

Patterning inconsistencies restrict the true potential of dopaminergic neurons derived from human induced pluripotent stem cells

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Human induced pluripotent stem cells (hiPSCs) are multipotent stem cells genetically reprogrammed using transcription factors, such as Sox2, c-Myc, Oct3/4 and Klf4 (Takahashi and Yamanaka, 2006) from fibroblasts, derived from either patient or control individuals. These factors are highly expressed in embryonic stem cells, and their overexpression can induce pluripotency in human somatic cells such as fibroblasts. Upon the generation of hiPSCs after reprogramming, these cells can be further differentiated into multiple neuronal cell types by using a strictly designed protocol. This process is known as patterning. Correct use of these hiPSCs derived neurons holds immense potential for researchers to uncover the underpinnings of disease pathophysiology and therefore is considered as a powerful tool. For example, in the context of Parkinson's disease (PD), numerous publications have highlighted the aggregation of an abnormally folded protein, α -Synuclein that forms intracellular inclusions in the cell body and neurite processes known as Lewy bodies. However, the mechanisms that cause neurodegeneration specifically in dopaminergic neurons as compared to other neuronal subtypes are still unknown. Unfortunately, it is rather difficult to culture genuine dopaminergic neurons from rodent embryos in sufficient amounts. Therefore, generating human dopaminergic or glutamatergic neurons from hiPSCs to determine the selective detrimental effect of α -Synuclein could offer an immensely valuable outlook. The use of hiPSCs derived dopaminergic neurons could enable us to decipher the pathophysiological mechanisms of this selective neurodegeneration in an *in vitro* culture system. However, there are several inconsistencies in the field of hiPSCs derived dopaminergic neurons, which need to be addressed in order to generate reliable, reproducible and efficient protocols for their patterning.

Importance of using multiple hiPSCs lines to determine the reproducibility of patterning protocols: One of the widely mentioned concern of using hiPSCs is the lack of reproducibility within different clones of the same hiPSCs line as well as within multiple hiPSCs lines. Based on the literature published to date, the majority of the patterning protocols use a single hiPSCs line to demonstrate the efficiency of their protocol for generating dopaminergic neurons. Moreover, only eight out of the twelve protocols previously published, have reported the number of dopaminergic neurons generated. The efficiency of dopaminergic neuronal generation ranges from 5–75% of the total cells in culture (Mak et al., 2012; Xi et al., 2012; Doi et al., 2014). Using the pharmacological

compounds for dopaminergic neuronal patterning as previously published (Kriks et al., 2011), we recently demonstrated significantly variable numbers of differentiated neurons and dopaminergic neurons generated from at least four different hiPSCs lines (Mahajani et al., 2019). Multiple publications, when employing a single hiPSCs line, have reported similar variation (ranging 8–85% of final cell number), but the variation could be attributed to the handling of hiPSCs lines among several labs. Various methods used to reprogram hiPSCs lines could explain their variable proliferation as well as differentiation potential. Therefore, we used four different hiPSCs lines (reprogrammed in a similar manner) to determine the efficiency of generating dopaminergic neurons by upregulation of specific transcription factors using adeno-associated viral vectors (AAVs). Even though the number of differentiated dopaminergic neurons generated, from one hiPSCs line, using AAV mediated protocol is lower than pharmacological compounds mediated patterning (~50% vs. ~80%), we observed comparable levels of differentiated neurons and dopaminergic neurons from all four hiPSCs lines. This is important because, in order to determine the specific toxicity induced by α -Synuclein overexpression in multiple patient and control hiPSCs lines derived differentiated dopaminergic neurons, it is essential to obtain similar amounts of dopaminergic neurons from each hiPSCs line. Along with the need to use multiple hiPSCs lines, future patterning protocols must also document the number of neurons generated, efficacy, the percentage of other cell types present in the culture as well as the genetic background of the donor.

Use of different patterning protocols: Out of the twelve protocols we focused on, ten of them employed pharmacological compounds to pattern hiPSCs into dopaminergic neurons (Chambers et al., 2009; Kriks et al., 2011; Kirkeby et al., 2012; Mak et al., 2012; Xi et al., 2012; Doi et al., 2014; Borgs et al., 2016). The remaining two protocols used lentiviral vectors (Friling et al., 2009; Theka et al., 2013) encoding transcription factors such as Lmx1a, Nurr1 and Ascl1 to generate dopaminergic neurons from either hiPSCs or human embryonic stem cells. Both these protocols reported ~50% of dopaminergic neurons in their culture, which is significantly lower than ~80% generated by Kriks et al. protocol using pharmacological compounds (Kriks et al., 2011). The absolute difference between these types of protocols is the use of pharmacological compounds to stimulate activity of specific transcription factors or upregulation of these transcription factors directly using viral vectors. Different combinations of pharmacological

compounds enable researchers to have control over patterning in a stage-specific manner. For example, Kriks and colleagues standardized their protocol to obtain floor plate progenitors before patterning them to generate dopaminergic neurons after testing multiple concentrations, combinations and durations of pharmacological compounds such as LDN, SB, CHIR99021 along with SHH and PUR (Kriks et al., 2011). Moreover, pharmacological compounds act in a fast and dose-dependent manner. However, it must be noted that a single pharmacological compound can affect the expression of multiple transcription factors and possess toxic effects at higher concentrations (Zhang et al., 2012). On the contrary, with the use of viral vectors, researchers can pinpoint certain specific transcription factors and study their efficiency in patterning hiPSCs to neurons. Studies that have employed the use of viral vectors to pattern dopaminergic neurons from hiPSCs report shorten time required for the differentiation and maturation of these neurons. However, a major disadvantage concerning lentiviral vector mediated patterning is their ability to integrate in the genome of the cell. However, we recently demonstrated that it is possible to pattern dopaminergic neurons from hiPSCs using AAV vectors overexpressing transcription factors under the control of HBA promoter which is self-limiting (Mahajani et al., 2019). Using qRT-PCR, we demonstrated that the mRNA expression of the induced transcription factors was upregulated at 5 days post transduction, but gradually returned to their endogenous expression levels by 10 days post transduction. Although, we are still trying to elucidate the molecular mechanism for the silencing of the HBA promoter, we speculate that targeted DNA methylation could have silenced the promoter (Mahajani et al., 2019). Taken together, it is important that researchers select a protocol that better suits their *in vitro* experimental setup. For example, researchers can opt to directly upregulate transcription factors via viral vectors if they want to study differences among dopaminergic neurons derived from multiple hiPSCs lines, in terms of differentiation or maturation. Viral vector mediated upregulation of transcription factors also reduces the time required for the differentiated neurons to exhibit electrical activity and release dopamine in culture (Theka et al., 2013; Mahajani et al., 2019). On the other hand, the researchers could use pharmacological compounds to generate dopaminergic neurons if their experiments focus of one hiPSCs line where highest dopaminergic neuronal yield is essential. Using pharmacological compounds also suits the researchers if they specifically want to generate long lasting brain organoids.

Standardization of coating methods before plating hiPSCs derived dopaminergic neurons: Most of the published protocols for the generation of dopaminergic neurons from hiPSCs use poly-L-ornithine and laminin to coat wells or coverslips for attachment of neurons after patterning. It has been previously described that stem cell fate commitment depends on the use of these coating substances (Mahajani et al. 2017). However, in preliminary unpublished experiments with the use of Poly-L-Ornithine and Laminin, we have observed aggregation (clustering) of neurons around 50 days *in vitro* (DIV 50). This may arise due to

the insufficient adhesion of the differentiated neurons to the well or coverslip or the lack of sufficient glia cells in the culture. Almost all the publications demonstrating a patterning protocol fail to mention whether they observe such 'clumping' at later time points. These neuronal clusters are connected together via fasciculated axons which render them unusable for experiments to determine neuronal network activity defects. Moreover, we have observed that increasing the concentration of Laminin as well as using multiple coating substances such as fibronectin, gelatin and matrigel did not significantly reduce the neuronal aggregation at DIV 50 (data not shown). It remains to be determined whether a co-culture of rodent primary astrocytes or hiPSCs derived glial cells with differentiated dopaminergic neurons reduces this effect in culture. This neuronal aggregation at later stages inhibits the long-term maintenance of the differentiated neurons to determine several factors affecting neuronal activity, maturation and survival. In terms of the protocols published hence forth, it is essential that the authors address this issue to increase awareness and thereby, motivating other researchers to study this limitation in detail.

The need to report time required for neuronal maturation post patterning: Upon differentiation, neurons require time and certain culture conditions to 'mature' and exhibit electrical activity. A neuron when is fully functional i.e. it shows activity, irrespective of its morphological structure, is said to be mature. This aspect is poorly represented in the patterning protocols published to date. Out of the twelve most cited dopaminergic neuronal patterning publications, only four of them reported (Kriks et al., 2011; Xi et al., 2012; Theka et al., 2013) the time it took the hiPSCs derived dopaminergic neurons to mature in culture. Electrophysiological experiments to determine whether these differentiated neurons show activity have helped researchers assess their maturity. Upon patterning hiPSCs to generate dopaminergic neurons, positive immunoreactivity signals for neuronal marker (β -tubIII) and dopaminergic neuronal marker (TH) could be observed after 3 days of plating these cells. Although, these plated cells demonstrate all morphological resemblance to neurons, they fail to show any electrical activity at such an early stage. To investigate this aspect in detail, we specifically designed an AAV vector expressing enhanced green fluorescent protein under the control of mature-neuronal 'hSyn1' promoter. The activity of hSyn1 promoter is inhibited by RE-1 silencing transcription factor and upon its downregulation in mature neurons (Mahajani et al., 2019); RE-1 silencing transcription factor cannot further inhibit hSyn1. After transducing the plated neuronal-like cells at DIV 15, we could pinpoint the time (DIV 25) when the cultured neurons would express enhanced green fluorescent protein, and therefore, be 'mature'. Calcium imaging demonstrated that these plated neurons showed spontaneous synchronous and asynchronous electrical activity at the same time point further confirming that these differentiated neurons are mature. Therefore, we established a surrogate marker system for neuronal maturity by determining the expressing of enhanced green fluorescent protein under the control of hSyn1 promoter. Another important aspect of dopaminergic neuronal maturation is their ability to

synthesize, package into vesicles, and release dopamine. However, only four out of the twelve publications demonstrated the ability of these hiPSCs derived dopaminergic neurons to release dopamine in the culture medium at different time points (Kriks et al., 2011; Theka et al., 2013; Doi et al., 2014). To obtain valuable insights from neurodegeneration or toxicity studies, it is imperative that these experiments are performed in mature dopaminergic neurons and resemble as closely as possible to the neurons observed *in vivo*. Therefore, as a rule of thumb, future dopaminergic neuronal patterning protocols need to demonstrate spontaneous electrical activity of differentiated neurons either by calcium imaging or electrophysiological experiments as well as report the levels of released dopamine in the culture medium using HPLC. Moreover, whenever possible, future patterning protocols should try to compare the non-stimulated spontaneous activity of hiPSCs derived dopaminergic or glutamatergic neurons with that of rodent primary neuronal cultures either from the midbrain or the cortex.

In conclusion, these four points listed above are few of the aspects that require immense attention while designing and validating patterning protocols for the generation of hiPSCs derived dopaminergic neurons. It is critical for the field to generate reproducible patterning protocols in order to determine the numerous variable factors involved in disease pathophysiology when comparing differentiated dopaminergic neurons derived from patient *versus* control hiPSCs. Moreover, it is also essential that researchers take note of the reprogramming method used for generating hiPSCs from different sources. There is a need for future patterning protocols to include responses to aspects such as reprogramming method, time required for neuronal maturation and different culture conditions in order to highlight the efficiency of their protocols. The patterning of hiPSCs derived dopaminergic neurons could act as a powerful tool to study neurodegeneration and its mechanisms in human neurons after the important aspects of hiPSCs proliferation, differentiation and maturation are addressed.

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