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Research Article

A scale out approach towards neural induction of human induced pluripotent stem cells for neurodevelopmental toxicity studies

Cláudia C. Miranda[a,b], Tiago G. Fernandes[a,b], Sandra N. Pinto[c,d], Manuel Prieto[c,d], M. Margarida Diogo[a,b], Joaquim M.S. Cabral[a,b,*]

[a] iBB - Institute for Bioengineering and Biosciences and Department of Bioengineering, Instituto Superior Técnico, Universidade de Lisboa, Av. Rovisco Pais, 1049-001 Lisbon, Portugal

[b] The Discoveries Centre for Regenerative and Precision Medicine, Lisbon Campus, Instituto Superior Técnico, Universidade de Lisboa, Av. Rovisco Pais, 1049-001 Lisbon, Portugal

[c] Centro de Química-Física Molecular and Institute of Nanoscience and Nanotechnology, Instituto Superior Técnico, Universidade de Lisboa, Av. Rovisco Pais, 1049-001 Lisbon, Portugal

[d] iBB - Institute for Bioengineering and Biosciences, Instituto Superior Técnico, Universidade de Lisboa, Av. Rovisco Pais, 1049-001 Lisbon, Portugal

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ABSTRACT

Stem cell's unique properties confer them a multitude of potential applications in the fields of cellular therapy, disease modelling and drug screening fields. In particular, the ability to differentiate neural progenitors (NP) from human induced pluripotent stem cells (hiPSCs) using chemically-defined conditions provides an opportunity to create a simple and straightforward culture platform for application in these fields. Here, we demonstrated that hiPSCs are capable of undergoing neural commitment inside microwells, forming characteristic neural structures resembling neural rosettes and further give rise to glial and neuronal cells. Furthermore, this platform can be applied towards the study of the effect of neurotoxic molecules that impair normal embryonic development. As a proof of concept, the neural teratogenic potential of the antiepileptic drug valproic acid (VPA) was analyzed. It was verified that exposure to VPA, close to typical dosage values (0.3 to 0.75 mM), led to a prevalence of NP structures over neuronal differentiation, as confirmed by analysis of the expression of neural cell adhesion molecule, as well as neural rosette number and morphology assessment. The methodology proposed herein for the generation and neural differentiation of hiPSC aggregates can potentially complement current toxicity tests such as the humanized embryonic stem cell test for the detection of teratogenic compounds that can interfere with normal embryonic development.

1. Introduction

Since their first isolation (Thomson et al., 1998), human pluripotent stem cells (hPSCs) have generated great interest in therapy-related areas such as regenerative medicine, disease modelling, drug discovery and toxicity screening. hPSCs main features include the capacity for self-renewal and concomitant proliferation capacity, as well as their ability to differentiate into cells from the three embryonic germ layers and, in the case of human induced pluripotent stem cells (hiPSCs), the capacity to generate patient-specific cells that allow bypassing rejection in cell therapy settings (Takahashi et al., 2007), as well as to create models of human diseases (Fernandes et al., 2015).

To fulfill the differentiation potential of hiPSCs, several protocols reporting the differentiation of hPSCs into specific cell types have emerged. In particular, we highlight the direct induction of hiPSCs into neural precursor cells (Elkabetz et al., 2008; Koch et al., 2009), either as adherent monolayer, or as suspension aggregates, using undefined or defined media compositions (Chambers et al., 2009; Shi et al., 2012; Miranda et al., 2015). For instance, the neural induction of hiPSC as three-dimensional (3D) aggregates allows better mimicking conditions during embryonic development, and neural rosettes act as an *in vitro* indicator of a successful closure of the neural tube (Zhang et al., 2001). These culture platforms are of foremost applicability and importance since, among other applications, it allow testing large numbers of molecules and their effects in human development and, specifically, their interference with the formation of cerebral tissue, which is in fact a growing need (Fernandes et al., 2014). As one example of such type of platforms, the embryonic stem cell test (EST) is used to determine the

* Corresponding author at: iBB - Institute for Bioengineering and Biosciences and Department of Bioengineering, Instituto Superior Técnico, Universidade de Lisboa, Av. Rovisco Pais, 1049-001 Lisbon, Portugal.
E-mail address: joaquim.cabral@tecnico.ulisboa.pt (J.M.S. Cabral).

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effects of chemicals in the viability of murine embryonic stem cells (mESCs) and their ability to differentiate into beating cardiomyocytes (Genschow et al., 2004). Still, the EST may fail to predict response in humans, and does not consider the effects on the development of other tissues, such as the brain (Marx- and Stoelting et al., 2009). Although studies on the neurotoxic effects of chemicals were already performed in cells derived from human embryonic stem cells (hESCs) (Bosnjak, 2012), the majority of the other reports have used different cell models like human embryonic carcinoma stem cells (hECSCs) (Cao et al., 2015). Moreover, an approach using a 3D microarray of encapsulated cells has been described for a high throughput analysis of neural cells to predict the effect of neurotoxic compounds that may affect neuronal development (Meli et al., 2014). Still, the challenge to develop systems capable to better mimicking early embryonic development, particular in 3D settings, still remains.

Suspension culture systems in particular are easily scalable (Olmer et al., 2012), since there is no need for an adherent support, such as extracellular matrices or microcarriers. Nonetheless, one of the limitations associated with aggregate culture is the coalescence of several aggregates, which can result in mass-transfer limitations leading for example to necrotic environments, and an overall difficulty in generating standardized tests. A possible solution to avoid this problem is the physical confinement of 3D aggregates in microwells. Microwells are an excellent alternative to obtain relevant numbers of cells for drug discovery and toxicity screening tests due to their intrinsic throughout capacities (Fernandes et al., 2009). Besides preventing agglomeration, by limiting the access of each aggregate to its respective microwell, spatial confinement can also regulate early commitment steps of hPSCs (Giobbe et al., 2012; Hwang et al., 2009; Warmflash et al., 2014). The accumulation of endogenous factors synthetized by hPSCs confined within microwells contributes to create a suitable environment for cell growth and differentiation, especially into the ectoderm lineage (Giobbe et al., 2012).

One particular area of interest for direct application of these culture platforms is the study of neurologic diseases. These conditions affect millions of people worldwide, and in many cases, like for epilepsy, advances in medicine offer some treatment possibilities, although presenting relevant side effects. For example, antiepileptic drugs direct their action towards targets located on the central nervous system (CNS), and their effects can induce negative effects on neurodevelopmental processes (Cotariu and Zaidman, 1991). The use of such compounds during pregnancy can increase the risk of malformations in the developing fetus, namely at the neural tube level, as well as growth retardation and microcephaly (Cotariu and Zaidman, 1991; 22). Valproic acid (VPA), for example, is an antiepileptic drug that is widely administered to treat neurologic disorders such as epilepsy and acute mania. Nevertheless, the intake of this drug by pregnant women can lead to problems in the developing fetus that include malformations, development delay, reduced cognitive functions and even autism-related behavior (Cotariu and Zaidman, 1991; Mitchell, 2005; Nau et al., 1991; Ornoy, 2009). VPA is also an inhibitor of histone deacetylase activity, interfering with chromatin remodeling (Phiel et al., 2001), being widely used in reprogramming of somatic cells into iPSCs (Wattanapanitch et al., 2014).

In the present study, we introduce microwells as an alternative for hiPSC derivation into the neural lineage that allows control over aggregate diameter and prevents aggregate agglomeration. Neural progenitors (NPs) derived inside microwells were able to generate typical neural structures, such as neural rosettes, and were able to further differentiate into neurons and astrocytes. Moreover, we demonstrated that this platform could be used as an alternative for screening drug toxicity effects during the embryonic neurodevelopment. For the purpose, we used VPA treatment as proof of principle. We demonstrate that this compound induces alterations at level of cell growth, and viability. We also observed alterations in neural rosette number and morphology, as well as in gene expression patterns.

2. Materials and methods

Culture of hiPSCs in adherent conditions The hiPSC line F002.1 A.13 (TCLab – Tecnologias Celulares para Aplicação Médica, Unipessoal, Lda.) was generated directly from fibroblasts through ectopic expression of a defined set of reprogramming factors, Oct4, Sox2, Klf4 and c-Myc, using a retroviral system. Cells were routinely cultured in Matrigel (1:30, BD Biosciences)-coated plates using mTeSR1 medium (StemCell Technologies) and passaged 1:5 using EDTA every 5 days (Beers et al., 2012).

Culture of hiPSCs in 3D conditions Cells were incubated with 10 μM ROCK inhibitor (ROCKi, Y-27632, StemGent) for 1 h at 37 °C and then treated with accutase for 5 min at 37 °C. Cells were seeded in microwell plates (AggreWell™, StemCell Technologies) at a density of 1.0×10^6 cells/mL to create aggregates containing approximately 5,000 cells each, using mTeSR™1 supplemented with 10 μM ROCKi for 24 h. As a control for aggregate formation, cells were seeded in 6-well ultra low attachment plates at the same density.

Neural commitment of hiPSCs After 24 h of culture inside microwells, mTeSR1 medium was replaced by 1:1 N2 and B27 medium, as previously described (Shi et al., 2012). The medium was replaced daily and supplemented with 10 μM SB431542 (SB, Sigma) and 100 nM LDN193189 (LDN, StemGent) throughout the 9 days of the neural commitment stage.

Neuronal differentiation of hiPSC After 9 or 12 days of neural commitment, NP aggregates were treated with 0.5 mM EDTA (Invitrogen) for 5 min at room temperature, gently dissociated into small clumps and replated in poly-L-ornithine (15 μg/mL, Sigma) and Laminin (20 μg/mL, Sigma)-coated plates at a density of 200,000 cells/cm^2. NP cells were further differentiated up to 125 days according to a previously described protocol (Miranda et al., 2015).

Drug exposure Valproic acid (VPA, Sigma) was dissolved in dimethyl sulfoxide (DMSO, Sigma) for a stock solution of 100 mM. Final concentration in the culture media ranged 0.01–1 mM VPA. Cells were exposed to VPA during the 9 days of neural commitment protocol. To verify the potential effect of DMSO alone during neural induction, we have included a DMSO control with a final concentration of 1% (v/v) DMSO at an equivalent volume of 1 mM VPA.

Immunostaining Cells were fixed with 4% (v/v) paraformaldehyde (PFA; Sigma) and stained according to a previously described protocol (Miranda et al., 2015). Further observation was performed under the fluorescence optical microscope (Leica DMI 3000B) and a digital camera (Nikon DXM 1200).

Flow cytometry Cells were fixed using 2% PFA and a minimum of 1×10^5 cells was tested per condition. Primary antibody and respective isotype were prepared in 3% FBS and cells were incubated for 15 min at room temperature in the dark. Cells were resuspended in PBS and analyzed in the flow cytometer (FACSCalibur, Becton Dickinson).

Fluorescence microscopy imaging Cells were imaged using a laser scanning confocal microscope (Leica TCS-SP5) equipped with a continuous Ar-ion laser (Multi-line LASOSs LGK 7872 ML05) and a Ti:sapphire laser (Spectra-Physics Mai Tai BB, 710–990 nm, 100 fs, 82 MHz). A 63 × 1.2 N.A. water immersion objective was used. Alexa-488 and 546 probes were excited with the 488 and 514 nm Ar^+ laser lines, and DAPI excitation was set at 780 nm using a Ti:sapphire laser. Images were collected with 512 × 512 pixels and at a scan rate of 100 Hz. 2D Image analysis was performed using the software ImageJ and 3D image analysis was performed using Fiji (http://pacific.mpi-cbg.de).

Antibodies for Immunocytochemistry Oct4 (Millipore, 1:750), Pax6 (Covance, 1:1000), Nestin (R&D Systems, 1:1000), ZO-1 (Invitrogen, 1:100), Ki67 (BD Pharmigen, 1:100), Nanog (Millipore, 1:5000), Sox2 (R&D Systems, 1:1000), β-III-tubulin (Tuj1, Covance, 1:20,000) and glial fibrillary acidic protein (GFAP, Millipore, 1:100) were used as primary antibodies whereas goat anti-mouse IgG Alexa Fluor–488 or 546 (1:500, Invitrogen), goat anti-rabbit IgG Alexa Fluor–488 or 546