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Generation and genetic modification of 3D cultures of human dopaminergic neurons derived from neural progenitor cells

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ABSTRACT

Central nervous system (CNS) disorders remain a formidable challenge for the development of efficient therapies. Cell and gene therapy approaches are promising alternatives that can have a tremendous impact by treating the causes of the disease rather than the symptoms, providing specific targeting and prolonged duration of action. Hampering translation of gene-based therapeutic treatments of neurodegenerative diseases from experimental to clinical gene therapy is the lack of valid and reliable pre-clinical models that can contribute to evaluate feasibility and safety.

Herein we describe a robust and reproducible methodology for the generation of 3D *in vitro* models of the human CNS following a systematic technological approach based on stirred culture systems. We took advantage of human midbrain-derived neural progenitor cells (hmNPC) capability to differentiate into the various neural phenotypes and of their commitment to the dopaminergic lineage to generate differentiated neurospheres enriched in dopaminergic neurons. Furthermore, we describe a protocol for efficient gene transfer into differentiated neurospheres using CAV-2 viral vectors and stable expression of the transgene for at least 10 days. CAV-2 vectors, derived from canine adenovirus type 2, are promising tools to understand and treat neurodegenerative diseases, in particular Parkinson's disease. CAV-2 vectors preferentially transduce neurons and have an impressive level of axonal retrograde transport *in vivo*.

Our model provides a practical and versatile *in vitro* approach to study the CNS in a 3D cellular context. With the successful differentiation and subsequent genetic modification of neurospheres we are increasing the collection of tools available for neuroscience research and contributing for the implementation and widespread utilization of 3D cellular CNS models. These can be applied to study neurodegenerative diseases such as Parkinson's disease; to study the interaction of viral vectors of therapeutic potential within human neural cell populations, thus enabling the introduction of specific therapeutic genes for treatment of CNS pathologies; to study the fate and effect of delivered therapeutic genes; to study toxicological effects. Furthermore these methodologies may be extended to other sources of human neural stem cells, such as human pluripotent stem cells, including patient-derived induced pluripotent stem cells.

1. Introduction

Neural stem cells (NSCs) are self-renewing, exist in specific regions of the developing and adult mammalian CNS, and have the multipotent ability to generate at least the three neural lineages – neurons, astrocytes and oligodendrocytes. Neural progenitor cells (NPC) have a limited capacity for self-renewal and may retain multipotency or present reduced differentiation potential. For the

Abbreviations: β3-tub, β3-tubulin; CNS, central nervous system; CAV-2, canine adenovirus type 2; DM, differentiation medium; DN, dopaminergic neurons; EGF, epidermal growth factor; EM, expansion medium; AM, aggregation medium; FDA, fluorescein diacetate; FGF-2, fibroblast growth factor-2; GFAP, glial fibrillary acidic protein; GFP, enhanced green fluorescent protein; hmNPC, human midbrain-derived neural progenitor cell; hiPS, human induce pluripotent stem; hNSC, human neural stem cell; hPS, human pluripotent stem; hpt, hours post-transduction; ip, infectious particles; MOI, multiplicity of infection; PCNA, proliferating cell nuclear antigen; PD, Parkinson's disease; PI, propidium iodide; PLOF, poly-L-ornithine-fibronectin; RPL22, ribosomal protein L22; TH, tyrosine hydroxylase; vg, viral genomes.

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last two decades multipotent NSC/NPC have been isolated from multiple brain regions and more recently, protocols have been developed for the *in vitro* derivation of human NSC (hNSC) from human pluripotent stem (hPS) cells, both embryonic and induced (iPS) [1–3]. The possibility of stable expansion, *in vitro* differentiation into neural phenotypes and genetic modification makes hNSC/NPC an attractive cell source for cell therapy as well as disease modeling of a wide variety of nervous system diseases, ranging from Parkinson's disease (PD) to stroke and brain trauma, with application in basic cell biology, drug discovery and toxicology [1]. Therefore, despite the significant body of research on hNSC/NPC, the field is still gaining momentum. To address specific questions in biomedical research or to generate therapeutically relevant effector cells for cell therapy, technologies for the culture, differentiation and modification of hNSC/NPC and derivatives are required.

Two well-described culture systems commonly used to expand hNSC/NPC include the adherent monolayer and the neurosphere culture system, where cells are cultured as free-floating aggregates. The neurosphere culture system, developed in the early nineties to identify neural stem cells, has been widely used for the isolation and expansion of embryonic and adult CNS stem cell. It is based on the ability of NSC/NPC to aggregate and proliferate under serum-free media conditions, in the presence of epidermal growth factor (EGF) and/or fibroblast growth factor-2 (FGF2) [4]. Numerous bioprocesses for the large-scale production of NSC/NPC isolated from different regions of murine and human brain have been developed based on both the classic and on the stirred suspension neurosphere culture systems [5–7]. The utility of the neurosphere formation assay as quantitative *in vitro* method for measuring NSC frequency was challenged by the discovery that the assay conditions allow the expansion of multipotent NSC/NPC but also of more committed progenitors, with the production of a heterogeneous cell population [4,8]. More recently, it has been argued that adherent monolayer culture conditions, using laminin or fibronectin as adherent substrate, in defined serum free media and exposure to EGF and FGF2 are better suited for long-term NSC expansion; these conditions allow cells to divide symmetrically retaining their tripotent neurogenic potential [1]. Concerning NSC *in vitro* differentiation, typically cells are plated on adhesive subtracts, such as laminin, EGF and FGF2 are withdrawn and neurotrophic factors added. Differentiation depends not only on the applied protocol but also on the developmental stage and region of the tissue of origin [9,10].

Several methods for the genetic modification of hNSC/NPC have been applied [11], including non-viral [12] and viral gene transfer. The transduction of proliferative neurospheres with human adenovirus vectors [13], was optimal only after dissociation of the neurospheres. Likewise, techniques for gene transfer into differentiated 2D cultures are abundant in the literature [14].

Amongst the viral vectors available, CAV-2 vectors, which are derived from canine adenovirus type 2, are promising tools for gene transfer due to the lack of immunological memory, long-term episomal expression and high cloning capacity [15]. Moreover, both in rat brain and *in vitro* 2D neural cultures, CAV-2 vectors preferentially transduce neurons and undergo efficient long-distance targeting via axonal transport [16], making them promising tools for the treatment of degenerative diseases, such as PD as well as for the development of robust human CNS cell models.

2. Materials and methods

2.1. Overview

In this study, we provide protocols for the generation of 3D *in vitro* models of the human CNS following an approach based on stirred culture systems (Fig. 1). As cell source we used human midbrain-derived neural progenitor cells (hmNPC) as these can be expanded *in vitro* and differentiated into tyrosine hydroxilase (TH)-positive cells, in 2D culture conditions [17]. In addition to the expression of TH, the key enzyme for dopamine synthesis, these neurons exhibited morphological and functional properties of dopaminergic neurons in culture, such as dopamine production and release [3]. We describe the generation of differentiated neurospheres enriched in TH-positive neurons. Furthermore, we established a protocol for efficient gene transfer of differentiated neurospheres using CAV-2 vectors that allowed long-term transgene expression.

We took advantage of a well-described 2D protocol for long-term proliferation of hmNPC based on the use of O_2 levels close to physiological conditions (3%) and serum-free media [17]. These conditions led to the expansion of EGF/FGF-2 responding cells for more than 1 year, while retaining their tripotency and the ability to differentiate into dopaminergic neurons [3]. This expanded population of hmNPC was used as starting point for a robust and reproducible methodology based on stirred culture systems for differentiation and transduction.

2.2. Cell isolation and expansion

All tissue procurement was performed with mother's consent and in accordance with the Ethics Committee of the University of Leipzig and with all German state and federal laws. Human neural progenitor cells derived from aborted fetal brain tissue 12–14 weeks post-fertilization were isolated as described previously [17–20]. In brief, prior to trituration, the tissue was incubated in 100 μg/mL Papain (Roche Diagnostics) and 10 μg/mL DNase in phosphate-buffered saline (PBS), for 30 min at 37 °C, followed by washing with PBS, and incubation with antipain (50 μg/mL; Roche) for 30 min at 37 °C.

Expansion of hmNPC was performed under 3% O_2, on poly-L-ornithine-fibronectin (PLOF)-coated surfaces and serum-free medium, as described previously [17,19,20]. Expansion medium (EM) was composed of Dulbecco's modified Eagle medium (DMEM) and Ham's F12 Nutrient Mix (both from Invitrogen) in a 1:1 ratio, 2% B27 supplement (Invitrogen) 20 ng/mL rhu-FGF2 and rhu-EGF (both from PrepoTech), 1 μg/mL Tocopherol (Fluka), 1 μg/mL Tocopherol Acetate (Sigma) and 10 μg/mL Gentamycin (Invitogen). Cells were split, typically every 10–14 days, at 90–100% confluency (Fig. 2), corresponding to a 3- to 5-fold increase in cell concentration.

For splitting or collecting cells, the monolayer was incubated with Accutase® (Sigma) up to 30 min at 37 °C and cells detached by gentle mechanical dislodgment using a cell scrapper. Cells were collected with PBS and sedimented by centrifugation at 300*g*, 5 min, with no brake setting. After resuspension in a small volume of EM (approximately 1 mL/T150 flask collected), a 1 mL pipette or a glass Pasteur pipette was used to obtain a homogeneous cell suspension, thus avoiding air bubbles. Viability was determined by Trypan blue exclusion assay: after incubation with 0.1% (v/v) Trypan blue (Invitrogen) in PBS, colorless (viable) and blue (unviable) cells were counted using a Fuchs–Rosenthal haemocytometer chamber.

To avoid cell death and spontaneous differentiation (i) media exchange should be performed every 3–4 days or whenever a drop in pH is detectable by visual inspection in the media (the use phenol red containing media is recommended unless it interferes with subsequent assays); (ii) avoid overgrowing the monolayers, to avoid cell migration, culture polarization and consequent spontaneous differentiation.

Using this protocol, hmNPC were stably expanded for long periods of time, >20 passages (at least 10–12 population doublings), in