Insights in spatiotemporal characterization of human fetal neural stem cells

Raquel Martín-Ibáñez a,b,c,d, Inés Guardia a,b,c,MónicaPardoa,b,c,1,CristinaHerranza,b,c,d,RikeZietlowe,2, Ngoc-Nga Vinh e, Anne Rosser e, Josep M. Canals a,b,c,d,

a Laboratory of Stem Cells and Regenerative Medicine, Department of Biomedical Science, Faculty of Medicine and Health Sciences, University of Barcelona, Casanova 143, 08036 Barcelona, Spain b August Pi i Sunyer Biomedical Research Institute (IDIBAPS), Rosselló 149-153, 08036 Barcelona, Spain

c Networked Biomedical Research Centre for NeuroDegenerative Disorders (CIBERNED), Spain

d Research and Development Unit, Cell Therapy Program, Faculty of Medicine and Health Sciences, University of Barcelona, Casanova 143, 08036 Barcelona, Spain

e Cardiff University Brain Repair Group, Schools of Biosciences and Medicine, University of Cardiff, UK

Abstract

Primary human fetal cells have been used in clinical trials of cell replacement therapy for the treatment of neurodegenerative disorders such as Huntington's disease (HD). However, human fetal primary cells are scarce and difficult to work with and so a renewable source of cells is sought. Human fetal neural stem cells (hfNSCs) can be generated from human fetal tissue, but little is known about the differences between hfNSCs obtained from different developmental stages and brain areas. In the present work we characterized hfNSCs, grown as neurospheres, obtained from three developmental stages: 4–5, 6–7and 8–9 weeks post conception (wpc) and four brain areas: forebrain, cortex, whole ganglionic eminence (WGE) and cerebellum. We observed that, as fetal brain development proceeds, the number of neural precursors is diminished and post-mitotic cells are in-creased. In turn, primary cells obtained from older embryos are more sensitive to the dissociation process, their viability is diminished and they present lower proliferation ratios compared to younger embryos. However, independently of the developmental stage of derivation proliferation ratios were very low in all cases. Improvements in the expansion rates were achieved by mechanical, instead of enzymatic, dissociation of neurospheres but not by changes in the seeding densities. Regardless of the developmental stage, neurosphere cultures presented large variability in the viability and proliferation rates during the initial 3–4 passages, but stabilized achieving significant expansion rates at passage 5 to 6. This was true also for all brain regions except cerebellar derived cultures that did not expand. Interestingly, the brain region of hfNSC derivation influences the expansion potential, being forebrain, cortex and WGE derived cells the most expandable compared to cerebellar. Short term expansion partially compromised the regional identity of cortical but not WGE cultures. Nevertheless, both expanded cultures were multipotent and kept the ability to differentiate to region specific mature neuronal phenotypes.

1. Introduction

Several clinical trials for both Parkinson's Disease and Huntington's Disease (HD) have demonstrated proof-of-principle that cell replacement therapy can provide significant alleviation of symptoms in both conditions (Bachoud-Lévi et al., 2006; Kelly et al., 2011; Lindvall et al., 1990; Mendez et al., 2008). Following transplantation, implanted primary neuroblasts from human fetal origin, can mature, innervate the surrounding neuropil, and contribute to the repair of circuitry that has been damaged as a result of the degeneration. However, the use of human primary fetal tissue for human transplantation carries with it many constraints. First, availability is severely limited, although this has been addressed to some extent through collection of medical terminations of pregnancy (MTOPs) rather than surgical terminations of pregnancy (STOPs) (Kelly et al., 2011). Despite this, variability in developmental stage between fetuses, heterogeneity of fetal samples that need to be pooled, very limited capacity for storage, and logistical problems in coordinating patient, surgery and tissue collection all mean that a source of cells that is more readily available and more amenable to quality control is required.

Thus a key challenge to the development of cell replacement therapies for neurodegenerative diseases is the identification of easily expandable multipotent cell sources with the capacity to differentiate into the specific types of neurons that have been lost to the disease pro-cess. One such source could be neural stem cells (NSCs) derived from the human fetal central nervous system (CNS). These cells can proliferate to give rise to an expanded and more homogeneous population. One technique for growing NSCs involves the generation of free floating spherical aggregates termed ‘neurospheres’. This method was developed for rodent tissues a number of years ago (Ostenfeld et al., 2002; Reynolds et al., 1992) and has been also adapted for the long-term growth of human cell neurospheres (Carpenter et al., 1999; Ostenfeld et al., 2002; Svendsen et al., 1998; Vescovi et al., 1999). Neurospheres consist of both multipotent stem cells and more restricted progenitors (Ostenfeld et al., 2002; Reynolds and Weiss, 1996) and, as such, are considered to comprise a heterogeneous population of neural precursor cells (NPCs) (Bez et al., 2003; Ostenfeld et al., 2002; Sun et al., 2011; Suslov et al., 2002).

However, a detailed characterization of the properties of hfNSCs of different origins and their progeny is required before considering application in patients. Currently, the expansion capacity and differentiation potential of NSCs isolated from human fetal brain are incompletely understood. Several studies have reported region-specific differences for hfNSCs expanded as neurospheres in vitro (Hitoshi et al., 2002; Horiguchi et al., 2004; Kallur et al., 2006; Kim et al., 2006; Ostenfeld et al., 2002). First, neurospheres from the forebrain grew faster than NSCs obtained from caudal brain areas (Horiguchi et al., 2004; Kim et al., 2006), and second, differences in the neurogenic potential and the neuronal phenotypes obtained after NSCs differentiation have been described, depending on the brain area of origin. In addition, these differences in neuronal differentiation appeared to be sustained during long-term passage of neurospheres in culture (Kim et al., 2006). In vivo characterization has also been carried out in several studies: hfNSCs have been isolated from the whole human fetal forebrain or cortex, expanded in vitro and grafted into the brains of neonatal and adult rodents (Burnstein et al., 2004; Caldwell et al., 2001; Englund et al., 2002a, 2002b; Le Belle et al., 2004; Svendsen et al., 1998; Tamaki et al., 2002; Uchida et al., 2000; Zietlow et al., 2012; Zietlow et al., 2005). However, it is still unclear how much the region of origin, site of implantation and host environment influence the fate of hfNSCs after intracerebral transplantation.

Here, we characterized a representative number of human fetal brain tissue samples derived from three developmental stages (4–5, 6–7and8–9 weeks post conception (wpc)) and four brain regions: fore-brain, cortex, whole ganglionic eminence (WGE) and cerebellum. All these samples were grown in short-term cultures as neurospheres and two main objectives were addressed: 1) the effect of the

gestational age of the tissue on survival, hfNSC proliferation and gene expression and 2) the effect of passage on rates of expansion and differentiation potential, especially the maintenance of regional identity.

2. Materials and methods

2.1. Human fetal tissue collection

Human fetal tissue was collected following the guidelines of the Polkinghorne (Polkinghorne J) and UK Department of Health reports as previously described (Kelly et al., 2011). Full consent was obtained from the maternal donor, following consent through the SWIFT human fetal tissue bank (http://www.biobankswales.org.uk/swift-research-tissue-bank), under UK Human Tissue Authority research li-cence (no. 12457) held by Cardiff University, and with ethical approval 02/4446 Post mortem human fetal tissue for neural transplantation in HD and PD from the Bro Taf research ethics committee. Fetal tissue ranged in age from 4 to 10 wpc (Table 1), assessed by ultra sound measurements and confirmed using fetal morphometric data upon termination (Evtouchenko et al., 1996; Hurelbrink et al., 2000). The age of the fetus was estimated as previously described (Kelly et al., 2011).

2.2. Medical termination of pregnancy (MTOP)

Human fetal tissue ranging in age from 4 to 10 wpc was collected ac-cording to (Kelly et al., 2011). Following expulsion of the products into a disposable cardboard receptacle, fetal material was inspected by the nursing staff, before being promptly transferred to a sterile 500-ml pot containing Hibernate E (Thermo Fischer Scientific. Waltham, MA USA), and was stored at 4 °C (usually for up to 3 h but for a maximum of 12 h) before being transported on ice. The time at which the products of conception were passed were recorded.

2.3. Dissection of human fetal brain tissue

A total of 23 embryos were collected on the day following MTOP and they were stored as whole embryos in Hibernate E overnight at 4 °C. The following morning tissues of interest, Forebrain, Whole Ganglionic Eminence (WGE), cortex and cerebellum, were dissected and tissue pieces were processed as described below (see Table 1 for summary of the embryos collected and tissues of interest dissected). Whole brains were dissected for immunohistochemistry characterization (see Section

Table 1

Human fetal brain samples included in the characterization study. Summary of brain human fetal samples collected and analyzed at different developmental stages and from several brain areas. Note that the identification of the samples includes 2 or 3 letters (CB for cerebellum, CTX for cortex, FB for forebrain, WGE for whole ganglionic eminence and WB for whole brain) and three-four numbers that account for each embryo. We also de-scribe gestational and post-conception age (weeks (wks) and days) and the crown rump length (CRL) for each sample.

Brain area	Samples	Gestational age	Post-conception age	CRL (mm)
Forebrain	FB511	6 wks 5 days	4 wks 5 days	–
	FB538	6 wks 5 days	4 wks 5 days	10,2
	FB514	8 wks 2 days	6 wks 2 days	14
	FB541	9 wks 2 days	7 wks 2 days	24
WGE	WGE520	7 wks 4 days	5 wks 4 days	–
	WGE523	9 wks 1 day	7 wks 1 day	24
	WGE534	10 wks	8 wks	23,6
	WGE539	9 wks 1 day	7 wks 1 day	26,1
	WGE545	10 wks 5 days	8 wks 5 days	37,5
	WGE517	11 wks 1 day	9 wks 1 day	46,5
CTX	Ctx501	7 wks 5 days	5 wks 5 days	16
	Ctx520	7 wks 4 days	5 wks 4 days	–
	Ctx497	9 wks 2 days	7 wks 2 days	26
	Ctx507	9 wks 3 days	7 wks 3 days	24
	Ctx523	9 wks 1 day	7 wks 1 day	24
	Ctx539	9 wks 1 day	7 wks 1 day	26,1
	Ctx498	10 wks	8 wks	–
	Ctx502	10 wks	8 wks	22,8
	Ctx534	10 wks	8 wks	23,6
	Ctx512	10 wks 3 days	8 wks 3 days	30,1
	Ctx526	10 wks 1 day	8 wks 1 day	30,9
	Ctx545	10 wks 5 days	8 wks 5 days	37,5
	Ctx510	11 wks 3 days	9 wks 3 days	25,7
	Ctx517	11 wks 1 day	9 wks 1 day	46,5
CB	CB507	9 wks 3 days	7 wks 3 days	24
	CB545	10 wks 5 days	8 wks 5 days	37,5
	CB517	11 wks 1 day	9 wks 1 day	46,5
Whole brain	WB1002	7 wks 1 day	5 wks 1 day	26
	WB1051	7 wks 2 days	5 wks 2 days	10
	WB880	8 wks 1 day	6 wks 1 day	–
	WB903	12 wks	10 wks	55.2
	WB896	10 wks 5 days	8 wks 5 days	43.3

2.12). A representative scheme in Supplementary Fig. 1 shows the developmental stages under study that were divided in three groups: 4–5, 6–7and 8–9 wpc, different brain regions and the parameters analyzed.

2.4. Preparation of primary fetal brain tissue cell suspension

Dissected tissue pieces were incubated in 1 ml of TrypLE express (Invitrogen TM Thermo Fischer Scientific, Fermont, USA) for 20 min at 37 °C, after which DNAse (Sigma-Aldrich Química SL, Madrid, Spain, UK, 0.01%) was added, and tissue incubated for a further 5 min at 37 °C. Tissue pieces of interest were washed twice with complete medium containing: Dulbecco's modified Eagle's medium (DMEM)/F-12, supplemented with 1% Glutamine and 1% PSF antibioticantimycotic (all from Invitrogen TM Thermo Fischer Scientific), and then collected by centrifugation at 1000 rpm for 3 min. The tissue was resuspended in 200 µl DMEM/F12, and then triturated to produce a single cell suspension. Then cell viability was determined and cells were plated in suspension cultures for expansion as explained below or collected in RNA later and conserved at −80 °C for gene expression analysis.

2.5. Assessment of cell viability

Cell suspensions obtained from each fetal brain region of interest or from growing neurospheres dissociated cultures were counted in a hemocytometer using the trypan blue exclusion method. The

proportions of live and dead cells were recorded and viability or % of dead cells expressed as the percentage of live or dead cells in the suspension, respectively.

2.6. Cell culture expansion

Fetal brain tissue derived neurosphere cultures were obtained by seeding 80.000 cells/cm2 in expansion medium containing: complete medium (DMEM/F-12, supplemented with 1% Glutamine and 1% PSF antibioticantimycotic) supplemented with 1% N2 supplement (Invitrogen TM Thermo Fischer Scientific), 20 ng/ml Recombinant Human fibroblast growth factor basic (FGF; R&D Systems Inc. Abingdon, UK) and 20 ng/ml epidermal growth factor (EGF; Sigma-Aldrich Química SL), 0,1% Human Leukemia Inhibitory Factor (LIF, Sigma-Al-drich Química SL.) Cells were maintained at 37 °C in a humidified atmosphere with 5% CO2. Expansion culture media was changed every 3–4 days. Every 7 to 10 days, depending on the size of the neurospheres, they were collected by centrifugation at 1000 rpm for 3 min, then incubated with 1 ml of Accutase (GE Healthcare Europe GmbH, Barcelona, Spain) at 37 °C for 30 min, spun down at 1000 rpm for 3 min and dissociated in 200 µl of fresh Accutase. Single dissociated cells were counted as previously described. Cells were re-plated in fresh expansion media at a density of 80.000 cells/cm2 in culture flasks for 6 passages.

A total of six passages (a total of about 8 weeks in culture) were performed for all the cultures that expanded properly. This short term passage took into account the work of Zietlow and Collabora-tors in which they demonstrated that long term passaged human fetal neural progenitors leads to reduced graft viability after trans-plantation (Zietlow et al., 2012). At each passage we analyzed viabil-ity, differentiation and expansion potential. Gene expression was analyzed at passage 0, 3 and 6.

In order to analyze the expansion potential of hfNSC two parameters were calculated:

- Ratio of Proliferation: total number of dissociated single cells after each passage divided by the number initially plated.

- Number of accumulated cells: an estimate of the number of cells that would be obtained if all the cells obtained after each passage were subcultured taking into account the ratios of proliferation obtained at each passage for each culture.

2.7. Cell density assay

Cell density was studied by plating primary fetal brain cells at: 20,000, 40,000, 80,000, 120,000 and 160,000 cells/cm2 in expansion media for 10 days. Afterwards, neurospheres were collected, dissociated and the number of single cells counted. For each cell density we analyzed the ratio of proliferation and the viability. Two different develop-mental stages were analyzed: 6–7and 8–9 wpc. Results are expressed as the mean of independent experiments ± SEM. Statistical analysis was performed by two way ANOVA and Tukey's multiple comparison test for the comparison of cell densities for each developmental stage.

2.8. Mechanical and enzymatic dissociation

In order to improve the expansion of hfNSC cultures, two splitting methodologies were compared: enzymatic dissociation using Accutase as previously described and mechanical dissociation using the chopping technique.

Mechanical dissociation was performed as described: medium was added to the pelleted neurospheres after each passage and they were transferred with a Pasteur pipette to a Petri dish. Neurospheres were chopped firmly with a sterile blade into 250 µm pieces (± 10 µm) and transferred back to a collecting tube with media. The Petri dish was washed several times with culture media to collect all the cells. Sphere pieces were split 1:1 in expansion media.

For this study, cultures derived from 6 to 7 and from 8 to 9 wpc were passaged using both techniques over 6 passages. For viability, the fold change in the number of cells and the number of accumulated cells was determined at passage 3 and 6. Fold change was determined as the fold change in the number of cells at passage 3 and 6 respect to the number of cells plated initially (at passage 0). In order to count the number of cells at passage 3 and 6, 1/10 of the initial neurosphere suspension was taken from the bulk and dissociated with accutase following the enzymatic dissociation protocol.

Results are expressed as log transformed individual values and the mean for the fold change parameter and square root transformed individual values and the mean for the number of accumulated cells. Statistical analysis was performed by one way ANOVA and Tukey's multiple comparison test.

2.9. Neurospheres sections

Proliferating neurospheres derived from Cortex, Forebrain, WGE and Cerebellum at passage 3 were fixed with 4% PFA/PBS for 1 h, cryoprotected with 20% sucrose in PBS, and frozen in dry-ice cooled isopentane. Sections (10 µm thick) were collected on silane coated slides and conserved at −20 °C. Afterwards, they were stained for Nestin, Ki67, β-III-tubulin and GFAP fluorescence immunostaining following the protocol described below.

2.10. Cell culture differentiation

To assess the differentiation potential along passages, dissociated cells were plated onto 13-mm, poly-L-lysine-coated cover- slips (0.1 mg/ml in distilled H2O) at a density of 25.000 cells/cm2. Cells were placed in the center of the coverslip in 30 µl of differentiation medium (complete medium supplemented with 1% fetal calf serum and 2%B27) for 1 h to allow cell attachment. Afterwards, 500 µl of differentiation media was added per well and cells were allowed to differentiate for either 7 days (short-term differentiation) or 21 days (long term differentiation). They were fed by replacing half of the medium with fresh one every 3 days. Cells were then washed using PBS and prepared for immunocytochemistry by fixation with 4% paraformaldehyde in 0.1 M PBS for 20 min. Differentiation potential was analyzed for cortical and WGE cultures.

2.11. Immunocytochemistry

Fluorescent immunocytochemistry was performed for proliferating neurospheres and differentiated cultures at 7 and 21 days in vitro. Standard protocols were used as previously described (Martín-Ibáñez et al., 2007) with primary antibodies directed against: anti-GFAP (Polyclonal 1:1000, Dako Diagnósticos, S.L.U.; Barcelona, Spain), anti-β III Tubulin (monoclonal and polyclonal 1:500; Sigma-Aldrich Química SL), anti-Ki67 (clone SP6, monoclonal 1:100 Abcam; Cambridge, UK), anti Nestin (monoclonal 1:200; Merck Chemicals and Life Science GesmbH; Ma-drid, Spain), anti-DARPP-32 (polyclonal 1:200; Abcam), anti-tyrosine hydroxylase (TH, polyclonal 1:300; Merck Chemicals and Life Science GesmbH), anti-MAP2 (2a + 2b) (Monoclonal 1:200 Sigma-Aldrich Química SL), anti-Ctip2 (rabbit 1:250; Abcam), anti-Calbindin (D-28K, rabbit 1:10,000, Swant® Swiss antibodies, Marly, Switzerland), anti-Calretinin (polyclonal 1:1500, Merck Chemicals and Life Science GesmbH), anti-

Vglut1 (rabbit 1:500, Synaptic Systems, Göttingen, Ger-many). The following secondary antibodies were used: Conjugated-Cy3 Donkey anti-Rabbit IgG (H + L) 1:500, Conjugated-Cy3 Donkey anti-Mouse IgG (H + L) 1:500, Conjugated-488 Donkey anti-Mouse IgG (H + L) 1:500, Conjugated-Alexa-488 Donkey anti-Rabbit IgG (H + L) 1:500 (all from Jackson Immuno Research Europe Ltd. Suffolk, UK). No signal was detected in control preparations from which the primary antibody was omitted. Fluorescent photomicrographs were taken on a Leica TCS SL laser scanning confocal spectral microscope (Leica Microsistemas S.L.U. Barcelona, Spain). All images were acquired as tiff files and adjustments of brightness and contrast were performed with ImageJ software.

2.12. Immunohistochemistry

Whole Brains, heads and/or whole human fetuses were frozen in Isopentane (Thermo Fischer Scientific) on dry-ice and kept at −80 °C until serial coronal sections (15 µm) were cut on a cryostat and collected on silanecoated slides and frozen at −20 °C.

Fetal sections were treated with fresh PFA 4% for 10 min. Then, 3 × 5 min washes with PBS were performed and sections were blocked for 1 h in blocking buffer containing: PBS with 0.3% Triton X-100 and 1%bovine serum albumin (BSA) to avoid non-specific binding. Afterwards, samples were incubated overnight at 4 °C in PBS containing 0.3% Triton X-100 and 1% BSA with the corresponding primary antibodies: antinestin (Rat 401; 1:50; polyclonal; Developmental Studies Hybridoma Bank; The University of Iowa), anti-Ki67 (clone SP6; 1:100; rabbit monoclonal; Thermo Fischer Scientific), anti-Doublecortin (DCX; 1:200; polyclonal; Abcam; Cambridge, UK), anti-NeuN (1:100; mono-clonal; Merck Chemicals and Life Science GesmbH; Madrid, Spain), anti-Ctip2 (1:200; polyclonal; Abcam). After three PBS washes, human fetal sections were incubated for 2 h at room temperature with the sec-ondary antibodies (Cy3-conjugated donkey anti-rabbit IgG (1:500), Cy2-conjugated donkey anti-mouse (1:200); Jackson Immuno Research Europe Ltd.). Human fetal sections were stained with Hoesch and mounted in Fluoromount-G (0100-01, SouthernBiotech, Birmingham, USA). No signal was detected in control preparations from which the primary antibody was omitted. Fluorescent photomicrographs were taken on a Leica TCS SL laser scanning confocal spectral microscope (Leica Microsistemas S.L.U. Barcelona, Spain). All images were acquired as tiff files and adjustments of brightness and contrast were performed with ImageJ software.

2.13. Quantitative PCR assays

In all the cases, tissues or cells were collected in RNA later and frozen at −20 °C. Expression of several genes was evaluated in each case by Q-PCR assays performed as previously described (Martín-Ibáñez et al., 2007), using the following qPCR gene expression assays:

Gene Symbol	Assay ID	Company
18s	Hs99999901_s1	Taqman gene expression assay
ASCL1 (Mash1)	N004316.1	IDT gene expression assay
BCL11B (Ctip2)	Hs.PT.49a.3425122	IDT gene expression assay
Calb1 (Calbindin)	Hs.PT.49a.2456406	IDT gene expression assay
CUX2	Hs.PT.56a.2585747	IDT gene expression assay
DLX 2	N004405.1	IDT gene expression assay
DLX 5	N005221.1.pt.DLX5	IDT gene expression assay
DLX1	N178120.1.pt.DLX1	IDT gene expression assay
EMX1	Hs.PT.58.4631352	IDT gene expression assay
EOMES (Tbr2)	Hs.PT.58.38662727	IDT gene expression assay
FEZF2	Hs.PT.56a.26212716	IDT gene expression assay
FOXG1	Hs.PT.42.3963926.g	IDT gene expression assay
FOXP1	N032682.1	IDT gene expression assay
FOXP2	Hs.PT.49a.2596830	IDT gene expression assay
GBX2	N001485.1	IDT gene expression assay
GSX2 (Gsh2)	Hs.PT.58.14623723	IDT gene expression assay
IKZF1 (Ikaros)	Hs.PT.56a.27332150.g	IDT gene expression assay
IKZF2 (Helios)	Hs.PT.56a.24792251	IDT gene expression assay
NESTIN	Hs.PT.49a.2056373738	IDT gene expression assay
NEUROG1 (Ngn1)	N006161.1	IDT gene expression assay
NEUROG2 (Ngn2)	N024019.1	IDT gene expression assay
NKX2.1	N001079688.1	IDT gene expression assay
OLIG2	N005806	IDT gene expression assay
PAX 6	N001127612.1	IDT gene expression assay
PENK (Enkephalin)	N001135690	IDT gene expression assay
SATB2	Hs.PT.56a.4186712	IDT gene expression assay
SLC17A7 (Vglut1)	Hs.PT.49a.4258143	IDT gene expression assay
SOX 2	Hs.PT.42.237897.g	IDT gene expression assay
TAC1 (Substance P)	N013997	IDT gene expression assay
TBR1	Hs.PT.51.3354411	IDT gene expression assay
TUBB 3 (β-III-tubulin)	Hs.PT.42.4.839.320	IDT gene expression assay
ZNF503 (Nolz1)	Hs.PT.42.4254663	IDT gene expression assay

To provide negative controls and exclude contamination by genomic DNA, the reverse transcriptase was omitted in the cDNA synthesis step, and the samples were subjected to the PCR reaction with each Takara gene expression assay.

Analysis and quantification was performed with the Comparative Quantitation Analysis program of the MxPro™ Q-PCR analysis software version 3.0 (Stratagene, La Jolla, CA, USA), using the 18S gene expression as internal loading control. All Q-PCR assays were performed in duplicate. The results are expressed as the log transformed relative levels with respect to the expression of the same gene in the control condition:

- 4–5 wpc samples in the analysis of Nestin expression in primary brain fetal tissue

- 4 wpc in the analysis of gene expression in primary forebrain samples at different developmental stages and

- Passage 0 in the analysis of expression along passages 3 and 6 for cortical and WGE samples, considered as 1.

2.14. Quantification and statistical analysis

For the cell density Assay see Section 2.7.

For the mechanical versus enzymatic dissociation study see Section 2.8.

The analysis of the % of cell death or viability was performed for primary brain fetal human tissue obtained from different developmental stages (4–5, 6–7and 8–9 wpc) and brain regions (Forebrain, Cortex, WGE and Cerebellum). In addition, Passage 1 neurospheres derived from these three developmental stages were also analyzed for this parameter. Results were expressed as individual values (4–8 samples) and the mean. Statistical analysis was performed by one way ANOVA and Tukey's multiple comparison test. The percentage of cell death was also analyzed along passages for: different developmental stages (4–5, 6–7and8–9 wpc) and brain regions (Forebrain, Cortex, WGE and Cerebellum). Results were expressed as the mean ± SEM of 3–11 hfNSC independent cultures. Statistical analysis was performed by two way ANOVA and Tukey's multiple comparison test.

The expansion potential was analyzed by two parameters: ratio of proliferation and number of accumulated cells. The ratio of proliferation was compared between hfNSC obtained from different developmental stages (4–5, 6–7 and 8–9 wpc) at passage 1. Results were expressed as individual values (4–11 samples) and the mean. Statistical analysis was performed by one way ANOVA and Tukey's multiple comparison test. A comparative study was also done between the log transformed ratios of proliferation along passages for: different developmental stages (4–5, 6–7 and 8–9 wpc) and brain regions (Forebrain, Cortex, WGE and CB). Results were expressed as the mean ± SEM of 3–11 hfNSC independent cultures. Statistical analysis was performed be-tween passages for each developmental stage or brain regions and be-tween developmental stages or brain regions for each passage. Two way ANOVA and Tukey's multiple comparison test was applied.

The number of accumulated cells was calculated along 6 passages for different developmental stages (4–5, 6–7 and 8–9 wpc) and brain regions (Forebrain, Cortex, WGE and CB). Results were expressed as the square root transformed mean ± SEM of 4–10 hfNSC independent cultures. Statistical analysis was done comparing the square root trans-formed numbers of accumulated cells at each passage for each developmental stage or brain region in order to know how many pas-sages were necessary to expand the cells significantly. Two way ANOVA and Tukey's multiple comparison test was applied. In addition, a comparison between developmental stages and brain regions was done for each passage. Two way ANOVA and Tukey's multiple comparison test was applied.

Gene expression was studied for different experiments:

a) Nestin mRNA levels in primary samples derived from fetal brain samples at three developmental stages: 4–5, 6–7and8–9wpc.

b) Neurogenic and neuronal markers in primary samples derived from forebrain tissue at three developmental stages (5, 6 and 7 wpc).

c) Changes in gene expression profiles in neurospheres along passages P0, P3 and P6 derived from Cortical and WGE fetal samples.

Results represent the mean of log transformed values ± SEM of 4–6 independent samples (except for forebrain samples that only 1–2sam-ples were used for each developmental stage). The results are expressed relative to 4–5 wpc (a), 4 wpc (b) and P0 (c), considered as 0. Statistical analysis was performed by one way ANOVA (a) or two way ANOVA (c) and Tukey's multiple comparison test.

Differentiation analysis was performed along passages for WGE and Cortical cultures regardless of the developmental stage of derivation. Cultures were allowed to differentiate for 7 days at each passage as previously described and the percentages of Nestin, Ki67, b-III-tubulin and GFAP were quantified. Results were expressed as the percentage of positive cells for each staining with respect to the total number of cells. The average of data obtained from three to seven independent cultures

for each brain region was calculated. Statistical analysis was performed by two way ANOVA and Tukey's multiple comparison test.

3. Results

3.1. Changes in the proliferative and post-mitotic populations along human fetal brain development determines the sensitivity of the tissue to the dissociation process

The limited availability of human fetal brain samples has meant that most reports in the literature are derived from one developmental age or from pooled samples of different ages. In the present study we characterized the differences between human fetal brain regions obtained at three developmental intervals: 4–5, 6–7and 8–9 wpc (see Supplemetary Fig. 1 and Table 1). Primary fetal tissue was dissociated into single cells and viability was analyzed before plating the cells in suspension cultures for expansion (see scheme of the procedure in Fig. 1A). The comparative analysis of samples obtained from different developmental stages, independently of the brain region of origin, showed that primary human fetal brain cells from 4 to 5 wpc presented a higher percentage of viability than 6–7 and 8–9 wpc cells. However, no differences were ob-served between the viability of primary brain cells derived from 6 to 7 and from 8 to 9 wpc embryos (% of viability: 87,26 ± 3,18 for 4–5 wpc, 60,08 ± 4,34 for 6–7 wpc and 63,49 ± 7,53 for 8–9wpc;Fig. 1B). These results suggested that fetal human brain cells derived from younger developmental stages were more resistant to the dissection and dissociation process than the ones obtained from older embryos. qPCR of the NSC marker Nestin showed that brain fetal human samples derived from 4 to 5 wpc embryos presented higher levels of Nestin expression than the ones obtained from 6 to 7 and from 8 to 9 wpc embryos (Fig. 1C). Interestingly, the expression of the neuronal markers β-III-tubulin, Enkephalin, Substance P, Calbindin and vesicular glutamate transporter 1 showed an increase with later stages of forebrain development (Fig. 1D). In addition, the levels of expression of specific transcription factors involved in telencephalic development such as Ikaros, Helios, Tbr1, Stab1, Fez2, Ctip2 and Foxp1 also increased in samples de-rived from later stage forebra Results represent the mean of log transformed values ± SEM of 4–6 independent samples (except for forebrain samples that only 1–2sam-ples were used for each developmental stage). The results are expressed relative to 4–5 wpc (a), 4 wpc (b) and P0 (c), considered as 0. Statistical analysis was performed by one way ANOVA (a) or two way ANOVA (c) and Tukey's multiple comparison test.

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The limited availability of human fetal brain samples has meant that most reports in the literature are derived from one developmental age or from pooled samples of different ages. In the present study we characterized the differences between human fetal brain regions obtained at three developmental intervals: 4–5, 6–7and 8–9 wpc (see Supplementary Fig. 1 and Table 1). Primary fetal

tissue was dissociated into single cells and viability was analyzed before plating the cells in suspensionins (6 and 7 wpc; Fig. 1E).

In order to corroborate these results, we next characterized by immunohistochemistry the presence and distribution of proliferative neural precursors and post-mitotic neurons in human fetal forebrains obtained from different developmental stages (Fig. 2). We observed that human embryos from 5 wpc presented more extensive positive areas for Nestin and Ki67, both in the cortical and the lateral ganglionic eminence (LGE), compared to the embryos analyzed at 10 wpc (Fig. 2A–F). Note that at 5 wpc the whole forebrain showed cells positive for Nestin and Ki67 (Fig. 2A–C), while these proliferative cells were restrict-ed to the germinal zone (GZ) adjacent to the ventricle at 10 wpc (Fig. 2D–F). The analysis of neuronal markers showed that Doublecortin (DCX), a marker of newly generated neurons, was emerging in the mantle zone (MZ) and cortical plate (CP) of the LGE and the cortex, respectively, at 5 wpc. Some NeuN positive staining was observed in the same areas, albeit less extensive, indicating that few mature neurons had been generated by this early stage (Fig. 2G–I). An increase in the expression of these two neuronal markers was observed at 8 wpc (Fig. 2J–L). Note that the expression of DCX was spread along the whole post-mitotic areas while NeuN positive cells were positioned in specific areas of the caudate-putamen and cortex. In the former, NeuN positive cells were localized in the most lateral part of the caudal putamen (Fig. 2J–L). However, in the cortex, two NeuN positive areas could be identified: one in the CP, the outer most layer, and a second one in the subventricular zone (Fig. 2J–L). Next, we analyzed the expression of Ctip2, a transcription factor involved in the neurogenesis of both the striatum and the cortex. Ctip2 expression was restricted to the subventricular zone and small neurogenic areas at 5 wpc (Fig. 2M–O). However, at 10 wpc it was spread through the caudate and putamen, and the intermediate zone, the subplate and the plate in the cortex (Fig. 2P–R).

3.2. The expansion potential of human fetal brain neurospheres is not influenced by the developmental stage of derivation

Dissociated cells derived from human fetal brain samples at different developmental stages were grown in suspension to form so-called neurospheres and were propagated over 6 passages, with analysis of cell survival and proliferation ratio at each passage. Primary neurospheres derived from 8 to 9 wpc fetal brain tissue presented a higher percentage of cell death than the ones obtained from 4 to 5 and from 6 to 7 wpc embryos (Fig. 3A). In addition, primary neurospheres derived from 4 to 5 wpc embryos presented higher proliferation ratios than the ones derived from older embryos (6–7 and 8–9 wpc; Fig. 3B). Note that the ratios of proliferation were very low in all cases (2.92 ± 1.32 for 4–5wpc,0.70±0.17for6–7wpcand 0.96 ± 0.22 for 8–9 wpc) pointing to a very low expansion rate before the first passage.

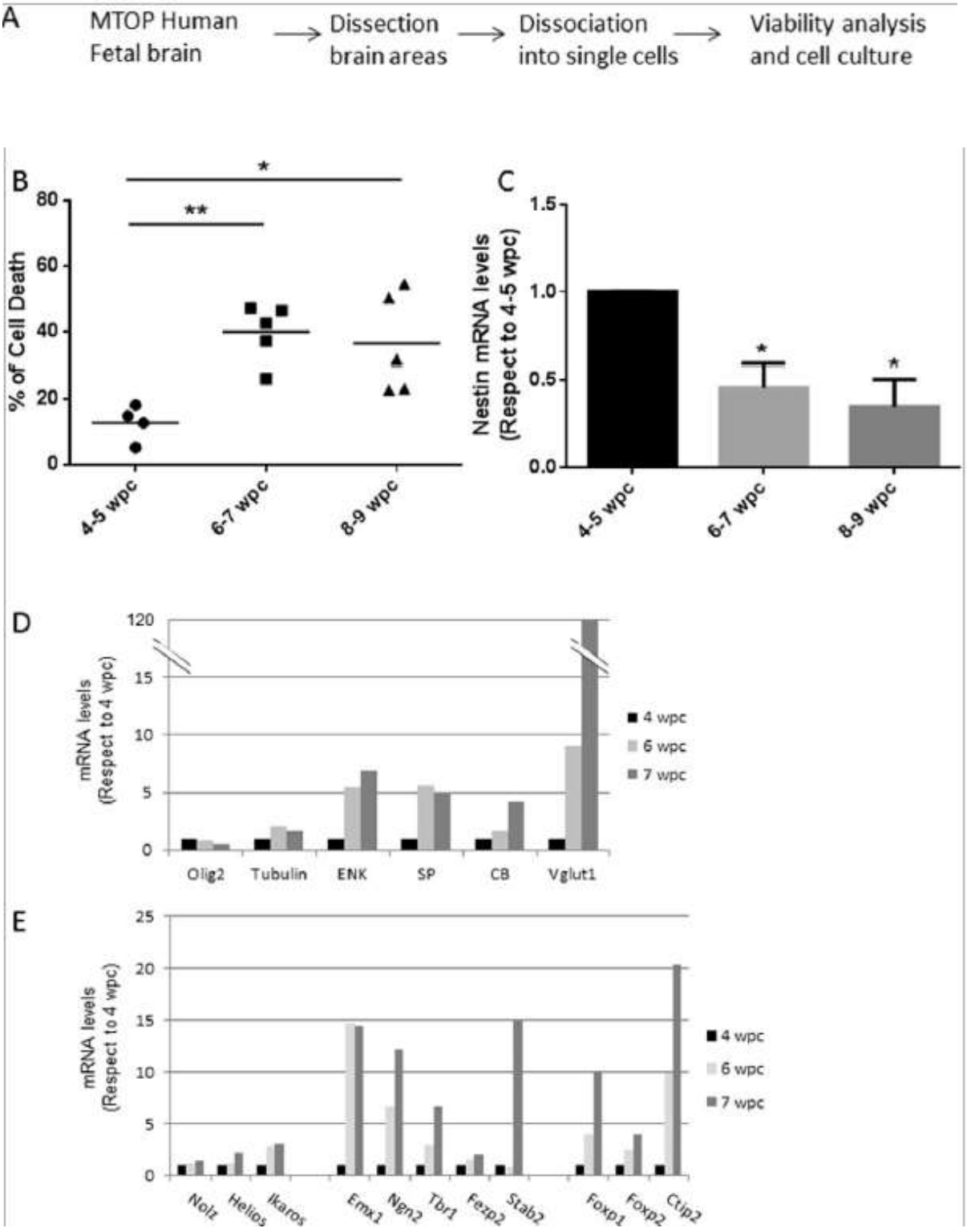

*Fig. 1. Primary brain human fetal samples derived from earlier developmental stages are less sensitive to the dissection and dissociation process. A) Schematic representation of the protocol followed for human fetal brain samples characterization. B) Cell death percentage after dissociation of human fetal brain primary samples from different developmental stages: 4–5, 6–7and8–9 wpc. Data are expressed as individual values and mean of 4–5 human brain fetal primary samples. Statistical analysis is performed by one way ANOVA and Tukey's multiple comparison test. *p b 0.05, **p b 0.005. C) RT-PCR analysis of Nestin mRNA levels of expression in human fetal brain primary samples derived from different developmental stages: 4–5, 6–7and 8–9 wpc. Results represent the mean ± SEM of 4–5 independent samples and are expressed relative to 4–5 wpc derived samples, considered as 1. Statistical analysis is performed by one way ANOVA and Tukey's multiple comparison test *p b 0.05. D–E) Q-PCR analysis of the mRNA levels of expression of specific genes implicated in differentiation performed in*

forebrain primary human fetal samples obtained at 4, 6 and 7 wpc. Results represent the levels of expression of 1–2 samples and are expressed relative to 4 wpc derived samples, considered as 1.

Next, we studied whether modifications in the initial cell density might be determinant to improve cell survival and proliferation. With this aim we cultured 6–7and 8–9 wpc primary human fetal brain cells at different densities ranging from 20.000–160.000 cells/cm2 (Fig. 3C–D) in suspension cultures. After 10 days, we did not ob-serve differences in the viability, nor in the ratio of proliferation between the cells densities studied for 6–7and 8–9 wpc derived cultures (Fig. 3C, D).

Taking into account the lack of differences between cell densities, we used the intermediate one (80.000 cells/cm2)to perform the expansion experiments. First, we analyzed if the differences found between develop-mental stages for the cell survival in primary neurospheres (P1) were maintained along the 5 following passages (Fig. 3E–F). Results showed that after the first passage no differences were observed between cultures (Fig. 3E). The study of the expansion potential along passages demonstrated differences in the proliferation ratios at passage 2 between 4–5 and 8–9 wpc derived cultures (Fig. 3F).However, from passage 3onwards a lack of differences between developmental stages were found neither in the proliferation ratios nor in the number of accumulated cells (Fig. 3F and Supplementary Fig. 2, respectively). Note that during the initial 3 pas-sages the percentages of cell death and the proliferation ratios were very variable for all the cultures. However, after the fourth passage the viability is stabilized between 70 and 80% and the mean proliferation rates in-creased above 2 (log transformed proliferation ratio above 0.3; Fig. 3E–F). These results point to the adaptation and/or stabilization of the hfNSC cultures after passaging. Accordingly, accumulated cell curves showed that cultures started to expand significantly after 6 passages for the three developmental stages under study (Supplementary Fig. 2).

3.3. Mechanical versus enzymatic dissociation: a key step in the expansion of hfNSCs

A general feature observed for the hfNSC neurosphere cultures was the low rates of proliferation over the passages studied. In order to study whether the mechanical dissociation could improve expansion rates, we compared enzymatic versus mechanic dissociation. Enzymatic dissociation of neurospheres was undertaken using accutase since it achieves higher viabilities and faster post dissociation recovery than trypsin, which is more prone to causing cell death and membrane disruption (Wachs et al., 2003). Mechanical dissociation was achieved by manually cutting the spheres into small pieces (chopping). The same cultures were passaged over 6 passages using these two techniques, and the expansion ratio and the survival after passaging were analyzed (Fig. 4). Our results showed that the fold change in the number of cells with respect to the initial number plated was significantly higher using the mechanic compared to the enzymatic dissociation after 3 and 6 passages (Fig. 4A). The number of accumulated cells was also higher in the chopped cultures compared to the enzymatically dissociated ones but only at passage 6 (Fig. 4B). However, the percentage of cell death at passage 6 was significantly higher for the mechanically-passaged cultures compared to the enzymatic dissociated ones (Fig. 4C). This result could be explained by the difference in the size of the spheres at the time of passage, which were significantly larger in the P6 mechanic dissociated cultures compared to the enzymatic dissociated ones (data not shown). Interestingly, we also observed differences between passage 3 and 6 in the fold change and number of accumulated cells for both enzymatic and mechanically-passaged cultures. Thus, after 6 passages cultures dissociated using both techniques expanded significantly more than at passage 3, even though the survival of passage 6 mechanic dissociated cultures is lower than at passage 3 (Fig. 4A–C).

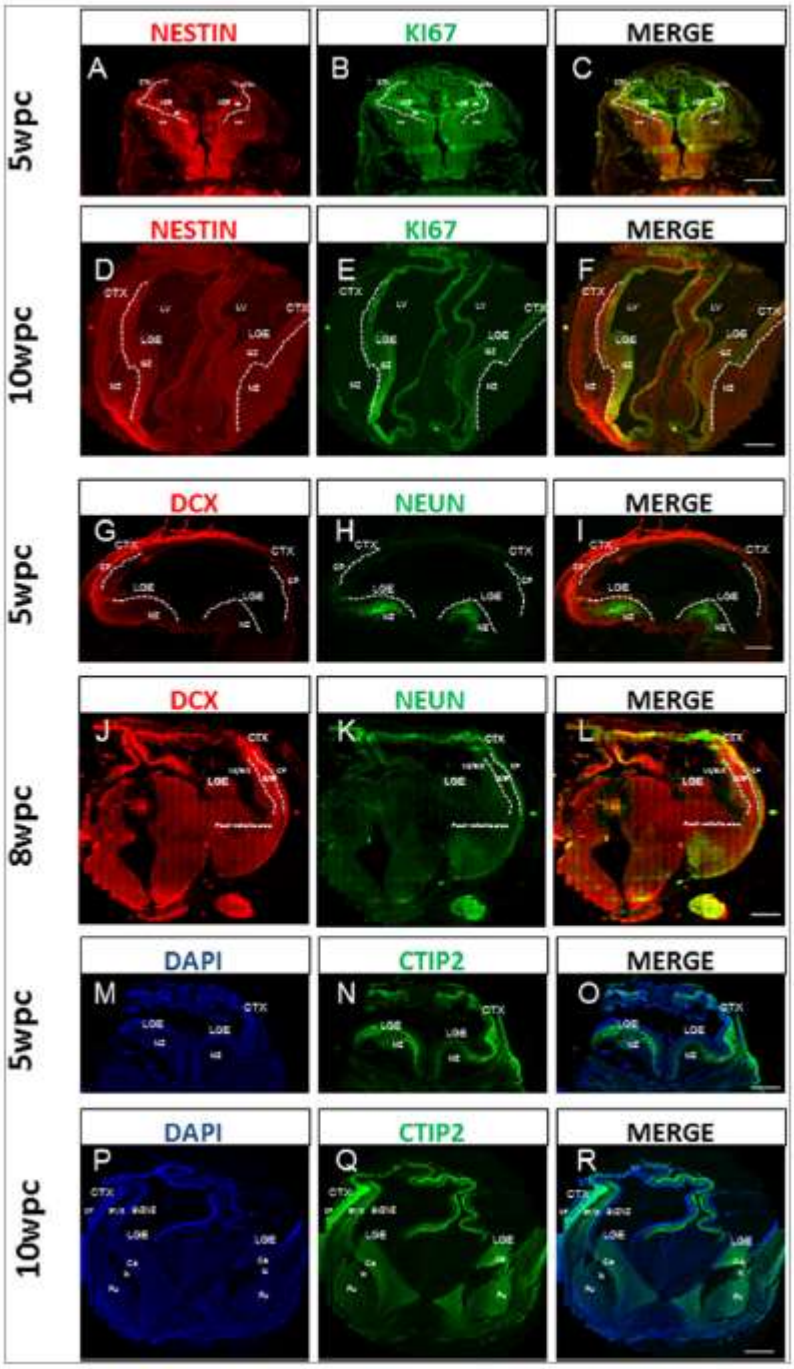

Fig. 2. Post-mitotic and neurogenic areas are observed in older fetal brain samples. A–R. Immunohistochemical characterization of human fetal coronal brain samples at 5, 8 and 10 wpc. A–F) Representative images of the neural stem cell markers Nestin and Ki67 at 5 wpc (A–C) and 10 wpc forebrains (D–F). G–L) The neuronal markers DCX and NeuN are mainly observed at the emerging mantel zone of the LGE and CP of the Ctx at 5 wpc (G–I). In contrast, they are extensively expressed at 10 wpc (J–L). M–R) Ctip2 positive cells are starting to emerge in 5 wpc human fetal brains (M–O), while they are spread through the intermediate zone, sub-plate and CP in the Ctx and caudate-putamen at 10 wpc (P–R). Ctx, cortex; LGE, lateral ganglionic eminence; MZ, mantel zone; GZ, germinal zone; VZ, ventricular zone; SVZ, subventricular zone; IZ, intermediate zone; SP, sub-plate; CP, cortical plate; Ca, caudate; Pu, putamen; ic, intermediate capsule. Scale bar: 500 µm.

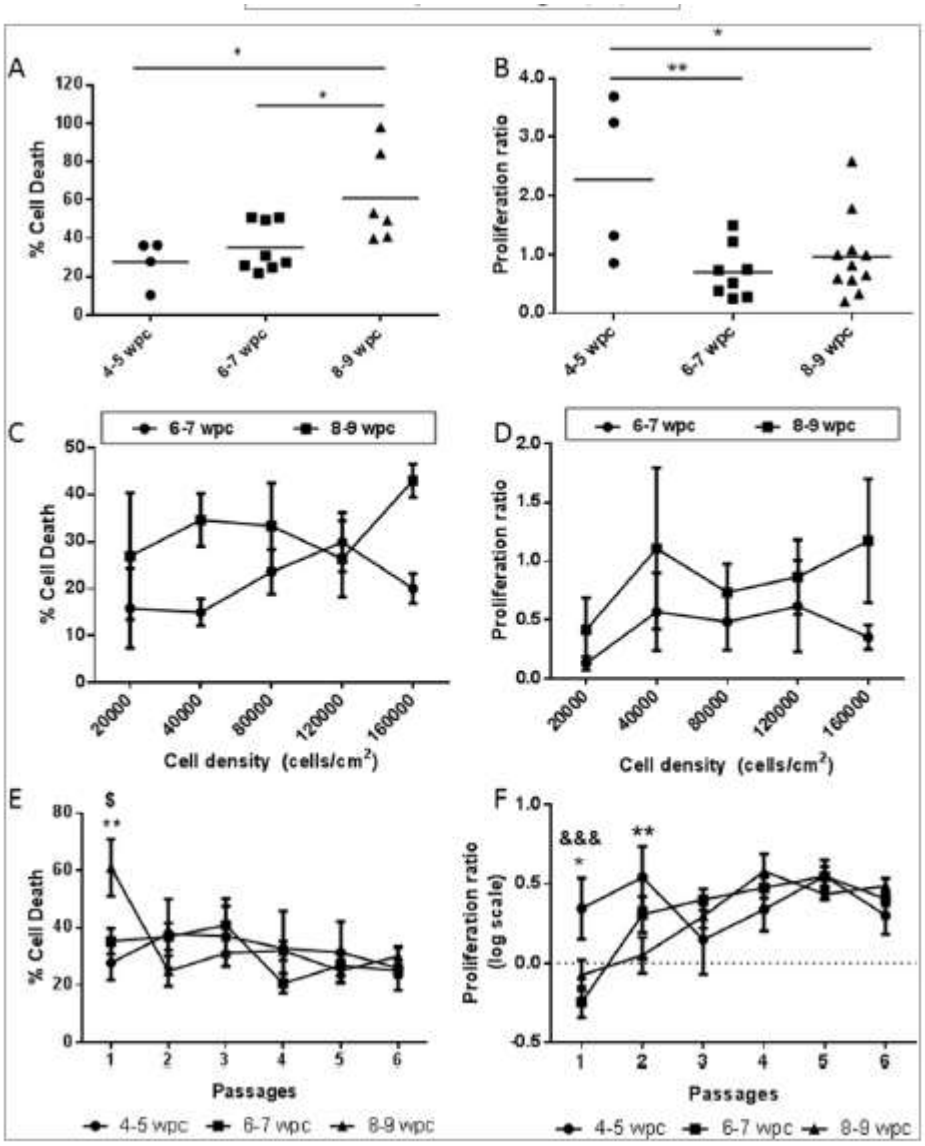

*Fig. 3. Human fetal neural stem cell cultures stabilize after several passages independently of the developmental stage of origin. A–B) Percentage of cell death (A) and ratio of proliferation (B) of hfNSC derived from different developmental stages (4–5, 6–7and8–9 wpc) and cultured for 1 passage. Data are expressed as individual values and mean of 4–11 human brain fetal primary samples. Statistical analysis was performed by one way ANOVA and Tukey's multiple comparison test. *p b 0.05, ** b 0.005. C-D) Study of the effect of the plating cell density in the survival (C) and proliferation ratios (D) of hfNSC cultures derived from 6 to 7 and from 8 to 9 wpc embryos. Results are expressed as the mean ± SEM of 3–5 hfNSC independent cultures. Statistical analysis was performed by two way ANOVA and Tukey's multiple comparison test for the comparison of cell densities for each developmental stage. E–F) Study of the percentage of cell death (E) and log transformed proliferation ratios (F) along passages for each developmental stage under study (4–5, 6–7and 8–9 wpc). Results are expressed as the mean ± SEM of 3–11 hfNSC independent cultures. Statistical analysis was performed by two way ANOVA and Tukey's multiple comparison test **p b 0.005 and *p b 0.05 for 4–5 wpc respect to 8–9wpc cultures and &&&p b 0.005 for 4–5 wpc respect to 6–7 wpc and $p b 0.05 for 6–7 wpc respect to 8–9 wpc cultures.*

3.4. The expansion potential of hfNSC is influenced by their brain region of origin

In order to study different brain sources of hfNSC for their potential use in cell therapy for HD we dissected out: WGE, cortex, cerebellum and forebrain (Table 1). Of note, MTOP permitted precise dissection of the regions of interest since this methodology provided us with intact whole brain.

No differences were found between brain areas in the survival of the primary tissue after dissociation (Supplementary Fig. 3). At 7 days most of the cultures formed neurospheres independently of the brain region of origin. Neurosphere number and growth rate was similar for fore-brain, WGE and cortical derived cultures, but cerebellum-derived neurospheres were the smallest as well as the lowest in number com-pared to the rest of brain regions at P0 (see a representative picture in Fig. 5A).

We next analyzed the phenotype and distribution of the neurospheres forming cells at 10 days after passage 1 using immunocytochemistry (see a representative picture in Fig. 5B). Nearly all the cells within the sphere were Nestin positive for all the brain regions studied and the distribution was homogeneous. We also observed positive cells for the proliferation marker Ki67, which were distributed throughout the neurosphere without differences between the four regions studied. Interestingly, in all the neurospheres examined, very few cells expressed the astrocyte-specific marker GFAP and the neuron-specific marker β-III-tubulin (Fig. 5B).

Next, we analyzed the expansion potential of hfNSCs derived from these four brain regions over 6 passages. For that, we studied cell survival, proliferation ratios, and number of accumulated cells (Supplementary Fig. 4 and Fig. 5C–D). Regardless of the brain areas of origin there was a substantial variability in the viability and the proliferation ratios during the initial 3 to 4 passages for all the cultures (Supplementary Fig. 4 and Fig. 5C). Nevertheless, after 4 passages they all stabilized with cell death values below 41% at passage 6 (% cell death at passage 6: fore-brain: 33.57 ± 7.28; WGE: 29.91 ± 3.63; Cortex: 23.94 ± 3.28 and Cerebellum: 29.91 ± 6.48). In addition, the proliferation ratios reached mean values of 2–3, except for cerebellar derived cultures, which reached mean ratios below 2 (proliferation ratios at passage 6: fore-brain: 2.34 ± 0.28; WGE: 3.39 ± 0.68; Cortex: 2.62 ± 0.48 and Cerebellum 1.73 ± 1.08; Fig. 5C shows log transformed values). No differences were found between the proliferation ratios at different passages for each brain region studied. However, significant differences were observed between WGE and Cortex derived cultures at passage 2 and between Forebrain, WGE and Cortex respect to Cerebellar derived cultures at passage 4 (Fig. 5C).

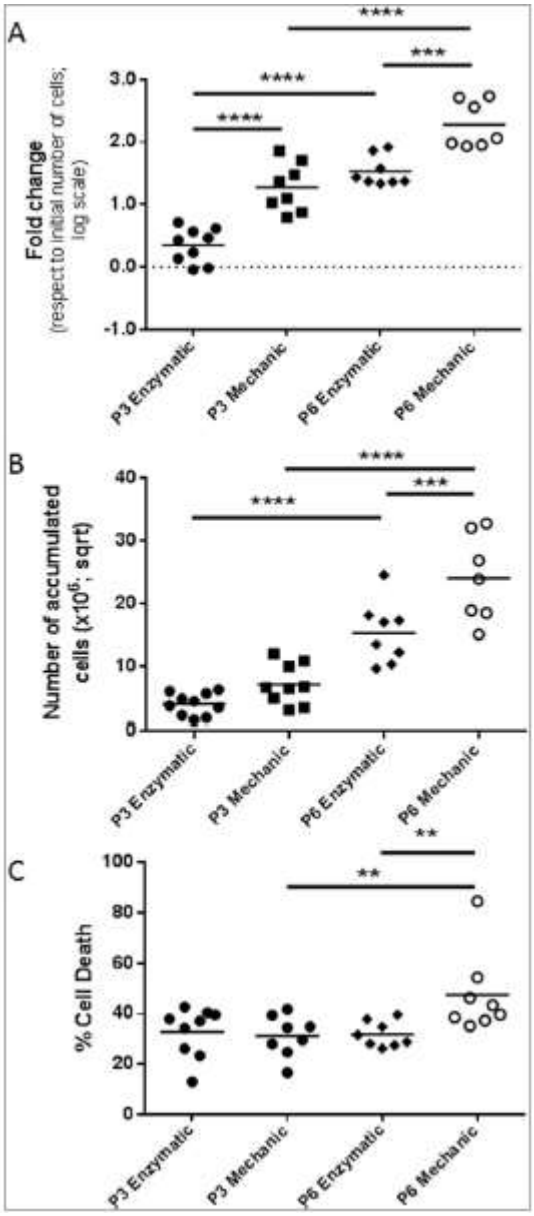

*Fig. 4. Effect of the splitting methodology on the expansion of neurosphere cultures derived from hfNSC. A–C) Expansion of neurospheres cultures is analyzed using two different dissociation techniques: mechanic (chopping) versus enzymatic (using accutase). A) Log transformed fold change in the number of cells at passage 3 (P3) and 6 (P6). Results are expressed individual values and the mean. Statistical analysis was performed by one way ANOVA and Tukey's multiple comparison test. ***p b 0.002 and ****p b 0.0001. B) Square root transformed number of accumulated cells for mechanically and enzymatic dissociated spheres at P3 and P6. Results are expressed as individual values and the mean. Statistical analysis was performed by one way ANOVA and Tukey's multiple comparison test. ***p b 0.002 and ****p b 0.0001. sqrt: square root transformed. C) Percentage of cell death after splitting mechanically or enzymatically at P3 and P6. Results are expressed as individual values and the mean. Statistical analysis was performed by one way ANOVA and Tukey's multiple comparison test. **p b 0.005. sqrt: square root transformed.*

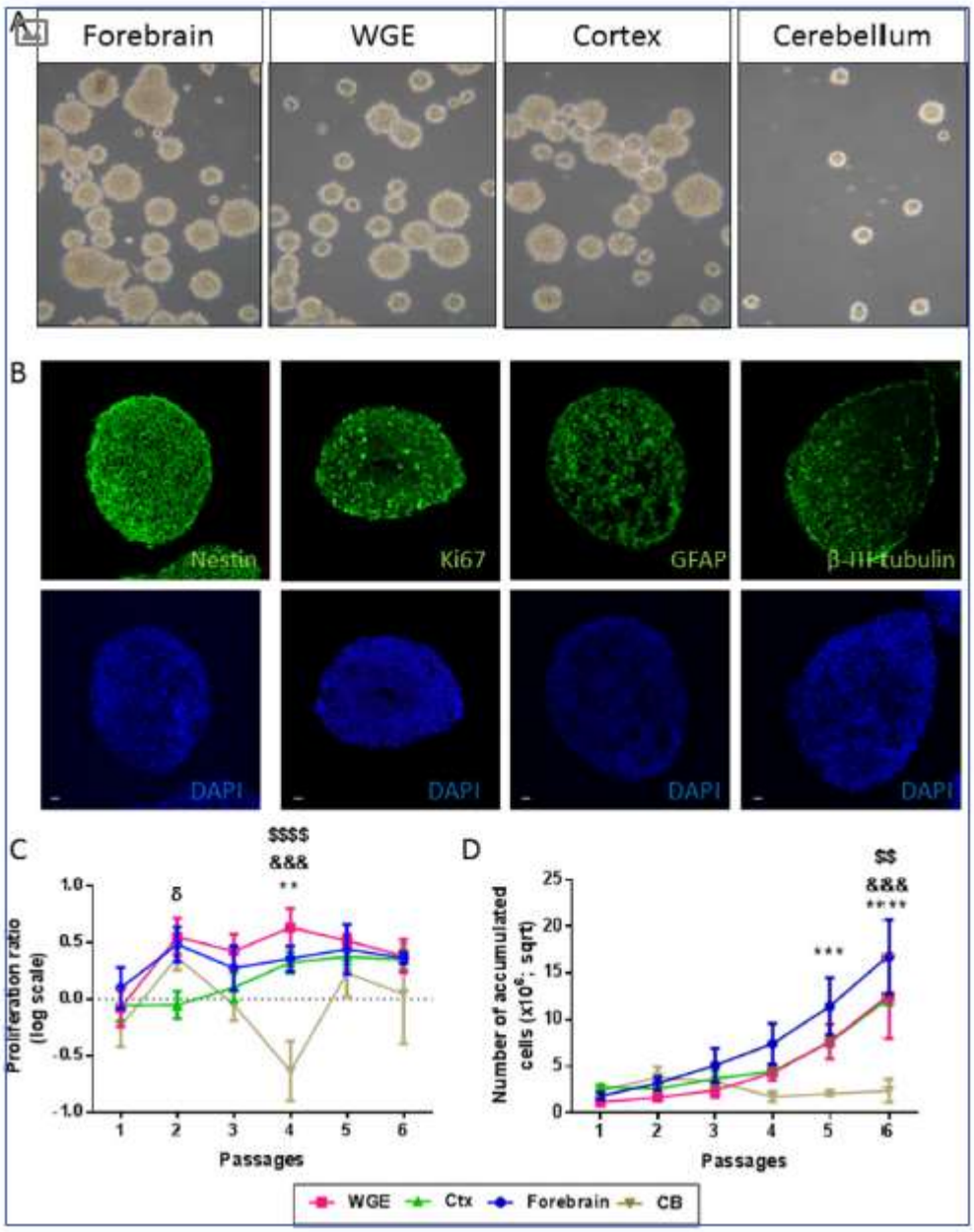

*Fig. 5. Neurospheres derived from several human fetal brain regions present differences in the expansion potential. A–D) Characterization of hfNSCs derived from several brain regions: Forebrain, Cortex, WGE and Cerebellum. A) Primary cells derived from all four regions are able to form spheres in suspension cultures. B) Representative pictures of immunocytochemistry for the neural marker Nestin, the proliferative marker Ki67, the astrocytic marker GFAP and the neuronal marker β-III-tubulin in forebrain derived spheres at passage 3. C) Log transformed proliferation ratios of cultures derived from the different brain regions under study through 6 passages. Results are expressed as the mean ± SEM. Statistical analysis was performed by two way ANOVA and Tukey's multiple comparisons test. δp b 0.05, **p b 0.005 &&&p b 0.002 and $$$$p b 0.0001 (δWGE respect to Ctx cultures. *Forebrain respect to CB cultures. &Ctx respect to CB cultures and $WGE respect to CB cultures. D) Representation of the square root transformed number of accumulated cells for cultures expanded from Forebrain, WGE, Ctx and CB. Results are expressed as the mean ± SEM. Statistical analysis is performed by two way ANOVA and Tukey's multiple comparison test. $$p b 0.005, ***,&&&p b 0.002 and ****p b 0.0001 (*Forebrain respect to CB cultures, $WGE respect to CB cultures, &Ctx respect to CB cultures). The comparison between passages of the sqrt number of accumulated cells for each brain region shows: forebrain ***p b 0.002 P5 respect P1, **p b 0.005 P5 respect P2 and *p b 0.05 P5 respect P3. ****p b 0.0001 P6 respect P1, P2 and P3 and ***p b 0.002 P6 respect P4. WGE: *** p b 0.002 P6 respect P1, P2 and P3 and **P b 0.005 P6 respect P4. Cortex: ****p b 0.0001 P6 respect P1, P2 and P3 and ***p b 0.002 P6 respect P4. WGE: whole ganglionic eminence; CB: cerebellum, Ctx: cortex. sqrt: square root transformed. Scale bar: 25 μmfor (B).*

The analysis of the proliferation curves, where the number of accumulated cells after sequential passaging is represented, showed that forebrain-derived NSCs were expanding significantly more than Cerebellar ones at passage 5 and 6 (Fig. 5D). In addition, WGE and Cortical-derived NSC expanded more than Cerebellar ones only at passage 6 (Fig. 5D). No differences were found in the expansion potential of Fore-brain, Cortex and WGE derived cultures along passages. However, fore-brain-derived NSC were found to expand significantly after 4 passages (passage 5 respect P1–P3 and passage 6 respect to P1–P4) whereas cortical and WGE derived cultures were significantly expanded at passage 6 (respect to P1–P5). In contrast, cerebellar derived hfNSCs were not able to expand even after 6 passages. All these results suggest that intrinsic differences exist in the expansion potential of hfNSC derived from several brain regions.

3.5. Region-specific gene expression profiles were partially lost for cortical hfNSCs after expansion

To determine whether human fetal neurospheres derived from different brain regions retain their regional identities over passages, we analyzed gene expression profiles at passage 0, 3 and 6. For this experiment we studied cortical and WGE-derived cultures. The analysis of early neural molecular markers showed that WGE derived cultures maintained the expression of Sox2 with sequential passages. This was also the case for specific regional genes such as Dlx1, 2 and 5, Gsx2 (Gsh2)and Ascl1 (Mash1). However, Foxg1 was down-regulated at 6 (Fig. 6A). Nolz1, Helios, Ikaros, Nkx2.1, Foxp1 and Ctip2 were analyzed as specific transcription factors involved in the generation of post-mitotic neurons in the WGE. All these molecular markers were down regulated after 6 sequential passages, except for Helios, which expression was not modified (Fig. 6B). Our results also showed that WGE derived neurosphere cultures presented very low levels of expression of cortical Pax6, Ngn2 and cerebellar Gbx2 region specific genes that were not modulated after sequential passaging (Fig. 6C).

In contrast, cortical derived cultures behaved differently. Sox2, Foxg1 and Pax6 expression were not modified with passages. In addition, most cortical specific early neural genes such as Ngn1, 2, Emx1 and Tbr2 were down regulated at passage 3 and 6 (Fig. 6D), as were the cortical specific neuronal markers(Tbr1, Cux2 and Fezp2) that we analyzed (Fig. 6E). When we examined WGE neural genes - Dlx2 and Gsx2 (Gsh2) - in cortical-derived hfNSCs, we observed that these genes were significantly up-regulated after 3 and 6passages(Fig. 6F). However, no changes were observed for Ascl1 (Mash1) and Gbx2 that were expressed at very low levels in cortical samples (Fig. 6F).

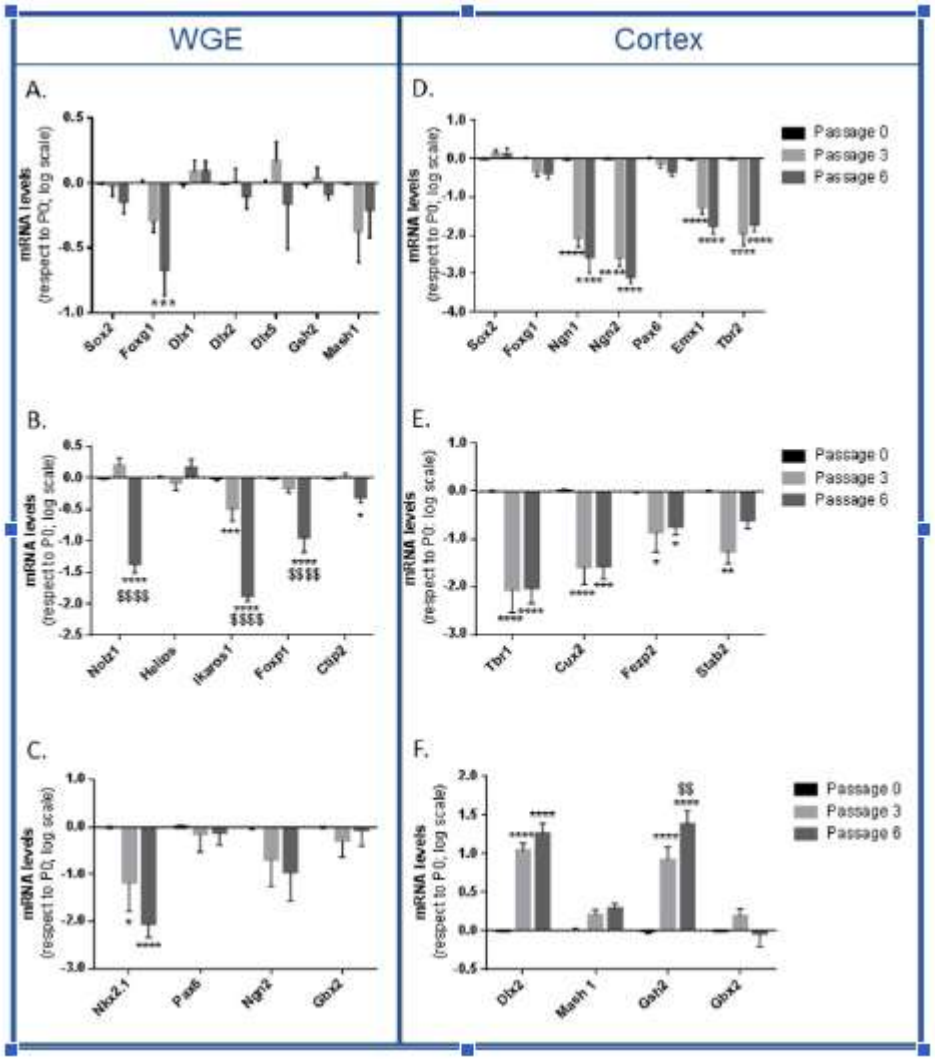

*Fig. 6. Gene expression analysis of WGE and Cortical derived cultures along passages. RT-PCR analysis of gene expression profiles along passages (P0, P3 and P6) of WGE and Cortical derived cultures. Three categories are studied: early neural precursor's genes (A–D), mature neuronal genes (B–E) both specific for each brain area and other brain regions genes (C–F). Results represent the log transformed mean ± SEM of 4–5 independent samples and are expressed relative to P0, considered as 0. Statistical analysis is performed by two way ANOVA and Tukey's multiple comparison test. *p b 0.05, **,$$p b 0.005, ***p b 0.002,****,$$$$p b 0.0001 (*Passage 6 respect to Passage 0 and $Passage 6 respect to Passage 3). P: passage.*

3.6. WGE and cortical derived neurosphere cultures maintained their differentiation potential after sequential passaging

It has been previously described that neurosphere cultures reduce their neurogenic differentiation potential along passages with increase in gliotic potential. Thus, we next evaluated the differentiation potential of WGE and cortical derived neurospheres cultures across passages. Single cell suspensions at different passages (P1–P6) were plated, and after seven days neural differentiation was evaluated. Quantification of the differentiated phenotypes showed that the number of Nestin positive cells was between 35 and 60% for both cortical and WGE (Fig. 7A, C, G, I). These results indicated that a high percentage of cells was not completely differentiated after 7 days.

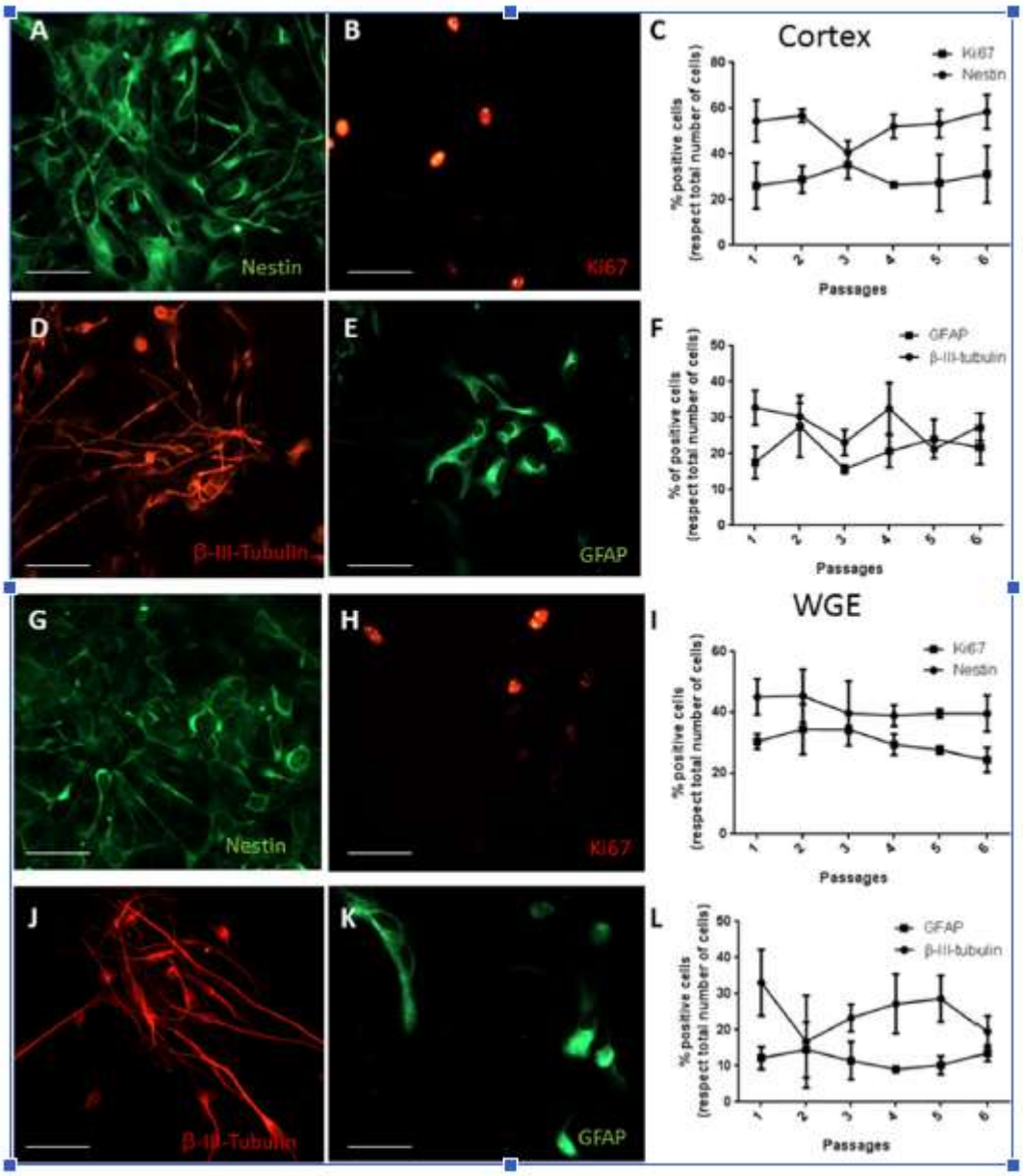

Fig. 7. WGE and Cortical cultures remain multipotent after 6 passages. A–L) Quantitative analysis of the percentages of Nestin, Ki67, GFAP and β-III-tubulin along passages for WGE and Cortical derived cultures. Representative pictures for Nestin (A) and Ki67 (B), β-III-tubulin (D) and GFAP (E) positive cells after 7 days of differentiation for cortical cultures at Passage 1. Representative pictures for Nestin (G) and Ki67 (H), β-III-tubulin (J) and GFAP (K) positive cells after 7 days of differentiation for WGE cultures at Passage 1. Results represent the mean ± SEM of 4–5 independent samples. Statistical analysis was performed by two way ANOVA and Tukey's multiple comparison test. Scale bars: 50 µm.

However, not all the Nestin positive cells were immunoreactive for Ki67 since the percentages of this proliferative marker (between 9 and 46%) were lower than the percent-ages of Nestin (Fig. 7B, C, H, I). These findings indicated that some Nestin positive precursors were out of the cell cycle and therefore, committed at the time of analysis. We next analyzed the number of neurons and astrocytes after differentiation. The mean values for β-III-tubulin positive neurons, were around 15–38% for cortical and WGE cultures. In addition, the mean values for the percentage of astrocytes, immunoreactive for GFAP, were found to be around 5–35% (Fig. 7D–F, J–L). No differences were found between passages in the percentages of any of the markers studied (Nestin, Ki67, β-III-tubulin and GFAP), neither for cortical nor for WGE cultures. In addition, no differences were found between cortical and WGE cultures in the percentages of all these markers. The potential to differentiate to

oligodendrocytes was also evaluated by the analysis of O4 immunoreactivity, which was detected in very few cells for cultures derived from both brain areas (b1%. Data not shown). Thus, neurosphere cultures derived from both the WGE and the cortex remained multipotent during 6 passages, being able to differentiate mainly into neurons and astrocytes and to a lesser extent to oligodendrocytes.

Next, we characterized the potential of neurospheres to give rise to different mature neuronal types after 6 passages. With this aim, P6 neurospheres were allowed to differentiate for 21 days and various neuronal markers were analyzed (Fig. 8). WGE and cortical differentiated cultures gave rise to a high percentage of MAP2 mature neurons (Fig. 8A), although the phenotypes of these neurons were slightly different. While WGE cultures showed a predominantly neuronal phenotype positive for DARPP32, Calbindin and Ctip2 (Fig. 8B, C and D, respectively), cortical cultures were more prone to differentiate to Vglut1 positive neurons in agreement with their region of origin (Fig. 8F). Similar differentiation potential towards Calretinin positive neurons was observed in cortical and WGE cultures (Fig. 8E). Interestingly, both of them were also able to differentiate to Tyroxine Hydroxylase (TH) positive cells, al-though with a low incidence (Fig. 8G).

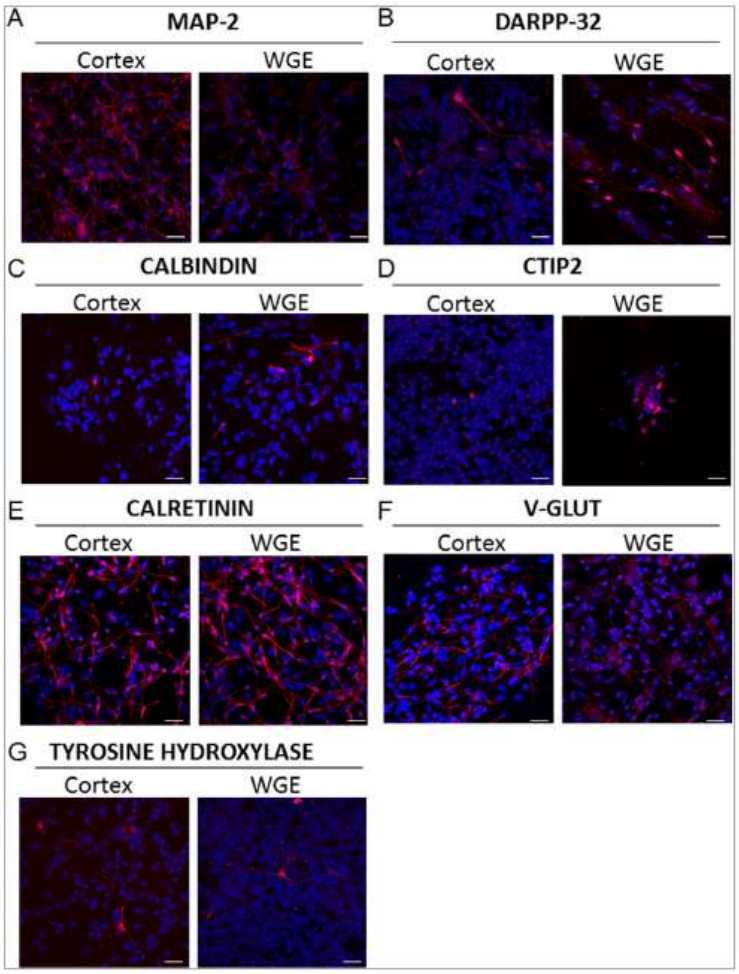

Fig. 8. WGE and Cortical cultures keep the potential to differentiate to mature neuronal phenotypes after expansion. Immunocyto chemical analysis of WGE and Cortical cultures, 21 days after differentiation. Both WGE and Cortical cultures are able to differentiate to MAP-2 (A), DARPP-32 (B), Calbindin (C), Ctip2 (D), Calretinin (E),V-glut1(F)and Tyrosine Hydroxylase(G). Scale bars: 25 µm.

4. Discussion

One of the challenges in the development of cell replacement therapies for neurodegenerative diseases is the identification and full characterization of easily expandable multipotent donor cell sources with the capacity to differentiate into the specific types of neurons lost to the dis-ease process. In the present work, we studied hfNSCs derived at different developmental stages from several brain areas as a source of renewable cells for clinical trials for HD. Our results show that the developmental stage of isolation is a critical factor for the capacity of primary cells to survive the dissection and dissociation process. As the brain develops neurogenesis takes place and the number of neural precursors is diminished while there are more post-mitotic neurons. Therefore, primary brain cells from older embryos present a higher sensitivity to the dissociation process and lower proliferation ratios. Nevertheless, the proliferation ratios were very low in all cases independently of the developmental stage. Improvements in the proliferation rates were obtained by dissociating the spheres mechanically instead of enzymatically, but not by modifications in the cell density. The comparison between spheres derived from several human fetal brain areas showed differences in the expansion potential, with forebrain, WGE and cortical derived cells being the most expandable. Expansion of hfNSC cultures partially modified the regional identity of cortical cultures, and to a lesser extent WGE derived ones. Nevertheless, both cultures kept their potential to differentiate to neurons, astrocytes and oligodendrocytes after 6 passages, as well as the ability to differentiate to mature neuronal phenotypes.

Clinical trials using primary human fetal brain tissue in HD involved the use of embryos from different developmental stages (6–12 wpc). However, the optimum gestational window for human donor fetal striatum has not yet been determined and little is known about the intrinsic differences between them. Our results show that the developmental stage of MTOP derivation is critical for the viability of the tissue after dis-section and dissociation. Although previous work reported higher viabilities for MTOP derived brain samples (Kelly et al., 2011), they included fewer samples from older embryos, which are the ones presenting lower viabilities (present results). Taking into account that the dissected tissue is composed of germinal zone containing neural precursors as well as their immediate progeny (neuronal, neuroglial, and glial progenitors) and newly post-mitotic cells (Maric and Barker, 2004; present results), we hypothesize that the decrease in viability and lower proliferation ratios observed for older derived samples might be due to the presence of a lower percentage of neural precursors that goes along with an increase in the number of post-mitotic neurons. This is in agreement with the enlargement of post-mitotic areas in the human fetal telencephalon observed at later developmental stages (8–11 wpc) (Onorati et al., 2014 and present results). These changes are ac-companied by modifications in the expression pattern of many transcription factors involved in neurogenesis such as: Nkx2.1, Ikaros, Tbr1, Ctip2, Foxp1 and Foxp2 in accordance with our RT-PCR results (present results).

Along the same lines, we postulate that neurospheres derived from older tissue might contain a higher percentage of post-mitotic cells than the younger ones, accounting for the lower proliferation ratio ob-served at P1. These post-mitotic cells might be decreasing over time in culture, as previously described (Sun et al., 2011). In agreement, we show that the levels of expression of post-mitotic neuronal genes de-crease after sequential passaging and the differences in the proliferation ratios disappear. These results indicate that the number of cells with neurosphere forming capacity might be similar between the develop-mental stages under study. Coincidentally, Piao and collaborators did not observe a correlation between the number of CD133 positive pro-genitors in P1 spheres and the developmental stage of forebrain spheres derivation (Piao et al., 2006). Moreover, our results also show that the developmental stage of derivation does not have any effect in the expansion potential along 6 passages. Thus, once the post-mitotic population is lost after initial passaging, the viability and the ratios of proliferation become stabilized and the cultures reach significant

expansion rates at passage 6. However, it is worth noting that there is a huge variability in the expansion potential within cultures derived from the same gestational age in agreement with previous work (Piao et al., 2006).

The expansion potential observed in the present work is significant-ly lower than those described in previous publications (Kallur et al., 2006; Kim et al., 2006). Methodological differences in human fetal brain tissue procurement (STOP or MTOP), culture conditions, passaging methodology and frequency may explain them (Jensen and Parmar, 2006). In fact, different disaggregation methodologies have been previously used for human neurosphere passaging: mechanic and enzymatic (Fan et al., 2014; Hitoshi et al., 2002; Kim et al., 2006; Ribeiro et al., 2013; Zietlow et al., 2005). Interestingly, our results demonstrate that mechanical disaggregation gave rise to higher proliferation ratios after 3 and 6 passages and therefore a higher expansion rate compared to enzymatic dissociation. These findings are in agree-ment with a previous report that define mechanical disaggregation as the least aggressive method (Gil-Perotín et al., 2013). However, neurospheres expanded by mechanical passage are bigger after 6 pas-sages and a higher cell death after complete dissociation is also ob-served. These findings could be explained by the fact that the cells in the inner layers have less access to the culture medium and therefore the availability of oxygen and nutrients is considerably reduced (Bez et al., 2003). Increasing the frequency of passage and refining the methodology are possible solutions to avoid neurosphere over-growth and improve hfNSC expansion significantly.

In order to explore different fetal brain sources of expandable hfNSCs obtained by MTOP for HD cell replacement therapies we compared hfNSCs from developing WGE, cortex, forebrain and cerebellum. In agreement with previous work, cells derived from all the regions under study were able to grow in suspension forming spheres (Darsalia et al., 2007; Fan et al., 2014; Kallur et al., 2006; Kim et al., 2006; Zietlow et al., 2005). Regardless of the region of origin, neurospheres show a heterogeneous composition containing proliferating neural progenitor cells, neurons and glia (Fan et al., 2014; Jensen and Parmar, 2006; Kallur et al., 2006; Kim et al., 2006). Interestingly, although most of the cells within the neurospheres are positive for the neural stem cell marker Nestin (Kallur et al., 2006; Kim et al., 2006), fewer cells are positive for the proliferative marker Ki67 (Jensen and Parmar, 2006; Reynolds and Rietze, 2005; Reynolds and Weiss, 1996). In agreement, the proliferation ratios are very low for all the brain regions studied. These results point to the notion that a small percent-age of cells holds neurosphere forming capacity.

Besides the general low proliferation ratios, analysis of the expansion potential show significant differences depending on the region of origin. Rostral derived hfNSC cultures are more expandable than caudal derived ones in agreement with previous reports (Horiguchi et al., 2004; Kim et al., 2006) and in this study forebrain, WGE and cortical derived hfNSC are the most expandable ones. Interestingly, no differences are found between the expansion potential of cortical and WGE derived samples as previously described (Kim et al., 2006). In our culture conditions cerebellar derived cultures were not able to expand efficiently enough to increase the population through passages. This result might be due to the early stage of derivation (7–9 wpc) for this brain region. In fact, Fan Y and col-laborators described an increase in the NSCs initiating colony efficiency for cerebellar derived samples with increasing gestation between 14 and 23 wpc, obtaining the highest efficiency at 23 wpc (Fan et al., 2014).

Next, we studied the regional identity and differentiation potential of hfNSC through passaging for WGE and cortical derived samples. Sox2 is expressed early in neuroepithelial development, and its expression persists in both mitotically competent NSCs and their transit-amplifying daughters (Graham et al., 2003; Pevny and Placzek, 2005; Pevny and Nicolis, 2010; Zappone et al., 2000). In

addition, Sox2 expression selectively identified multipotential and self-renewing neural progenitor cells in dissociates of human fetal fore-brain (Wang et al., 2010). Taking into account these previous results, the present findings indicate that a Sox2 posgoes along with an increase in the number of post-mitotic neurons. This is in agreement with the enlargement of post-mitotic areas in the human fetal telencephalon observed at later developmental stages (8–11 wpc) (Onorati et al., 2014 and present results). These changes are ac-companied by modifications in the expression pattern of many transcription factors involved in neurogenesis such as: Nkx2.1, Ikaros, Tbr1, Ctip2, Foxp1 and Foxp2 in accordance with our RT-PCR results (present results).

Along the same lines, we postulate that neurospheres derived from older tissue might contain a higher percentage of post-mitotic cells than the younger ones, accounting for the lower proliferation ratio ob-served at P1. These post-mitotic cells might be decreasing over time in culture, as previously described (Sun et al., 2011). In agreement, we show that the levels of expression of post-mitotic neuronal genes de-crease after sequential passaging and the differences in the proliferation ratios disappear. These results indicate that the number of cells with neurosphere forming capacity might be similar between the develop-mental stages under study. Coincidentally, Piao and collaborators did not observe a correlation between the number of CD133 positive pro-genitors in P1 spheres and the developmental stage of forebrain spheres derivation (Piao et al., 2006). Moreover, our results also show that the developmental stage of derivation does not have any effect in the expansion potential along 6 passages. Thus, once the post-mitotic population is lost after initial passaging, the viability and the ratios of proliferation become stabilized and the cultures reach significant expansion rates at passage 6. However, it is worth noting that there is a huge variability in the expansion potential within cultures derived from the same gestational age in agreement with previous work (Piao et al., 2006).

The expansion potential observed in the present work is significant-ly lower than those described in previous publications (Kallur et al., 2006; Kim et al., 2006). Methodological differences in human fetal brain tissue procurement (STOP or MTOP), culture conditions, passaging methodology and frequency may explain them (Jensen and Parmar, 2006). In fact, different disaggregation methodologies have been previously used for human neurosphere passaging: mechanic and enzymatic (Fan et al., 2014; Hitoshi et al., 2002; Kim et al., 2006; Ribeiro et al., 2013; Zietlow et al., 2005). Interestingly, our results demonstrate that mechanical disaggregation gave rise to higher proliferation ratios after 3 and 6 passages and therefore a higher expansion rate compared to enzymatic dissociation. These findings are in agreement with a previous report that define mechanical disaggregation as the least aggressive method (Gil-Perotín et al., 2013). However, neurospheres expanded by mechanical passage are bigger after 6 pas-sages and a higher cell death after complete dissociation is also ob-served. These findings could be explained by the fact that the cells in the inner layers have less access to the culture medium and therefore the availability of oxygen and nutrients is considerably reduced (Bez et al., 2003). Increasing the frequency of passage and refining the methodology are possible solutions to avoid neurosphere over-growth and improve hfNSC expansion significantly.

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most of the cells within the neurospheres are positive for the neural stem cell marker Nestin (Kallur et al., 2006; Kim et al., 2006), fewer cells are positive for the proliferative marker Ki67 (Jensen and Parmar, 2006; Reynolds and Rietze, 2005; Reynolds and Weiss, 1996). In agreement, the proliferation ratios are very low for all the brainitive population might be the one giving rise to self-renewing neurospheres in WGE and cortical-derived samples. Moreover, these expanded neurosphere cultures remain multipotent over 6 passages. Despite these similarities, neurosphere expanded cultures derived from WGE and cortex behave differently in the maintenance of region identity. WGE ex-panded cultures keep their regional identity through the expression of early transcription factors involved in striatal fate determination such as Dlx family members, Gsx2 (Gsh2)and Ascl1 (Mash1)(Anderson et al., 1997; Stenman et al., 2003; Toresson et al., 2000; Wang et al., 2009). In agreement, long-term differentiation gives rise to a high percentage of DARPP-32 and Ctip2 positive cells as well as Calbindin and Calretinin positive neurons. This is coincident with the main striatal neuronal populations that are medium-sized spiny neurons which are positive for DARPP-32 and/or Calbindin (Deacon et al., 1994; Olsson et al., 1998) and some striatal interneurons that express Calretinin (Kawaguchi et al., 1995). In agreement with Kallur et al. we also obtained a low number of glutamatergic neurons in WGE cultures. Although contamination of WGE samples with cortical tissue during dissection could not be completely ruled out, it might be possible that striatal expanded cells can differentiate into glutamatergic neurons in vitro in agreement with previous authors (Kallur et al., 2006). In contrast, cortical-derived hfNSCs down-regulate most of its region specific early genes after expansion, acquiring a ventral identity as previously described (Hitoshi et al., 2002; Parmar et al., 2002). Long term differentiation of cortical-derived neurospheres generate a substantial population of glutamatergic neurons (positive for vglut1) and a significant number of interneurons most of which are positive for Calretinin and a smaller number for Calbindin, which correlates with the main cortical neuronal populations (Jones, 1993). Thus, although there is a ventralization at the level of cortical progenitors, they keep the potential to acquire a cortical neuronal fate. We also obtain a small population of DARPP-32 and Ctip2 positive neurons when differentiating cortical-derived neurospheres (Deacon et al., 1994; Olsson et al., 1998). Although these markers have been broadly considered specific of striatal medium-spiny neurons, Onorati and collaborators have recently described positive cells for these two markers during cortical development at 8–11 weeks (Onorati et al., 2014).

5. Conclusions

Our results show that MTOP derived human fetal forebrain, WGE and cortex from 4 to 10 wpc have potential to be used as a source of expandable hNSCs for cell therapy replacement. hfNSC cultures derived from the three brain regions expand significantly after 6 passages while keeping their neurogenic potential. However, from our studies, WGE derived hfNSCs present the best potential as a donor cell source for HD treatment since they demonstrate the highest efficiency of differ-entiation to mature striatal neurons through the maintenance of the regional identity after expansion.

Conflict of interest

The authors confirm no potential conflict of interest.

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Subdirección General de Evaluación and European Re-gional Development Fund (ERDF) [RETICS to JMC (RD12/0019/0002; Red de Terapia Celular)], Spain; CIBERNED; and CHDI Foundation (A-7332 and A-12076 to JMC), USA. This work has been developed in the context of AdvanceCat with the support of ACCIÓ (COMRDI15-1-0013) (Catalonia Trade & Investment; Generalitat de Catalunya) under the Catalonian ERDF operational program 2014–2020. The South Wales fetal tissue bank was supported by the UK Medical Research Council (RCBM523), a grant from the Welsh Government, and by Health and Care Wales (RCBM763). The funding sources had no involvement in study design, collection, analysis and interpretation of data, neither in the writing or decision to submit the article for publication.

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