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A robust and reproducible human pluripotent stem cell derived model of neurite outgrowth in a three-dimensional culture system and its application to study neurite inhibition

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ABSTRACT

The inability of neurites to grow and restore neural connections is common to many neurological disorders, including trauma to the central nervous system and neurodegenerative diseases. Therefore, there is need for a robust and reproducible model of neurite outgrowth, to provide a tool to study the molecular mechanisms that underpin the process of neurite inhibition and to screen molecules that may be able to overcome such inhibition. In this study a novel in vitro pluripotent stem cell based model of human neuritogenesis was developed. This was achieved by incorporating additional technologies, notably a stable synthetic inducer of neural differentiation, and the application of three-dimensional (3D) cell culture techniques. We have evaluated the use of photostable, synthetic retinoid molecules to promote neural differentiation and found that 0.01 μM EC23 was the optimal concentration to promote differentiation and neurite outgrowth from human pluripotent stem cells within our model. We have also developed a methodology to enable quick and accurate quantification of neurite outgrowth derived from such a model. Furthermore, we have obtained significant neurite outgrowth within a 3D culture system enhancing the level of neuritogenesis observed and providing a more physiological microenvironment to investigate the molecular mechanisms that underpin neurite outgrowth and inhibition within the nervous system. We have demonstrated a potential application of our model in co-culture with glioma cells, to recapitulate aspects of the process of neurite inhibition that may also occur in the injured spinal cord. We propose that such a system that can be utilised to investigate the molecular mechanisms that underpin neurite inhibition mediated via glial and neuron interactions.

1. Introduction

During neuronal development, dynamic processes involving cytoskeletal remodelling occur, known as neuritogenesis. Long cytoskeletal processes, known as neurites, project from the cell body of the developing neuron and ultimately form distant neural connections that become the axon and dendrites of a mature neural network (Da Silva and Dotti, 2002). The growth cone is situated at the distal tip of the developing neurite and is rich in actin filaments (Khodosevich and Monyer, 2010) and microtubules (Dent et al., 2011), along with adaptor proteins, as it undergoes significant cytoskeletal rearrangements during neurite elongation. Primarily the remodelling of the actin cytoskeleton drives protrusion and motility of the growth cone; therefore actin dynamics play an important role during neuritogenesis (Dent et al., 2011).

Complex neuronal networks are essential for nervous system function, and depend on the successful formation of neurite projections from the developing neuron. Accordingly, neuritogenesis is an integral process essential to the proper functioning of the nervous system (Da Silva and Dotti, 2002). Inhibition of neurite outgrowth is common to many nervous system disorders including central nervous system trauma (Rolls et al., 2009; Yiu and He, 2006a; Niederost et al., 2002; Bush et al., 1999) and

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neurodegenerative diseases (Petratos et al., 2008; Postuma et al., 2000; Takenouchi et al., 2001; Winner et al., 2011). This highlights the importance of *in vitro* models of neuritogenesis to enhance our understanding of the process and to screen potential therapeutic molecules.

Many of the popular current models of neurite outgrowth include the isolation and *in vitro* culture of primary mammalian cell types derived from the nervous system of rats and mice. Dorsal root ganglia (DRG) neurons and explants derived from mammalian or chick embryos are commonly isolated and cultured *in vitro* without the need for differentiation and form the basis of many neurite outgrowth studies (Clagett-Dame et al., 2006; Balgude et al., 2001; Fitzgerald et al., 1993). Although the use of primary cells avoids the limitations associated with immortalised cell lines, the physiology of animal derived cells may differ significantly from that of human cells and impact experimental outcomes. This becomes particularly important when applying such models to human neurological diseases. Human cell lines such as SH-SY5Y are commonly used to study neurite outgrowth (Ferrari-Toninelli et al., 2004; Tucholski et al., 2001; Ross et al., 1983). SH-SY5Y cells are a popular neuroblastoma cell line that originated from a metastatic bone biopsy that require differentiation with *all*-trans retinoic acid (ATRA) (Kovalevich and Langford, 2013). However, due to their neoplastic nature, mixed morphology, and somewhat limited capacity for neuritogenesis, questions have arisen as to the consistency and appropriateness of the use of such cells in neurite outgrowth models, particularly when the application may be to test potential therapeutics (Kovalevich and Langford, 2013).

Human pluripotent stem cells are recognised models of neural development. The embryonal carcinoma (EC) stem cell line, TERA2, readily differentiates into neuronal subtypes when exposed to ATRA. EC cells are the malignant counterpart of embryonic stem cells, and cells from the TERA2 lineage have provided the basis for many *in vitro* models of neural differentiation, function and neurite outgrowth (Tegengem et al., 2011; Satoh et al., 1997; Pewsey et al., 2010; Roloff et al., 2015; Przyborski, 2001; Przyborski et al., 2000, 2003, 2004; Stewart et al., 2004; Coyne et al., 2011). While such models are valuable, there is significant scope to enhance their reliability in terms of robustness and reproducibility.

Cell technologies are becoming available which are designed to improve current practice, and enhance the development and application of *in vitro* assays. Such techniques can be used to improve the robustness and reproducibility of neurite outgrowth assays and enhance their physiological significance. For example, ATRA is a derivative of vitamin A, which is important during the patterning and differentiation of the developing nervous system *in vivo* (Magden, 2007). However, the use of ATRA *in vitro* is limited, as it readily breaks down when exposed to light and heat. Stable, synthetic forms of retinoic acid such as, EC23 and AH61 offer more practical *in vitro* use, as they contain a non-isomerisable conjugated linker unit that stabilises the molecule, and have previously been described as tools for stem cell differentiation studies (Clemens et al., 2013; Christie et al., 2008). In recent years there has been growing interest in 3D culture techniques to enhance the physiological relevance of *in vitro* models. Many existing models of neurite outgrowth use conventional two-dimensional (2D) cell culture, rather than culturing developing neurites in a more physiological three-dimensional (3D) system. 3D scaffolds have been developed to enhance neurite development (Hayman et al., 2004, 2005). Another limitation of current 2D neurite outgrowth models is that they are difficult to quantify, as monolayers of developing neurons intertwine and form complex neuronal networks within the cell population, making it difficult to identify individual neurites emanating from individual perikarya. We propose that quantification of neurite outgrow can be simplified using a neurosphere model, whereby all neurites radiate from a central point aiding quantification and significantly reducing neurite network complexity.

In this study we have combined our stem cell model of human neuritogenesis with synthetic retinoids and 3D cell culture technologies to produce an enhanced model of neurite development and outgrowth. This provides a powerful new tool to study neurite inhibition and to investigate the molecular processes involved in the context of different neurological disorders. Here, we present an example application of this system, to study the process of neurite inhibition and the ability of small molecules to overcome such inhibition. Such research tools will be important to help elucidate the mechanisms that underpin neurite inhibition to enable intervention and recovery of neurite outgrowth.

2. Material & methods

2.1. Cell line maintenance

2.1.1. Human pluripotent stem cells

The EC cell line TERA2.cl.SP12, was maintained in maintenance medium consisting of Dulbecco's modified Eagles medium containing high glucose (DMEM-HG, Lonza, Basel, Switzerland), 10% heat treated foetal bovine serum (FBS, ThermoFisher Scientific, Cramlington, UK), 2 mM L-glutamine (Lonza) and 20 active units of penicillin and streptomycin (Lonza). EC cells were maintained at high confluence and passaged using acid washed glass beads in a 2–3 ratio into 75 cm (Khodosevich and Monyer, 2010) culture flasks (BD Falcon, Erembodegem, Belgium) to ensure their pluripotent phenotype. A Leica DFC 310FX with digital camera DMI 3000B was used to record phase contrast images of live cells.

2.1.2. U-118 MG glioma cells

U-118 MG cells were maintained in DMEM-HG, 10% FBS, 2 mM L-glutamine and 100 active units of penicillin and streptomycin. Cells were maintained in 75 cm^2 culture flasks (ThermoFisher Scientific) and passaged at high confluence in a 3–6 ratio using 0.25% trypsin/2 mM EDTA (Lonza).

2.2. Induction of cell differentiation in conventional 2D culture

TERA2.cl.SP12 stem cells were seeded at 250,000 cells per 25 cm (Khodosevich and Monyer, 2010) culture flask (BD Falcon) and incubated for 24 h to allow cells to adhere. Following 24 h incubation, the culture medium was replaced with media containing ATRA Sigma-Aldrich, EC23 (ReproCELL Europe, UK) or AH61 (Chemistry Department, Durham, UK) in dimethyl sulfoxide (DMSO) at concentrations of 0.001 μM, 0.01 μM, or 1 μM. Cells were cultured for a further 7 days with a media change on the 4th day prior to analysis of cell surface markers by flow cytometry or lysis for Q-PCR.

2.3. Analysis of protein expression by flow cytometry

Following the 7 day differentiation period a single cell suspension was obtained through the trypsinisation of monolayers and cells were resuspended in blocking buffer (0.1% bovine serum albumin (BSA, Sigma-Aldrich) in phosphate buffered saline (PBS)) at a density of 0.2×10^6 cells per well of a 96 well plate (Greiner Bio-one, Stonehouse, UK). The plate was centrifuged at 1000 rpm for 3 min at 4 °C, and the pellet was resuspended in the appropriate primary antibody (anti-p3X (gift from Prof. Peter Andrews, Sheffield University, UK), anti-SSEA3 (Developmental Studies Hybridoma Bank, Iowa, USA), anti-TRA-160 (Millipore, Darmstadt, Germany) or anti-A2B5 (R&D Systems, Abingdon, UK)) for 60 min.