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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.