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Characterization of an established endothelial cell line from primary cultures of fetal sheep hypothalamus

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ABSTRACT

Immortalized cell lines from fetal brain are an experimental model for studying the *in vitro* molecular pathways regulating neural cell differentiation and the development of neural networks. The procedures are described to obtain an established cell line from the 90-day old fetal sheep hypothalamus. Viral oncogene LT-SV40 transformation was used to isolate a stable cell line (ENOS-01) that was characterized immunocytochemically. Immortalized cells can be classified as an endothelial cell line of hypothalamic microvasculature. Furthermore, mRNA expression and immunocytochemical of estrogen receptors α and β were also evaluated. Since it is known that cerebral vessels are directly targeted by sex steroids, our established cell line represents an alternative system to study estradiol/receptor interactions during brain development. Our *in vitro* model can provide a tool to investigate the complex relationships among the cell types forming the blood–brain barrier, which is known to be involved in the pathogenesis of sheep transmissible neurological diseases.

1. Introduction

The use of *in vitro* models greatly improved the knowledge of many cellular processes otherwise difficult to explore by *in vivo* research. So far, most studies have been conducted in rodents (Rigter et al., 2010), whereas few data are reported about brain-derived cell cultures from sheep and other domestic ruminants (Peruffo et al., 2004, 2008). Protocols necessary to obtain primary cultures of sheep glial cells (Anderson et al., 1987; Elder et al., 1988; Duittoz and Hevor, 2001) or neurons (Richard et al., 1998) are available in the literature, whereas, to the best of our knowledge, there is only one published report dealing with sheep established cell lines, relative to astrocytes (Vilette et al., 2000). In this paper, we present the development of *in vitro* methods suitable to study the properties of the endothelial cells and their possible involvement in many neurological disorders of ruminants (Weissmann and Aguzzi, 2005). Existing *in vitro* models of the blood–brain barrier (BBB) often consist of brain microvascular endothelial cells that are co-cultured with other cells of the neurovascular unit, such as astrocytes and neurons, to enhance BBB properties (Lippman et al., 2011). In the present investigation, we propose a protocol suitable to obtain an established endothelial cell line derived from the ovine hypothalamus.

2. Materials and methods

2.1. Tissue collection

Fetuses were available at local abattoirs, when sheep were slaughtered casually pregnant. Among them, one male 90-day fetus was collected and quickly transported to lab hood on ice. Under sterile conditions, the cranial cavity was opened, the brain visualized and the hypothalamus isolated. The whole process of tissue sampling was carried out in 2 h from slaughtering. Fetal age was established measuring crown-rump length using a standard reference table (McGeady et al., 2006). The sample was minced into small fragments, and stored in liquid nitrogen in sterile conditions.

2.2. Primary cultures from hypothalamus

A papain dissociation system kit (Worthington Biochemical Co., Lakewood, NJ, USA) was used to dissociate cells following Manufacturer's instructions. Cells were then suspended in basal medium consisting of a 1:1 mixture of DMEM and Ham's F-12, supplemented with penicillin (30 mg/l), streptomycin (50 mg/l), sodium bicarbonate (2.4 g/l), insulin (10 μg/ml), transferrin (10 μg/ml), sodium selenite (10^{-8} M) and 10% fetal calf serum. Cells were placed on glass coverslips, previously coated with poly-L-lysine at a

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density of 5×10^5/ml medium and incubated at 37 °C (Peruffo et al., 2004).

2.3. Transfection of primary cultures

Primary cultures at 70% confluence were transfected following the Manufacturer's instructions via lipofectamine LTX and PLUS Reagents (Invitrogen, Carlsbad, CA, USA) with 2 μg/μl plasmid construct pSV3neo (LCG Promochem, Teddington, UK) containing the SV40 sequence coding large T-antigen and G418 resistance genes. After 48 h, G418 (neomycin, 400 mg/ml, Gibco) was added and subsequently maintained for clonal selection. Microscopic G418-resistant colonies were picked using cloning rings after 2–5 weeks and expanded in new Petri dishes.

2.4. Immunocytochemistry

The hypothalamic primary cultures and the established cell line at 70% confluence were fixed with 4% paraformaldehyde at room temperature for 20 min. After fixation, cells were washed with PBS, then permeabilized with 0.1% Triton X-100 for 10 min at 4 °C, treated with 5% PBS/BSA for 30 min to block endogenous non-specific sites and incubated overnight with primary antibodies against: GFAP (Sigma Aldrich, St. Louis, MO, USA) raised in rabbit, diluted 1:500 to identify astrocytes; β III-tubulin (Sigma Aldrich), raised in mouse, diluted 1:200 to identify neurons; vimentin (Sigma Aldrich) raised in mouse, diluted 1:200, Vascular Endothelial Growth Factor (Sigma Aldrich) raised in rabbit, diluted 1:200 and Von Willebrand factor (Sigma Aldrich) raised in rabbit, diluted

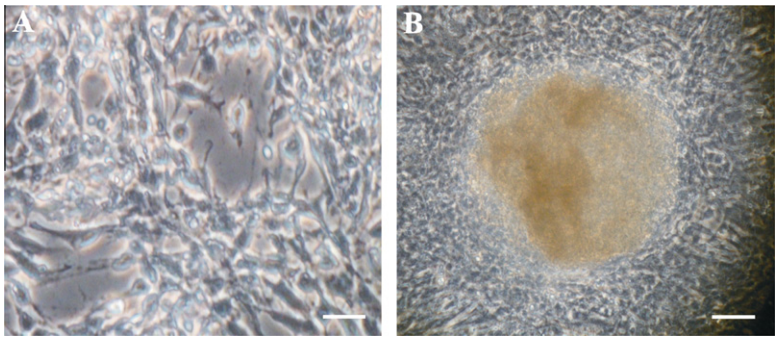

Fig. 1. Primary cultures before and after transfection from sheep fetal hypothalamus: (A) Primary culture from 90-day old fetal sheep hypothalamus. Scale bar = 80 μm. (B) Established cell line obtained 5 days after transfection. Scale bar = 300 μm.

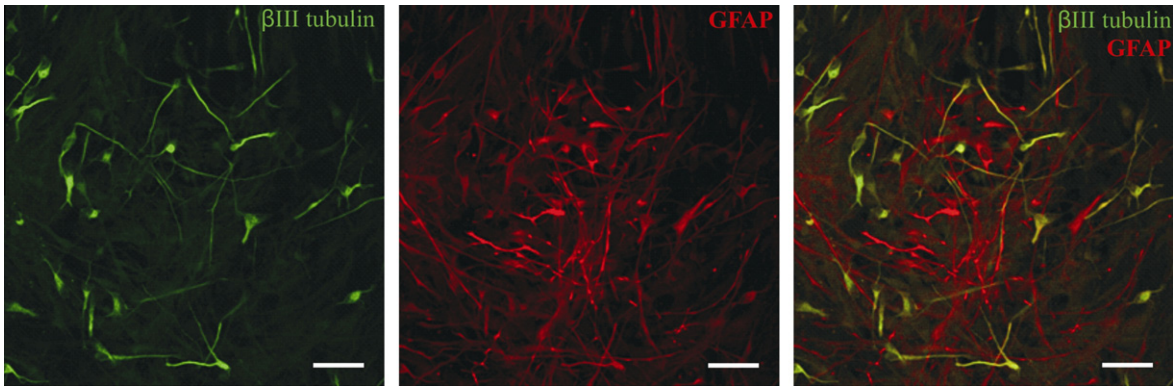

Fig. 2. Cells characterization before transfection. Immunocytochemical detection of β-III tubulin and GFAP in primary cell cultures by confocal microscopy. β-III tubulin-immunoreactive neurons (left panel), GFAP-immunoreactive cells (central panel), superimposed confocal images (right panel). Scale bar = 100 μm.

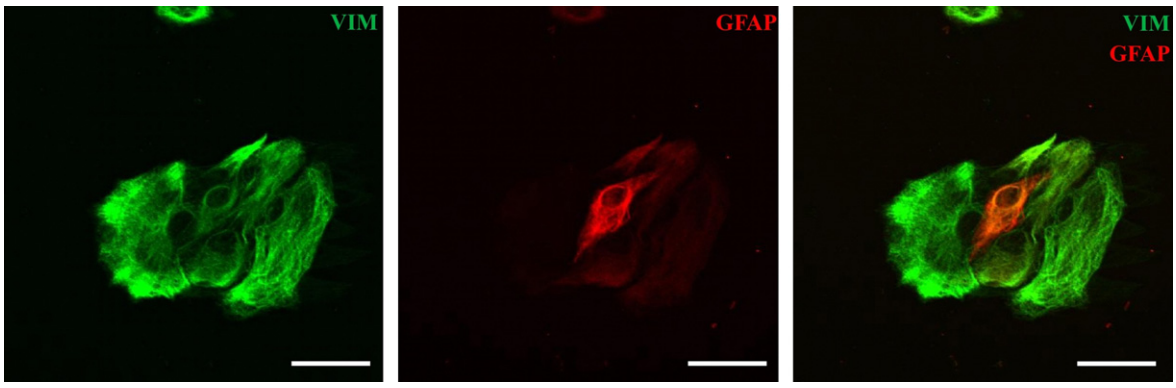

Fig. 3. Cells characterization before transfection. Immunocytochemical detection of vimentin and GFAP in primary cell cultures by confocal microscopy. Vimentin-immunoreactive cells (left panel), GFAP-immunoreactive cells (central panel), superimposed confocal images (right panel). Scale bar = 100 μm.