Fully integrated closed-system expansion and differentiation of pluripotent stem cells towards mesencephalic dopaminergic progenitor cells

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Introduction

Pluripotent stem cell (PSC)–derived cell products hold great promise for future clinical use in a variety of indications such as type I diabetes, cardiomyopathies, macular dystrophies or Parkinson's disease. Increasing regulatory requirements for such advanced-therapy medicinal products (ATMPs) imply the need for standardized reagents and highly reproducible production procedures. Automation of PSC expansion, differentiation, and potentially product optimization through cell sorting may contribute to successful and cost-effective innovative therapies. Using our versatile and integrated GMP-compliant cell processing platform CliniMACS Prodigy® we previously developed a cultivation and expansion workflow for iPS cells. Now we have implemented the differentiation of PSCs into mesencephalic dopaminergic (mesDA) progenitor cells on the device. Adapting this differentiation protocol from embryonic body–based[1] to fully adherent cultivation[2] enabled straightforward upscaling of a lab protocol to a medium-scale production process within the closed system. Additionally, we designed a concise marker panel (patent pending) for flow cytometry–based quality control (QC), i.e., characterization of the resulting mesDA progenitors.

Methods

1 Experimental setup and workflow

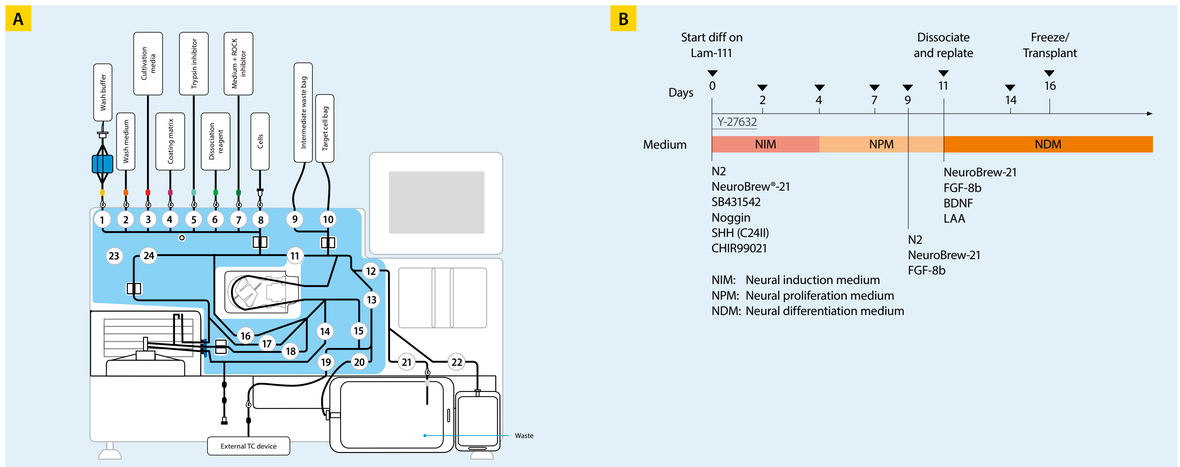

Figure 1

Day –1: Device setup	Day 0: Culture start	Day >1: Culture	Day 5: Split to external TC device	Day >5: Differentiation
• Install tubing set • Connect all media and solutions • Coat centrifugation and cultivation unit (CCU)	• Inoculate culture in CCU	• Automated media exchange	• Automated harvest • Storage of cells in Target cell bag (possible QC and cell count sampling)	• Closed system media exchange • "Semi-automated", depending on external TC device

The CliniMACS Prodigy provides a range of ports for connecting bags containing buffer, media, and reagents. Various tubing sets allow for a multitude of applications. For the cultivation and differentiation process, we chose the tubing set CliniMACS Prodigy TS 730, which provides up to eight connections for buffer and media and the possibility to prewarm solutions during transfer from an external 4 °C storage compartment to the cultivation and centrifugation unit (CCU). Bags can be connected in a sterile manner prior to the installation procedure, or later via sterile welding (fig. 1A).

Following an initial expansion step the differentiation is performed in an external tissue culture (TC) vessel which is placed in an incubator next to the CliniMACS Prodigy. All liquid handling steps during culture, including washing the CCU, coating, removal of matrix protein, inoculation, medium exchange, and cell harvest can be performed automatically, except potential handling of the external culture vessel. To adapt the manual differentiation protocol for generation of mesDA neurons to an automated process, a protocol[2] was modified to achieve adherent differentiation (fig. 1B).

Results

1 GMP-compliant expansion of hPSCs prior to differentiation

Expansion of hPSCs was started with 1 million cells in the laminin-521–coated Centrifugation and Cultivation Unit (CCU) within the closed and controlled system of the CliniMACS Prodigy. Cells were cultured in iPS-Brew GMP Medium. Cell yields were highly reproducible in different experiments (6.23×10^5 cells/cm² on average; n = 6). The number of cells obtained was significantly higher on the CliniMACS Prodigy compared to the manual protocol (3.5×10^5 cells/cm²) as shown in figure 2A. Flow cytometric analysis revealed comparable pluripotency marker profiles for hPSCs expanded on the instrument and on TC plates. Cells showed high co-expression of the pluripotency markers TRA-1-60, SSEA-4, and SSEA-3, while SSEA-1 expression was below 5% (fig. 2B).

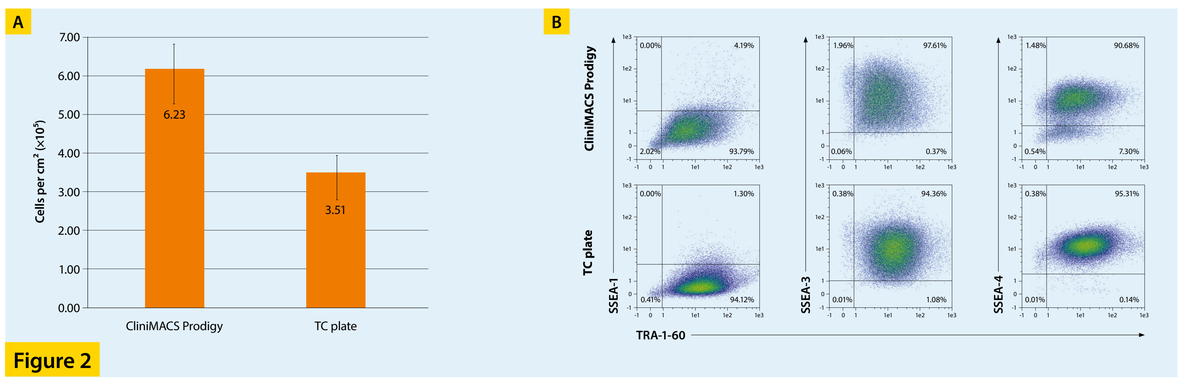

Figure 2

2 Flow cytometry-based quality control assay for mesDA cell preparations

To characterize the differentiated mesDA progenitors, we developed a panel for flow cytometry–based QC, which enables determination of the correct identity, purity, and potential tumorigenicity of the cells (fig. 3). Rostro-caudal patterning was driven by increasing CHIR99201 concentrations, dorso-ventral patterning by increasing hSHH concentrations (fig. 3A). We chose the positive markers IAP (integrin-associated protein, CD47), FoxA2, OTX2, and Nkx2.1 to identify the ventral midbrain region of the floor plate (fig. 3B). Expression of Pax-6 is indicative of a dorsal phenotype (fig. 3C), whereas Nkx6.1 and loss of OTX2 expression are associated with a caudal phenotype (fig. 3D). Sox1 identifies a dorsal phenotype as well as primitive neuroectoderm (fig. 3A,C,D). Ki-67 was included to monitor the proliferation capacity, Oct3/4 serves as marker to identify residual pluripotent cells (fig. 3B).

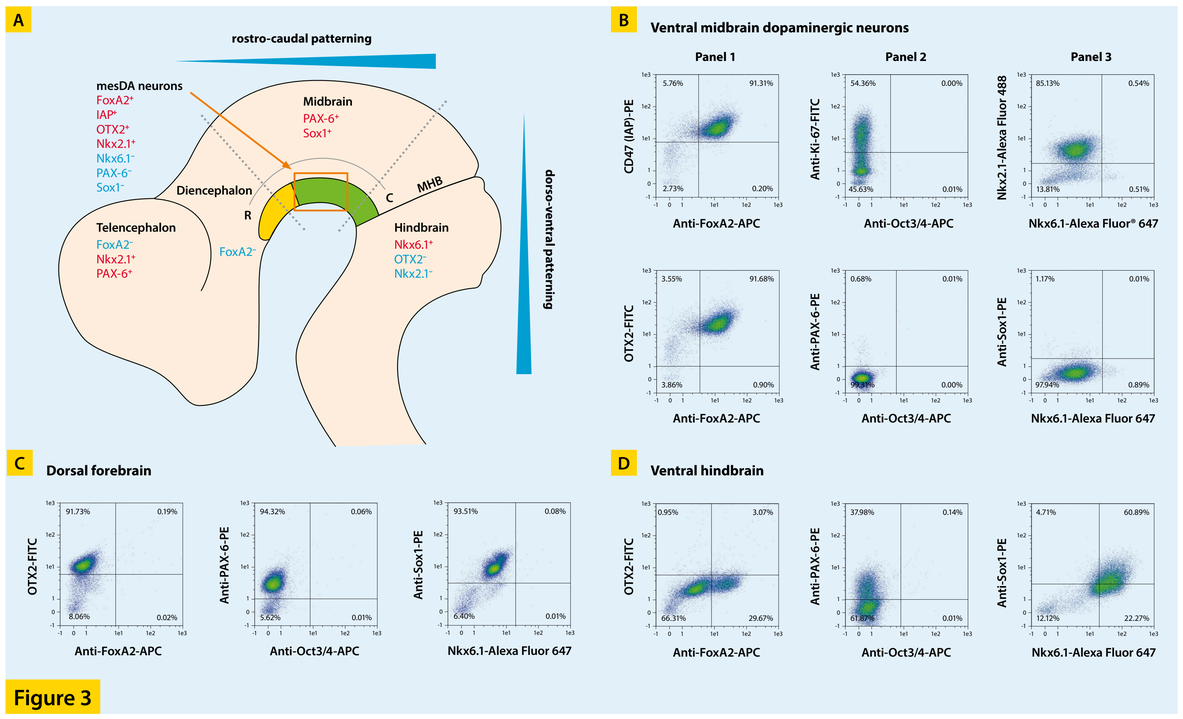

Figure 3

3 Comparability of manual and CliniMACS Prodigy–based hES differentiation into mesDA progenitors

Following the modified protocol[2] based on adherent cultures (fig. 1B) we found that cells differentiated with the CliniMACS Prodigy developed a mesencephalic dopaminergic phenotype, comparable to cells differentiated manually in 12-well plates at a lab scale, with a high frequency of OTX2⁺IAP⁺FoxA2⁺ cells (>90%) lacking the expression of PAX-6, Sox1, and Oct3/4 and a decreased proliferative capacity compared to the original pluripotent cells (fig. 4).

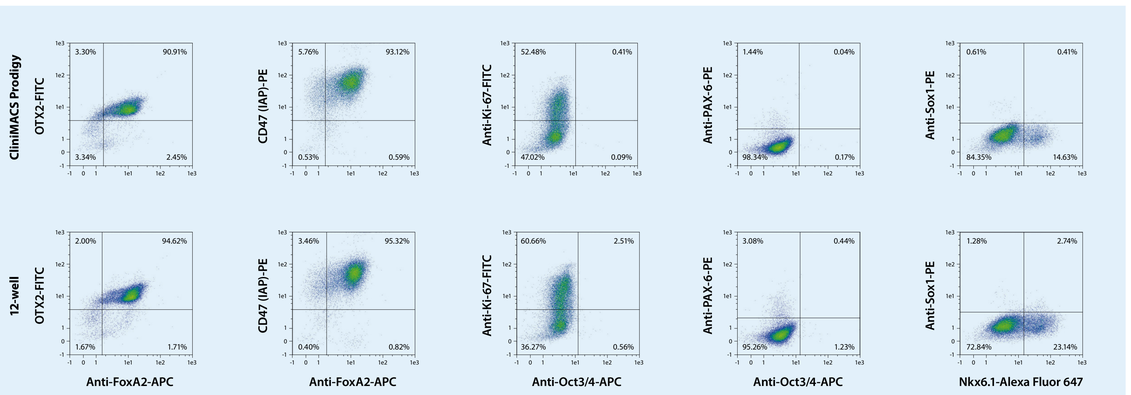

Figure 4

Conclusion and outlook

We developed a method for adherent, closed-system cultivation of PSCs and subsequent differentiation into dopaminergic progenitor cells. In addition, we designed a concise marker panel for flow cytometry–based quality control (QC).
Extrapolating the cell numbers obtained from the differentiation of one million PSCs and assuming that a cryopreserved unit would contain five million cells, the yield of our differentiation runs would correspond to 500–800 theoretical patient doses. However, the process holds further potential for upscaling. In a next step, we will take requirements from GMP facilities into account, such as quality and sterility controls as well as final formulation, to further enhance the process. Furthermore, an optimized tubing set will be developed to ease usability under GMP lab conditions.

References
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2. Kirkeby, A. *et al.* (2017) Cell Stem Cell 20: 135–148.
3. Lehnen, D. *et al.* (2017) Stem Cell Reports (in press).

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