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Isolation, characterization, and differentiation of multipotent neural progenitor cells from human cerebrospinal fluid in fetal cystic myelomeningocele

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ABSTRACT

Despite benefits of prenatal in utero repair of myelomeningocele, a severe type of spina bifida aperta, many of these patients will still suffer mild to severe impairment. One potential source of stem cells for new regenerative medicine-based therapeutic approaches for spinal cord injury repair is neural progenitor cells (NPCs) in cerebrospinal fluid (CSF). To this aim, we extracted CSF from the cyst surrounding the exposed neural placode during the surgical repair of myelomeningocele in 6 fetuses (20 to 26 weeks of gestation). In primary cultured CSF-derived cells, neurogenic properties were confirmed by in vitro differentiation into various neural lineage cell types, and NPC markers expression (TBR2, CD15, SOX2) were detected by immunofluorescence and RT-PCR analysis. Differentiation into three neural lineages was corroborated by arbitrary differentiation (depletion of growths factors) or explicit differentiation as neuronal, astrocyte, or oligodendrocyte cell types using specific induction mediums. Differentiated cells showed the specific expression of neural differentiation markers (βIII-tubulin, GFAP, CNPase, oligo-O1). In myelomeningocele patients, CSF-derived cells could become a potential source of NPCs with neurogenic capacity. Our findings support the development of innovative stem-cell-based therapeutics by autologous transplantation of CSF-derived NPCs in damaged spinal cords, such as myelomeningocele, thus promoting neural tissue regeneration in fetuses.

1. Introduction

Myelomeningocele is a severe type of spina bifida that represents one of the most debilitating birth defects in humans (Copp et al., 2003; Copp and Greene, n.d.; Dias and McLone, 1993; Dias, 1999; Hunt, 1990; Lary and Edmonds, 1996; Meuli et al., 1997), with an estimated incidence of 3.3 in 10,000 live births. Myelomeningocele harshly affects both spinal cord and encephalic structures during gestation (Lary and Edmonds, 1996) resulting in disabilities that include paraplegia, skeletal deformities, hydrocephalus, hindbrain herniation, impaired mental development, and fecal, urinary, and sexual dysfunction (Dias and McLone, 1993; Dias, 1999; Hunt, 1990). A deficiency on primary neurulation leads to a deficient neural tube closure and myelomeningocele formation (Copp et al., 2003; Dias, 1999), which then induces the progressive neurodegeneration of the exposed spinal cord during pregnancy (Meuli et al., 1997). In humans, the two most common neural tube defects are myelomeningocele and myelocele; neural tissues are surrounded by a thin cystic sac in the former (spina bifida cystica) or exposed directly to the amniotic fluid in the latter (Copp and Greene, n.d). The myelomeningocele cyst around the exposed neural tissue contains CSF. When damaged or perforated during pregnancy, these contents can be released toward the amniotic space (Meuli and Moehrlen, 2014).

Existing treatments for myelomeningocele repair consist of pre- or post-natal surgical closure of the defect. Prenatal intervention has been shown to be more effective than postnatal surgery because it can prevent or ameliorate sequels (Adzick et al., 2011; Adzick et al., 1998; Meuli et al., 1997; Meuli et al., 1995). Clearly, in utero repair of

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myelomeningocele is neither a complete remedy nor free of risk for mother and fetus (Adzick et al., 2011; Meuli and Moehrlen, 2014). Despite the benefits of surgical repair in preventing further injury, neurological outcomes remain a critical concern (Gebb et al., 2015) because of the damage incurred before closure of the defect. In spite of the low 3% perinatal mortality rate and possible benefits of in-utero repair of myelomeningocele, 36% of patients after surgery had mild to severe impairment (Danzer et al., 2010; Hisaba et al., 2012). Therefore, new therapeutic approaches concomitant to fetal surgery are needed to improve locomotive and cognitive functions in these babies.

During the last 10 years, researchers made great efforts toward the development of neural stem cell-based therapeutic approaches for spinal cord regeneration (Barnabé-Heider and Frisén, 2008; Li and Lepski, 2013; Mothe and Tator, 2013; Parati et al., 2004; Sandner et al., 2012). Even with CSF's low cellularity (de Graaf et al., 2011), recent studies have reported that embryonic CSF contains neurogenic growth factors (Buddensiek et al., 2010; Zappaterra and Lehtinen, 2012). In pre-term babies with post-hemorrhagic hydrocephalus, CSF samples showed some circulating cells with neural progenitor properties (Krueger et al., 2006). Although technical difficulties exist in CSF sampling during gestation, the findings of neural progenitor cells (NPCs) in CSF strongly support its potential as an important source of stem cells for the new regenerative medicine-based therapeutic approaches for spinal cord injury repair. During prenatal surgical repair of myelomeningocele (Enriquez et al., 2012; Peiró et al., 2009a; Peiró et al., 2009b; Pellicer et al., 2007; Ruano et al., 2013a, 2013b), collection of fresh and well-preserved CSF samples from the cyst was feasible by gentle aspiration, thus permitting examination of its properties and cellular composition.

To further this strategy, we evaluated the NPC types in CSF samples collected from the lumbar cyst that covered the defect in a small sample of patients undergoing repair of myelomeningocele. We then determined the neurogenic properties of CSF-derived cells by in vitro characterization and differentiation into various neural lineage cell types.

2. Materials and methods

During the surgical repair of myelomeningocele in 6 fetuses between 20 and 26 weeks of gestation, CSF samples were extracted from the cyst surrounding the exposed neural placode. The study was approved by the IRB Ethics Committees of CCHMC (IRB 2013-5324) and IRB of the Vall d'Hebron University Hospital (protocol ID: PR(AMI)65/2013). Patients received detailed information about the experimental protocol and gave written consent for their participation.

2.1. Human cerebrospinal fluid samples

Before the actual surgical repair of the myelomeningocele, the cyst was punctured to gently aspirate 1.5–2 ml of CSF using a 5-ml (BD Luer-Lok™) syringe with 22-gauge detachable needle (Becton Dickinson, Franklin Lakes, New Jersey). These samples were then used to establish the CSF-derived primary cell cultures designated as CSF-fc1 to fc6: fc1, 2, 3, and 5 (male fetuses of 20, 20, 21, and 25 weeks of gestation, respectively) and fc4 and 6 (female fetuses of 25 and 26 weeks of gestation, respectively).

2.2. Primary cell culture of human CSF-derived stem cells

Immediately after CSF collection, the cell culture was begun under sterile conditions. First, 1.5–2 ml of CSF were mixed with 15 ml of proliferation medium composed of high glucose Dulbecco's modified Eagle's medium (DMEM):M-199 medium (3:1), supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine (Sigma), 25 ng/ml fibroblast growth factor (FGF2), 10 ng/ml epidermal growth factor (EGF) (Becton Dickinson), and antibiotics/antimycotics (Life Technologies). Seeded on 75-cm^2 cell culture flasks (Nunc), the first medium change was made 10–14 days later when the colonies of adherent cells appeared; non-adherent cell debris was removed by changing the medium. Next, CSF cell cultures were trypsinized and again plated until the cultures reached 60%–70% confluence. Finally, cells were harvested using trypsin and seeded: in 60-mm culture dishes for RNA extraction and RT-PCR analysis, or onto cover slips on 12-well plates for immunocytofluorescence analysis. All experiments were performed on cells between passages 1 and 2.

2.3. Cell proliferation assay

Human CSF-derived primary cultured cells were seeded at 75,000 cells/well in 24-well culture plates and incubated with proliferating medium, which was changed every 2–3 days. Quadruplicate wells were counted at days 3 and 5 using a Neubauer chamber.

2.4. Immunocytofluorescence analysis

For immunocytofluorescence analysis, cells were fixed with 4% paraformaldehyde for 30 min at room temperature, washed three times with phosphate-buffered saline (PBS, Life Technologies), incubated for 20 min with 0.1% triton X-100 in PBS with agitation, and blocked with PBS with 3% bovine serum albumin (BSA, Sigma) for 30 min before antibody incubation. Primary antibodies against TBR2 (ab23345, Abcam), CD15 (ab119844, Abcam), SOX2 (ab97959, Abcam), βIII-tubulin (ab41489, Abcam), GFAP (Z0334, Dako), Oligo-O1 (MAB5540, Chemicon) and CNPase (ab6319, Abcam) were diluted 1:100 in PBS with 1% BSA and incubated in a humid chamber for 16 h at 4 °C. Next, cover slips were washed three times with PBS and incubated with the secondary antibodies diluted 1:1000 in PBS with 1% BSA containing 1 μg/ml Hoechst 33,342 (Invitrogen) in a dark humid chamber for 45 min at room temperature. Secondary antibodies used for single or triple immunocytofluorescence analysis were the Alexa Fluor® 568 anti-mouse (A-11004, Invitrogen), Alexa Fluor® 488 anti-rabbit (A-11008, Invitrogen), Alexa Fluor® 488 anti-mouse (A-11001, Invitrogen), Alexa Fluor® 647 anti-rabbit (A-21245, Invitrogen), Alexa Fluor® 647 anti-chicken (A-21449, Invitrogen), and Alexa Fluor® 568 anti-chicken (A-11041, Invitrogen). Finally, cover slips were washed three times with PBS and mounted with Fluoromount-G mounting medium (Southern Biotech). Microphotographs were obtained by using a BX-61 microscope (Olympus).

2.5. Analysis of gene expression by quantitative real-time PCR

Human CSF-derived NPCs were cultured on 60-mm dishes. When confluence reached 50%–60%, total RNA was extracted using TRIzol (GIBCO BRL Life Technologies) and purified on RNeasy Micro kit columns (Qiagen, Hilden, Germany). Reverse-transcriptase (RT) reaction was performed using up to 5 μg of total RNA with the Superscript II RT kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions; to each RT reaction, we added 100 ng of Random primers (Promega, Southampton, UK) and 20 units of SUPERase-in RNase inhibitor (Ambion, Huntingdon, UK) to prevent RNA degradation.

The gene expression levels of CD15, TBR2 and SOX2 were analyzed by quantitative real-time PCR. Each data point was normalized by the mRNA abundance of the housekeeping PES1 control gene. All oligonucleotide primer pairs used for each gene in this study (Supplementary Table 1) were designed according to the sequences derived from GenBank (accession numbers in parenthesis). Complementary DNA (25 ng) from the RT reaction was used as a template for each PCR in a 10-μl reaction volume. All qRT-PCR reactions were performed using the LightCycler® 480 SYBR Green I Master kit and a LightCycler® 480 II Thermocycler (Roche, Indianapolis, IN) following the manufacturer's instructions.

2.6. Random differentiation of CSF-derived NPCs into neural lineage cell subtypes by growth factors deprivation

CSF-derived cells (10^5 cells/well) were seeded onto cover slips on 12-well plates and cultured with proliferation medium (DMEM:M-199 medium, 3:1, supplemented with 10% FBS, 2 mM glutamine (Sigma), 25 ng/ml fibroblast growth factor (FGF2), 10 ng/ml epidermal growth factor (EGF) (Becton Dickinson), and antibiotics/antimycotics (Life Technologies) until the confluence reached 40%–50%. Cells were then washed twice with PBS and induced to arbitrary differentiation into neural lineage cell subtypes by incubation for 10 days with StemPro® NSC SFM medium (Life Technologies) in the absence of external growth factors addition. Cell culture medium was replaced every 2–3 days.

2.7. Specific differentiation of CSF-derived NPCs into neural lineage cell subtypes

CSF-derived NPCs were plated onto cover slips on 12-well plates and cultured. When the confluence reached 40%–50%, cultures were induced to differentiate into three neural lineage cell types as neurons, astrocytes, or oligodendrocytes by incubating with explicit differentiation mediums as follows:

2.7.1. Neuron-specific differentiation protocol

NPCs were plated onto polyornithine and laminin-coated cover slips on 12-well plates before cell seeding. Cover slips in culture vessels were incubated with 20 μg/ml poly-L-ornithine (P3655, Sigma) solution for 1 h at 37 °C, rinsed twice with sterile water, incubated with 10 μg/ml laminin (Life Technologies) solution for 2 h at 37 °C, and rinsed with PBS without calcium or magnesium (Life Technologies). The cells (10^5 cells/well) were then seeded and cultured with proliferation medium (DMEM:M-199 medium, 3:1, supplemented with 10% FBS, 2 mM glutamine (Sigma), 25 ng/ml fibroblast growth factor (FGF2), 10 ng/ml epidermal growth factor (EGF) (Becton Dickinson), and antibiotics/antimycotics (Life Technologies). When the confluence reached 40%–50%, cells were washed twice with PBS before incubation with differentiation medium.

Differentiation into neurons was induced by incubating for 7 days with neural differentiation medium composed of Neurobasal Medium with 2% of B-27 Serum-Free supplement, 2 mM GlutaMAX-I, and antibiotic-antimycotic solution (all from Life Technologies). At day 7, dibutyryl cAMP (Sigma) was added to the medium, to reach a final concentration of 0.5 mM, and incubated up to day 10. Differentiation medium was replaced every 2–3 days.

2.7.2. Astrocyte-specific differentiation protocol

NPCs were plated onto Geltrex-coated cover slips on 12-well plates. Before cell seeding, cover slips in culture vessels were incubated with Geltrex (P3655, Sigma) working solution (diluted 1:200 with DMEM/F-12 following manufacturer's instructions) for 1 h at room temperature and washed once with D-PBS with calcium and magnesium (Life Technologies). Cells (10^5 cells/well) were seeded and cultured with proliferation medium (DMEM:M-199 medium, 3:1, supplemented with 10% FBS, 2 mM glutamine (Sigma), 25 ng/ml fibroblast growth factor (FGF2), 10 ng/ml epidermal growth factor (EGF) (Becton Dickinson), and antibiotics/antimycotics (Life Technologies). When the confluence reached 40%–50%, cells were then washed twice with PBS before incubation with differentiation medium.

Differentiation into astrocytes was induced by incubating with astrocyte-differentiation medium composed of DMEM with 1% of N-2 supplement, 2 mM GlutaMAX-I, 1% FBS, and antibiotic-antimycotic solution (all from Life Technologies) for 10 days. Differentiation medium was replaced every 2–3 days.

2.7.3. Oligodendrocyte-specific differentiation protocol

NPCs were plated onto polyornithine and laminin-coated cover slips on 12-well plates before cell seeding as above. Then, cells (10^5 cells/well) were seeded and cultured with proliferating medium (DMEM:M-199 medium, 3:1), supplemented with 10% FBS, 2 mM glutamine (Sigma), 25 ng/ml fibroblast growth factor (FGF2), 10 ng/ml epidermal growth factor (EGF) (Becton Dickinson), and antibiotics/antimycotics (Life Technologies) until the confluence reached 40%–50%. Cells were washed twice with PBS before incubation with differentiation medium.

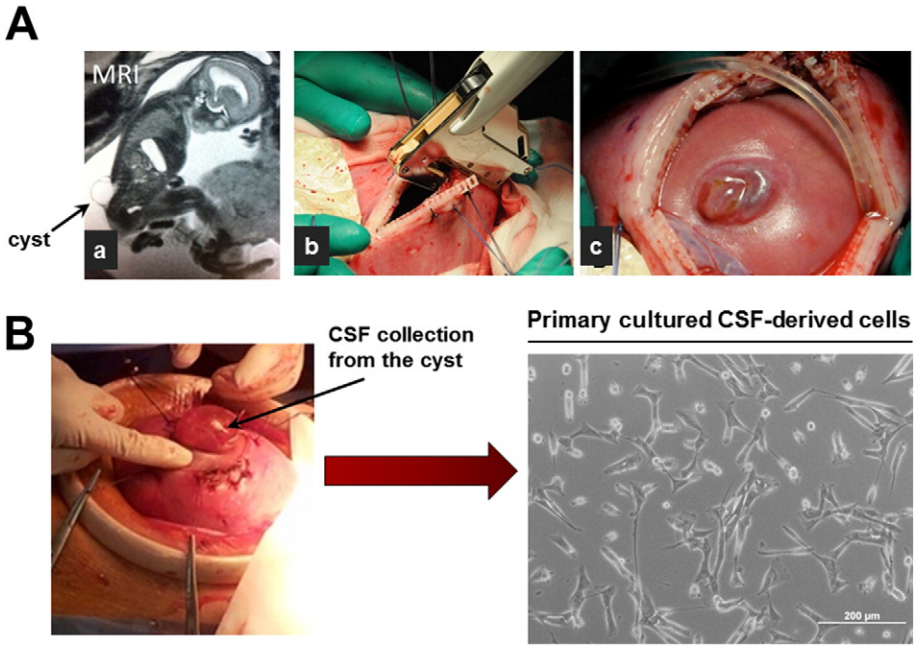

Fig. 1. Human cerebrospinal fluid (CSF) collection during the prenatal surgical repair of myelomeningoceles (MMC). (A) CSF collected from the cysts over the defect (a) before repair. After infraumbilical laparotomy and hysterotomy in the pregnant mother (b), the fetal lumbar region was exposed (c). (B) CSF samples were gently aspirated with a sterile syringe and immediately used to establish CSF-derived primary cell cultures. Scale bars = 200 μm.

Differentiation into oligodendrocytes was induced by incubating with oligodendrocyte-specific differentiation medium for 10 days. It contained Neurobasal medium with 2% of B-27 Serum-Free supplement (Life Technologies), 2 mM GlutaMAX-I (Life Technologies), 30 ng/ml Triiodo-L-Thyronine (T3, Sigma), and antibiotic-antimycotic solution (Life Technologies). Differentiation medium was replaced every 2–3 days.

2.8. Flow cytometry

Cells were dissociated with Tripsin 0.25% (Life Technologies) and resuspended in stain buffer (BD Pharmingen) by adding 5 mM EDTA. Cells were stained live on ice for 20 min with a specific antibodies panel as previously reported by Yuan SH et al. (Yuan et al., 2011). Cells were washed and NSC/NPC (CD45 −/CD184 +/CD44 −/CD271 −/CD24 +/CD15 +), neurons (CD45 −/CD184 −/CD44 −/CD24 +/CD15Low), and glia cell (CD45 −/CD184 +/CD44 +/CD271 −) populations were analyzed by flow cytometry on a LSRII Flow Cytometer system (BD Pharmingen). All fluorochrome-conjugated antibodies were from BD or BioLegend unless otherwise noted: Fixable viability staining (eBioscience), CD45 (557059), CD184 (306516), CD271 (345110), CD44 (103043), CD24 (555427) and CD15 (125614). Data were analyzed by BD FACSDiva™ (BD Pharmingen) software for generation of heat maps.

2.9. Statistical analysis

For immunocytofluorescence images, the average signal intensity was calculated as: normalized mean signal intensity to exposure time divided by number of nuclei. Signal intensities and nuclei count obtained using Image J software (version 1.45s; NIH, Bethesda, MD), averaging 3 or 4 images. Student's *t*-test was used to determine statistical significance throughout the study. Differences were considered significant with a p value < 0.05. Data are presented as mean ± standard deviation (SD).

3. Results

By successful extraction of CSF samples from the cyst of the myelomeningocele during fetal surgery at mid gestation (Fig. 1), our protocol detected the presence of NPCs in CSF-derived primary culture by the expression of neural and pluripotent specific markers TBR2,

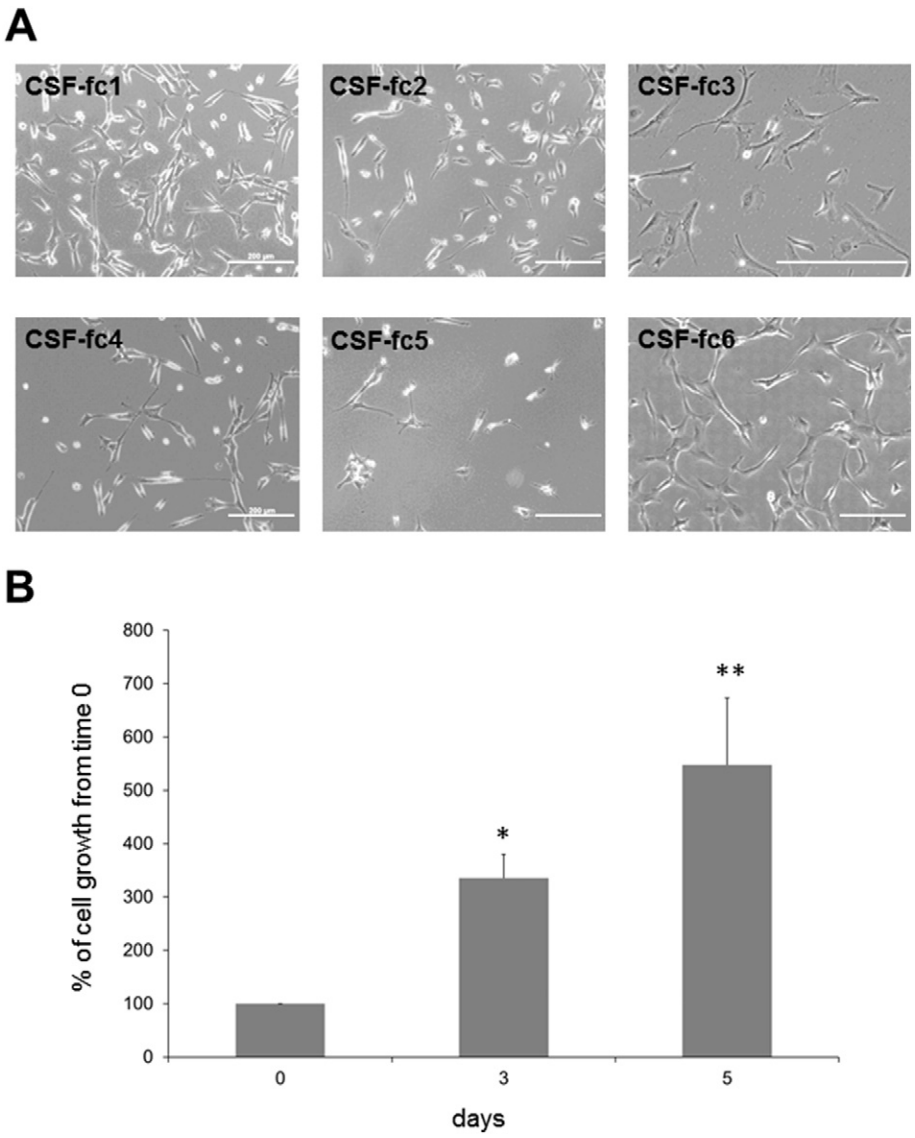

Fig. 2. Human CSF-derived primary cell cultures from MMC patients. (A) Human CSF-derived cells from 6 fetal patients aged 20 to 26 weeks of gestation were successfully expanded in primary culture. Scale bars = 200 μm. (B) Primary cultured cells were seeded at 75,000 cells/well in 24-well culture plates and incubated with proliferating medium. Quadruplicate wells were counted on days 3 and 5. Values mean ± standard deviation (SD). * indicates $p < 0.05$ and ** indicates $p < 0.01$ respecting cell number at time 0. CSF, cerebrospinal fluid.

SOX2, and CD15 using immunocytofluorescence, quantitative real-time PCR, and flow cytometry analysis.

3.1. Establishment of primary cell cultures from human MMC patients CSF samples

From the CSF samples extracted from 6 fetuses, CSF-derived cells were successfully grown in primary culture (Fig. 2A). Proliferation rates at day 3 and 5 (Fig. 2B) exhibited linear growth. Compared with baseline day 0, cell numbers increased 335 ± 44% ($p = 0.026$) and 547 ± 125% ($p = 0.0027$) at days 3 and 5, respectively.

3.2. Human CSF-derived primary cultured cells show neural progenitor cell characteristics

To determine the presence of NPCs in CSF-derived primary culture, we studied the expression of the neural and pluripotent specific markers TBR2 (Hodge et al., 2012; Hodge et al., 2008), SOX2 (Pevny and Nicolis, 2010; Tanaka et al., 2004; Wegner and Stolt, 2005; Wells et al., 2011), and CD15 (Capela and Temple, 2002; Doetsch, 2003; Dromard et al., 2008) by immunocytofluorescence and quantitative real-time PCR analysis. CSF-derived cultured cells showed robust expression of the specific neural progenitor markers CD15, SOX2, and TBR2 (Fig. 3A); specifically, percentages of positive cells were 45.4 ± 5.4%, 27.6 ± 7.8% and 31.3 ± 4.2%, respectively (Fig. 3B). qPCR analysis demonstrated the presence of NPC markers expression in CSF-derived primary cultured cells (Fig. 3C). Compared with control gene PES1, mRNA abundance for the genes CD15, SOX2, and TBR2 was 0.72 ± 0.16, 0.73 ± 0.27, and 0.46 ± 0.15 (based on relative expression units), respectively. Thus, a cell subpopulation with neural progenitor characteristics was found in the CSF of myelomeningocele fetuses during the course of the disease.

3.3. In vitro random differentiation of CSF-derived NPCs into neural lineage cell subtypes

CSF-derived NPCs in primary culture were induced to differentiate by growth factor deprivation to determine their arbitrary capacity to differentiate into neural lineage cell subtypes. In primary cultured cells at a confluence of 40–50% incubated for 10 days with the StemPro® NSC SFM medium in the absence of growth factors, human CSF-derived NPCs had a strong capacity to differentiate into neural lineage cells as neurons, astrocytes, and oligodendrocytes. This was demonstrated by the expression of the neuronal (βIII-tubulin) and glial (GFAP for astrocytes, CNPase and oligo-O1 for oligodendrocytes) specific markers, which were detected by triple immunocytofluorescence after differentiation (Fig. 4). Cells were monitored for morphological changes (Fig. 4A) and expression changes in specific neural lineage markers (Fig. 4B). Cells induced to differentiate with StemPro® NSC SFM medium without

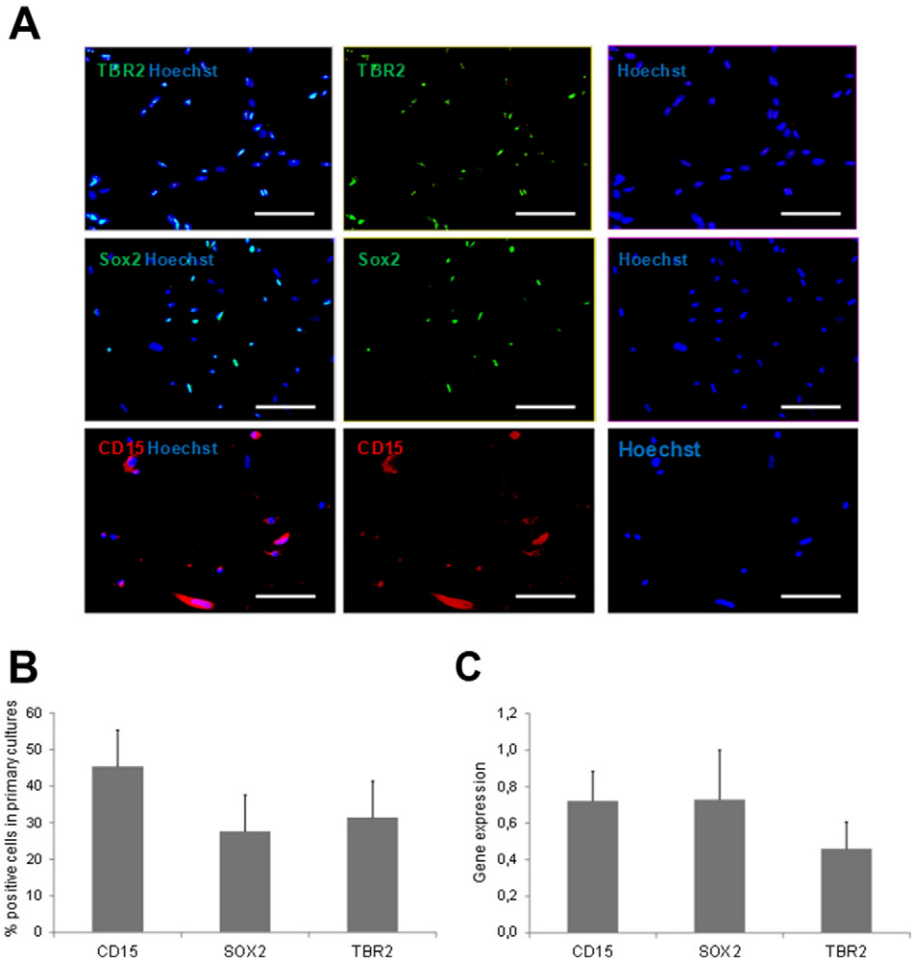

Fig. 3. NPC markers expression in MMC patient's CSF-derived primary cultured cells. Analysis by immunocytofluorescence and real-time RT-PCR. (A) Immunocytofluorescence demonstrated positive staining of the NPCs markers CD15, SOX2 and TBR2 in CSF-derived cells. Cell cultures from CSF-fc1 patient. Scale bars = 200 μm. (B) Percentage of positive cells for CD15, SOX2, and TBR2 markers defined as total number of cells in CSF-derived cell cultures. (C) qPCR values of CD15, SOX2, and TBR2 mRNA abundance were quantified and normalized by the PES1 mRNA content. Data means ± SD obtained from 3 independent experiments. NPC, neural precursor cell; CSF, cerebrospinal fluid; MMC, myelomeningocele; CD15, 3-fucosyl-*N*-acetyl-lactosamine; SOX2, SRY (Sex Determining Region Y)-Box 2; TBR2, T-box transcription factor Eomes/T-box brain protein 2; PES1, Pescadillo ribosomal biogenesis factor 1.

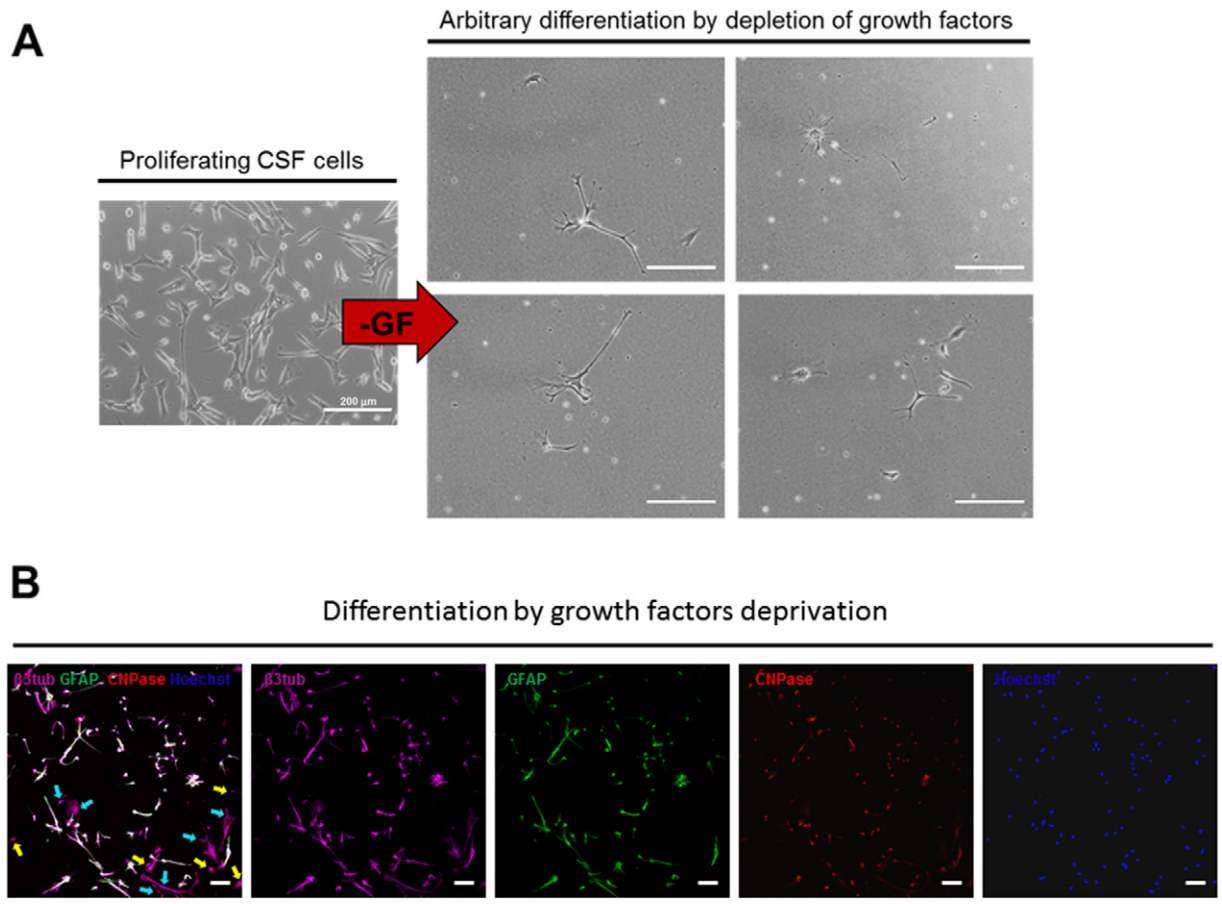

Fig. 4. Arbitrary neural differentiation of human CSF-derived cells in primary culture. CSF-derived cells seeded onto cover slips on 12-well plates and cultured by incubation with proliferation medium. When confluence reached 40%–50%, cells then induced to differentiate by deprivation of growth factors by incubating with StemPro® NSC SFM medium without addition of EGF and bFGF growth factors for 10 days. Neural differentiation analyzed by morphological changes monitored under optical microscopy (A) or by expression of specific neural differentiation markers GFAP (glial marker), βIII-tubulin (neuronal marker) and CNPase (oligodendrocyte marker) determined by triple immunocytofluorescence (B). β3Tub+/GFAP- (light blue arrow) and CNPase+/GFAP- cells (yellow arrow). Microphotographs of 10-day differentiated CSF-derived cells from CSF-fc1 patient. Scale bars = 200 µm. CSF, cerebrospinal fluid; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; GFAP, glial fibrillary acidic protein; βIII-tubulin, class III β-tubulin; CNPase, 2′,3′-Cyclic-nucleotide 3′-phosphodiesterase.

growth factors acquired other morphologies (i.e., thin elongated protrusions and abundant neurites or large rounded cell bodies).

3.4. In vitro specific differentiation into neural lineage cell subtypes of CSF-derived NPCs

In evaluating CSF-derived NPCs differentiation into neurons, astrocytes, or oligodendrocytes during a 10-day incubation with specific differentiation mediums, NPCs effectively differentiated into the three neural lineages. Specific morphological changes identified included spindle shape with growing neurite protuberances for neurons and oligodendrocytes; and round-flat shape for astrocytes. The expression of specific markers for neuron (βIII-tubulin), astrocyte (GFAP), and oligodendrocyte (oligo-O1) lineages was noted in triple immunocytofluorescence (Fig. 5). When compared with CSF-derived proliferating cells, cells differentiated into neurons showed a robust 5.7-fold increase ($p = 0.014$) in the neuronal marker βIII-tubulin (Fig. 6A) and clear expression of the neuronal markers MAP2 and nestin (Supplementary Fig. 1). Astrocyte-specific differentiation of CSF-derived NPCs induced the strongest upregulation (8.6-fold) of the GFAP astrocyte marker ($p = 0.0022$) (Fig. 6B) and lesser increased expression of the neuronal and oligodendrocyte markers βIII-tubulin (2.3-fold, $p = 0.0017$) and oligo-O1 (6.5-fold, $p = 0.0057$). Additionally, oligodendrocyte-specific differentiation induced the strongest 12.1-fold upregulation of the oligodendrocyte marker oligo-O1 ($p = 2.0e\text{-}4$); increases of the βIII-tubulin (5.2-fold, $p = 2.5e\text{-}5$) and GFAP (5.3-fold, $p = 0.0017$) markers were also detected following the specific oligodendrocyte differentiation process (Fig. 6C).

3.5. Flow cytometry evaluation of CSF-derived cell populations

We used a previously described (Yuan et al., 2011) flow cytometry strategy to corroborate the presence of NSC/NPC, neuronal and glial cell populations in CSF-derived primary cultured cells (Fig. 7). Cells were first selected on the basis of viability staining and the absence of CD45 staining. NSC/NPC cell populations were selected for CD184+ and then negative selected for CD44- and CD271- and co-expression of CD24+ and CD15+. The percentage of CD184+/CD271−/CD44−/CD24+/CD15+ cells in CSF-derived cell cultures was 1.4% of total. However, glial cells were selected for CD184+ and then for CD44+ and CD271−, which represented the 0.1% of total CSF-derived cells. Cells committed to the neuronal lineage were first selected for depletion of CD44 and CD184 and then for CD24+ and CD15low staining. Thus, neuronal population (CD184−/CD44−/CD15Low/CD24+) represented the 0.3% of the total CD45- and live cells.

4. Discussion

Our novel approach for potential cell therapy used a previously uncharacterized class of progenitor cells extracted during in utero repair of myelomeningocele. With successful extraction of NPCs from the CSF of myelomeningocele's cyst in midgestation fetuses, the cells

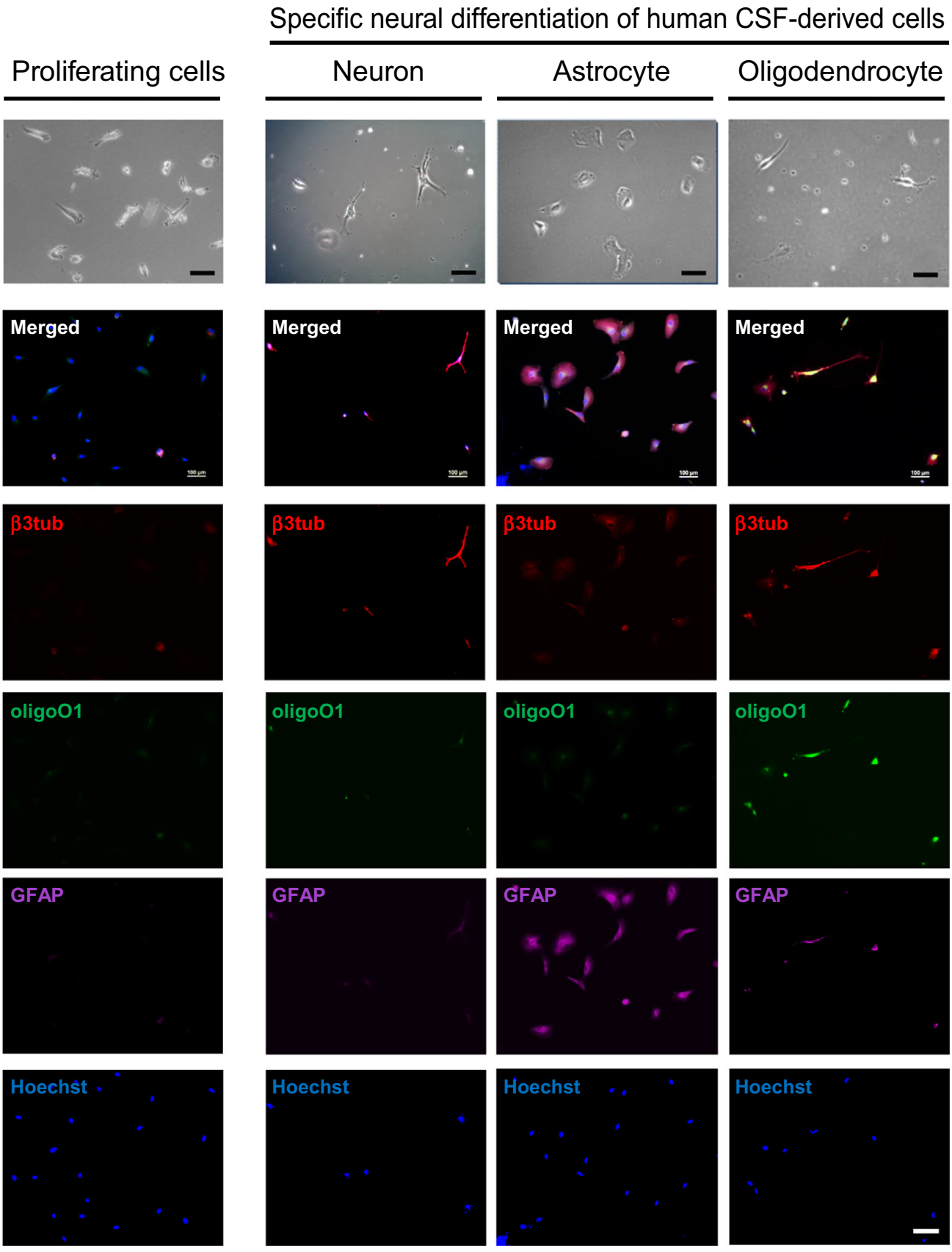

Fig. 5. Specific differentiation of CSF-derived NPCs into neural lineage cell subtypes. CSF-derived cells were seeded onto cover slips on 12-well and cultured by incubation with proliferation medium. When confluence reached 40%–50%, cells were induced to differentiate into the three neural lineage cell types, neurons, astrocytes and oligodendrocytes, by incubating with specific differentiation mediums for 10 days. Expression of the neural differentiation markers GFAP (astrocyte marker), βIII-tubulin (neuron marker) and Oligo-O1 (oligodendrocyte marker) was determined by triple immunocytofluorescence before (proliferating cells) and after (differentiated cells) the 10-days differentiation period. Pictures show representative images of cell cultures from CSF-fc3 patient. Scale bars = 100 μm. CSF, cerebrospinal fluid; GFAP, glial fibrillary acidic protein; βIII-tubulin, class III β-tubulin; Oligo-O1, oligodendrocyte marker O1.

proliferated in vitro and had the ability to differentiate into neuronal and glial cells, specifically neurons, astrocytes, or oligodendrocytes. Their differentiation into various neural lineage cell subtypes along with morphological changes and expression for specific markers may further the development of new therapeutics based on NPCs for spinal cord repair, such as autologous cell transplantation in spina bifida patients. Cell therapy-based approaches could be particularly interesting because of the current limitations of prenatal surgical treatment of myelomeningocele in restoring neural damage or preventing further damage to the spinal cord pre- or post-natal.

4.1. Human CSF-derived NPCs expansion and neurogenic properties

The presence of neural stem and progenitor cells have been found in adult spinal cord tissuein animal models (Shihabuddin et al., 1997; Weiss et al., 1996) and humans (Dromard et al., 2008). Spinal-cord-derived cells have the ability to proliferate in vitro and differentiate into neurons and glial cells (Dromard et al., 2008). Demonstrating a high neurogenic potential in vitro, these multipotent cells could represent an excellent resource in the development of new therapeutic approaches for repair of spinal cord injury. The presence of neural stem and progenitor cells in adult human spinal cords suggests the possibility for their potential migration or liberation into the circulating CSF across the central canal. Thus, these cells could be isolated and expanded in culture for use in therapeutic approaches to spinal cord tissue repair.

In myelomeningocele disease, development of new prenatal therapeutic approaches for repair of spinal cord injury using autologous cell transplantation during gestation becomes possible based on the ability to isolate a cell population with neurogenic properties from fetal CSF. In this scenario, the progenitor cells needed to regenerate damaged spinal cord tissue could be obtained from each patient and developed as a personalized cell-therapy-based treatment.

Despite considerable research directed to localize neurogenic cells in the adult spinal cord (Dromard et al., 2008; Shihabuddin et al., 1997; Weiss et al., 1996), few publications have addressed the presence of pluripotent cells in human fetal CSF. A 2006 study revealed the presence of NPCs in CSF samples of preterm patients with post-hemorrhagic hydrocephalus (Krueger et al., 2006), suggesting the potential of human preterm CSF as a source of NPCs.

After confirming the presence of NPCs in CSF samples collected from the lumbosacral cysts of myelomeningoceles in fetuses, we showed that these cells had the ability to proliferate in vitro and to differentiate into neuronal and glial cells. These findings then suggest the possibility of in vivo differentiation. Cell culture of neural precursor cells from human fluids requires the use of fresh and well-preserved samples, which pose a technical challenge in obtaining fetal samples during gestation. We overcame this obstacle by extracting the CSF during prenatal surgery, specifically from the lumbar cyst surrounding the myelomeningocele defect (Fig. 1). Eventually, collection of fetal CSF in this clinical scenario could be by ultrasound-guided tap of the myelomeningocele cyst during the amniocentesis procedure, weeks before the prenatal myelomeningocele intervention. This technique could ensure sufficient time for cell culture, expansion of the NPCs before pre- or post-natal surgical repair, and finally autologous NPCs transplantation for spinal cord repair.

Previous studies have shown that embryonic CSF contains both neurogenic growth factors (Buddensiek et al., 2010; Zappaterra and Lehtinen, 2012) and some circulating cells with neural progenitor properties (Krueger et al., 2006). Despite low cellularity (de Graaf et al., 2011), we successfully established primary cultures with small extractions of 1.5–2 ml of CSF fluid from the cyst of the fetal myelomeningoceles (Fig. 2). Our flow citometry results of fresh CSF samples showed clearly its low cellularity, and the need for primary culture expansion to achieve relevant numbers of NPCs.

Once expanded in culture, the expression of specific NPC markers (i.e., TBR2, SOX2, CD15) was corroborated to show the potential

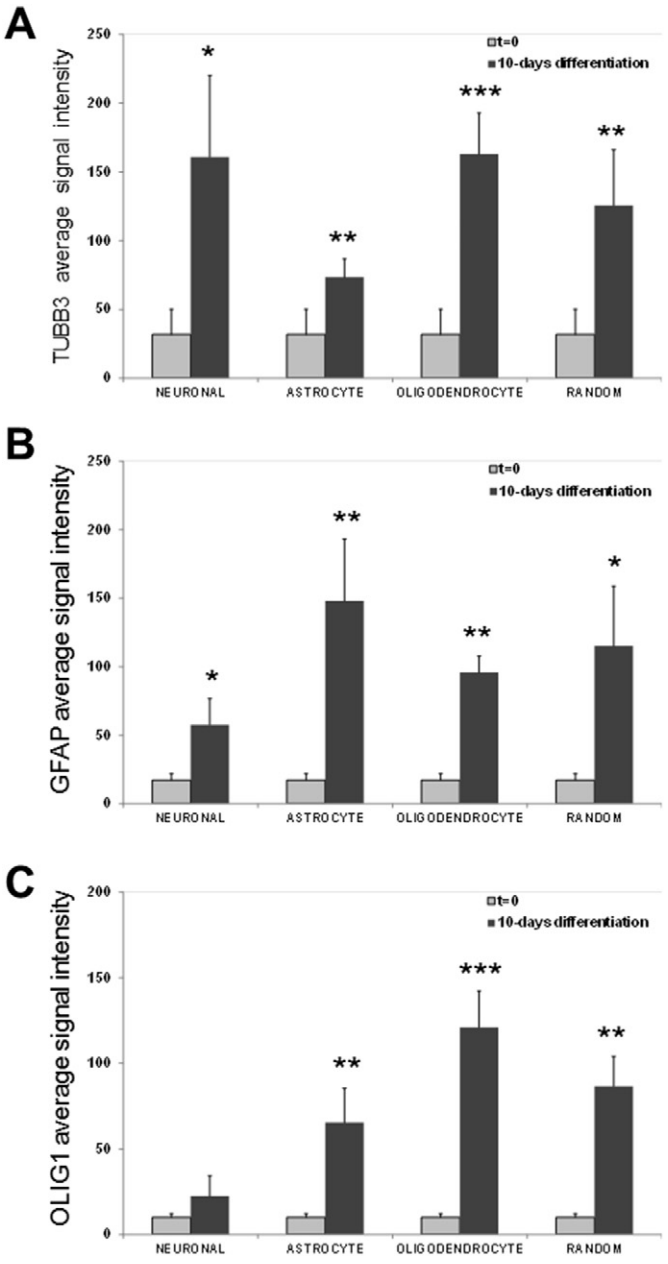

Fig. 6. Neuronal, astrocyte and oligodendrocyte differentiation of CSF-derived NPCs alters markers expression. Relative expression of βIII-tubulin (A), GFAP (B) or oligo-O1 (C) as neuronal, astrocyte and oligodendrocyte differentiation markers, respectively, was analyzed by qPCR in CSF-derived cells before (light gray bars) or after (dark gray bars) 10-days of random or specific differentiation into neuronal, astrocyte or oligodendrocyte lineage cells. Relative expression is shown as the average signal intensity graphs from the images obtained by immunocytofluorescence analysis. Error bars = SD, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

neurogenic properties of CSF-derived primary cultured cells (Fig. 3). TBR2, SOX2, and CD15 are well known NPC markers that have been expressed in neural stem/progenitor cells. Specifically, TBR2, a marker of intermediate neuronal progenitors, plays a key role driving the progression from neural stem cells to intermediate progenitors and neurons (Hodge et al., 2012; Hodge et al., 2008). SOX2, a neural stem cell pluripotency marker (Pevny and Nicolis, 2010; Tanaka et al., 2004; Wells et al., 2011), is directly involved in the maintenance of adult and embryonic neural progenitor and stem cells (Wegner and Stolt, 2005). CD15 (SSEA-1), a marker of neural stem cells (Capela and Temple, 2002; Doetsch, 2003), is also detected in the immature neural precursor cells and heterogeneously expressed by a large fraction of

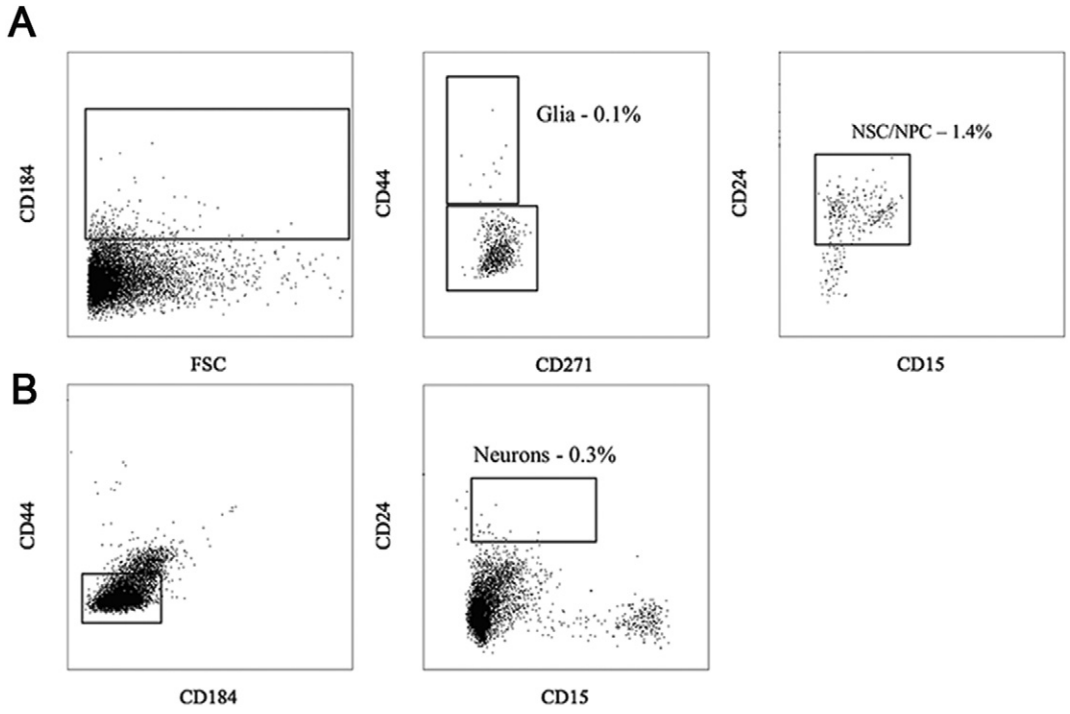

Fig. 7. Flow Cytometry analysis of cell populations in CSF-derived primary cultured cells. Human CSF-derived cells from cystic MMC fetal patients were expanded in primary culture and used for cell population analysis by flow cytometry. (A) Cells were first selected base on Viability Staining and CD45. NSC/NPC were selected for CD184+ and then negative selected for CD44− and CD271 − and co-expression of CD24+ and CD15+. Glial cells were selected for CD184+ and then for CD44+ and CD271 −. (B) Neuronal population was first selected for depletion of CD44− and CD184− and for CD24+ and CD15low.

dorsal ependymal cells and some subependymal cells (Dromard et al., 2008).

Our findings of CD15, SOX2, and TBR2 expression correlated with previous studies that described >90% of SOX2(+) and 35–55% of CD15(+) cells in lumbar samples of adult human spinal cords (Dromard et al., 2008). Their presence in CSF-circulating cells suggest their possible origin by migration from the cell layer along the central canal in human spinal cord (Brill et al., 2009; Dromard et al., 2008). However, more research is needed to define their possible origins (e.g., ependymal, subependymal, and subventricular zones or choroid plexus).

4.2. Human CSF-derived NPCs multi-lineage capacity

In confirming human CSF-derived NPCs multipotency, our analysis included the neurogenic properties of primary cultured cells from the fetal myelomeningocele cysts. Inducing in vitro differentiation into neural and glial cell subtypes demonstrated their strong potential to differentiate into neurons, astrocytes and oligodendrocytes either by random differentiation in the absence of growth factors (Fig. 4) or by specific differentiation (Fig. 5).

Myelomeningoceles CSF-derived cells not only generated a large number of differentiated cells with characteristic neural cell morphologies (spindle shaped with growing neurite protuberances for neurons and oligodendrocytes, and round-flattened for astrocytes) but strongly expressed specific differentiation markers for neuronal (βIII-tubulin), astrocyte (GFAP), and oligodendrocyte (CNPase, oligo-O1) cell lineages (Fig. 6). Our findings showing the neurogenic properties of fetal CSF-derived cells from the cystic myelomeningocele defect confirms previous in vitro findings for human neural stem/precursor cells from various origins (Barnabé-Heider and Frisén, 2008; Li and Lepski, 2013; Mothe and Tator, 2013; Parati et al., 2004; Sandner et al., 2012).

5. Conclusion

To the best of our knowledge, our findings provide the first evidence of NPCs present in the CSF of the lumbar cyst of myelomeningocele-affected fetuses. The effective expansion of CSF-derived cells in primary culture and their differentiation into neuronal and glial cells suggests their potential for future therapeutic approaches in spinal cord regeneration. Thus, our findings could help in the development of innovative progenitor/stem cell-based therapies for the regeneration of damaged neural tissue by using NPCs obtained from the patient's own CSF sample and later autologous cell transplantation in the injured spinal cord. Further studies in myelomeningocele animal models will analyze in vivo ability to colonize, integrate, and restore the damaged neural tissue, as well as to promote regeneration at the spinal cord level. Future studies will ultimately develop new fetal or postnatal therapeutic approaches for myelomeningocele patients.

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