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METHODS AND REAGENTS

EZ spheres: A stable and expandable culture system for the generation of pre-rosette multipotent stem cells from human ESCs and iPSCs

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Abstract We have developed a simple method to generate and expand multipotent, self-renewing pre-rosette neural stem cells from both human embryonic stem cells (hESCs) and human induced pluripotent stem cells (iPSCs) without utilizing embryoid body formation, manual selection techniques, or complex combinations of small molecules. Human ESC and iPSC colonies were lifted and placed in a neural stem cell medium containing high concentrations of EGF and FGF-2. Cell aggregates (termed EZ spheres) could be expanded for long periods using a chopping method that maintained cell–cell contact. Early passage EZ spheres rapidly down-regulated OCT4 and up-regulated SOX2 and nestin expression. They retained the potential to form neural rosettes and consistently differentiated into a range of central and peripheral neural lineages. Thus, they

Abbreviations: AP2, activating protein 2; BLBP, brain lipid binding protein; BMP, bone morphogenetic proteins; CNS, central nervous system; DA, dopamine; DACH1, dachshund homolog 1; EB, embryoid body; EGF, epidermal growth factor; ESCs, embryonic stem cells; FGF-2, fibroblast growth factor; FOXG1, forkhead box protein G1; GBX2, gastrulation brain homeobox 2; HB9, homeobox gene Hb9; hESCs, human embryonic stem cells; HNK, human natural killer-1; HOXB4, homeobox B4; iPSCs, induced pluripotent stem cells; MAP2, microtubule-associated protein 2; MEF, mouse embryonic fibroblast; NG2, chondroitin sulphate proteoglycan; OCT4, octamer-binding transcription factor 4; OLIG2, oligodendrocyte transcription factor 2; OTX2, orthodenticle homeobox 2; PAR3, polarity complex gene 3; PAX6, paired box gene 6; PAX7, paired box gene 7; PCR, polymerase chain reaction; PDGFRα, platelet-derived growth factor alpha; PLZF, promyelocytic leukemia zinc finger; PNS, peripheral nervous system; SMAD, Sma and Mad related family; SOX1, SRY-related HMG-box 1; SOX2, SRY-related HMG-box 2; SSEA3, stage specific embryonic antigen 3; TGFβ, transforming growth factor beta; TH, tyrosine hydroxylase; Tuj1, βIII-tubulin; ZO-1, zona occludens protein 1

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represent a very early neural stem cell with greater differentiation flexibility than other previously described methods. As such, they will be useful for the rapidly expanding field of neurological development and disease modeling, high-content screening, and regenerative therapies based on pluripotent stem cell technology.

Introduction

Human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) have provided a platform for studying basic human development and disease mechanisms and hold great potential for future cell therapies (Murry and Keller, 2008). However, biomedical application of hESCs and iPSCs depends on the availability of robust cell expansion and differentiation protocols. Undifferentiated colonies of hESCs and iPSCs can be technically challenging to maintain and expand. For example, they thaw from frozen samples with low efficiency and then require co-culture with mouse embryonic fibroblasts (MEF), or expensive media and matrix proteins, in order to remain undifferentiated. Furthermore, they need daily medium changes, examination, and manual selection to ensure the cultures remain in an undifferentiated state. Finally, >10% of hESC and iPSC cultures develop karyotypic anomalies (Ben-David et al., 2011; Peterson et al., 2011; Taapken et al., 2011), which should be monitored as they could impact differentiation capabilities (Graf and Stadtfeld, 2008) and clinical applicability. Clearly, less time consuming and labor intensive culturing techniques would be advantageous.

Research into embryogenesis and nervous system development has been instrumental to the identification of factors required for cell specification. Using information gleaned from these studies, many groups have developed induction protocols to instruct hESCs and iPSCs to become a variety of neural cell types (Zhang et al., 2001), including motor neurons, dopamine neurons, striatal neurons, and oligodendrocytes (Aubry et al., 2008; Delli Carri et al., 2013; Li et al., 2005; Nistor et al., 2005; Perrier et al., 2004). Some of these traditional differentiation protocols use embryoid body (EB) formation as the first step of lineage restriction to mimic early human embryogenesis (Zhang et al., 2001), which is then followed by manual selection of neuroepithelial precursors. Interestingly, the efficiency of EB formation and subsequent differentiation can vary among hESC and iPSC lines, and in some instances fail using the same culturing conditions (Boulting et al., 2011; Hu et al., 2010; Osafune et al., 2008). While the mechanisms underlying these differences remain to be determined, this observation suggests that progressing through an EB step may not always be optimal. Additionally, EBs cannot be robustly expanded, so one must start with a large number of undifferentiated hESCs or iPSCs to generate enough EBs to push through the various differentiation steps for each experimental or therapeutic use, therefore increasing batch-to-batch variations among differentiation procedures.

A technique that efficiently expands neural stem cells from hESCs or iPSCs and allows consistent differentiation of neural tissue is of great interest, and there are a number of published protocols that have been developed (Chaddah et al., 2012; Elkabetz et al., 2008; Koch et al., 2009; Nemati et al., 2011). Elkabetz et al. (2008) used an extended EB formation period and sorting methods to isolate rosette stage neuroepithelial cells that allowed them to generate a transient population of expandable neural stem cells that retain differentiation potential. In contrast to other reports (Falk et al., 2012; Koch et al., 2009), only when these cells were grown in the presence of signaling molecules (e.g. sonic hedgehog and notch) were they able to retain rosette formation and structure, induce proliferation, and subsequently differentiate into motor neurons, dopamine neurons, and neural crest progenitor cells (Elkabetz et al., 2008). However, further expansion in the presence of growth factors resulted in the loss of rosette formation and in vitro regionalization capacity and biased the culture toward gliogenic differentiation (Elkabetz et al., 2008). Also, Koch et al. (2009) described a protocol in which neuroepithelial stem cells were mechanically isolated following EB formation and expanded in the presence of EGF and FGF-2 to successfully generate a variety of neural subtypes. However, cells became regionally restricted after ~15 in vitro passages (Falk et al., 2012; Koch et al., 2009).

In the current study we have devised a method that generates pre-rosette stem cells directly from hESCs and iPSCs in a free-floating aggregate system in the presence of EGF and FGF-2. Due to their ease of expansion and differentiation, we have termed these cultures "EZ spheres". Using our previously described method of a mechanical, non-enzymatic chopping technique (Svendsen et al., 1998), EZ spheres can be expanded for at least 30 passages while maintaining chromosomal stability. Given the proper neural differentiation conditions, rosettes appear within whole spheres and upon plate-down indicating that EZ spheres retain rosette properties after long-term exposure to EGF and FGF-2. Longitudinal analysis of neural gene expression patterns in EGF and FGF-2 culture conditions showed consistent and sustained expression of nestin and SOX2 for all lines, with more varied expression of region specific markers including FOXG1, GBX2, PAX7, and OTX2. Nevertheless, EZ spheres could be taken at any passage and placed into appropriate differentiation conditions to generate specialized neuronal and glial subtypes, such as dopamine neurons, motor neurons, striatal neurons, peripheral sensory neurons, astrocytes, and oligodendrocytes, with similar efficiencies between hESCs and iPSCs. Importantly, EZ spheres do not acquire regionally restricted differentiation potential over successive passages. As a result, the EZ sphere method eliminates the need for EB formation and manual selection, allows for exponential expansion of pre-rosette multipotent neural stem cells, is amenable to healthy and disease-specific iPSCs, and increases versatility of lineage specification over other published techniques.

Materials and methods

Cell culture

hESCs (H9 WiCell Research Institute) and iPSCs were grown on irradiated MEF as previously described (Thomson et al.,