Accelerated maturation and improved functionality of neurons with CultureOne Supplement

Introduction

Generating functional and mature neurons from human pluripotent stem cell (hPSC)–derived neural stem cells (NSCs) offers great promise for *in vitro* disease models for neurodegenerative disorders, such as Alzheimer's disease and Parkinson's disease [1]. However, during neuronal differentiation using conventional methods, NSCs overgrow and result in highly dense, mixed cultures of neurons and progenitor cells that prevent long-term maintenance and sufficient maturation of neurons, and lead to a diluting effect, which contaminates downstream assays carried out on the cultures. Gibco™ CultureOne™ Supplement is a xeno-free reagent that inhibits proliferation of NSCs without inducing cell death, which results in significantly enriched cultures of functional neurons at the end of differentiation and alleviates the problems previously mentioned with conventional methods.

The expression of voltage-gated ion channels is an important indicator of neural maturity because it represents a major route for calcium entry into neurons [2], regulating key elements of synaptic transmission and membrane excitability. During and after differentiation, neurons express voltage-dependent calcium channels on their membranes. As a major factor driving excitability, the concentration of calcium ions is maintained several thousand times higher outside the neurons than inside [3]. At resting membrane potential, these channels are closed and the concentration of cytosolic calcium is low. Upon depolarization, these voltage-gated calcium channels open and calcium influx is induced, which can be detected either electrophysiologically or with a sensitive indicator like Invitrogen™ Fluo-4 AM dye. In this study, we demonstrate how neurons, differentiated with CultureOne Supplement, generate higher calcium influx responses per cell at earlier time points relative to untreated control cells. We interpret this effect as an indication that CultureOne Supplement also accelerates the maturation and functionality of neurons differentiated from hPSC-derived NSCs.

Materials and methods

The materials used for cell culture and neuronal differentiation, cell proliferation assay, and cell imaging assay are listed in Table 1.

Table 1. Materials used in functional assay experiments.

Product name	Cat. No.
PSC Neural Induction Medium	A1647801
Neurobasal Medium	21103049
B-27 Supplement (50X)	17504044
CultureOne Supplement	A3320201
GlutaMAX Supplement	35050061
L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate (Sigma-Aldrich)	A8960
BDNF Recombinant Human Protein	PHC7074
GDNF Recombinant Human Protein	PHC7044
Mouse laminin	23017015
DPBS, no calcium, no magnesium	14190094
CyQUANT Direct Cell Proliferation Assay	C35011
Live Cell Imaging Solution (LCIS)	A14291DJ
Fluo-4 Calcium Imaging Kit	F10489

Cell culture and neuronal differentiation

The human NSCs, generated with Gibco™ PSC Neural Induction Medium from H9 embryonic stem cells (ESCs), were thawed in phosphate-buffered saline (PBS) and then split in half. Thawed cells were centrifuged and resuspended at 200,000 cells/mL in neuronal differentiation medium with CultureOne Supplement (Table 2) or without the supplement (control). Diluted NSCs were plated at a density of 20,000 cells/well (100 µL/well) onto 96-well poly-D-lysine–coated microplates treated with laminin. Cells were allowed to attach for one hour at room temperature and then moved to a cell culture incubator in 5% CO_2 at 37˚C. Two days after incubation, an additional 100 µL of neuronal differentiation medium with or without CultureOne Supplement was added to each well. Every third day following this, 100 µL of spent medium was removed from the wells and replaced with an equal volume of fresh medium with or without CultureOne Supplement.

Table 2. Neuronal differentiation medium with CultureOne Supplement.

Reagent	Volume
Neurobasal Medium	96 mL
B-27 Supplement (50X)	2 mL
GlutaMAX Supplement (100X)	1 mL
CultureOne Supplement	1 mL
Ascorbic acid	100 µL

Glial cell–derived neurotrophic factor (GDNF) at 10–20 ng/mL and brain-derived neurotrophic factor (BDNF) at 10–20 ng/mL were added into neuronal differentiation medium with CultureOne Supplement to improve neuron survival. Optional use of these reagents depends on the NSC line being used.

Neurite outgrowth analysis

At various time points after differentiation, label-free cells were imaged by phase-contrast microscopy. To detect and compare changes in neurite outgrowth between the two culture conditions, multiple images were captured from each well of the 96-well plate using the IncuCyte ZOOM™ live-cell imaging system (Essen Bioscience). For each condition, 3–4 wells were imaged. These images were then analyzed using the IncuCyte™ NeuroTrack™ analysis software to calculate total neurite length per image area, expressed as mm/mm^2 image area.

CyQUANT Direct Cell Proliferation Assay

The Invitrogen™ CyQUANT™ Direct Cell Proliferation Assay was used to determine cell density in the two culture conditions. This method generates a fluorescence signal in direct proportion to the cell numbers based on staining of cellular nuclear DNA, and is independent of a cell's metabolic state. Since each cell has one nucleus, cultures with cells that are dividing generate larger fluorescence signals than the cultures with fewer dividing cells. Changes in nucleic acid content can therefore serve as a sensitive indicator of the overall cell density. On the day of testing, a 2X stock solution of CyQUANT™ Direct detection reagent was prepared per the manufacturer's instructions [5] in Invitrogen™ Live Cell Imaging Solution (LCIS). An equal volume of the 2X detection reagent was then added directly to the control and CultureOne Supplement–treated wells, respectively, and the cell plates were incubated at 37°C for 1 hour. Following incubation, fluorescence was measured at an excitation/emission maxima of 490/530 nm on a Thermo Scientific™ Varioskan™ LUX Multimode Plate Reader. Fluorescence was expressed in relative fluorescence units (RFU).

Calcium imaging assay

On the day of measurement, cells were prepared using the kinetic calcium influx assay per the manufacturer's instructions (Invitrogen™ Fluo-4 Calcium Imaging Kit) [4] as follows: To a 15 mL tube, 10 mL of a 2X Fluo-4 loading solution was prepared for each assay plate by adding these components in the following order: 200 µL of PowerLoad™ Concentrate (Component B), 20 µL of Fluo-4 AM dye (Component A), 7.6 mL LCIS, 200 µL water-soluble probenecid solution—prepared per the kit's instructions, and 2 mL Neuro Background Suppressor (Component C). In preparation for loading the cells with the green-fluorescent Fluo-4 calcium indicator, 100 µL of culture media was removed and replaced with 100 µL of 2X Fluo-4 loading buffer to make a total volume of 200 µL solution in each well of the plate. Cells were placed in the cell culture incubator at 37˚C for 30 minutes and then moved to the benchtop incubator at room temperature for 30 minutes. During this time, the potassium chloride (KCl) stimulus buffer series (see below) was prepared. At various time points after differentiation, depolarization with 0–30 mM KCl was used to analyze the activity of voltage-gated calcium channel–mediated calcium influx in Fluo-4 dye–loaded cells. A stimulus plate was prepared so that each well of the cells (containing 200 µL of dye and culture medium) received 50 µL of stimulus buffer during the assay. Culture plates were loaded into an FDSS6000 kinetic imaging microplate reader (Hamamatsu) and cells were illuminated in bottom-read mode with an excitation/emission maxima of 488/525 nm collected at 1-second intervals. After collecting 20 seconds of baseline signal, 50 µL of KCl stimulus buffer was gently transferred by a robotic pin tool onto the cells. The fluorescence signal from the Fluo-4 AM dye was measured at 1 Hz and peak responses were plotted as a running average of multiple wells as "fold-increase".

Preparation of potassium chloride (KCl) stimulus buffers

To introduce a graded depolarization stimulus to the cells, a series of isotonic potassium chloride buffers were prepared and added in a ratio of 1:5 to the cells (i.e., 40 µL KCl stimulus added to 200 µL cells), while collecting fluorescence imaging data at 1-second intervals. Five KCl-based stimulus solutions were prepared from mixing isotonic potassium chloride buffer (140 mM KCl, 2 mM NaCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES; pH set to 7.4 with KOH; osmolarity 300–310 mOsm) at various ratios with LCIS.

Results

Neurite outgrowth analysis

Neuronal morphology is an important indicator of neuronal maturation and function. It has been shown that neurons with more complex and enriched dendritic branches integrate synaptic inputs and form improved communication networks [6,7]. Over two weeks of neuronal differentiation, control cultures contained increasingly dense populations of progenitor and neuronal cells lacking neurite processes. CultureOne Supplement–treated neurons had more extensive processes than control cells and showed significantly fewer contaminating progenitor cells (Figure 1).

CyQUANT Direct Cell Proliferation and Fluo-4 calcium imaging assays

Subsequent cell density and calcium imaging assays provided additional evidence to support improved functionality and accelerated maturation of neurons differentiated with CultureOne Supplement. RFU values from the CyQUANT Direct detection reagent were measured at 4, 14, and 21 days of differentiation in control and CultureOne Supplement–treated neuronal cell cultures. CultureOne Supplement–treated cultures had considerably lower cell densities at each time point compared to the control cultures due to the inhibition of progenitor cell proliferation by the supplement. Over time, CultureOne Supplement–treated cultures maintained a consistent cell density ranging from 0.36 RFUs on day 4 to 0.30 RFUs on day 21, while control cultures showed increasing cell densities at each time point, ranging from 0.93 RFUs on day 4 to 21.5 RFUs on day 21 (Figure 2A). Results from the Fluo-4 calcium assay at each corresponding time point revealed that CultureOne Supplement–treated cultures with significantly lower cell densities had considerably higher calcium response signals at every concentration of KCl stimulus (Figure 2B).

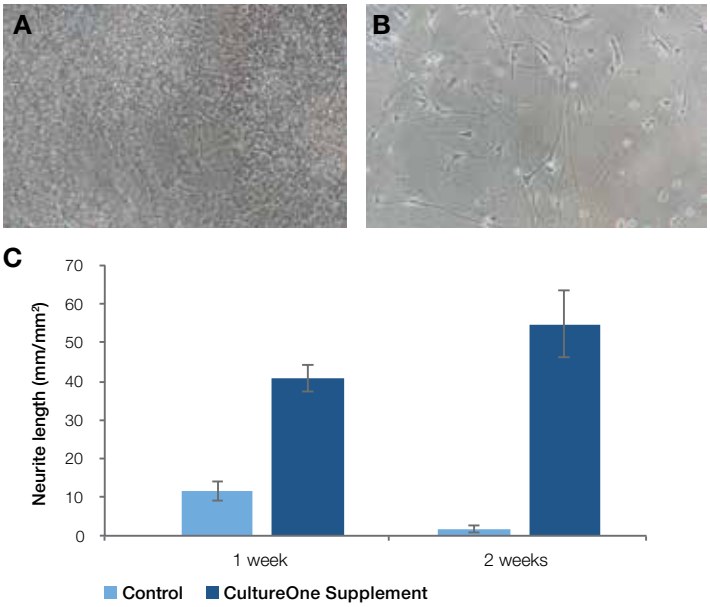

Figure 1. Phase-contrast images and neurite lengths of control and CultureOne Supplement–treated neuronal cell cultures. On day 14, **(A)** control cultures contain highly dense cells with limited neurite processes; **(B)** CultureOne Supplement–treated cultures have significantly fewer contaminating neural progenitor cells and extended neurite processes. **(C)** Average total neurite length per image area (mm/mm²) of control neurons and CultureOne Supplement–treated neurons on day 7 and day 14.

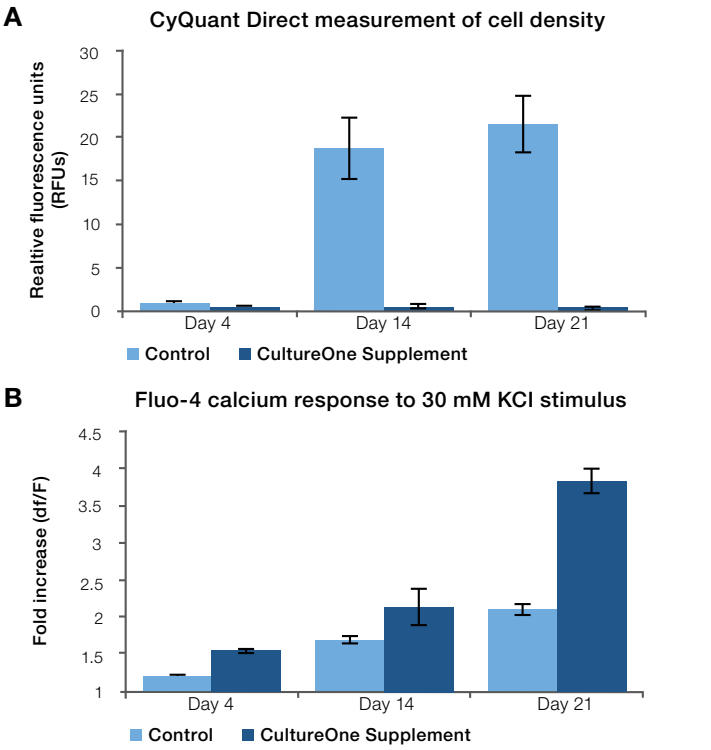

Figure 2. Cell proliferation and calcium imaging results of control and CultureOne Supplement–treated neuronal cell cultures on different days of differentiation. (A) CyQUANT Direct fluorescence indicated that control cultures had significantly higher cell densities relative to CultureOne Supplement–treated cultures at corresponding days of differentiation. **(B)** Averaged calcium responses (n = 4 each) after addition of 30 mM KCl depolarizing stimulus at different days of differentiation show CultureOne Supplement–treated neurons had higher Fluo-4 calcium responses than control cells cultured in conventional differentiation medium.

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This data indicates that neurons differentiated from PSC-derived NSCs with CultureOne Supplement express significantly higher numbers of active voltage-gated calcium ion channels earlier in differentiation than conventional differentiation methods.

Conclusion

During differentiation of hPSC-derived NSCs, developing neurons undergo a series of morphological changes as they mature, including the emergence of neurites from cell bodies and increased expression of voltage-gated ion channels. Functional assays performed with the Fluo-4 Calcium Imaging Kit demonstrated significantly higher voltage-gated calcium channel expression per NSC-derived neuron earlier in the differentiation process with CultureOne Supplement, as compared to the conventional neuronal differentiation medium. Moreover, differentiation of NSCs with CultureOne Supplement prevents the overgrowth of contaminating progenitor cells while efficiently maintaining a population of neurons that continue to form extended neurites over time. Together, these compelling findings demonstrate that CultureOne Supplement accelerates maturation and enhances functionality of neurons differentiated from NSCs compared with conventional differentiation medium. Thus, this supplement can provide a much-needed solution for the reliable production of PSC-derived neuronal disease models for high-throughput screening or basic research applications.

References

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