Short Communication

Evaluation of Epidermal Neural Crest Stem Cells in Organotypic Spinal Cord Slice Culture Platform

(spinal cord injury / epidermal neural crest stem cells / organotypic slice culture)

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Abstract. Among various strategies employed for spinal cord injury, stem cell therapy is a potential treatment. So far, a variety of stem cells have been evaluated in animal models and humans with spinal cord injury, and epidermal neural crest stem cells represent one of the attractive types in this area. Although these multipotent stem cells have been assessed in several spinal cord injury models by independent laboratories, extensive work remains to be done to ascertain whether these cells can safely improve the outcome following human spinal cord injury. Among the models that closely mimic human spinal cord injury, the *in vitro* model of injury in organotypic spinal cord slice culture has been identified as one of the faithful platforms for injury-related investigations. In this study, green fluorescent protein-expressing stem cells were grafted into injured organotypic spinal cord slice culture and their survival was examined by confocal microscope seven days after transplantation. Data obtained from this preliminary study showed that these stem cells can survive on top of the surface of injured slices, as observed on day seven following their transplantation. This result revealed that this *in vitro* model of injury can be considered as a suitable context for further evaluation of epidermal neural crest stem cells before their application in large animals.

Introduction

Spinal cord injury (SCI) is a devastating condition that leads to persistent neurological dysfunction. Currently, various therapeutic options are employed to cure SCI, including use of pharmacological treatments, surgical intervention and rehabilitative care. Also, diverse promising cell-based strategies, neuroregenerative materials and neuroprotective agents have been tested for SCI (Silva et al., 2014).

Among all aforementioned SCI therapies, cell transplantation strategies have been proved to be an attractive option. Rationales for using stem cells for SCI mainly include replacement of injured neurons and glia, secretion of nutrient factors, attenuation of gliosis and scar formation, and enhancement of axon elongation (Sahni and Kessler, 2010).

Based on the fact that any therapeutic strategy in the treatment of SCI has to be examined in animal models that closely mimic the human SCI condition, currently there are various models that all have aimed to recreate features of human SCI (Dunham and Floyd, 2011; Zhang et al., 2014). The *in vitro* model of injury in spinal cord organotypic slice culture is one of the models that has been identified as a robust approach for SCI-related investigations (Pandamooz et al., 2016).

The long-term slice cultures, by providing an ideal platform between dissociated cell cultures and animal models, have revolutionized numerous experimental studies. In these *ex vivo* cultures, the main neural connectivity and cytoarchitectural features are preserved, and they encompass major hurdles that animal models are facing in experiments (Stoppini et al., 1991; Cho et al., 2007; Ravikumar et al., 2012). Recently, this model has been acknowledged as a suitable context for evalua-

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Abbreviations: DIV – day *in vitro*, EPI-NCSC – epidermal neural crest stem cell, GM – grey matter, GFP – green fluorescent protein, HBSS – Hanks' balanced salt solution, MEM – minimum essential medium, PI – propidium iodide, SCI – spinal cord injury, WM – white matter.

tion of stem cell therapies following injury (Kim et al., 2010; Sypecka et al., 2015).

Although a variety of SCI models, from chemically to mechanically induced, have been simulated in organotypic slice culture so far, only the drop weight contusion model can mimic both primary mechanical damage and secondary reactive phase of injury (Krassioukov et al., 2002). Due to its remarkable features, this model is a suitable context to define the underlying biology of SCI, and it can also serve as a screening platform to study the impact of different SCI treatments such as cell therapies (Park et al., 2010; Weightman et al., 2014).

Among various types of stem cells that have been assessed in the treatment of SCI, epidermal neural crest stem cells (EPI-NCSCs) hold great promise (Amoh et al., 2008; Hu et al., 2010; Liu et al., 2011). This population of ectodermal stem cells, due to its capacity to generate different neural lineages, has attracted great attention in the last few years. Although the role of EPI-NCSCs has been evaluated in various *in vivo* models of SCI, little is yet known about their accurate fate and function following the injury. Beside all its advantages over *in vivo* models, the organotypic spinal cord slice culture allows testing survival of the transplanted stem cells, tracing them in the grafted slice, addressing their differentiation and assessing their role in SCI recovery.

Until the present, various stem cells such as neural stem cells (Kim et al., 2010), human mesenchymal stem cells (Park et al., 2010) and olfactory ensheathing cells (Riggio et al., 2013) have been investigated in different spinal cord organotypic slice cultures. Since all employed slices were obtained from neonatal animals, they can hardly simulate features of the adult spinal cord context.

A prominent step in evaluation of EPI-NCSC impacts following spinal cord injury is to provide a faithful context that fully mimics human SCI. In addition, another debatable issue in all cell-based therapies is survival of the grafted cells. Therefore, after transplantation of EPI-NCSCs, the main factor that should be determined prior to assessment of their role in SCI recovery is their survival in the inhospitable environment of the injury. Thereby, this study was designed to define the presence of EPI-NCSCs in spinal cord slice culture excised from adult rat following the injury.

Material and Methods

All procedures on the rats were approved and performed according to the recommendations of the Animal Care Committee of Kharazmi University.

Spinal cord organotypic slice culture

Organotypic spinal cord slice culture was prepared from an adult Wistar rat (weighing 200–230 grams) according to the regular interface method (Stoppini et al., 1991). The rat was deeply anesthetized with CO_2, the back skin was removed and the vertebral column was dissected. Once exposed, the spinal cord was flushed out with ice-cold PBS by using a 60-ml syringe and placed into a petri dish. Then 400 micrometer spinal cord slices were cut by a vibratome and the slices were transferred to inserts with 0.4 micrometer pore size (Millipore, Darmstadt, Germany) placed in a six-well plate with 1 ml of culture medium consisting of 50 % minimum essential medium (MEM) with L-glutamine (Sigma, St. Louis, MO), 25 % heat-inactivated horse serum, 25 % Hanks' balanced salt solution (HBSS, Sigma), 6.4 mg/ml D-glucose (Sigma) and 1 % penicillin/streptomycin (Invitrogen, Waltham, MA) and incubated in a 5 % CO_2 humidified incubator at 37 °C.

PI staining

Identification and quantification of dead cells in the cultured spinal cord slices was performed using propidium iodide (PI, Invitrogen) as a fluorescent indicator for the loss of plasma membrane integrity. At various time points of culture, the slices were introduced to 1 ml PI solution at 5µg/ml concentration, prepared in serum-free medium. The slices were then placed back into the incubator for 30 min to allow permeation of PI. Four different regions of grey matter in each slice were evaluated under a fluorescent inverted microscope. The average number of PI^+ cells counted in four quadrants of each slice was considered as a single mean (Fig. 1A).

Protocol for spinal cord contusion

The injury was induced on day 7 (DIV: 7) using a weight drop device. Briefly, an impactor with a 3-mm diameter head and 0.5 gram total weight was dropped from a height of 3 cm on the entire slice surface, and the slices were then returned to the incubator (Fig. 2A).

EPI-NCSC isolation and transduction

The EPI-NCSCs were isolated from the bulge of whisker hair follicles dissected from adult rats and characterized as described earlier (Fig. 3) (Sieber-Blum et al., 2004; Pandamooz et al., 2013). Here, the migrated stem cells derived from primary culture of explanted hair bulge were trypsinized and collected in less than a week. Afterward, the population of stem cells was transduced with lentiviral particles (pGreenPuro, SBI, Boston, MA) produced in LentiX-293T (Clontech, Mountain View, CA) to express green fluorescent protein (GFP) to be traced following transplantation (Fig. 2B). Also, in order to have a pure population of transduced stem cells, cultured cells were treated with 2 µg/ml puromycin (Sigma) to kill non-transduced cells.

Stem cell transplantation

One hour after injury, GFP-expressing, multipotent EPI-NCSCs were trypsinized, and almost 3×10^4 cells in 3 µl medium were transplanted on top of the surface of slices (Fig. 2C).

Confocal microscopy

Seven days after cell therapy, injured slices were fixed with 4% paraformaldehyde and assessed with a

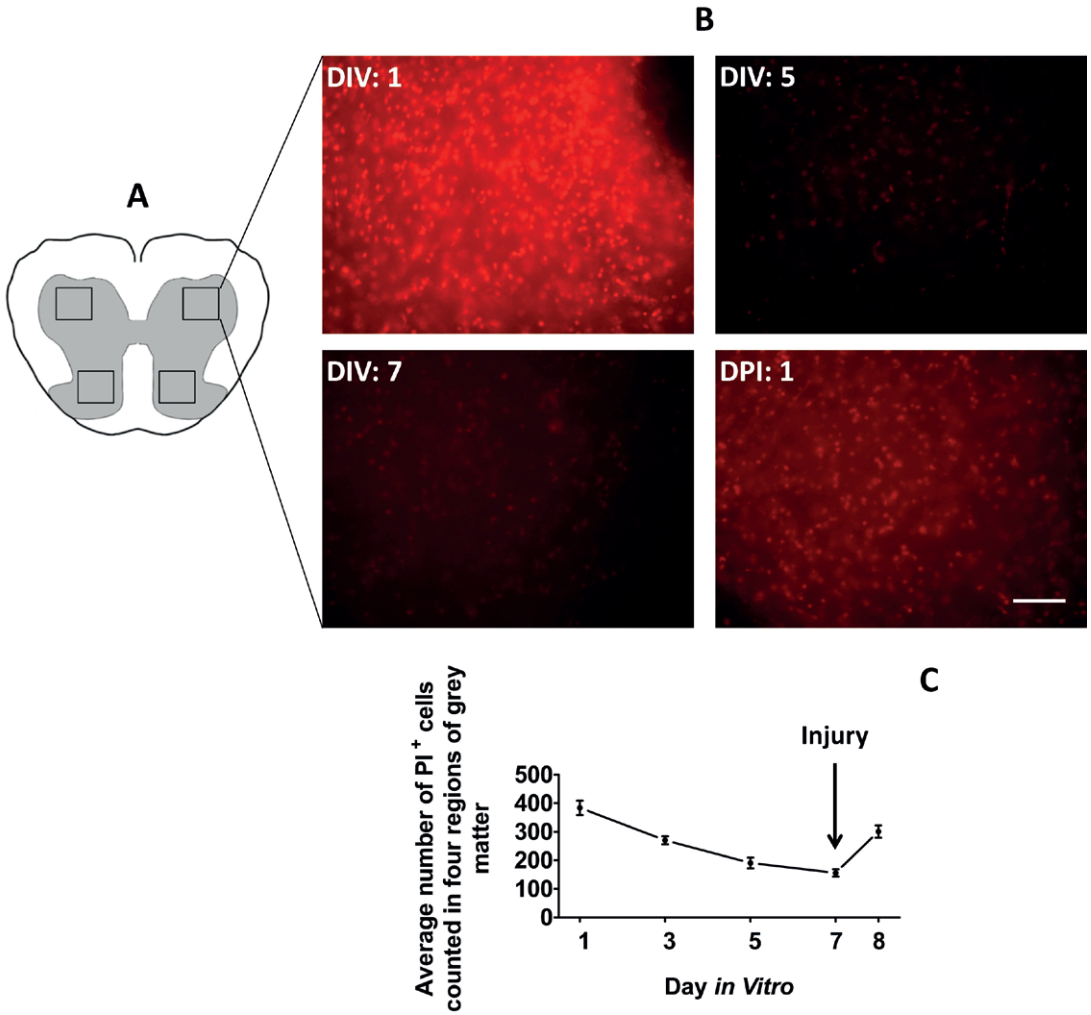

Fig. 1. Evaluation of cell death in cultured slices of spinal cord. The number of PI^+ cells was counted in four regions of grey matter (**A**). Here, the representative micrographs of DIV: 1,5,7 and DPI:1 taken from the same region of grey matter revealed a high rate of cell death on the first day *in vitro* and first day post injury (**B**). Statistical analysis of PI^+ cells at various time points has shown that although the rate of cell death decreases by seven days *in vitro*, injury induces a high rate of cell death on day one post injury (**C**).
DIV – day *in vitro*, DPI – day post injury.

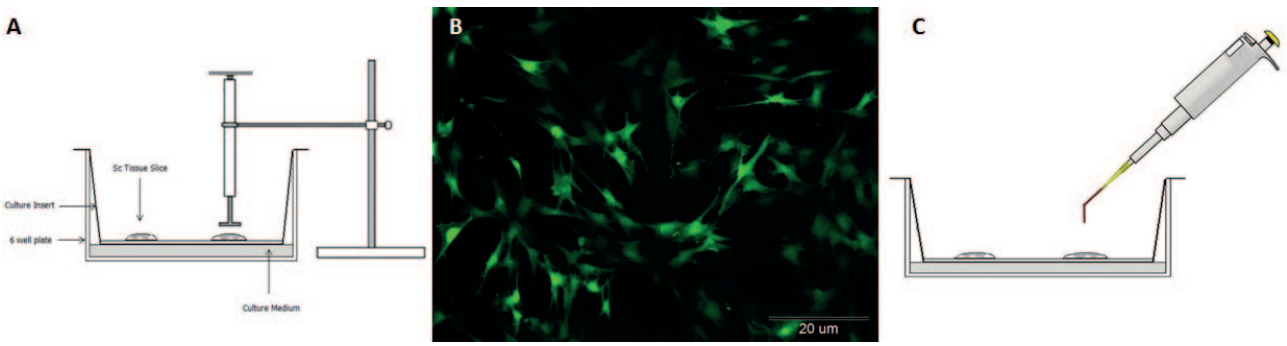

Fig. 2. Modelling injury in organotypic spinal cord slice culture and stem cell transplantation: contusion was induced in the organotypic slice culture via dropping a weight on top of the surface of slices (**A**). Transduced epidermal neural crest stem cells (**B**) were grafted on top of the surface of injured slices using a fire-polished capillary tube connected to a pipette tip (**C**).

confocal microscope (Nikon, Tokyo, Japan) to check the presence of GFP-expressing stem cells. It is worth noting that the GFP-expressing EPI-NCSCs were detected on top of the surface of spinal cord slices, as the confocal microscope was set to observe the upper 20 micrometer layer of slices (excitation peak at 395 nm and emission peak at 509 nm).

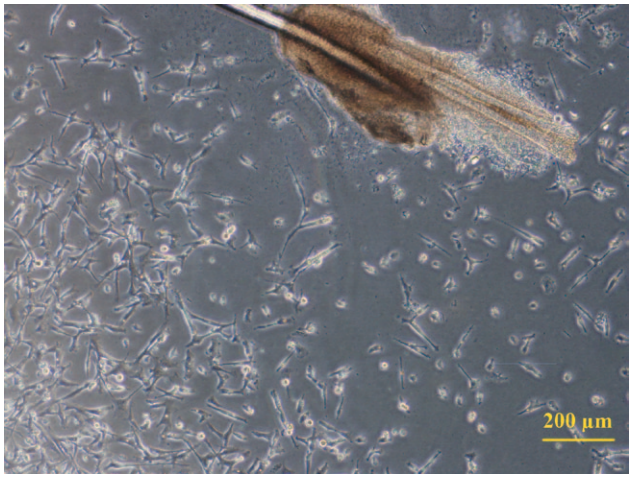

Fig. 3. Morphology of epidermal neural crest stem cells. Within two to three days following hair bulge explantation, EPI-NCSCs migrate and their stellate morphology appears.

Results and Discussion

Our findings revealed survival of GFP-expressing EPI-NCSCs on the *ex vivo* model of spinal cord injury. This result shed light on the claim that slice culture is an appropriate context for assessments of EPI-NCSCs.

Induction of injury in the organotypic slice culture via the drop weight method produced the contusion model of injury in slices. This model has been adopted from the introduced model of Krassioukov et al. (2002). Here, PI staining of slices at various time points of culture defined that following five days *in vitro*, the rate of cell death decreases as slices recover from the harsh procedure of slicing and adopt to culture conditions. Therefore, the day seven of *in vitro* was selected for induction of contusion in the slices. Also, this staining elucidated the increased rate of cell death in the grey matter of slices on day one post injury (Fig. 1B, C).

In addition, within two to three days after hair bulge explantation, cells with stellate morphology emigrated from the bulges with an increasing number over time (Fig. 3). Here, transduction of migrated stem cells with lentiviral expression vector carrying GFP and subsequent treatment with puromycin resulted in stable expression of GFP in a pure population of transduced EPI-NCSCs.

In our study, a confocal microscope was used to illuminate the presence of GFP-positive EPI-NCSCs in the upper layer of slices. Since EPI-NCSCs share so many identical markers with spinal cord cells, use of antibodies and immunohistochemistry was not an appropriate choice to evaluate the presence of grafted stem cells. Evaluation of spinal cord slices with a confocal microscope seven days subsequent to cell transplantation revealed survival of grafted cells. To ascertain that the observed green cells under the confocal microscope were the putative transplanted stem cells, slices were evaluated at different wavelengths, and only at a proper wavelength the desired stem cells were detected. Also, based on the data obtained from the confocal microscope, not only the number of GFP-positive cells in the grey matter exceeded that in the white matter of spinal cord slices, but also the grafted cells displayed stellate morphology in the grey matter while being more spherical in the white matter. This result can be due to the presence of cell bodies in the grey matter and the attachment that can be formed between transplanted stem cells and resident spinal cord cells (Fig. 4).

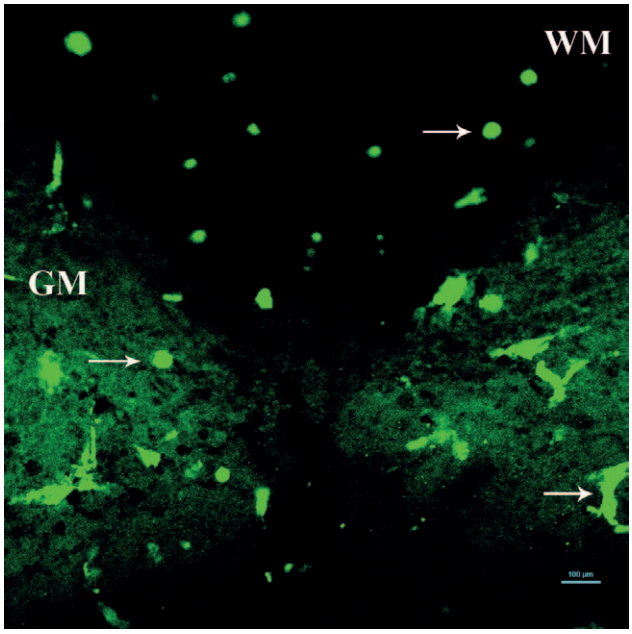

Fig. 4. Survival of GFP-expressing stem cells on top of the surface of injured organotypic spinal cord slice culture seven days after transplantation (arrow) in grey and white matter
GM – grey matter, WM – white matter.

Beside survival of stem cells, their differentiation can be easily addressed in this platform, as the slice culture provides the possibility of direct real-time observation of grafted stem cells. Moreover, testing spatial and temporal expression of various inflammatory, anti-apoptotic markers and trophic factors can be performed at the gene and protein levels in this model (Daviaud et al., 2013; Pakan and McDermott, 2014).

Taken together, this finding indicated that the organotypic spinal cord slice culture is a proper platform to evaluate EPI-NCSCs alone or in combination with therapeutic enhancers following induction of injury. Since these stem cells can survive on top of the surface of slices after seven days, this context can be employed as a faithful and useful model for more accurate evaluation of these stem cells before their transplantation in large animals.

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Disclosure of conflicts of interest

The authors declare that they have no conflict of interests.

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