Development and Functional Applications of Human iPSC-derived Spinal Motor Neurons

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Abstract

The aim of this study was to produce spinal motor neurons from human iPSCs with sufficient purity for use in a multitude of downstream assays. In particular, we wanted to produce motor neurons that could be cultured in defined conditions over long periods of time, without being hampered by outgrowth from proliferative cell types. Using an optimized differentiation protocol that improves upon published methods, we were able to produce motor neurons from iPSCs at greater than 60% purity as measured by Isl1/2 and Tuj1 positive staining. These cells can be stored frozen, thawed, and cultured in media without glia for extended periods. We collected ICC, qPCR and MEA data to characterize the motor neuron cells and used iPSC lines from multiple donors to demonstrate a robust protocol that produces motor neurons independent of donor iPSC line.

These data show the characteristics and utilization of motor neurons produced from iPSC.

Process Development

A step-wise approach to the differentiation process was taken by adapting and optimizing techniques described in the literature. Directed differentiation was used to neuralize and caudalize the iPSC cells. Motor neuron specification was initiated after caudalization and cells were cryopreserved. Maturation of cells post thaw was accomplished using specific growth factors for 1-2 weeks before characterizing the motor neurons.

Cell Staining

Immunocytochemistry staining of neuronal markers. BIII-tubulin (Tuj1) stains the microtubule element exclusively in neurons. Isl1/2 is a pan marker specific for motor neurons. VACHt stains vesicular acetylcholine transporter located in presynaptic secretory vesicles, synaptophysin stains synaptic vesicles and cells that participate in synaptic transmission, peripherin stains neurofilaments in peripheral neurons and Smi-32R selectively stains non-phosphorylated neurofilaments found mainly on motor neurons. Images were taken with either a confocal microscope or a high content imager.

Morphology

Day 1 post thaw  Day 7 post thaw  Day 14 post thaw

Morphology of wild type motor neurons. Phase images of cells plated at 1x10⁶ cells per well in 6-well poly-D-lysine and laminin-coated plates. Images were taken with a 10x objective.

Purity

<table>
<thead>
<tr>
<th>Sample</th>
<th>iPSC Clone</th>
<th>Isl1/2</th>
<th>BIII tubulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor 1</td>
<td>A</td>
<td>72%</td>
<td>92%</td>
</tr>
<tr>
<td>Donor 1</td>
<td>B</td>
<td>79%</td>
<td>96%</td>
</tr>
<tr>
<td>Donor 2</td>
<td>C</td>
<td>85%</td>
<td>94%</td>
</tr>
<tr>
<td>Donor 2</td>
<td>D</td>
<td>94%</td>
<td>95%</td>
</tr>
<tr>
<td>Donor 3</td>
<td>E</td>
<td>59%</td>
<td>93%</td>
</tr>
</tbody>
</table>

Multiple iPSC lines were differentiated into motor neurons using the same protocol. At 14d post-thaw, purity was analyzed by flow cytometry. Motor neurons from all lines demonstrated ≥55% Isl1/2 and >90% BIII-tubulin.

Functional Characterization

Functional characterization by calcium-induced acetylcholine (ACh) release assay. Motor neurons were plated into 96wp (6.4 x 10⁴ cells/well) and assayed for ACh release using a choline/ACh detection kit (abcam ab65345). To induce ACh release, cells were incubated with 10mM CaCl₂ and 10μM A23187 (calcium ionophore) for 3 mins. At both 8 and 11 days post-thaw (dpt), cells released increased amounts of ACh in response to CaCl₂ with A23187 (**p<0.05* p<0.001).

Conclusions

- Optimized directed differentiation was used to efficiently derive spinal motor neurons with >90% cholinergic neuron purity and >55% motor neuron purity.
- Our motor neuron differentiation protocol is robust, consistently producing high purity cells from different clones and multiple donors.
- Gene expression analysis shows high expression of motor neuron and cholinergic neuron markers.
- iPSC-derived motor neurons exhibit characteristic functional activities, including acetylcholine release, electrical activity, and induced contraction of skeletal myotubes.

Electrical Activity

Electrophysiological characterization using multielectrode array (MEA). Motor neurons were plated in a 48-well Axion MEA plate (6 x 10⁴ cells/well). Cells displayed spontaneous action potentials as early as 4 days post-thaw (dpt), and continued to increase over a 2 week recording period. Increased electrical activity is reflected by high mean firing rate, burst frequency and intensity. Activities then plateaued and started to decrease after 4 weeks (data not shown). Three different batches of motor neurons derived from the same iPSC line showed consistent electrophysiological activity.