Low-Density Neuronal Cultures from Human Induced Pluripotent Stem Cells

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Abstract

Induced pluripotent stem cell (iPSC)-based technologies offer an unprecedented possibility to investigate defects occurring during neuronal differentiation in neuropsychiatric and neurodevelopmental disorders, but the density and intricacy of intercellular connections in neuronal cultures challenge currently available analytic methods. Low-density neuronal cultures facilitate the morphometric and functional analysis of neurons. We describe a differentiation protocol to generate low-density neuronal cultures (~2,500 neurons/cm²) from human iPSC-derived neural stem cells/early neural progenitor cells. We generated low-density cultures using cells from 3 individuals. We also evaluated the morphometric features of neurons derived from 2 of these individuals, one harboring a microdeletion on chromosome 15q11.2 and the other without the microdeletion. An approximately 7.5-fold increase in the density of dendritic filopodia was ob-

served in the neurons with the microdeletion, consistent with previous reports. Low-density neuronal cultures enable facile and unbiased comparisons of iPSC-derived neurons from different individuals or clones.

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Introduction

The experimental approaches to model central nervous system infection and neurological and neurodegenerative diseases profoundly changed with the advent of induced pluripotent stem cells (iPSCs) [1]. For the first time, it is possible to generate and manipulate virtually limitless numbers of live iPSC-derived neuronal lineage cells originating from specific individuals. Thus, iPSC-based models offer the potential to investigate the molecular mechanisms underlying the pathogenesis of numerous neuropsychiatric and neurodevelopmental disor-

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ders. Neuronal cultures generated from patient-specific iPSCs have been employed to model schizophrenia [2, 3], Parkinson disease [4], Alzheimer disease [5], autism [6], and bipolar disease [7].

Low-density 2D neuronal cultures are suitable for analyses of individual neurons, dendritic spine morphology and number, synapse formation, and signaling networks [8], for analyses of changes in synaptic/network function in response to pharmacological treatments, and to investigate subcellular localization and neuronal protein trafficking [9]. Low-density neuronal cultures have been generated starting from primary neurons using several strategies, such as (1) coculture of primary neurons with glial cells, (2) sandwich cultures in 3D PuraMatrix [10], and (3) sandwich cultures where a layer of neurons at low density faces a layer of neurons at high density [11, 12]. To our knowledge, similar approaches have not been employed for iPSC-derived neurons. We describe a differentiation procedure that enables the generation of low-density neuronal cultures from iPSCs-derived neural stem cells/early neural progenitor cells (NSCs/NPCs).

Materials and Methods

Cell Lines

Six cell lines from 3 individuals (ID# 73-56010-01, clones SB and SD, ID# 73-56010-02, clones SG, SC, and SF, and ID# 71-500-9001) were used in this study. Human fibroblast cells were reprogrammed into iPSCs via Sendai viral vectors using the transcription factors Sox2, c-Myc, Klf4, and Oct4 at the NIMH-funded Rutgers University Cell and DNA Repository (<http://www.rucdr.org/mental-health>). Human iPSCs were maintained in supplemented mTeSRTM1 medium (Stem Cell Technologies) under standard conditions (5% CO₂, 37°C, and 100% humidity). Karyotyping and array comparative genomic hybridization were used to check for chromosomal abnormalities and to confirm deletions in the chromosome 15q11.2 region as described [13].

Generation of Low-Density Neuronal Cultures

Monolayer cultures of NSCs/NPCs were generated from iPSCs as previously described [14]. NSCs/NPCs were dissociated with Accutase, resuspended in mTeSRTM1 medium, and seeded at the same density on uncoated or Matrigel-coated glass coverslips (4 × 10⁵ cells/well in 12-well plates). After 90 s, the cell suspension was removed gently, fresh mTeSRTM1 medium was added, and the plates were incubated overnight under standard culture conditions. These conditions were established following pilot experiments to determine the best time for seeding the cells at clonal density. Four different time points were tested: 30, 60, 90, and 120 s. The duration of 90 s was found to be optimal to generate low-density neuronal cultures (data not shown). Following overnight incubation, the mTeSRTM1 medium was replaced by Neurobasal medium supplemented with 2% B27 (Gibco), 10 ng/mL brain-derived neurotrophic factor (PeproTech), 50 U/mL penicil-

lin G, and 50 mg/mL streptomycin. Attached cells were differentiated in Neurobasal medium for 4–6 weeks. The culture medium was changed every other day.

Immunocytochemistry

Immunocytochemistry was performed as previously reported [15]. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X. The primary antibodies used were mouse monoclonal anti- β -tubulin III antibody (clone TUJ1; R&D Systems; 1:50 dilution), mouse monoclonal anti-microtubule-associated protein 2 (MAP2; EMD Millipore; 1:1,000 dilution), chicken polyclonal anti-GFAP (EMD Millipore; 1:200 dilution), rabbit polyclonal anti-PSD-95 antibody (Abcam; 1:500 dilution), rabbit polyclonal anti-vimentin (Abcam; 1:1,000), mouse monoclonal anti-nestin (R&D Systems; 1:1,000 dilution), mouse monoclonal anti-synaptophysin (BD Biosciences; 1:1,000 dilution), and rabbit monoclonal anti-S100 β (Abcam; 1:200 dilution). Alexa Fluor 488 goat anti-mouse, anti-rabbit, and anti-chicken secondary antibodies as well as Alexa Fluor 594 goat anti-mouse and anti-rabbit secondary antibodies (Life Technologies; 1:200 dilution) were used for detection. Counterstaining was performed with Hoechst 33342. A Leica IL MD LED inverted fluorescence microscope was used for image acquisition. Additionally, a Leica SP5 CW-STED confocal microscope (in normal confocal mode) was used to collect a virtual stack of optical sections taken from the samples. The series of images was then reconstructed into 3D images using the Volocity imaging software (Improvision). Dendritic filopodia (membranous protrusions without a bulbous head protruding from neuronal dendrites that were longer than 2 μ m [16]) were counted on 35 randomly selected neuronal processes within a distance of 30 μ m from the cell soma.

Electrophysiological Recordings

NSCs/NPCs were differentiated on uncoated 15-mm coverslips for 6 weeks in Neurobasal medium before recording. Neuronal cells were visualized with a Zeiss inverted microscope. The external recording solution contained 140 mM NaCl, 2.8 mM KCl, 1 mM CaCl₂, 0.01 mM EDTA, 10 mM HEPES, pH 7.2 $\pm$ 0.05 adjusted with NaOH, osmolality 290 $\pm$ 10 mmol/kg adjusted with sucrose, and agonists added at indicated concentrations. The pipette solution contained 130 mM K-gluconate, 10 mM NaCl, 1 mM CaCl₂, 1.1 mM EGTA, 10 mM HEPES, 2 mM ATP-Na₂, 2 mM MgCl₂, pH 7.2 $\pm$ 0.05 adjusted with KOH, osmolality 275 $\pm$ 10 mmol/kg. Membrane potentials were corrected for liquid junction potential.

Whole-cell recordings were performed on iPSC-derived neurons with pipettes pulled from borosilicate glass and fire-polished (3.2–5.5 M Ω). Membrane currents were recorded with an Axopatch 1D amplifier (Molecular Devices) in voltage clamp mode, low-pass filtered at 5 kHz, and digitized at 10 kHz with a Digidata 1440a (Molecular Devices). Series resistance was compensated at 70–80%. For the data presented here, recordings were rejected if the holding current at –70 mV was more negative than –300 pA. Rapid solution exchange was achieved using an in-house-fabricated fast perfusion system connected to gravity-fed reservoirs. All experiments were performed at room temperature. Peak currents were measured relative to the baseline current prior to agonist application using Clampfit 10.3 (Molecular Devices). Mean values $\pm$ standard error of the mean are given.

Results

Generation of Low-Density Neuronal Cultures from NSCs/NPCs

We utilized a strategy that enables large-scale generation of NSCs/NPCs from iPSCs [14]. NSCs were distributed at clonal density on both uncoated and Matrigel-coated coverslips as described in Materials and Methods (Fig. 1). During the first 24 h, cells began to proliferate and extended lamellipodia (Fig. 2). A drastic difference in cellular distribution was observed between cultures grown on uncoated and on Matrigel-coated glass coverslips (Fig. 2). In the former, cell differentiation resulted in the generation of low-density neuronal cultures ($\sim 2,500$ neurons/cm²) (Fig. 2; online suppl. Fig. 6; see www.karger.com/doi/10.1159/000476034 for all online suppl. material). In some areas, differentiating cells self-organized into three-dimensional (3D) structures (online suppl. Fig. 1). Cultures differentiating on Matrigel-coated coverslips displayed a confluent monolayer of cells (Fig. 2).

Our differentiation protocol to generate low-density neuronal cultures was applied to 6 different iPSC lines derived from 3 individuals, including cell line 71-500-9001 from an individual carrying the 15q11.2 (BP1-BP2) deletion, cell lines 73-56010-01-SB and 73-56010-01-SD derived from a patient with schizophrenia, and cell lines 73-56010-02-SA, 73-56010-02-SC, and 73-56010-02-SF derived from a control individual without schizophrenia or the microdeletion. Differentiation and culture was feasible for 4–6 weeks, but cells derived from 73-56010-02-SC cultures detached starting during the third week of NSCs/NPCs differentiation.

Immunocytochemistry analysis was performed to investigate the composition of cell cultures following differentiation of NSCs/NPCs in Neurobasal medium for 4 weeks on uncoated coverslips. The neuronal phenotype of the differentiating cells was confirmed by staining with TUJ1 and MAP2, indicating a neuronal lineage (Fig. 2, 3).

Immunocytochemistry also indicated the presence of GFAP-positive cells with bipolar morphology resembling radial glial cells, i.e., an unbranched long process and a short process [17] (Fig. 2; online suppl. Fig. 2). Expression of VIMENTIN, S100 β , and NESTIN was also observed in these unbranched bipolar-shaped radial glia-like cells (RGLCs; Fig. 2; online suppl. Fig. 2). TUJ1- or MAP2-positive neurons were frequently observed to tightly adhere to the processes of these RGLCs (Fig. 2a–c). The frequency of GFAP-positive cells with astrocyte morphology varied among the cell lines (data not shown).

Analysis of Dendritic Spine Formation during the Early Stage of Neuronal Differentiation of iPSC-Derived NSCs/NPCs in Low-Density Cultures

Low-density areas in our culture system enabled analysis of dendritic spines without the need to express a fluorescent reporter construct in cultures, which is required for neurons in high-density neuronal cultures [13]. Dendritic filopodia and dendritic spines were identified by staining with FITC-phalloidin (Fig. 3; online suppl. Fig. 3). Interestingly, FITC-phalloidin [18] and anti-MAP2 antibody signals showed colocalization in most but not all dendritic filopodia and dendritic spines (Fig. 3; online suppl. Fig. 3). Fluorescence was more intense in dendritic spines than in dendritic filopodia (Fig. 3; online suppl. Fig. 3), suggesting that MAP2 is present in the dendritic spines during early neuronal maturation. Colocalization of MAP2 and PSD-95 was observed in a fraction of dendritic filopodia and dendritic spines (Fig. 3; online suppl. Fig. 3). Co-immunostaining with PSD-95 and synaptophysin is depicted in online supplementary Figure 4.

Analysis of Dendritic Spines during Neuronal Differentiation of iPSC-Derived NSCs/NPCs Generated from Cell Lines with the Chromosome 15q11.2 Deletion

We have previously reported on iPSC-derived neurons from 2 individuals with a microdeletion in the chromosome 15q11.2 region that leads to haploinsufficiency of *TUBGCP1*, *NIPA1*, *NIPA2*, and *CYFIP1* [13]. Altered differentiation patterns in neuronal and glial lineages of human iPSCs bearing the 15q11.2 (BP1-BP2) deletion have been reported by other groups [19]. This deletion is associated with several neuropsychiatric disorders, including autism, schizophrenia, and intellectual disability [20]. Cells that carry the deletion or that are haploinsufficient for *CYFIP1* exhibit an increased number of dendritic filopodia, indicating their immaturity [13, 21]. To investigate whether such neuronal differentiation defects could be detected in our low-density neuronal culture system, we employed an iPSC line (71-500-9001) harboring the 15q11.2 (BP1-BP2) deletion (Fig. 4). The low-density neuronal cultures were generated using NSCs/NPCs derived from this individual and a healthy control individual without the deletion (73-56010-02-SF). After 4 weeks of differentiation, cells were stained with MAP2 and the number of dendritic filopodia per neuronal process was analyzed. Dendritic filopodia were analyzed by the localization of MAP2 to dendritic protrusions longer than 2 μ m. The number of dendritic filopodia per process was counted on 35 randomly selected processes in both

Fig. 1. Schematic representation of the method for generation of low-density neuronal cultures. **a** NSCs/NPCs are seeded at the density of 4×10^5 cells/well on uncoated coverslips. After 90 s, cell suspension is withdrawn and NSCs/NPCs are cultured overnight in mTeSRTM1 medium followed by culture in Neurobasal medium. **b** During the first 24 h, cells start proliferating, extending lamellipodia and migrating. **c** Cells are differentiated for at least 4 weeks in Neurobasal medium (see Materials and Methods).

Fig. 2. a Generation of low-density neuronal cultures. Top left panels: Microphotographs depicting differentiation of NSCs/NPCs at indicated time points in uncoated coverslips generating low-density cultures. Top right panels: Microphotographs of NSCs/NPCs differentiating on Matrigel-coated coverslips at the indicated time points. Scale bars, 50 μ m. Center panels: Immunocytochemistry analysis of differentiating cells in low-density cultures (left panels) and on Matrigel-coated coverslips (right panels). Scale bars, 50 μ m, with the exception of the left microphotograph in the upper panel depicting co-immunostaining with TUJ1/GFAP, where the scale bar is 100 μ m. Bottom panels: Low-density neuronal areas in 2D-3D cultures allow for a high-resolution analysis of the association of neurons with the radial glia-like cells (RGLCs). **b** TUJ1- and MAP2-positive neurons tightly adhered to the supportive fibers of RGLCs expressing GFAP and VIMENTIN. Scale bars, 25 μ m. Nuclei were counterstained with Hoechst 33342.

Fig. 3. Visualization of dendritic filopodia and dendritic spines in low-density regions. Dendritic filopodia and dendritic spines can be directly analyzed in low-density regions by immunocytochemistry, without the need for transfection with a construct expressing a fluorescent reporter gene, which is required to highlight isolated neurons in high-density neuronal cultures. **a** Immunostaining with postsynaptic marker PSD-95 on a MAP2-positive dendrite. **b** Immunofluorescence-stained cells using phalloidin as F-actin stain to visualize dendritic filopodia and dendritic spines. Scale bars, 5 μ m.

cell lines. The difference in the frequency of dendritic filopodia per process was statistically significant (cell line 71-500-9001: 2.6 ± 0.3 , mean $\pm$ standard deviation; cell line 73-56010-02-SF: 0.3 ± 0.1 ; $p < 0.0001$; Fig. 4).

Electrical Recordings

Currents were recorded from ligand-gated channels after 6 weeks of neuronal differentiation via whole-cell patch clamp experiments. Glutamate receptor-mediated currents were recorded at a holding potential of -70 mV, with α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor currents recorded in response to application of 100μ M AMPA, and *N*-methyl-D-aspartate (NMDA) receptor currents recorded in response to the application of 100μ M NMDA + 100μ M glycine. Gamma-aminobutyric acid (GABA) receptor currents were recorded at a holding potential of 30 mV in response to the application of 1 mM GABA. In low-density neuronal areas, 0 of 7 cells showed NMDA receptor currents, 6 of 7 cells showed AMPA receptor currents (the peak current range in responding cells was -46 to -217 pA, average -107 pA for all cells), and 5 of 7 cells showed GABA receptor currents (peak current range 98 – 442 pA, average 175 pA for all cells) (online suppl. Fig. 5). The average time to peak for AMPA receptor currents in AMPA-responsive cells was 20.5 ± 0.8 ms; the average time to peak for GABA receptor currents in GABA-responsive cells was 15 ± 1 ms.

Discussion

Low-density neuronal cultures are well suited for analysis of dendritic morphogenesis, axonal growth, and synaptogenesis during development, protein trafficking, subcellular distribution, trafficking, and expression changes of neuronal proteins [12]. In the present study, we describe a differentiation protocol for the generation of low-density neuronal cultures that enables rapid characterization of isolated neurons without the need for transfection with a fluorescent reporter construct [13]. The low-density cultures also enable facile electrophysiological recordings and unbiased neuronal morphological analysis. Importantly, the distribution of NSCs/NPCs at clonal density on coverslips facilitates the real-time tracking of individual cell division, behavior, and the characterization of their progeny. Furthermore, our differentiation protocol does not require coculture with glial cells, reducing the complexity of the culture system and facilitating interpretations. The relatively simple protocol is also significantly less expensive.

The low-density areas in our culture system facilitated characterization of dendritic spines and dendritic filopodia. Staining with FITC-phalloidin and mouse monoclonal anti-MAP2 showed distribution of MAP2 in the dendritic filopodia and dendritic spines, which are thought to be devoid of MAP2 [22–24] (Fig. 3; online suppl. Fig. 3). Whether MAP2 is detectable in dendritic spines during early neuronal maturation is controversial [25].

this deletion). Dendritic filopodia (immature spines) are indicated by arrowheads. Scale bars, 5 μm . **c** An approximately 7.5-fold increase in dendritic filopodia was observed in cell line 71-500-9001 neurons ($n = 35$, t test, $p < 0.0001$; for 71-500-9001, mean = 2.6 ± 0.3393 ; for 73-56010-02-SF, mean = 0.3429 ± 0.1293 ; error bars show the standard deviation).

spectrum disorders [26, 27]. This microdeletion affects genes associated with neural development and cytoskeleton dynamics, notably *CYFIP1*, which plays a critical role in dendritic spine maturation. In rat models, *CYFIP1* haploinsufficiency leads to an increase in the density of immature dendritic spines (dendritic filopodia) [13, 21]. Recently, this phenotype was also observed in iPSC-derived neurons carrying 15q11.2 BP1-BP2 microdeletion

grown on Matrigel-coated coverslips at a high density and visualized by transfection with a fluorescent reporter construct [13]. As a proof of principle, we tested whether our differentiation procedure could facilitate quantitative analyses of neuronal morphology. We generated low-density neuronal cultures using NSCs derived from an iPSC cell line harboring the 15q11.2 deletion (71-500-9001) and a healthy control line (73-56010-02-SF) and compared the density of dendritic filopodia. In our culture system, cells were sufficiently spaced so that dendritic filopodia could be analyzed without the need to transfect neurons with a construct carrying a fluorescent reporter gene. Thirty-five randomly selected MAP2-positive processes from each cell line were analyzed. A significant increase in dendritic filopodia (7.5-fold) in 71-500-9001-derived neurons carrying the 15q11.2 BP1-BP2 microdeletion when compared with the control was observed in low-density regions (Fig. 4). Neurons in low-density regions were also analyzed using whole-cell patch clamp recordings (online suppl. Fig. 5). Consistent with previous work using Matrigel-coated coverslips [14], most cells in low-density regions responded to AMPA receptor and GABA receptor stimulation (online suppl. Fig. 5). However, in the present study, responses to NMDA receptor activation were not observed.

Some limitations of this protocol should be noted. Like in other current differentiation protocols for iPSCs, mature and myelinated neurons cannot be generated [28–30]. Another disadvantage of our protocol is that cells cannot be maintained in culture for more than 4–6 weeks. Subsequently, cells in the low-density areas begin to detach. The detachment could be due to the lack of paracrine trophic support of proximal cells [10] or relatively weak cell adhesion to the uncoated coverslips. The latter imposes the need for particular care during the change of culture media and when performing immunocytochemical and electrophysiological assays.

There are a number of possible modifications to improve the utility of this protocol. Foremost, improved visualization and quantification of dendritic spines could be achieved by dual staining for proteins at synaptic density. In the present paper, we show examples using PSD-95 and MAP2 (Fig. 3; online suppl. Fig. 3), but other combinations are also feasible. Methods for faster differentiation of iPSCs could also be explored. For example, we observed that the differentiation proceeds faster only when NPCs self-aggregate, forming 3D aggregates in both regular and low-density neuronal cultures.

In conclusion, we present a novel iPSC differentiation strategy that enables the generation of low-density neuronal cultures. We foresee applications in studies aimed at unraveling the mechanisms underlying the onset of neurodevelopmental disorders.

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Statement of Ethics

The participants provided written informed consent.

Disclosure Statement

The authors have no conflicts of interest to disclose.

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