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Efficient neural differentiation of mouse pluripotent stem cells in a serum-free medium and development of a novel strategy for enrichment of neural cells

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ABSTRACT

Pluripotent stem cells (PSCs) offer an excellent model to study neural development and function. Although various protocols have been developed to direct the differentiation of PSCs into desired neural cell types, many of them suffer from limitations including low efficiency, long duration of culture, and the use of expensive, labile, and undefined growth supplements. In this study, we achieved efficient differentiation of mouse PSCs to neural lineage, in the absence of exogenous molecules, by employing a serum-free culture medium containing knockout serum replacement (KSR). Embryoid bodies (EBs) cultured in this medium predominantly produced neural cells which included neural progenitors (15–18%), immature neurons (8–24%), mature neurons (10–26%), astrocytes (27–61%), and oligodendrocytes (~1%). Different neuronal subtypes including glutamatergic, GABAergic, cholinergic, serotonergic, and dopaminergic neurons were generated. Importantly, neurons generated in the KSR medium were electrically active. Further, the EB scooping strategy, involving the removal of the EB core region from the peripheral EB outgrowth, resulted in the enrichment of PSC-derived neural cells. Taken together, this study provides the evidence that the KSR medium is ideal for the rapid and efficient generation of neural cells, including functional neurons, from PSCs without the requirement of any other additional molecule.

1. Introduction

Pluripotent stem cells (PSCs) are defined by two important properties *i.e.* unlimited self-renewal capacity and the ability to generate all the differentiated cell types present in the body. PSCs include both embryonic stem cells (ES-cells, ESCs) and induced pluripotent stem cells (iPS-cells, iPSCs) (Kuijk et al., 2011). These cells can be easily genetically manipulated (Zhu and Huangfu, 2013) and hence, they provide a useful system to study the cellular and molecular mechanisms of neural development and function. Further, the *in vitro* neural differentiation of PSCs recapitulates *in vivo* neural development (Abranches et al., 2009; Mertens et al., 2016), thereby making these cells a suitable model for neurodevelopmental studies. PSC-derived neural cells have been employed for toxicological studies, disease modeling, and drug screening (Avior et al., 2016; Marchetto et al., 2011; Telias and Ben-Yosef, 2014; Yap et al., 2015). Most importantly, PSCs serve as an excellent source of cells for potential cell replacement for the treatment of various neurodegenerative diseases and disorders

Abbreviations: Actb, beta-actin; AMED, amniotic membrane matrix-based ES-cell differentiation; AP, action potential; DMEM, Dulbecco's modified eagle medium; DPBS, Dulbecco's phosphate-buffered saline; EB, embryoid body; Egfp, enhanced green fluorescent protein; ES-cell, ESC, embryonic stem cell; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; FWHM, full width at half maximum; Gad1, glutamate decarboxylase 1; Gfap, glial fibrillary acidic protein; ICC, immunocytochemistry; iPS-cell, iPSC, induced pluripotent stem cell; KSR, knockout serum replacement; LAS AF, Leica application suite advanced fluorescence lite; LIF, leukemia inhibitory factor; MACS, magnetic-activated cell sorting; Mag, myelin-associated glycoprotein; Map2, microtubule-associated protein 2; Mbp, myelin basic protein; MEF, mouse embryonic fibroblast; MEM NEAA, minimum essential medium non-essential amino acids; Mit C, mitomycin C; Mitf, microphthalmia-associated transcription factor; Nes, nestin; Oct4, octamer-binding protein 4; PSC, pluripotent stem cell; qRT-PCR, qPCR, quantitative reverse transcription polymerase chain reaction; RMP, resting membrane potential; RPE, retinal pigmented epithelium; RT, reverse transcriptase; Sert, serotonin transporter; SFEB, serum-free floating culture of EB-like aggregates; SFEBq, quick SFEB; Sox1, sex determining region Y-box 1; Ssea1, stage-specific embryonic antigen 1; TE, trypsin-EDTA; TEA, tetraethylammonium; Th, tyrosine hydroxylase; TTX, tetrodotoxin; Tubb3, betaIII-tubulin; UDG, uracil-DNA glycosylase; Vacht, vesicular acetylcholine transporter; Vglut2, vesicular glutamate transporter 2; Zo1, zona occludens 1

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(Jongkamonwiwat and Noisa, 2013).

Many protocols have been developed to direct the differentiation of PSCs into neural lineage. These include protocols based on the embryoid body (EB) culture, coculture with stromal cells, and default differentiation into neural cells (Studer, 2004). By employing them, differentiation of PSCs to different neural cell types including glutamatergic, GABAergic, cholinergic, serotonergic, and dopaminergic neurons, astrocytes, and oligodendrocytes has been achieved (Engel et al., 2016; Liu and Zhang, 2011; Nikoletopoulou and Tavernarakis, 2012; Petros et al., 2011). However, many of the reported protocols are limiting in terms of low efficiencies of generation of neural cells (Maroof et al., 2010; Tropepe et al., 2001), long culture durations (Lee et al., 2000), requirement of exogenous molecules for induction and enhancement of neural differentiation (Wang et al., 2015; Yao et al., 2013), and supplementation with serum, a highly variable media component (Carpenter et al., 2001; Chatzi et al., 2009). Therefore, attempts have been made to achieve efficient differentiation of PSCs to neural lineage under serum-free culture conditions.

An important serum-free supplement that has been employed in many protocols aimed at generating neural cells from PSCs is knockout serum replacement (KSR) (Price et al., 1998), whose composition has been described elsewhere (Garcia-Gonzalo and Izpisúa Belmonte, 2008). KSR-containing medium is used in the neural differentiation protocols based on the coculture of the mouse (Joshi et al., 2016), primate (Kawasaki et al., 2002), and human ES-cells (Vazin et al., 2008) with the PA6 stromal cell line. The KSR medium is also used in the amniotic membrane matrix-based ES-cell differentiation (AMED) of the mouse and human cell lines (Ueno et al., 2006). KSR is a component of the DFK medium which is utilized for the motoneuron differentiation of mouse (Yao et al., 2013) and human (Egawa et al., 2012) PSCs. The KSR-containing medium is used in the protocols based on the dual inhibition of SMAD signaling for neural differentiation of human PSCs (Chambers et al., 2009; Fasano et al., 2010). It is also employed in the neural differentiation protocols based on the serum-free floating culture of EB-like aggregates (SFEB) and quick SFEB (SFEBq) (Egawa et al., 2012; Watanabe et al., 2005). The KSR-supplemented medium has been successfully utilized for the derivation of various neural cell types including telencephalic precursors (Watanabe et al., 2005), dopaminergic progenitors (Doi et al., 2014), serotonergic neurons (Shimada et al., 2012), hypothalamic-like neurons (Wang et al., 2015), cortical interneurons (Maroof et al., 2013), and nociceptors (Chambers et al., 2012). These studies highlight that KSR is an important media supplement in various neural differentiation protocols for generating a variety of neural cell types.

However, in most of the neural differentiation protocols, KSR is used in the initial stages of the culture. Subsequently, it is replaced with N2 (Cho et al., 2006; Suter et al., 2009), B27 (Doi et al., 2014; Egawa et al., 2012), or N2B27 (Sachdeva et al., 2010; Wataya et al., 2008) supplements. Also, KSR is generally used along with other growth factors (Akanuma et al., 2012; Andersson et al., 2013) and inhibitors (Egawa et al., 2012; Wang et al., 2015) to achieve generation of neural cells from PSCs. In some of the KSR-based neural differentiation protocols, serum is included in the media to facilitate the adhesion of the ES-cells or EBs to the culture surface (Lenka and Ramasamy, 2007; Miles et al., 2004). It is observed that very few studies are conducted where the KSR medium is employed throughout the neural differentiation protocol in the absence of exogenous factors. These include studies based on the adherent monolayer (Fico et al., 2008; Incitti et al., 2014; Parisi et al., 2010) and EB-based (Parisi et al., 2007; Taha et al., 2014) culture of mouse ES-cells. Of relevance, here, is our own study on the differentiation of mouse GS-2 ES-cells in the KSR medium using EB-based approach, where we characterized the generated neural cells solely on the basis of their morphology (Singh et al., 2012). However, these studies lacked quantitation of the efficiencies of generation of various neural cell types and assessment of the functionality of these cells. Also, there is no report available on neural differentiation of a mouse iPS-cell line in the KSR medium in the absence of exogenous factors.

Because of the applicability of the KSR supplement in various neural differentiation protocols, we realized the need to have a comprehensive and comparative assessment of neural differentiation potential of a few PSC lines in the KSR medium in the absence of exogenous factors. For this, we analyzed three PSC lines which included both ES- and iPS-cell lines. Additionally, we attempted to enrich PSC-derived neural cells by employing a simple scooping method involving the removal of the EB core region from the neural cell-enriched peripheral EB outgrowth.

2. Materials and methods

2.1. Culture and differentiation of PSCs

PSC lines used in this study were well-characterized mouse GS-2 ESCs (Singh et al., 2012), N9 iPSCs (Seshagiri et al., 2011), and D3 ESCs (Doetschman et al., 1985). GS-2 and N9 are our in-house-derived enhanced green fluorescent protein (Egfp)-transgenic cell lines, whereas D3 is a standard cell line cultured in many laboratories. PSCs were maintained on mitomycin C (mit C)-treated mouse embryonic fibroblasts (MEFs) in 0.1% gelatin (Sigma)-coated 35 mm tissue culture dishes (Greiner). The PSC culture medium contained Dulbecco's modified eagle medium (DMEM, Invitrogen) supplemented with 15% ESC-qualified fetal bovine serum (FBS, Invitrogen), 2 mM glutamax (Invitrogen), 0.1 mM minimum essential medium non-essential amino acids (MEM NEAA, Invitrogen), 0.1 mM β-mercaptoethanol (β-ME, Sigma), 50 U/ml penicillin and 50 μg/ml streptomycin (penicillin-streptomycin solution, Invitrogen), and 1000 U/ml recombinant mouse leukemia inhibitory factor (LIF, Chemicon). PSC colonies attained about 80% confluency after 48 h and were passaged in 1:4–1:10 ratios by treatment with 0.25% trypsin-EDTA (TE, Invitrogen) for 3 min at 37 °C. Subsequently, about one-fourth to one-tenth of the PSCs were plated over the fresh layer of MEFs and the remaining cells were cryopreserved. Throughout the culture, the cells were maintained at 37 °C and 5% CO_2 in an incubator.

Differentiation of PSCs (passage 13–45) was induced by EB formation using the hanging drop method (Guan et al., 1999). On day 0 of culture, PSC colonies were trypsinized and the cells were suspended in the PSC differentiation medium at the density of 2×10^4 cells/ml. Drops (20 μl volume containing ~400 cells) of this cell suspension were placed on the underside surface of the lids of 94 mm bacteriological petri dishes (Greiner) containing about 20 ml of Dulbecco's phosphate-buffered saline (DPBS). PSC differentiation medium (also referred to as the KSR medium) contained DMEM supplemented with 20% KSR, 2 mM glutamax, 0.1 mM MEM NEAA, 0.1 mM β-ME, 50 U/ml penicillin, and 50 μg/ml streptomycin. The lids were then inverted to form the hanging drops and were maintained in this state for 48 h. On day 2 of culture, the generated EBs were collected and were kept for further growth for 72 h in the suspension culture in 60 mm bacteriological petri dishes (Greiner). On day 5, the EBs were transferred from the suspension to adherent culture condition; Geltrex (Invitrogen) was used as the matrix for the attachment of EBs. For RNA sampling and flow cytometry analysis, EBs were plated in 60 mm tissue culture dishes (Greiner) at the density of 20–30 EBs/dish. EBs were also plated in 24-well plates (Greiner), at the density of 1 EB/well, for regular assessment of their proliferation and differentiation. Additionally, EBs were plated on micro cover glasses (Bluestar) for immunocytochemistry (ICC) and patch clamp recording. Tissue culture dishes and 24-well plates were coated with 1:800 dilution of Geltrex, while micro cover glasses were coated with 1:100 dilution of Geltrex. Medium change was performed after every 48 h.

PSCs and PSC-derived EBs were observed using an inverted stage microscope (IX70-S8F2, Olympus optical Co. Ltd., Japan) and the imaging was performed using Image Pro version 4.0 software (Media cybernetics, Silver Spring, MD, USA) with the help of a color video