factors. The most widely used therapy, levodopa, is administered to synthesize more dopamine, offering temporary relief but failing to address the underlying loss of dopaminergic neurons. Gradually, patients will experience diminishing responsiveness to medications, possibly sustaining other unwanted therapeutic effects such as dyskinesia. Thus, implementing a suitable, long-term, disease-modifying approach in resolving PD is crucial. In recent years, stem cell-based therapy has emerged as a promising alternative, particularly through the use of induced pluripotent cells to synthesize fully functional dopaminergic neurons, mimicking their functions within the basal ganglia, altering the course of PD.

The Princeton Journal of Interdisciplinary Research
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The development of neural stem cells (NSCs), a multipotent type of stem cell, is a notable process, as they can subsequently differentiate into dopaminergic neurons. An alternative to embryonic stem cells (ESCs) was founded by Shinzo Yamanaka and Kazutoshi Takahashi, who introduced induced pluripotent stem cells (iPSCs) from somatic cells under ES cultures and reprogramming factors (Oct3/4, Sox2, c-Myc, and Klf4). Due to its undifferentiated nature, excellent self-renewability, and elimination of ethical risk, medical advances broaden the possibility of not only synthesizing endless iPC-derived somatic cells in vitro to develop cell-based therapies, but also reducing the risk of immune rejection between allogeneic transplants.

For the development of hESCs (Human embryonic stem cells), ensuring that iPSCs cell culture systems are well developed to naturally possess their differentiation capabilities: gene expression profiles, epigenetic marks, differentiation potential, and high self-renewability. Moreover, the application of iPSCs is aimed at providing therapeutic potential, exerting effectiveness once differentiated in regenerative medicine, with research preventing minimal side effects such as incomplete tissues or the risk of teratoma formation. Hence, its pluripotency - capability to differentiate into ectoderm, mesoderm, and endoderm- is pivotal for the start of developing iPC-derived dopaminergic cells to combat one of the main neurological disorders.

Comparing the differences between embryonic stem cells (ESCs) and iPSCs, ESCs are derived from the inner cell mass of a blastocyst, while iPSCs are reprogrammed adult somatic cells to develop their pluripotency. Highlighting ethical concerns, ESCs are controversial due to the destruction of human embryos; however, when comparing tumor risks, the frequency of

karyotypic abnormalities in iPSCs seems to be higher than in ESCs, with anecdotal evidence emphasizing that teratomas are prone in iPSCs due to reprogramming.

To ensure that iPSCs can efficiently differentiate into fully functioning somatic cells, most are directed to differentiate into neural progenitor cells with the exposure to dual SMAD inhibition. By blocking the two signaling pathways, BMP and TGF-β, it promotes efficient neural induction and is directed towards a neural fate.

Although iPSCs' therapeutic potential has been explored in animal models, particularly in mice, by successfully inducing stem cells to form other types of neurons, the effects of applying and mimicking procedures with iPSCs remain largely unknown (Wernig et al., 2008). Moreover, induced neural cells often show inconsistent expression of key midbrain markers, limiting their clinical potential. Ultimately, it suggests variable identity or mispatterning within cells, limiting clinical applications and the replicability of actual dopaminergic cells while increasing the risk of tumorigenesis. However, animal models also have limitations, such as their limited genotypic variability, high cost, ethical implications, and the high complexity of the human brain, leading to different therapeutic effects.

This study not only aims to discover more about the possibilities and reasoning for implementing iPSCs sourced from different case studies, in hopes of creating an evaluation and an analysis of cell behaviour, methodologies in vitro and vivo, manufacturing practice requirements, ethical viewpoints, safety regulations, and their progress towards therapies in PD.

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Methodology

Derivation Of Ipscs

Human induced pluripotent stem cells (hiPSCs) were sourced from the collection of somatic cells, with most existing research extracting skin biopsies, peripheral blood samples from healthy individuals, or urine epithelial cells. This study heavily relied on secondary data published in reputable scientific journals, laboratory protocols, and peer-reviewed research findings, which were recommended by a Harvard professor studying regenerative medicine and stem cells. Papers were reviewed for four weeks before this analytical research paper was conducted.

An analysis shows that participants were required to consent, acknowledging that some experiments may pose risks in surgical stimulations (Schweitzer, 2020). According to Takahashi and Yamanaka. (2006), the reprogramming factors OCT4, SOX2, KLF4, and c-MYC initiate the transition from an originally differentiated cell type to a pluripotent-like state.

A variety of gene editing techniques were implemented in most research studies, including integrating and non-integrating methods. In a case, Sameehan Mahajani, who is the author of a study regarding Homogenous generation of dopaminergic neurons from multiple hiPSC lines by transient expression of transcription factors, used a healthy-free gingival fibroblast to generate iPC lines by Sendai virus reprogramming; it efficiently delivers reprogramming genes without entering the genome. Furthermore, some sources also combine

microRNAs with reprogramming factors to effectively and equally generate quality-controlled iPSCs for further integrations.

Induction Of Dopaminergic Differentiation

The sources used have shown the relevance in ensuring normal karyotypic traits, with several sporadic PD and familial PD iPSC lines acting as a control. To mimic the full functions of neural cells, several protocols have been conducted. The cells were cultured in a medium of different concentrations of pharmacological compounds, including knockout serum replacement, l-Glutamine/GlutaMAX, with SMAD inhibitors such as LDN193189 and SB431542, actively accelerating the process of inducing neural fates within iPSC colonies. Although these methods were emphasized most of the time, DA progenitor cells may be restored or isolated using floor plate marker cells (CORIN+), proven to be relevant in differentiating into dopaminergic neurons within the midbrain section (Sawamoto et al*.,* 2025).

Early Neural Markers

To confirm the stable progression of iPSC colonies into DA progenitor identity, a stepwise evaluation of molecular markers was performed, including but not limited to: Single-cell quantitative PCR with reverse transcription (RT–qPCR) analysis (Sawamoto, 2025), verification by the upregulation of early neuroectodermal markers such as SOX1, PAX6, NESTIN (Momcilovic et al., 2016).

Midbrain Patterning Markers

Furthermore, a trend was commonly observed within days 7-20, depending on the progression of iPSC colonies from different articles, as culture systems were constantly changing due to the addition of Sonic Hedgehog (SHH C2H5ii) in mice samples; this promotes ventral identity, similar to the dopaminergic neurons that originate. The resulting populations after day 20 were further matured with BDNF, GDNF, and ascorbic acid to generate neurons expressing markers such as LMX1A, FOXA2, NURR1, etc.

Lmx1a, a transcription factor, is often utilized in studies of stem cell differentiation into somatic cells, such as embryonic stem cells (ESCs), to acquire a true midbrain dopaminergic (mDA) fate (Cai et al., 2009). One of its key features in contributing to that fate is by specifying ventral midbrain identity during early neurodevelopment, as it indicates proper patterning and further initiation of the mDA lineage. Alongside Lmx1a, FoxA2 similarly maintains the midbrain phenotype and characteristics by initiating floor patterning, or functioning as a floor plate marker, properly aligning regional identity, similarly to the substantia nigra for iPSCs to differentiate into DA neurons.

Following regional identification, Nurr1 is both transcription factors that specialize in the maturation of the mDA lineage into DA neurons, vital for the maintenance and differentiation of iPSCs, and regulation of gene expressions involved in the neurotransmitter dopamine.

These markers are commonly positive 7 days after the detection of neuroectodermal markers, representing early midbrain patterning markers, which indicate a ventral midbrain DA fate (Kriks et al., 2011).

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Dopaminergic Neuron Markers

As midbrain neurons mature, DA-specific markers are analyzed. One of the major markers includes Tyrosine hydroxylase, which is a key enzyme for dopamine production from synaptic vesicles in the axon. The nuclear receptor NURR1 (NR4A2) is essential for the DA neuron phenotype; immunostaining for Tyrosine hydroxylase, a vital rate-limiting enzyme in dopamine biosynthesis, and NURR1 is responsible for confirming bona fide midbrain DA neurons (Kriks et al., 2012; Bin Song et al., 2019).

Additionally, a separate study created iPSC-derived organoids from PD patients and healthy patients (Chong-Lei Fu, 2023). This alternative showcases medicinal benefits for further drug discovery, disease modelling, and many other potential applications. However, its extended timespan of approximately 60 days for slow differentiation requires immunofluorescence analysis of cell markers and plate marker cells to ensure a stable development.

Electrophysiological recordings, such as patch-clamp techniques, were also employed to evaluate functional integrity by producing high-resolution images of the neurons' synaptic activity. As a result, this assessment further confirms their maturation and excitability. An example given by a group of researchers was conducted in an external laboratory, where they showed necessary electrophysiological characterization, including a controlled variable of a temperature chamber, and a low-pass filter at 3 kHz - 10 kHz with patch electrodes and their specific electrical resistance (Gilmozzi et al., 2021).

Overall, this research paper directly discusses only a few out of many more methods in the induction process, with different variables and periods from a branched set of sources collected, but many were successful in generating either early neurons or matured neurons capable of producing dopamine with their relative evidence all connected with the expression markers mentioned above.

In Vivo Procedures

With the already produced and recorded dopaminergic neurons derived from iPSCs, applications within models are a fundamental next step for a deeper understanding. Following in vitro processes, the neuronal cells were cultured to monitor potential differentiation while controlling certain variables that may affect the growth of these cells, such as nutrient availability and oxygen levels. Most importantly, before directly injecting it into a patient/model, undifferentiated or unstable cells may cause issues such as tumor formation.

Following the successful validation of iPSC-derived DAs, with the help of underlying research, scientists have started to tailor in vivo transplantation to assess their therapeutic potential. Deciphering the causes of dopaminergic neurodegeneration within animal models may lead to further molecular pathology analysis. Hence, this phase involves many to use lab rats or rodents, with and without a PD history. Although ethical concerns remain a challenge, this step is necessary for the development of future regenerative medicine, with its target of effectively assessing safety and efficacy surrounding iPSCs. However, several questions must be prioritized before conducting the striatal/substantia nigra transplantation. i) Do the iPSC-derived DAs

effectively restore the lost dopamine levels in vivo? ii) Which cells will instinctively produce the DAs, the iPSCs, or the other repaired neural cells by the transplantation?

These questions were notably a key element in understanding the procedures and optimizing for accurate representations with a fair comparison. Answered themselves by Stanford medicine students, Xinjiang Kang, Huadong Xu, Sasa Teng, and Zhuan Zhou, they reported that, "Although electrical impulses were present and rescued using similar therapies, amperometry/chronoamperometry alone is unable to distinguish DA from other monoamines in vivo, like norepinephrine (NE) and serotonin."

Moreover, a clash between different sources suggests that DA is most likely to be released from grafted cells (LM Bjorklund et al., 2002), while others suggest that the grafted stem cells may restore striatal DA levels by regenerating neural tissues (Ourednik et al., 2002; Redmond et al., 2007). Hence, question 2 must be investigated further.

In vivo transplantations often need biochemical and behavioral assays to evaluate the DA neurons. Some include an apomorphine-induced rotational test in 6-hydroxydopamine (6-OHDA)-lesioned models, assessing dopamine depletion by measuring rotational behaviour. With a fall in rotations, it implies functional recovery; microdialysis and HPLCs are also applied to measure dopamine levels within the medial forebrain bundle (MFB).

Lee & Kwon. (2022) demonstrated that microdialysis acts as a process to sample free unbound chemical substances from a medium. In other words, neurotransmitters such as dopamine, in this case, are continuously perfused through a semi-permeable membrane and

isolated from other particles, confirming DA levels within the neurons. One of its major advantages is its sensitivity and specificity in detecting neurochemical differences, making it extremely effective in affirming dopamine from the transplanted neurons. Under the monitoring of physiological conditions, it is sensitive to various stimuli (pharmacological agents). However, its major disadvantage is its process, as it requires a surgical probe implantation, which poses an extreme risk, especially if in vivo processes are conducted on a patient. The risk of tissue damage or accidental inflammation is high, with temporal resolution being relatively low. Inaccurate measurements may also be produced due to a delay in diffusion and collection. Furthermore, generalized results may be challenging as they sample from a limited brain area (striatum). Although these risks must be considered, microdialysis remains a relevant, standardized method in validating the neurochemical function of substances such as the iPSC-derived DA neurons within clinical models of Parkinson's disease.

According to MDBiosciences, lesion size within one side of the nigrostriatal pathway may influence motor behavioral impairments. Due to the dopamine depletion, the 6-OHDA rats would show asymmetric movements, particularly in rotating towards one direction, which is towards the lesioned side. The experiment starts with the animal placed in a round open arena after transplantation, where it rotates towards the lesioned side as the intact side releases dopamine, stimulating motor activity, while the lesioned side is unable to respond. This practice not only secures consistency and an observable variable to measure, but it also correlates with the severity of dopamine depletion. As a result, linking back with iPSC-derived DA neurons, a

reduction in the number of rotations emphasizes that dopamine function has been restored, directly assessing functional integration and efficacy of transplanted neurons in vivo.

This rotational test is classified as *ipsiversive rotation* because administration of external DA neurons into the striatum presynaptically stimulates the grafted terminals, enhancing the area where DA levels were originally low. In contrast, if Apomorphine or L-DOPA were used as dopamine receptor agonists, they act as drugs that activate dopamine receptors in the lesioned side. As a result, it causes the animal to rotate away from the lesioned side (contraversive rotation).

The advantage of implementing the rotation test for behavioural assays is that, firstly, it is simple and quantifiable, as the number of rotations can be easily measured and analyzed, providing a clear representation of results depending on the independent variables. Secondly, it is sensitive to unilateral lesions such as 6-OHDA lesions, which mimic Parkinson's disease-related dopamine depletion on the lesioned side of the striatum. Finally, one of the major advantages within the laboratory is the cost-effectiveness of the setup, as it requires minimal equipment — often just a rotation bowl with the animal inside. However, applying human models will differ, as the rotation test is ineffective in bilateral PD models. Furthermore, it is difficult to distinguish and reflect complex motor functions due to the measurement of rotational behavior.

Safety Considerations

Establishing safety protocols within the process of iPSC derivation, differentiation, and transplantation in animal models proves pivotal due to the clinical potential of the cells in

treating Parkinson's disease and the potential risks following the complex procedures. One already mentioned risk includes teratoma formation, which may occur if the undifferentiated iPSCs uncontrollably divide, forming malignant tumours at various stages in vivo. Hence, scientists and clinicians should be able to develop ways to mitigate this risk factor, such as using stable and quality-controlled markers (Oct4 and Nanog), or even by thoroughly reexamining reagents and assays used to lower percentage errors during the experiment.

Another key safety consideration involves genetic instability during reprogramming cultures, which can lead to karyotypic abnormalities and mutations. Researchers struggle to maintain genetic stability during in vivo procedures, as it poses a dangerous risk to human models if implemented. Although this study mainly focuses on 6-OHDA lesioned models, such as roaches or mice, it is relevant to discuss all possible in vivo examinations in most methods used.

Elaborating further on human models, scientists often encounter immunogenicity, which is defined as activating the patients' immune system to fight or respond to foreign substances. In this case, it refers to the iPSC-derived DA neurons. Although generally somatic cells were extracted from the same patient, or the term autologous, reprogramming mutations or culture conditions may still contribute to evoking immune responses, making immunosuppressive strategies like tacrolimus administration often necessary.

In response, insertional mutagenesis, involving integration of retroviral DNA, may alter the activity of nearby genes, sometimes affecting subsequent cell growth (Bushman., 2020).

Normal cell activity, differentiation pathways in the striatum and substantia nigra, cell proliferation from a tumor suppressor gene, and the skewing of data may all contribute to inefficiency and unexpected outcomes, creating possible anomalies and misinterpretation of results. Non-integrating vectors, such as Sendai virus or episomal plasmids, have recently been a favourite in studies, simultaneously screening for insertion sites and removing undifferentiated or genetically unstable cells before application in vivo.

Discussion

This study aimed to explore the different therapeutic approaches in creating a promising avenue in regenerative medicine for Parkinson's disease (PD), potentially using induced pluripotent stem cells (iPSCs) to generate DA neurons. Navigating step by step in this study is a vital process, covering the main gist of the neurodegenerative disease, its relevance to extracting somatic cells, using transition markers, explaining molecular pathways, exploring its efficiency and safety considerations, and finally implementing it and achieving results both in vitro and in vivo. While in vitro protocols, including dual SMAD inhibition and midbrain patterning through SHH and Wnt signaling, greatly impacted the specificity and efficiency of dopaminergic differentiation, making it clinically viable and reproducible across further studies within the brain. While in vivo protocols, including the 6-OHDA rotational test or microdialysis, further contribute to isolating results, reliability, and their accuracy towards different studies, making it a definitive source for conclusions or comparing hypotheses.

With all these different approaches, this study section aims to discuss perspectives, opinions, and attitudes regarding the topic presented. With the scientific promise of iPSC-based therapies, ethical and practical concerns arise during application and must be acknowledged. Starting with the main ethical advantages, iPSCs contain several benefits that may be considered during clinical applications over ESCs, such as preventing embryo destruction. With debates regarding embryo destruction, iPSCs are extracted from somatic cells while mimicking ESCs' renewability and pluripotent state, proving effective and sustainable throughout clinical uses, simultaneously alleviating moral objections related to the origin of most pluripotent stem cells.

On the other hand, ethical controversies persist within the research community, mainly surrounding patients' informed consent, privacy, genetic data, and misuse of allogeneic reprogrammed human cells. With animal models emerging in the research community, it is crucial to validate hypotheses preclinically; however, this raises significant ethical concerns regarding procedures involving animal welfare, as they are subjected to invasive surgeries or neurological impairment, indicating neurodegeneration or neurological dysfunction within the models' brains.

Even with the specification of tissues being from the same patient/model, autologous transplants can still lead to unforeseen complications, such as unintended immune responses and excessive tissue formation. These are the risks that are associated with iPSCs that oppose the many benefits presented. Hence, the ethical and scientific concerns must be carefully managed through proper oversight, constant reprogramming/refinement, and studies that penetrate the surface of this topic.

Although the focus is directed towards its methodologies and therapeutic potential of implementing iPSCs to generate DA neurons, this study has certain limitations. As mentioned above, this study is unable to provide a definitive conclusion on the effectiveness of iPSCs in combating Parkinson's Disease, as most information is compiled from secondary sources, and even with the preclinical models providing valuable insights, they cannot fully replicate human pathology nor predict long-term efficacy in the patient's health. For example, the efficiency of DA generation also depends on the spatial and temporal signals within the brain; wavelengths constantly changing over time, patterns mimicking over space, etc. Another crucial information that must be considered is that the quantitative generation is often dependent on the hiPSC line, with different lines from PD patients creating fewer DA neurons than normal patients. Addressing the range of these variables proves difficult to implement in vitro, resulting in differing results from one patient to another.

The complex endeavor of Parkinson's disease further poses a problem within iPSCs' nature, as external causes of PD are not always signified and taken into account. For example, once the iPSCs successfully differentiate into complete dopamine-producing nerve cells, most cases are considered idiopathic, which means the cause of the disease may be unknown. Toxins, pesticides, genetic mutations, age groups, and environmental factors suggest a possibility of developing PD at a higher risk, instead of the lack of dopaminergic neurons that affect their cognitive function and mobility (Kouli et al., 2018). With research emphasizing other factors such as α-synuclein-containing Lewy bodies, disrupting cellular functions within neurons, and

being associated with the malfunction of the Nigrostriatal pathway that is critical for facilitating movement, other causes of PD may be neglected and not mitigated in the treatment of PD.

The need to recognize these concerns is increasingly momentous within this controversial topic, as iPSCs deliberately act as one of the most important resources towards developing cell-based therapies for not only Parkinson's Disease, but also drug discovery, disease modelling, gene editing, and finally, regenerative medicinal approaches.

Conclusion

In conclusion, the generation of DA neurons from iPSCs presents a transformative approach in addressing the core pathology of Parkinson's Disease by replacing the dopaminergic neurons lost to degeneration. By exploring the key molecular mechanisms, methods, and pathways, it provided a promising platform for personalized and regenerative approaches to combat not only PD but also other neurological diseases. This research directly explored the methods involved in neural induction of iPSCs, midbrain patterning, and the generation of DA neurons, while briefly describing functional assays to validate the therapeutic potential both in vitro and in vivo. However, it comes with both benefits and challenges, with ethical considerations, safety procedures, and long-term efficacy. While current studies demonstrate great outcomes with great potential, refinement in differentiation techniques will be essential to realize the full therapeutic potential of iPSC-based interventions in PD by improving clinical-grade iPSC differentiation and standardization of in vivo protocols.

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