Cytotherapy, 2013; 15: 201–208

International Society for Cellular Therapy
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Transplantation of porcine embryonic stem cells and their derived neuronal progenitors in a spinal cord injury rat model

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Abstract

Background aims. The purpose of this study was to investigate therapeutic potential of green fluorescent protein expressing porcine embryonic stem (pES/GFP$^+$) cells in A rat model of spinal cord injury (SCI). *Methods.* Undifferentiated pES/GFP$^+$ cells and their neuronal differentiation derivatives were transplanted into the contused spinal cord of the Long Evans rat, and *in situ* development of the cells was determined by using a live animal fluorescence optical imaging system every 15 days. After pES/GFP$^+$ cell transplantation, the behavior functional recovery of the SCI rats was assessed with the Basso, Beattie, and Bresnahan Locomotor Rating Scale (BBB scale), and the growth and differentiation of the grafted pES/GFP$^+$ cells in the SCI rats were analyzed by immunohistochemical staining. *Results.* The relative green fluorescent protein expression level was decreased for 3 months after transplantation. The pES/GFP$^+$-derived cells positively stained with neural specific antibodies of anti-NFL, anti-MBP, anti-SYP and anti-Tuj 1 were detected at the transplanted position. The SCI rats grafted with the D18 neuronal progenitors showed a significant functional recovery of hindlimbs and exhibited the highest BBB scale score of 15.20 ± 1.43 at week 24. The SCI rats treated with pES/GFP$^+$-derived neural progenitors demonstrated a better functional recovery. *Conclusions.* Transplantation of porcine embryonic stem (pES)-derived D18 neuronal progenitors has treatment potential for SCI, and functional behavior improvement of grafted pES-derived cells in SCI model rats suggests the potential for further application of pES cells in the study of replacement medicine and functionally degenerative pathologies.

Key Words: *embryonic stem cells, porcine, spinal cord injury, xenotransplantation*

Introduction

Spinal cord injury (SCI) in which fragments of broken vertebrae and ligaments compress the cord is one of the leading causes of disability. Axonal regeneration, delayed death of neurons, oligodendrocytes and glial cells and axonal demyelination of intact fiber traced around the injured site then cause remarkable morbidity and mortality and restrict therapeutic options (1–3). Embryonic stem cell transplantation has been considered a cell therapy approach for promoting neural repair and functional recovery from SCI. Optimal SCI approaches minimize the progression of secondary injury, manipulate the neuroinhibitory spinal cord environment, replace lost tissue with transplanted cells, remyelinate denuded axons and maximize the intrinsic regenerative potential of endogenous progenitor cells (4). Embryonic stem cell-based transplantation has been studied several times in SCI animal models. Results indicated that the grafting of embryonic stem cell-derived oligodendrocytes might have remyelinated fibers along the neuronal axons of long projection neurons and did not cause harm (5–7), that embryonic stem cell-derived neurons could survive and integrate after injection into the injured rat spinal cord, and that long-term therapy could be achieved by embryonic stem cell transplantation (8,9). Also, embryonic stem cell-derived

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(Received 3 January 2012; accepted 17 September 2012)

http://dx.doi.org/10.1016/j.jcyt.2012.09.001

gliogenic neural stem/progenitor cell transplantation improved functional recovery after SCI (10).

In an earlier study, we demonstrated that grafted porcine embryonic stem (pES) cells improved decreased asymmetric rotation in a Parkinson disease rat model after transplantation (11). In this study, we tried to investigate the therapeutic potential of pES cells and their derived cells in the SCI rat model. We also determined the growth and differentiation of the grafted pES cells *in vivo* and the functional recovery of the SCI animals.

Methods

In vitro culture of green fluorescent protein-expressing pES cells

The green fluorescent protein-expressing pES (pES/GFP$^+$) cells used in this study were obtained from green fluorescent protein (GFP) electroporation of pES M215-3, which was derived from the pre-implantation blastocysts of the Livestock Research Institute Black Pig No. 1 and nominated as pES/GFP$^+$ 10 (Figure 1) (11,12). The pES/GFP$^+$ cells were maintained in embryonic stem cell culture medium and propagated on a feeder layer of mitomycin C (Sigma-Aldrich, St. Louis, MO, USA) inactivated STO cells (mouse embryonic fibroblasts, ATCC, CRL-1503, Manassas, VA, USA) in gelatin-coated Multidish 4 Wells (Nunc No. 176740; Nunc Roskilde, Denmark) as previously described (12). These pES/GFP$^+$ cell lines have been maintained in culture for >90 passages in a period of 20 months, and >90% maintained a normal 36+XX karyotype by regular G-banding analysis (12).

In vitro induction of neural differentiation

In vitro neural differentiation of pES/GFP$^+$ cells was induced by suspension culture in medium containing different combinations of neurogenic stimulators and re-plated onto gelatin-coated four-well dishes in expanding medium. The neurogenic stimulators used for neural induction in the present study were retinoic acid (RA, 1 μM; Sigma-Aldrich), sonic hedgehog (Shh, 200 ng/mL; R&D Systems, Minneapolis, MN, USA) and fibroblast growth factor (FGF, 100 ng/mL; Sigma-Aldrich) (11). The pES/GFP$^+$ colonies were subjected to a two-step *in vitro* induction protocol. They were first taken into a single-cell suspension culture at a concentration of 1×10^5 cells/mL in an Easy Flask (Nunc No. 169900; Nunc) with serum-free embryonic stem cell culture medium containing RA + Shh + FGF. After 12 days, the pES cell-derived cells were re-plated onto gelatin-coated four-well dishes in 5×10^3 cells/mL in the medium containing DMEM/F12 (Sigma-Aldrich), 1:50 B27 supplementation (Invitrogen, Grand Island, NY, USA), 2 mM L-glutamine, 50 units/mL penicillin G and 50 μg/mL streptomycin sulfate (Invitrogen) and supplemented with 20 ng/mL human recombinant epidermal growth factor (hrEGF; Invitrogen), 20 ng/mL human recombinant basic fibroblast growth factor (hrbFGF; Invitrogen), and 1:100 N2 (Invitrogen).

In vivo tracking of grafted pES/GFP$^+$ cells in non-SCI rats

In situ monitoring of these grafted pES/GFP$^+$ cells in non-SCI rats was accomplished by using a live animal fluorescence optical imaging system, the In Vivo Imaging System (IVIS 50; Xenogen Corporation, Alameda, CA, USA) for non-invasive tracking every 15 days for the 3-month experimental period. The relative fluorescence values of treatment groups were calculated as the fluorescence intensity detected from the region of interest on rats in each treatment group divided by that of the control group at the same time window.

SCI model, pES/GFP$^+$ cell transplantation and functional recovery assessment

All animal experiments in this study were carried out in accordance with ethical guidelines and following

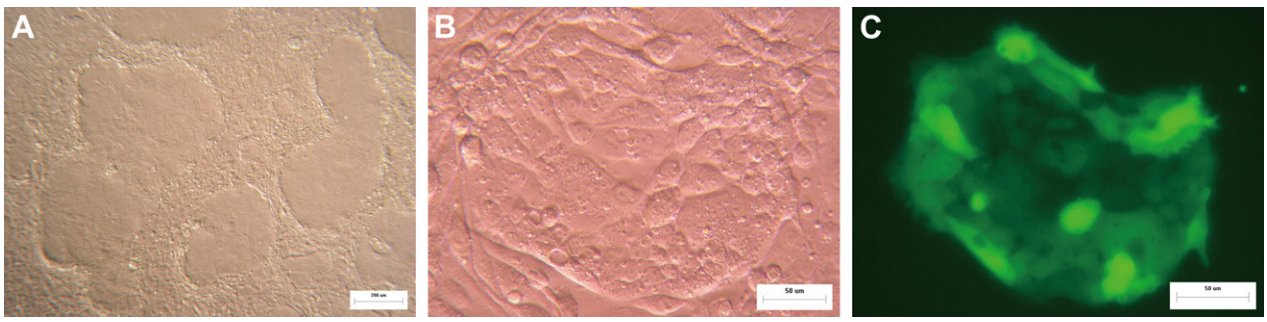

Figure 1. The colony morphology of undifferentiated pES/GFP$^+$ cells maintained on mitomycin C inactivated STO feeder layers. (A–C) Light (A, magnification ×50, scale bar = 200 μm; B, magnification ×200, scale bar = 50 μm) and fluorescent (C, magnification ×200, scale bar = 50 μm) micrographs of undifferentiated pES/GFP$^+$ cells.