

Research Highlight

A Novel Method for the Generation of Region-Specific Neurons and Neural Networks from Human Pluripotent Stem Cells

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Abstract

Stem cell-based neuronal differentiation has provided a unique opportunity for disease modeling and regenerative medicine. We have reported a novel culture condition and method for generating neuronal progenitors and neural networks from human embryonic and induced pluripotent stem cells without any genetic manipulation. Neurospheres generated under 10% CO₂ with Supplemented Knockout Serum Replacement Medium (SKSRM) had doubled the expression of NESTIN, PAX6 and FOXG1 genes compared to the neurospheres generated under 5% CO₂. Furthermore, an additional step (AdStep) was introduced to fragment the neurospheres, which increased the expression of neuronal progenitor genes NEUROD1, NEUROG2, TBR1, TBR2, and NOTCH1 and the formation of the neuroepithelial-type cells. With the supplements, neuronal progenitors further differentiated into different layers of cortical, pyramidal, GABAergic, glutamatergic, cholinergic, dopaminergic and purkinje neurons within 27-40 days, which is faster than traditional neurodifferentiation protocols (42-60 days). Furthermore, our *in vivo* studies indicated that neuronal progenitors derived under our culture conditions with “AdStep” showed significantly increased neurogenesis in Severe Combined Immunodeficiency (SCID) mouse brains. This neurosphere-based neurodifferentiation protocol is a valuable tool for studies neurogenesis, neuronal transplantation and high throughput screening assays.

Stem cell based neuronal differentiation has provided the tools to generate large populations of neurons for “disease-in-a-dish” for neurological disorder research and regenerative medicine. There are two basic methods for generating neurons from human embryonic and induced Pluripotent Stem Cells (h/iPSCs): adherent (neuroectoderm) and non-adherent (embryoid body or neurosphere) culture conditions. Neuroectoderm use dual inhibition of SMAD signaling to promote efficient neuronal differentiation [1-4]. Although neuroectoderm is a well established method, utilizing neurospheres has many advantages over neuroectoderm. Neurospheres can be maintained and expanded without losing the expression of

neural progenitor markers for more than 10 passages [5-7]. Furthermore, reports suggest that neurospheres derived from iPSCs with disease-specific genetic backgrounds can be used for many disease modeling studies, such as Timothy syndrome, Fragile X syndrome or Niemann Pick Disease (NPD), Alzheimer’s Disease (AD), Parkinson’s Disease (PD) [8-11]. Recently, we reported a new defined culture medium and conditions, namely, SKSRM medium and 10% CO₂ for deriving neurospheres. Use of 10% CO₂ culture conditions which increased the expression of NESTIN, PAX6, and FOXG1 compared to the traditional 5% CO₂ culture conditions [12].

Relatively few studies describe the function of CO₂ on neurogenesis. The physiological CO₂ concentration in many tissues is far greater than that in which cells are typically cultured *in vitro*. This may influence the proliferation and differentiation potential of many cell types. Neurogenesis is also tightly regulated via both extrinsic environmental influences and intrinsic genetic factors. A large number of transcription factors have been implicated in the proliferation of neural progenitors, which ultimately influence the number of neurons in the developing brain [13,14]. Studies suggest that PAX6, Tbr1, Tbr2, and NeuroD are expressed sequentially during neurogenesis. They regulate diverse developmental mechanisms including regional identity, neuronal fate, cell cycle kinetics, cell migration, cell adhesion, axonal growth and guidance [15-17]. We investigated the influence of CO₂ concentration on neurogenesis by comparing neurospheres derived from human Embryonic Stem Cells (hESCs) under two different culture conditions. The results showed that round and bright-edged neurospheres were generated in supplemented SKSRM with 10% CO₂. In addition, the neurospheres generated under 10% CO₂ were more stable, with double the expression of NESTIN, PAX6 and FOXG1 genes compared to those cultured with 5% CO₂. However, the molecular mechanism of CO₂ on controlling neurogenesis is still not clear. As previously reported, this could be due to reduced oxygenation or hypoxia [18]. There are several groups that have reported that hypoxia or reduced oxygenation enhances neural stem cell colony survival and increased NESTIN, SOX1, SOX2, and FOXG1 expression [19,20]. CO₂ concentration appears to could be a fundamental factor in recreating the micro-environmental niche of the developing brain by altering the expression of key signaling proteins during early time-points of induced differentiation. Further investigation is needed to fully understand the molecular mechanisms of CO₂ control of neurogenesis.

Neurospheres are a good source of neural progenitors for neural transplantation due to their easy delivery and ability to migrate [2,5]. However, clumping has been a challenge for neurodifferentiation both *in vivo* and *in vitro*, which results in less efficiency and a longer time period to generate subtype and region-specific functional neurons [7,21]. A procedure termed "AdStep" was introduced to broke neurospheres into smaller fragments which increased the efficiency of neuronal production. Neurospheres were collected and resuspended in the dissociation solution (Stem Cell Technologies), and then use 5 ml polystyrene serological pipette mechanical broken down into smaller fragments by 20-30 pounding motions. The pellet was collected and transferred to matrigel-coated plates. The monolayer neuroepithelium formed after 3-5 days cultures at 5% CO₂, 37°C culture condition [12]. Our gene expression study result indicated that the "AdStep" increased the expression of NEUROD1, NEUROG2, TBR1, TBR2, MYT1, and NOTCH1. When compared to other current available neurodifferentiation methods, this procedure has allowed us to

increase the number of neuroprogenitor cells and also enabled us to rapidly generate sub-type or region-specific neurons and neural networks. Using this protocol, cortical, glutamatergic, cholinergic, and GABAergic neurons were generated within 27 days. Supplementing with Sonic Hedgehog (SHH) and Fibroblast Growth Factor 8 (FGF8) allowed us to generate mid/hindbrain dopaminergic neurons and cerebellar purkinje neurons as well. In addition, the neurons derived from our protocol have strong ongoing spontaneous Ca²⁺ activities as measured by Fluo-4, Ca²⁺ indicator dye. Time lapse Ca²⁺ imaging demonstrated that neurons derived from neurospheres with AdStep have higher networking neurons compared to the neuroectoderm derived neurons [22].

We then injected the AdStep-fragmented and the non-fragmented neurospheres into Severe Combined Immunodeficiency (SCID) mouse brains to investigate the effect of "AdStep" on neurogenesis *in vivo*. Six weeks after neurosphere transplantation, the mice were sacrificed and histological analysis was conducted with antibodies against Human-Specific Nuclear Antigen (HumN) and β III-Tubulin in order to identify the transplanted human cells. The results suggested that the engrafted AdStep-fragmented neurospheres differentiated into multipotent neural stem cells and mature neurons *in vivo* at a higher amount compared to the non-fragmented neurospheres. It is important to note that these results may have significant impacts on neuronal transplantation and regenerative medicine.

In conclusion, this novel and viral-free method for rapidly generating neurons from neurospheres provides a valuable model system for studying neurogenesis and understanding the molecular mechanisms associated with neurodegenerative diseases. This neurodifferentiation method is an excellent protocol for transplantation studies of various neurological disorders and regenerative medicine.

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References

1. Chambers SM, Fasano CA, Papapetrou EP, Tomishima M, Sadelain M, et al. (2009) Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. *Nat Biotechnol* 27: 275-280.
2. Matigian N, Abrahamsen G, Sutharsan R, Cook AL, Vitale AM, et al. (2010) Disease-specific, neurosphere-derived cells as models for brain disorders. *Dis Model Mech* 3: 785-798.
3. Koehler KR, Tropel P, Theile JW, Kondo T, Cummins TR, et al. (2011) Extended passaging increases the efficiency of neural differentiation from induced pluripotent stem cells. *BMC Neurosci* 12: 82.
4. Bez A, Corsini E, Curti D, Biggiogera M, Colombo A, et al. (2003) Neurosphere and neurosphere-forming cells: morphological and ultrastructural characterization. *Brain Res* 993: 18-29.
5. Jensen JB, Parmar M (2006) Strengths and limitations of the neurosphere culture system. *Mol Neurobiol* 34: 153-161.

6. Reynolds BA, Weiss S (1992) Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* 255: 1707-1710.
7. Reynolds BA, Rietze RL (2005) Neural stem cells and neurospheres--re-evaluating the relationship. *Nat Methods* 2: 333-336.
8. Kumari D, Swaroop M, Southall N, Huang W, Zheng W, et al. (2015) High-Throughput Screening to Identify Compounds That Increase Fragile X Mental Retardation Protein Expression in Neural Stem Cells Differentiated From Fragile X Syndrome Patient-Derived Induced Pluripotent Stem Cells. *Stem Cells Transl Med* 4: 800-808.
9. Macauley SL, Sidman RL, Schuchman EH, Taksir T, Stewart GR (2008) Neuropathology of the acid sphingomyelinase knockout mouse model of Niemann-Pick A disease including structure-function studies associated with cerebellar Purkinje cell degeneration. *Exp Neurol* 214: 181-192.
10. Pasca SP, Portmann T, Voineagu I, Yazawa M, Shcheglovitov A, et al. (2011) Using iPSC-derived neurons to uncover cellular phenotypes associated with Timothy syndrome. *Nat Med* 17: 1657-1662.
11. Paşca AM, Sloan SA, Clarke LE, Tian Y, Makinson CD, et al. (2015) Functional cortical neurons and astrocytes from human pluripotent stem cells in 3D culture. *Nat Methods* 12: 671-678.
12. Begum AN, Guynes, Cho J, Hao J, Lutfy K, et al. (2015) Rapid generation of sub-type, region-specific neurons and neural networks from human pluripotent stem cell-derived neurospheres. *Cell Res* 15: 731-741.
13. Espósito MS, Piatti VC, Laplagne DA, Morgenstern NA, Ferrari CC, et al. (2005) Neuronal differentiation in the adult hippocampus recapitulates embryonic development. *J Neurosci* 25: 10074-10086.
14. Egeland M, Zunszain PA, Pariante CM (2015) Molecular mechanisms in the regulation of adult neurogenesis during stress. *Nat Rev Neurosci* 16: 189-200.
15. Kroll TT, O'Leary DD (2005) Ventralized dorsal telencephalic progenitors in Pax6 mutant mice generate GABA interneurons of a lateral ganglionic eminence fate. *Proc Natl Acad Sci USA* 102: 7374-7379.
16. Hevner RF, Miyashita-Lin E, Rubenstein JLR (2002) Cortical and thalamic axon path finding defects in Tbr1, Gbx2, and Pax6 mutant mice: evidence that cortical and thalamic axons interact and guide each other. *J Comp Neurol* 447: 8-17.
17. Liu M, Pleasure SJ, Collins AE, Noebels JL, Naya FJ, et al. (2000) Loss of BETA2/NeuroD leads to malformation of the dentate gyrus and epilepsy. *Proc Natl Acad Sci USA* 97: 865-870.
18. Putnam RW, Filosa JA, Ritucci NA (2004) Cellular mechanisms involved in CO(2) and acid signaling in chemosensitive neurons. *Am J Physiol Cell Physiol* 287: 1493-1526.
19. Morrison SJ, Csete M, Groves AK, Melega W, Wold B, et al. (2000) Culture in reduced levels of oxygen promotes clonogenic sympathoadrenal differentiation by isolated neural crest stem cells. *J Neurosci* 20: 7370-7376.
20. Clarke L, van der Kooy D (2009) Low oxygen enhances primitive and definitive neural stem cell colony formation by inhibiting distinct cell death pathways. *Stem Cells* 27: 1879-1886.
21. Xie Y, Zhang J, Lin Y, Gaeta X, Meng X, et al. (2014) Defining the role of oxygen tension in human neural progenitor fate. *Stem Cell Reports* 3: 743-757.
22. Shi Y, Kirwan P, Livesey FJ (2012) Directed differentiation of human pluripotent stem cells to cerebral cortex neurons and neural networks. *Nat Protoc* 7: 1836-1846.