*OSPEDALE Casa Sollievo della Sofferenza*
ISTITUTO DI RCOVERO E CURA A CARATTERE SCIENTIFICO
OPERA DI SAN PIO DA PIETRELCINA
Viale Cappuccini snc -SAN GIOVANNI ROTONDO (FG)

**DICHIARAZIONE DI UNICITA'**

PER PROGETTI FINANZIATI DAI FONDI SIE-FSC-FESR E DA ENTI NAZIONALI ED INTERNAZIONALI

San Giovanni Rotondo,

Al Responsabile Amministrativo
Dott. Michele Giuliani

**DICHIARAZIONE DI UNICITA'**
**(resa ai sensi del D.P.R. n. 445/2000)**

Il sottoscritto Dott.ssa Jessica Rosati C.F. RSTJSC73S66H501G in qualità di Responsabile Scientifico del Progetto: "Pathogenic role of myelin loss in focal seizures and epilepsy: an integrated approach from neurons to patients", ID:PNRR-MAD-2022-12376068, PNRR2022, codice commessa RPNRR22MIP11, CUP I23C22000690001, con riferimento alla richiesta di acquisto n.29660 presentata in data 25/11/2024

nel rispetto dei principi di trasparenza, non discriminazione, pubblicità ed effettiva concorrenza previsti dalla normativa nazionale e comunitaria applicabile a tutti i soggetti, anche di natura privata, che accedono a progetti finanziati:

☐ **dai fondi afferenti al Piano nazionale di ripresa e resilienza, PNRR 2022;**

a fronte dello svolgimento di un'apposita indagine di mercato volta a verificare l'esistenza di operatori economici in grado di fornire il prodotto con le caratteristiche tecniche richieste;

**dichiara che la ditta**

Agilent Technologies Italia S.p.A.

***si è rivelata essere l'unica in grado di poter fornire i seguenti prodotti*** ***(materiale di consumo):***

- z033401-2 Glial Fibrillary Acidic Protein

*(N.B. La descrizione dei prodotti di consumo/attrezzature/strumenti deve coincidere con quella della richiesta d'acquisto. Quando possibile si prega di inserire il dettaglio delle caratteristiche tecniche del prodotto di consumo/attrezzatura/strumento del quale si attesta l'unicità ovvero di allegare la relativa scheda tecnica debitamente sottoscritta dal responsabile del progetto ed approvata dal Direttore Scientifico)*

***per le seguenti motivazioni:***

a) la produzione è garantita da privativa industriale, come dalle allegate dichiarazioni di esclusività allegate, comprovanti quindi che la fornitura è oggetto di diritti di esclusiva, quali diritti d'autore, brevetti, marchi;

**inoltre:**

b) trattasi di prodotti utilizzati per e/o direttamente citati nei protocolli di ricerca standardizzati ed autorizzati dall'autorità competente ai sensi della normativa vigente in materia che non

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possono essere modificati in corso di sperimentazione (i.e. sperimentazione clinica etc.) e/o di protocolli pubblicati su riviste scientifiche da utilizzarsi per replicare dati e/o protocolli standardizzati a seguito di opportune fasi di verifica e validazione, raccolti nell'elenco protocolli di ricerca del laboratorio e conservati e gestiti come documenti del sistema di gestione qualità;

c) trattasi di beni di consumo collegati a tecnologia;

**Il sottoscritto dichiara, inoltre, che quanto affermato corrisponde a verità e di essere a conoscenza della responsabilità penale prevista, dall'art. 76 del D.P.R. 445/2000, per le ipotesi di falsità in atti e dichiarazioni mendaci ivi indicate in merito alla presente dichiarazione.**

Il Responsabile del Progetto
Dott.ssa Jessica Rosati

Dott.ssa Jessica Rosati
CM 87340
Istituto CSS Mendel

**Si Allega:**

- Documentazione comprovante i diritti di privativa industriale ricevuta dal fornitore;
- Protocollo in cui viene citato il prodotto dichiarato infungibile.

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Cell Death & Disease

ARTICLE Open Access

# Establishment of stable iPS-derived human neural stem cell lines suitable for cell therapies

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**Abstract**

Establishing specific cell lineages from human induced pluripotent stem cells (hiPSCs) is vital for cell therapy approaches in regenerative medicine, particularly for neurodegenerative disorders. While neural precursors have been induced from hiPSCs, the establishment of hiPSC-derived human neural stem cells (hiNSCs), with characteristics that match foetal hNSCs and abide by cGMP standards, thus allowing clinical applications, has not been described. We generated hiNSCs by a virus-free technique, whose properties recapitulate those of the clinical-grade hNSCs successfully used in an Amyotrophic Lateral Sclerosis (ALS) phase I clinical trial. Ex vivo, hiNSCs critically depend on exogenous mitogens for stable self-renewal and amplification and spontaneously differentiate into astrocytes, oligodendrocytes and neurons upon their removal. In the brain of immunodeficient mice, hiNSCs engraft and differentiate into neurons and glia, without tumour formation. These findings now warrant the establishment of clinical-grade, autologous and continuous hiNSC lines for clinical trials in neurological diseases such as Huntington's, Parkinson's and Alzheimer's, among others.

## Introduction

Cell therapy remains one of the most promising approaches for the treatment of neurological disorders. Recent observations of improved motor function in Parkinson's patients as elicited from transplanted mesencephalic dopaminergic neurons, suggest that the harnessing of the healing potential of these techniques may finally be within our reach[1]. However, many of the currently accessible cell systems present us with serious hurdles, pertaining to donor tissue procurement, heterogeneity, availability and related technical or ethical concerns[2–5].

Many of these issues could be alleviated by the use of stem cells, whose inherent expansion ability and functional plasticity could respectively increase availability and trigger therapeutic actions, such as the replacement of dead cells, immunomodulation, anti-inflammatory, trophic and homeostatic activities[6–13]. For a systematic clinical use of neural stem cells (NSCs)[14–18], manipulation systems and preparations must guarantee the broad availability of donor cells with reproducible cell behaviour and therapeutic effects through (1) expression of the full complement of stem cell functional characteristics and (2) stable and extensive self-renewal properties.

We have recently stated that stable human NSCs (hNSCs) can satisfy these requirements. Having obtained current good manufacturing practices (cGMP)

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with serum-free neurosphere growth medium[8]. After 10 days, cells were collected, mechanically dissociated into single cells and replated at high density in the same medium. In this phase, a single cell produces a sphere that can be routinely split every 10–15 days: up to 20–30 times. The mycoplasma contamination test and karyotype analysis were performed with the same protocols as those used for iPS cells; the only difference, for karyotype analysis, was the plating of neurosphere cells, which were attached onto cultrex and grown in the neurosphere medium to amplify cells. CNV analysis was performed using an SNP-array platform (Cytoscan HD; Affymetrix, Santa Clara, CA), following the manufacturer's instructions. Labelled DNA was hybridised for 16–18 h at 49 °C in a GeneChip Hybridisation Oven 645 (Affymetrix). The chip was washed, stained in the GeneChip Fluidic Station 450, and scanned with a 3000 7 G (Affymetrix) scanner. Copy number analysis was performed using Affymetrix Chromosome Analysis Suite software (ChAS v3.0; Affymetrix). CNVs were filtered as follows: 25 markers and 500 kb of minimal size.

### hiNSC cryopreservation protocol

Neurospheres were collected in a 15 mL tube and pelleted by centrifugation. The supernatant was discarded, the pellet was gently re-suspended in 1.5 mL of growth medium supplemented with 10% dimethylsulphoxide and transferred to a cryovial. The cryovial was placed into a freezing jar containing isopropyl alcohol, kept for at least 4 h at −80 °C and finally transferred to the liquid nitrogen tank.

To thaw hiNSCs, the cryovial was transferred in a 37 °C bath until thawed. Cells were transferred into a 15 mL tube containing 5 mL of growing medium and pelleted by centrifugation. The supernatant was discarded and cells gently re-suspended in growing medium and transferred to a cell culture flask to allow for further expansion[36]. Vitality of thawed cells was evaluated at the first amplification passage by counting viable cells out of the total cell number.

### Neural differentiation

Neurospheres were mechanically dissociated to yield a single-cell suspension and transferred onto cultrex-coated glass coverslips at the density of 10 000 cells/coverslip in neurosphere growth medium consisting exclusively of fibroblast growth factor 2 (20 ng/mL). Cultures were shifted after 72 h to a mitogen-free medium containing 2% FBS (default differentiation protocol). Differentiated cells were cultured for up to 5 weeks to obtain a mixture of neural cells containing astrocytes, neurons and oligodendrocytes. Immunofluorescence analysis was performed at different stages of differentiation: 10, 17, 24 and 31 days after plating (see below).

The spontaneous differentiation of hiNSCs in our protocol was used exclusively to demonstrate their multipotency; in fact, the neurons and astrocytes were not intended for subsequent utilisation in humans.

### Immunocytochemistry and immunohistochemistry

Cultures were fixed for 10 min in freshly buffered 4% paraformaldehyde at room temperature, followed by two 1× PBS washes. After blocking with 10% normal goat serum (NGS), the cultures were incubated overnight at 4 °C with the following antibodies: anti-TUBB3 (1:400—BioLegend); GFAP (1:200—Dako); GALC (1:200—Merk Millipore); anti-GLU; and anti-GABA. After rinsing with PBS, cultures were incubated with the following secondary antibodies: anti-rabbit Alexa Fluor 488 and anti-mouse Alexa Fluor 555 (Invitrogen). Cultures were then stained with Hoecht 33342 (Invitrogen) or 49,6-diamidino-2-phenylindole (DAPI) for nuclear staining. Microphotographs were taken, using a Nikon C2 fluorescence microscope and NIS Elements 1.49 software. Data are reported as percentages of labelled cells over the total number of nuclei ± SEM. Each value represents the average of at least three independent experiments.

### RT and qRT-PCR

Total RNAs were isolated from fibroblasts, hiPSCs and hiNSCs of each patient using TRIzol reagent (Life Technologies), following the manufacturer's instructions. RNA quality was assessed by determining ultraviolet 260/280 absorbance ratios at Nanodrop 1000 (Thermo Scientific) and examining RNA size distribution on RNA 6000 Nano LabChips (Agilent Technologies), processed on the Agilent 2100 Bioanalyzer, using the total RNA electrophoresis programme. Only RNAs with a RNA integrity number ≥ 8 were used for subsequent analysis.

Reverse transcription was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), following the manufacturer's instructions, after digestion with DNAse I (Life Technologies).

qRT-PCR was performed using a 7900HT Fast Real-Time PCR system (Applied Biosystem). For each gene of interest, qRT-PCR was performed as follows: each RNA sample was tested in duplicate and B-ACTIN was used to normalise transcript abundance and calculations were performed with the 2^DeltaDeltaCt method. Statistical analyses were performed on at least three independent experiments.

Sybr green reactions were performed using Power SYBR Green PCR Master Mix (Applied Biosystem) with the following PCR programme: denaturation 95 °C for 10 min; amplification 95 °C for 10 s, 60 °C for 10 s, 72 °C for 30 s, all of which was repeated for 50 cycles; final elongation 72 °C for 7 min; final dissociation step 95 °C for 15 s, 60 °C for 15 s and 95 °C for 15 s. TaqMan reactions