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Transcription Factor-Mediated Differentiation of Human iPSCs into Neurons

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Michael S. Fernandopulle[1], Ryan Prestil[1], Christopher Grunseich[1], Chao Wang[2], Li Gan[2], Michael E. Ward[1]

[1]National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland; [2]Gladstone Institute of Neurological Disease, Gladstone Institutes, San Francisco, California

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Tech. support email: **ndcn-help@chanzuckerberg.com**

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Abstract

Accurate modeling of human neuronal cell biology has been a long-standing challenge. However, methods to differentiate human induced pluripotent stem cells (iPSCs) to neurons have recently provided experimentally tractable cell models. Numerous methods that use small molecules to direct iPSCs into neuronal lineages have arisen in recent years. Unfortunately, these methods entail numerous challenges, including poor efficiency, variable cell type heterogeneity, and lengthy, expensive differentiation procedures. We recently developed a new method to generate stable transgenic lines of human iPSCs with doxycycline-inducible transcription factors at safe-harbor loci. Using a simple two-step protocol, these lines can be inducibly differentiated into either cortical (i^3Neurons) or lower motor neurons (i^3LMN) in a rapid, efficient, and scalable manner (Wang et al., 2017). In this manuscript, we describe a set of protocols to assist investigators in the culture and genetic engineering of iPSC lines to enable transcription factor-mediated differentiation of iPSCs into i^3Neurons or i^3LMNs, and we present neuronal culture conditions for various experimental applications. © 2018 by John Wiley & Sons, Inc.

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Guidelines

Introduction

Neurons are the primary information-processing cells in higher eukaryotes, performing essential functions in stimulus reception, signal transmission, and adaptive responses. In humans, neuronal dysfunction can cause a variety of clinical disorders, including developmental conditions such as autism, psychiatric illnesses such as schizophrenia, and degenerative diseases such as Alzheimer's dementia. Neurons are unique among cell types in their large and functionally polarized structure, post-mitotic state, and electrochemical activity. The human nervous system is unique in both its complexity and susceptibility to disease, and animal models often fail to recapitulate key characteristics of human neuronal disorders (D'Alton et al., 2014; Kitazawa, Medeiros, & Laferla, 2012). In order to understand normal human neuronal physiology as well as how cells and networks malfunction in disease, better experimental models of neuronal cell biology are needed.

Historically, the two main *in vitro* model systems for studying cellular and molecular neuroscience have been rodent primary neurons and human immortalized cell lines. Primary rodent neurons have specialized machinery unique to neurons, but, as they originate from another species, these cells may not recapitulate relevant aspects of human genetics or disease pathophysiology (Kloskowska et al., 2010; Nuber et al., 2013). Practically, primary neurons are time-consuming to isolate, can vary in quality from preparation to preparation, are difficult to scale for some applications, and are difficult to genetically engineer once isolated. Human immortalized lines such as HeLa, HEK293T, and U2OS, along with neuroblastoma lines such as SH-SY5Y, circumvent many of these challenges; they are easily cultured, relatively homogenous, scalable, and readily manipulated genetically.
However, they have widespread and unstable genotypic abnormalities and lack a truly neuronal phenotype, so they are poorly suited to study neuron-specific biology such as axonal or synaptic phenomena.

The first derivation of human embryonic stem cell lines in 1998 was soon followed by techniques to manipulate developmental pathways in order to promote differentiation into cell types of interest (Bain & Gottlieb, 1998; Kawai et al., 1998; Shimazaki, Arsenijevic, Ryan, Rosenfeld, &Weiss, 1999; Thomson et al., 1998). The scalability and genetic tractability of stem cells finally permitted large populations of normal human cells to be grown *in vitro*, and for the first time human neuronal cell culture became a viable model system. The landmark development of induced pluripotent stem cell (iPSC) technology further enabled the reprogramming of patient-derived cells to establish stem cell lines capable of differentiating and recapitulating cellular disease phenotypes in culture (Takahashi & Yamanaka, 2007). The field quickly embraced these transformative tools (Chambers et al., 2009; Wernig et al., 2008), and, together, their success revolutionized the study of human neurobiology; no longer were scientists limited to choosing between cell-type and species specificity, and the use of stem cells greatly facilitated genetic engineering. Still, initial methods for differentiating iPSCs to neurons or neuralprecursors were far from simple; all involved complex media formulations and lengthy protocols (Hu & Zhang, 2009; Karumbayaram et al., 2009; Zeng et al., 2010). Recent approaches have simplified the process by employing primarily small molecules, and the use of overexpressed or inducibly-expressed transcription factors have simplified it further.

Most small molecule-based differentiation approaches rely on a combination of pathway inhibitors (e.g., noggin, SB431542) to drive ES or iPS cells toward neuroectodermal development. This process results in neural progenitor

cells (NPCs), which must be coaxed to differentiate further with different small molecules and growth factors to finally produce the desired neuronal subpopulation. There are three major drawbacks with these strategies: small molecules are not highly efficient, individual cells transduce signals at different rates, and different iPSC clones (even from the same patient) can respond differently to the same small molecules. Together, this leads to a mixed population of neural progenitors and various neural and glial cell types, producing batch-to-batch and line-to-line variability. Particularly for long-term cultures, proliferative cells can quickly outcompete post-mitotic neurons of interest, and the presence of multiple cell types complicates downstream analysis, especially for high-throughput microscopy screens or "-omics" applications. Finally, small molecule-based methods are often laborious, expensive, and slow. From a survey of articles describing small molecule-mediated iPSC differentiation to neurons in the past year (Cao et al., 2017; Kikuchi et al., 2017; Qi et al., 2017), timelines extended from 13 to 70 days, and involved between four and six medium-formulation changes over those periods. This technical and time burden decreases laboratory output, increases the likelihood of contamination, and creates barriers to entry for new users.

Transcription factor overexpression is a new approach to neuronal differentiation from iPSCs that circumvents many challenges associated with small molecule pathway inhibitors. Initially demonstrated by Zhang et al. (2013), overexpression of the master neuronal transcriptional regulator neurogenin-2 (NGN2) results in rapid, one-step differentiation of iPSCs to functionally mature glutamatergic cortical neurons. Similar results were independently obtained by the study of Busskamp et al. (2014), in which NGN2 was virally delivered and inducibly expressed in iPSCs, resulting in synaptically mature cells in 14 days. Both studies recorded upwards of 90% differentiation efficiency and purity, as measured by immunostaining of characteristic cortical neuron markers. With fewer medium changes and relatively rapid differentiation time, NGN2 overexpression offers an appealing alternative to small molecule differentiation strategies.

The set of protocols described here follows an improvement to the NGN2 method by Wang et al. (2017). In that study, the neurogenin-2 transgene was stably integrated into a safe-harbor locus in iPSCs under a doxycycline-inducible promoter. Clonal isolation of this stably integrated line enables near 100% efficiency and purity of differentiation to glutamatergic cortical neurons within the previously observed 14-day timeline, and simplifies differentiation to a two-step protocol. These cells, termed i^{3}Neurons (integrated, inducible, and isogenic), offer a substantial improvement in efficacy and ease-of-use over other existing iPSC-to-neuron differentiation strategies.

Here we provide a detailed set of protocols for the generation and use of i^{3}Neurons, and a related technique to generate lower motor neurons (i^{3}LMNs), which also includes overexpression of the transcription factors Islet-1 (ISL1) and LIM Homeobox 3 (LHX3) along with NGN2. The described techniques mostly require only basic laboratory instrumentation and reagents, and can be completed without any specific training beyond mammalian cell culture proficiency, making them appealing to a wide range of laboratories. Basic Protocols 1 to 4 provide an update on the state of the art of human iPSC culture and transgenic-line generation, with Support Protocol 1 describing a genotyping strategy for confirming stably integrated lines. Basic Protocols 5 to 8 discuss the differentiation and culture of i^{3}Neurons and i^{3}LMNs, with Support Protocols 2 to 5 providing specific instructions on immunocytochemistry, transfection, transduction, and live imaging of differentiated neurons. Support Protocols 6 to 7 provide instructions on assessing induction efficiency, as well as optional culture supplementation with astrocytes.

Background Information

The use of transcription factors (TFs) to specify iPSC differentiation follows naturally from the use of Yamanaka factors (Oct4/Sox2/Klf4/c-Myc) to generate iPSCs from differentiated cells. Whereas small molecules elicit inconsistent effects based on cell-to-cell variability in uptake and metabolism, stably integrated transcription factors enable uniformly efficient differentiation. These strategies are gaining momentum in the iPSC field, with recent reports detailing TF-based strategies for iPSC-derived motor neurons, oligodendrocytes, and even pancreatic beta cells (Goto et al., 2017; Major, Powers, & Tabar, 2017; Zhu, Liu, Zhou, & Ikeda, 2017). Ultimately, TF-based differentiation offers higher efficiency, higher purity, and a shorter timeline for producing the desired cell model when compared to small molecule differentiation methods. For laboratories with expertise in iPSC culture, TF-based methods have the potential to accelerate productivity, while for laboratories new to iPSC technologies, TF-based methods offer a low barrier to entry. The i^3Neuron and i^3LMN strategies described in this article express these TFs in an integrated and inducible fashion, offering the user maximal facility and control. For instance, multi-transgenic iPSC lines can be constructed piecemeal over time, with no concerns over loss of hNGN2 or hNIL expression. These lines can then be differentiated on a small or large scale, matured and assayed experimentally, or cryopreserved in a convenient pre-differentiated stock.

The main limitation with our strategy is the unavailability of the Tet-On promoter system for precise control of other transgenes, such as toxic genes. The 3-day neuronal differentiation period with doxycycline will also induce any other genes controlled by the Tet-On promoter, and overexpression of toxic genes (e.g., TDP-43 overexpression for ALS modeling) at this critical timemight compromise the quality of the resulting neurons.Methods that we have used to counter this challenge include implementing a shorter differentiation period (terminal differentiation is induced within 24 hr of continuous doxycycline exposure; Busskamp et al., 2014), inserting Kozak sequences with short upstream open reading frames (uORFs) to attenuate expression from the Tet-On promoter, and employing orthogonal inducible systems.

Critical Parameters and Troubleshooting

DNA quality for transfections

Efficient transfections are critical for facilitating downstream enrichment and clonal isolation when constructing a new iPSC line. Low efficiency transfections will require several weeks of enrichment, whereas an exceptionally high efficiency transfection could result in clonal isolation after the initial serial dilution or FACS enrichment of transfected cells.We have observed that the best indicators of DNA quality (apart from proper 260/280 and 260/230 spectrophotometric ratios) are concentration and preparative scale. Concentrating DNA, either through a new prep or ethanol precipitation, almost always improves efficiency. DNA for transfection should ideally be at a concentration higher than 1 µg/µl, and should almost certainly be concentrated if below 300 ng/µl. Miniprep DNA tends to produce less efficient transfections than either midi- or maxiprep DNA at the same concentration, likely

due to higher bacterial endotoxin levels in smaller preps. Since endotoxins cannot be removed from existing preps, DNA to be used for transfection should ideally be prepared in a maxiprep.

Coating and freshness of medium for neurons

Plate coating for neurons (PLO for hNGN2 neurons and PLO/PEI with laminin for hNIL neurons) is the single most important parameter determining long and short-term neuronal health. Coating solutions should always be freshly prepared, and any stocks should be used within 1 week. Coated and washed dishes may also be stored covered at 4°C for only 1 week. Though we have not observed a striking cutoff point for coating viability, we have observed a steady decline in neuronal health when older coating solutions have been used. Likewise, neuronal media (IM, CM, or MM) should be prepared fresh with supplements in small scale batches (i.e., 50 to 100 ml), and supplemented media should be used within 1 week.

FACS conditions for iPSC sorting

FACS conditions must be carefully selected to ensure iPSC viability. Microfluidic flow sorting machines (e.g., Sony SH800S, NanoCellect WOLF Cell Sorter) employ low pressures for sorting, and are therefore ideal to preserve iPSC viability. If only conventional sorting machines are available, the nozzle should be adjusted to 100 µm (versus the 30-µm standard nozzle) in order to lower the sorting pressure. For bulk sorting, iPSCs should be resuspended for sorting in E8 + ROCK inhibitor, and they should be sorted into Matrigel-coated dishes with E8 + ROCK inhibitor. Improved survival and outgrowth from single cells may be accomplished by resuspending in StemFlex medium (Gibco, cat. no. A3349401) with ROCK inhibitor and plating on wells that have been coated with rhLaminin-521 (Gibco, cat. no. A29249). If routine passaging was done in E8 and E8 + ROCK inhibitor, these cells should be pre-equilibrated to StemFlex for at least 2 passages.

Immunocytochemistry

Neurons can be stained in situ, but special care must be taken to prevent dissociation of individual cells or sheets of neurons from the dish. It is therefore critical to reduce the total number of washes (e.g., using 2× fixative solution added directly to the neuronal medium) and carry out washes slowly (see Support Protocol 2).

Lentiviral vectors for i3Neuron and i3LMN transduction

We have found that iPSCs and iPSCderived neurons (i3Neurons and i3LMNs) silence certain viral elements in transgenic constructs. In particular, CMV promoters and IRES elements consistently fail to express.We suggest replacing CMV promoters with CAG, PGK, or EF-1α promoters, and replacing IRES elements with T2A linkers for polycistronic gene expression.

Enhancing stable cell line generation

Establishing stable lines of iPSCs using the safe-harbor integration strategy requires induction of double-stranded breaks at defined genomic loci (e.g., AAVS1 or CLYBL). One of the major roadblocks in the clonal enrichment and isolation process is the inefficiency of stable transgene integration (<1%). In addition to the low frequency of

homologous recombination events between the donor plasmid and the genomic integration locus, a high frequency of p53-mediated death has been observed in cells that successfully undergo TALEN- or CRISPR/Cas9-mediated doublestranded breaks (Ihry et al., 2017). To counter the latter problem, we co-transfect a nonintegrating dominant negative p53 construct (Addgene #41856) along with donor DNA and TALENs when establishing a new line. We have seen major improvements in donor construct integration through this strategy, as measured by increased numbers of fluorescent cells both 24 hr after transfection and 1 week after FACS.

Anticipated Results

The construction of i^{3}Neuron and i^{3}LMN iPSC lines enables versatility in studying various aspects of neuronal cell biology through additional gene knockouts, knockdowns, or overexpression systems. The integrated, inducible, and isogenic transgene system allows the production of a more pure and mature population of neurons in less time than neurons differentiated by growth factors and small molecules alone. These cells are furthermore easily amenable tomultiple experimental applications, including microscopy, biochemistry, -omics, and electrophysiology (with addition of glia).

Time Considerations

The estimated time to generate a stable hNGN2 or hNIL-expressing iPSC line is approximately 1 month (transfection, manual or FACS fluorophore enrichment, clonal isolation, genotyping, karyotyping). The doxycycline differentiation period is 3 days, and culture to mature neurons takes an additional 11 days (mature d14 neurons). These neurons can also be cultured longer, as we have observed good viability and morphology through 30 days after initial doxycycline induction.

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NAME

ASSESSING RTTA ACTIVITY (Support Protocol 6)

VERSION 1

CREATED BY

Julia Rossmanith
protocols.io

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Protocol

iPSCs

NAME

ASTROCYTE PRODUCTION (Support Protocol 7.1)

VERSION 1

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Protocol

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REAGENTS AND SOLUTIONS (Support Protocol 7.2)

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