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Transplanted choroidal plexus epithelial cells can integrate with organotypic spinal cord slices into a new system

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Abstract: This study aimed to evaluate the integration of transplanted choroidal plexus epithelial cells with organotypic spinal cord slices. Organotypic spinal cord slices, normally cultured for 6 days, were divided into control group (Ctrl) and transplanted group (T). The choroidal plexus epithelial cells were dissociated and primary cultured (C group). The choroidal plexus epithelial cells cultured for 6–7 days were labeled by 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanineperchlorate (CM-Dil), and were identified by transthyretin (TTR) in immunocytochemistry. They were adjusted to the density of 0.5–1 × 10^7/ml, then 2 μl cells suspension were transplanted to the spinal cord slices in the T group. The same amount of basal medium was dripped on the spinal cord slices in the Ctrl group. After 14 days of transplantation, the differentiations into neurons and astrocytes, and the synapses were identified by immunofluorescence histochemistry. At the same time, the ratios of cell differentiations and synapses in new system, and the changes of MAPK signaling pathway were tested by western blotting. The choroid plexus epithelial cells were well labeled by CM-Dil and were immune-stained by TTR in immunocytochemistry. The choroid plexus epithelial cells bodies were small when transplanted on the spinal cord slices, but big when transplanted on the polyester membrane inserts. The transplanted cells could differentiate into astrocytes, and possibly differentiate into neurons, and there were a large number of synaptophysin positive vesicles between transplanted cells and organotypic spinal cord slices in immunofluorescence histochemistry. The levels of GFAP, TUB-III and synaptophysin in the T group were higher than which in the Ctrl and C groups in western blotting ($P < 0.05$). And the ratios of p-JNK/JNK and p-P38/P38 in the T group were significantly lower than which in the Ctrl and C groups ($P < 0.05$). But the ratio of p-ERK/ERK in the three groups was of no significant difference. The transplanted choroidal plexus epithelial cells can integrate with organotypic spinal cord slices into a new system.

Introduction

Choroidal plexus epithelial cells have stem cells characteristics with markers of nestin (Huang *et al.*, 2011; Huang *et al.*, 2013a; Hashemi *et al.*, 2017). They had the ability to differentiate into neurons (Itokazu *et al.*, 2006; Bolos *et al.*, 2013) and astrocytes (Kitada *et al.*, 2001). Thus they can be used for repairing damaged central nervous system (Matsumoto *et al.*, 2010; Ide *et al.*, 2016; Aliaghaei *et al.*, 2016; Kanekiyo *et al.*, 2016; Xu *et al.*, 2021). In addition, they can produce numerous growth factors and neurotrophic factors such as TGF-β, GDF-15, GDNF, BDNF, NGF, VEGF, etc. (Ikeda *et al.*, 1999; Emerich *et al.*, 2007; Huang *et al.*, 2014; Ide *et al.*, 2016; Zhao *et al.*, 2018). As seed cells, they can change the host's local microenvironment by secreting neurotrophic factors (Kitada *et al.*, 2001; Borlongan *et al.*, 2007; Ide *et al.*, 2016; Eslami *et al.*, 2021), which are helpful for axon regeneration and repairment. Therefore, choroidal plexus epithelial cells are a good choice for cell transplantation, and there is a potential value in the treatment of spinal cord injury and neurodegenerative diseases.

Microenvironment regulates seed cells survivals, differentiation, and synaptogenesis (Hofer *et al.*, 2012; Shamloo *et al.*, 2015). At the same time, the seed cells change the host's microenvironment through their own activities. Thus, the seed cells and host tissue interact and integrate with each other to generate a new system (Jäderstad *et al.*, 2010; Hofer *et al.*, 2012). In the new

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system, there is not only behavioral and morphological change of seed cells, but also behavioral and microenvironmental changes of host's tissue. Currently, studies about the change of new system are comparatively fewer.

The organotypic spinal cord slices preserve the basic tissue cytoarchitecture at utmost, and closely mimic complex tissue microenvironment (Kim *et al.*, 2010; Cifra *et al.*, 2012; Sypecka *et al.*, 2015; Dionne *et al.*, 2021). Importantly, it is advantageous for being host tissue, and it is an ideal platform to study the transplant new system. There is a growing body of literatures reporting the transplantation of choroidal plexus epithelial cells in several animal models or cell lines (Kitada *et al.*, 2001; Matsumoto *et al.*, 2010; Aliaghaei *et al.*, 2016; Ide *et al.*, 2016). However, there is no literature reporting the transplantation in organotypic spinal cord slices. The interaction and integration of choroidal plexus epithelial cells with organotypic spinal cord slices have not paid attention. The survivals, differentiations, synaptogenesis and intracellular signaling pathway changes in new system are not clear.

Thus, we conducted the current study to understand the above issues better. Our goal was to identify the changes of new system.

Materials and Methods

Animals

The Xi'an Jiaotong University Animal Experimentation Committee approved protocols for animal care and experimental management. Ethical approval for the study was obtained from the Medical Ethics Committee of the Second Affiliated Hospital of Xi'an Jiaotong University. Postnatal male Sprague-Dawley 5–7-day-old rats (N = 12) weighed 22–26 g and neonatal male Sprague-Dawley 1-day-old rats (N = 6) weighed 5–6 g were applied by the Center of Experimental Animals, Xi'an Jiaotong University. The 5–7-day-old rats were used for organotypic spinal cord slices, which were divided into control group (Ctrl) and transplanted group (T). In the T group, organotypic spinal cord slices were normally cultured for 6 days firstly, then were transplanted by choroidal plexus epithelial cells which were labeled by 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanineperchlorate (CM-Dil). In the Ctrl group, organotypic spinal cord slices were normally cultured for 6 days, then the same amount of basal medium was dripped on the organotypic spinal slices. The organotypic spinal cord slices in the two groups both were cultured for 20 days in total, including 6 days before transplantation and 14 days after transplantation. The 1-day-old rats were used for dissociation and primary culture of choroidal plexus epithelial cells (C group).

Materials and reagents

Vibratome, razor blades, ophthalmic scissors, corneal scissors, microforceps and operating knife blades were used in this experiment. 0.4-μm-pore polyester membrane inserts (Transwell 3450) were provided by Corning Costar (New York, USA). Dulbecco's modified Eagle's medium (DMEM/F12, DMEM/low glucose), fetal bovine serum (FBS) and CM-Dil were purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA). Rabbit monoclonal anti-GFAP, anti-beta III Tubulin (TUB-III), anti-Synaptophysin (SYN) and anti-transthyretin (TTR) were purchased from Abcam (Cambridgeshire, UK). Rabbit polyclonal or monoclonal anti-caspase 3, anti-caspase 3 active, anti-ERK, anti-p-ERK, anti-P38, anti-p-P38, anti-JNK and anti-p-JNK were purchased from Cell Signaling Technology (Danvers, MA, USA). 4',6-diamino-2-phenylindole (DAPI) was purchased from Roche (Basel, Switzerland). Anti-rabbit HRP, RIPA buffer, protease inhibitor cocktail, BCA assay and ECL were purchased from KPL (Milford, USA). Fluorescein-conjugated goat anti-rabbit IgG, biotin-conjugated goat anti-rabbit IgG and peroxidase streptavidin were provided by CWBIO (Beijing, China). Recombinant rat epidermal growth factor (EGF) was obtained from PeproTech (Rocky Hill, USA).

Preparation of organotypic spinal cord slices

The organotypic spinal cord slices were prepared according to the following method (Liu *et al.*, 2017). Lumbar spinal cord was extracted because there were more neurons, and the meninges were removed. The spinal cord was embedded by two agarose blocks. Then they were transverse sectioned into 350-μm slices. The slices were placed on the surface of the membrane inserts and cultured for 20 days at 37°C in a 5% CO_2 humidified incubator. The growth medium (DMEM/F12 supplemented with 10% FBS) was changed the day after plating, and then was changed every 3 days.

Choroidal plexus epithelial cells primary culture

The neonatal 1-day-old rats were used. The procedure is as follows (Huang *et al.*, 2013b). Briefly, 6 rat brains were removed and kept in chilled DMEM/low glucose medium. The choroid plexus tissues were extracted from both lateral ventricles, transferred into a beaker containing chilled DMEM/low glucose medium. The tissue pieces were mechanically triturated by repeated passages through a 1 mL pipette. After centrifugation, the growth medium (DMEM/low glucose supplemented with 10% FBS and 10 ng/mL EGF) was added. Then, cells suspended homogeneously in the growth medium were seeded in Petri dishes. They were then cultured in a 5% humidified CO_2 incubator at 37°C. The growth medium was changed 48–72 h later, and then changed every 3 days.

Transplantation of choroid plexus epithelial cells

Choroidal plexus epithelial cells cultured for 6–7 days were applied. The medium in Petri dishes was discarded, and CM-Dil (1 μg/mL) was added. The choroidal plexus epithelial cells and CM-Dil were incubated together at 37°C for 30 min, then at 4°C for 15 min. The choroidal plexus epithelial cells were washed by phosphate-buffered saline (PBS) for two times, then were mechanically dissociated into cells suspension. Next, they were centrifuged at 2000 × *g* for 5 min. The supernatant was discarded, and PBS was added to modulate cells density to 0.5–1 × 10^7/mL. A 2-μL cell suspension was dripped onto organotypic spinal cord slice cultured for 6 days in the T group. In the Ctrl group, the

same amount of basal medium was dripped. The CM-Dil labeled choroidal plexus epithelial cells were observed by fluorescence microscopy (Olympus, Japan).

Immunocytochemistry

The choroidal plexus epithelial cells cultured for 6–7 days were fixed by 4% paraformaldehyde for 30 min, incubated with 0.5% Triton X for 15 min, blocked with 0.3% H_2O_2 for 15 min, and 10% normal goat serum for 40 min. They were subsequently incubated with rabbit monoclonal TTR (1:1000) at 4°C overnight. After 3 washes in PBS, they were incubated in the biotin-labeled secondary antibody followed by a further treatment of avidin-biotin-peroxidase complex. The nuclei were counterstained with Mayer's hematoxylin. Microscopy was performed (Olympus, Japan). Negative controls were performed, with primary antibodies omitted.

Immunofluorescence histochemistry

The organotypic spinal cord slices cultured for 20 days in total in the T group were fixed with 4% paraformaldehyde for 30 min, incubated with 0.5% Triton X for 30 min and 10% normal goat serum for 40 min. Then they were incubated with rabbit monoclonal anti-GFAP (1:300), anti-beta III tubulin (1:500), and anti-synaptophysin (1:100), respectively, at 4°C overnight. Subsequently, they were incubated with fluorescein-conjugated goat anti-rabbit IgG for 2 h. Cell nuclei were counterstained using DAPI. The slices were then rinsed and cover-slipped with fluorescent mounting media. Laser confocal microscopy (Leica, Wetzlar, Germany) was performed. Negative controls were performed, with primary antibodies omitted.

Western blotting

There were 6 samples in each group. Samples in Ctrl, T and C groups were collected and centrifuged at 2000 × *g*. Then they were homogenized in ice-cold RIPA lysis buffer supplemented with protease inhibitors cocktail. Homogenates were centrifuged at 12,000 × *g*, and the supernatant were collected and measured using BCA protein assay. Protein samples were normalized and loaded for SDS-PAGE, then transferred to a PVDF membrane. Membranes were then incubated for 2 h in blocking buffer (5% skim milk, tris-buffered saline, 0.1% Tween 20). Subsequently, they were incubated in primary antibodies over night at 4°C: rabbit monoclonal or polyclonal anti-GFAP (1:5000), anti-beta III tubulin (1:5000), anti-synaptophysin (1:2000), anti-ERK (1:3000), anti-p-ERK (1:2000), anti-p-JNK (1:1000), anti-P38 (1:2000), anti-caspase 3 (1:3000), anti-caspase 3 active (1:1000), anti-JNK (1:1000) and anti-p-P38 (1:1000), followed by incubation with HRP coupled anti-rabbit IgG for 1 h. Then they were incubated in ECL solution. The images were taken using EPSON V300 camera system (Epson, Japan), and the immunoreactive bands were measured by Alphaview software (Alpha Innotech, San Leandro, CA).

Statistics

SPSS 26.0 software package was used. All data were reported as means ± SD. Group data were compared using One-way ANOVA with the Dunnett's post-hoc test. Statistical significance was assessed at $P < 0.05$.

Results

The morphology of choroidal plexus epithelial cells

The choroidal plexus epithelial cells were well labeled by CM-Dil. The cytomembrane was emitted strong red fluorescence when observed by fluorescence microscopy (Fig. 1A). And they were well labeled by transthyretin (TTR) in immunohistochemistry. In which, the cytoplasm was manifested as brown (Fig. 1B). Because TTR is specifically expressed in choroidal plexus epithelial cells (Nilsson *et al.*, 1992), in our experiment, the cells were identified definitely as choroidal plexus epithelial cells. The morphology of

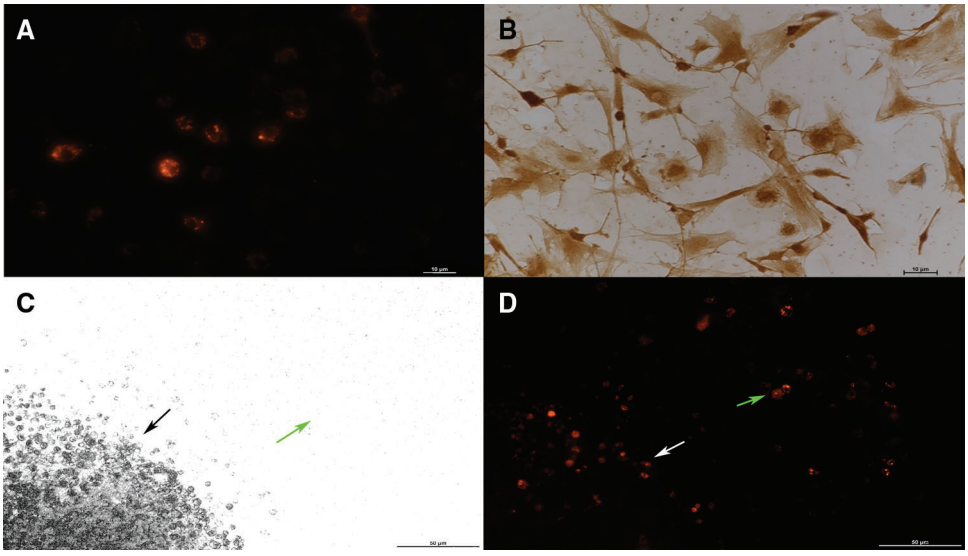

FIGURE 1. The morphology of choroidal plexus epithelial cells (A and B × 200, C and D × 100). (A) The cytomembrane of CM-Dil labeled choroidal plexus epithelial cells was emitted strong red fluorescence (Scale bar: 10 μm). (B) The primary-cultured choroidal plexus epithelial cells were immune-stained with an antibody against TTR in immunocytochemistry (Scale bar: 10 μm). (C) Organotypic spinal cord slices (black arrow) and polyester membrane inserts (green arrow). (D) Choroidal plexus epithelial cells transplanted into the organotypic spinal cord slices had small cell bodies (white arrow), and choroidal plexus epithelial cells transplanted to the polyester membrane inserts had big cell bodies (green arrow, Scale bar: 50 μm).

choroidal plexus epithelial cells transplanted at different positions was different. The cell bodies were small when transplanted on the organotypic spinal cord slices (Fig. 1D, white arrow), but were big when transplanted on the polyester membrane inserts (Fig. 1D, green arrow).

The differentiations of transplanted choroidal plexus epithelial cells

The differentiations of choroidal plexus epithelial cells which were transplanted into organotypic spinal cord slices for 14 days, were identified by immunofluorescence staining. The transplanted choroidal plexus epithelial cells could possibly differentiate into neurons (Fig. 2C, white arrow), and differentiate into astrocytes (Fig. 2F, white arrow).

The synapses in the transplant new system

The organotypic spinal cord slices and transplanted choroidal plexus epithelial cells formed a transplant new system. The synapses in the new system were tested by immunofluorescence staining. The synaptophysin positive vesicles were located between the neurons of organotypic spinal cord slice and transplanted choroidal plexus epithelial cells (Fig. 3D, white arrow).

The cells differentiations and synaptogenesis in the new system

The ratios of different types of cells in the new system were tested by Western blotting. The levels of GFAP and TUB-III in the T group were higher than which in the Ctrl and C groups (Figs. 4A–4C). The new system promoted the cell differentiations into neurons and astrocytes. The synapses in the new system were also tested by Western blotting. The synaptophysin level in the T group was significantly higher as compared with in the Ctrl and C group (Figs. 4A and 4D). There were new synaptogenesis in the new system.

The changes of MAPK signaling pathway in the new system

The ratios of p-JNK/JNK and p-P38/P38 in the T group were significantly lower than those in the Ctrl and C groups. But the ratios of p-ERK/ERK in the three groups were of no significant difference (Figs. 5A–5D).

Discussion

The choroidal plexus epithelial cells can be used as seed cells, and organotypic spinal cord slices can be used as host tissue. In the present experiment, we investigated the integration between choroidal plexus epithelial cells and organotypic spinal cord slices. We found that the transplanted choroidal plexus epithelial cells integrated excellently with organotypic spinal cord slices into a new system. The new system promotes transplanted cells differentiating into neurons and astrocytes, and it promotes the synaptogenesis. In the new system, there were changes of MAPK signaling pathway. The phosphorylation level of MAPK may be related with the cell survival, apoptosis, and differentiation.

Organotypic spinal cord slices leave the structural integrity essentially intact. This allows detailed studies of cellular responses and cell–cell interactions. The culture

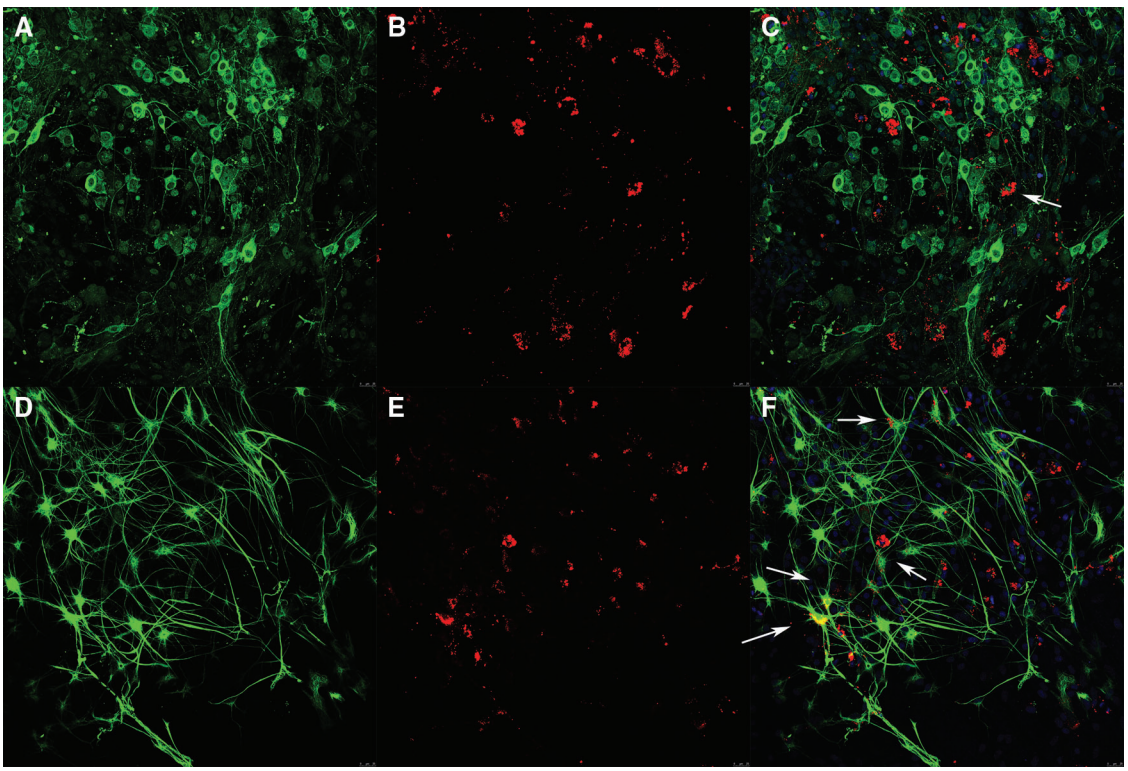

FIGURE 2. The differentiations of transplanted choroidal plexus epithelial cells (×200). (A) Green: neurons of the organotypic spinal cord slice. (B) Red: choroidal plexus epithelial cells transplanted onto the organotypic spinal cord slice. (C) Merged. Only one transplanted choroidal plexus epithelial cell possibly differentiated into neuron (white arrows). (D) Green: astrocytes of the organotypic spinal cord slice. (E) Red: choroidal plexus epithelial cells transplanted onto the organotypic spinal cord slice. (F) Merged. Part of transplanted choroidal plexus epithelial cells differentiated into astrocytes (white arrow, Scale bar: 25 μm).

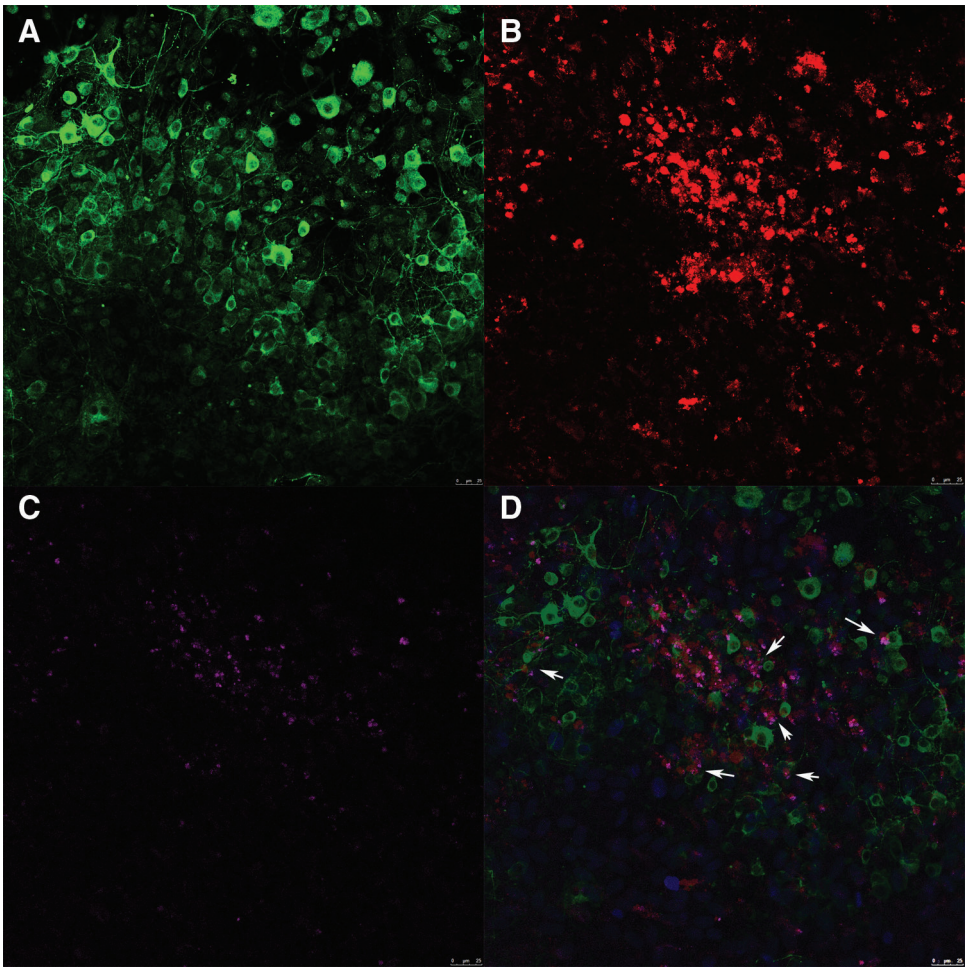

FIGURE 3. The synapses in the new system (×200). (A) Green: neurons of the organotypic spinal cord slice. (B) Red: choroidal plexus epithelial cells transplanted onto the organotypic spinal cord slice. (C) Pink: synaptophysin positive vesicles in the transplant new system. (D) The synaptophysin (white arrow) between neurons and choroidal plexus epithelial cells (Scale bar: 25 μm).

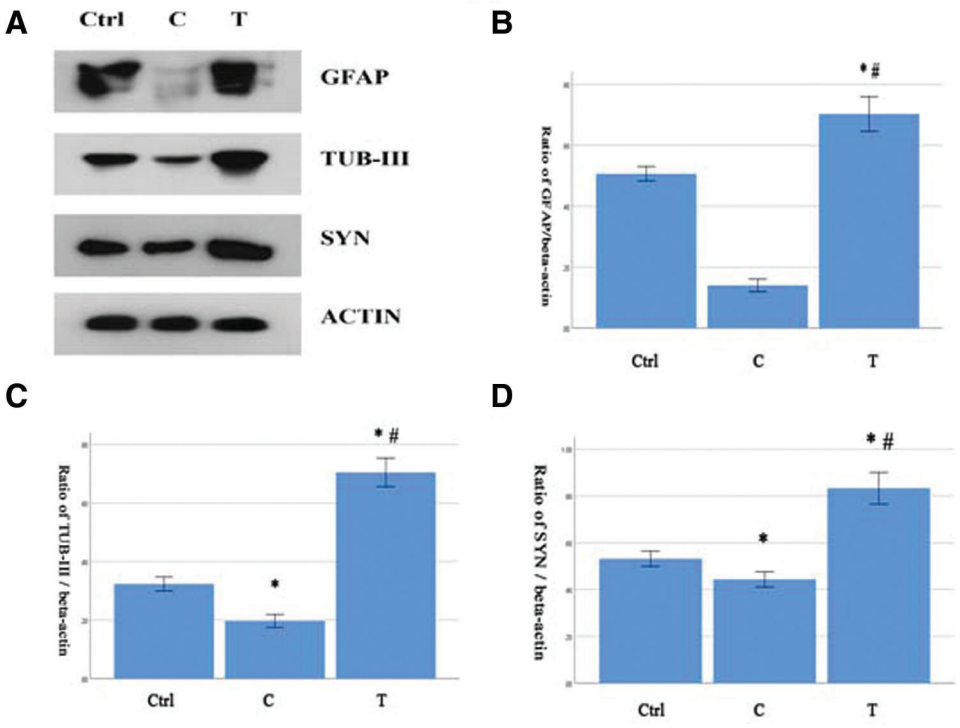

FIGURE 4. The cells differentiations and synaptogenesis in the new system (n = 6). (A–D) The levels of GFAP, TUB-III and synaptophysin were significantly higher in the T group. Compare with the Ctrl group, $^{*}P < 0.05$. Compare with the C group, $^{\#}P < 0.05$.

system has been successfully established in our previous work (Liu *et al.*, 2017). So organotypic spinal cord slices as an excellent platform was used in our experiment.

CM-Dil is always used for fluorescent labeling. The cytomembranes which are dyed manifest stable red fluorescence. In addition, the cellular viability is not

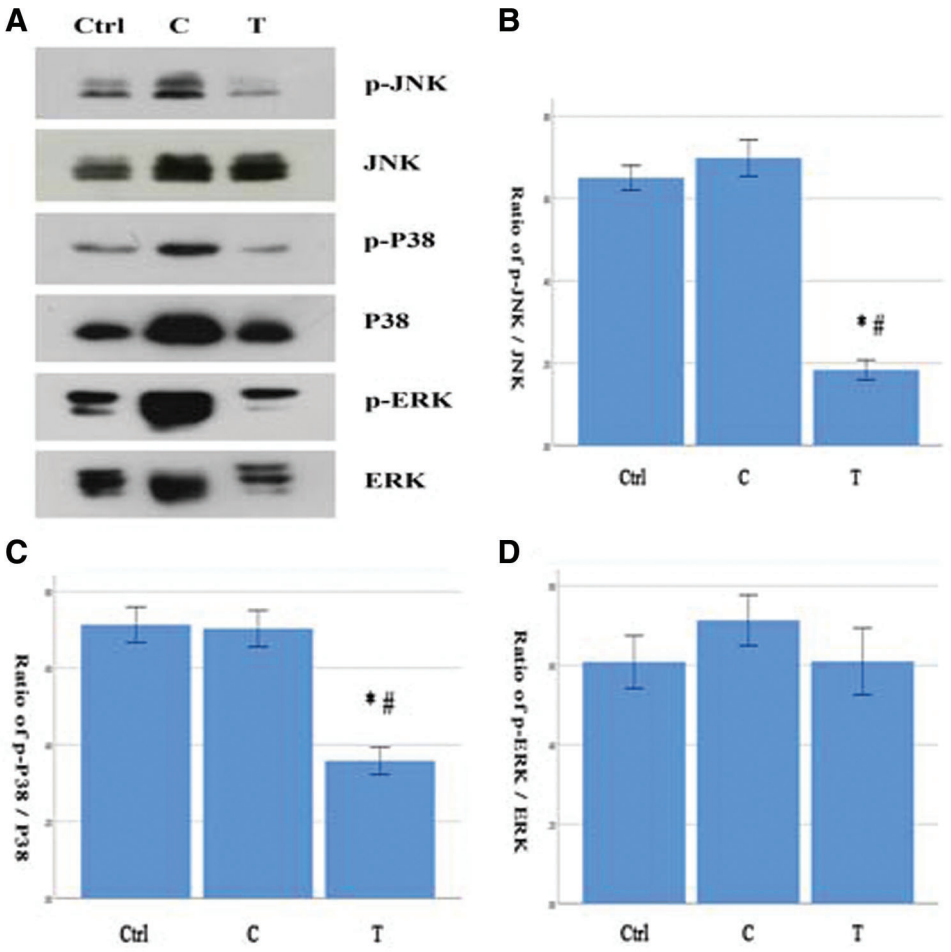

FIGURE 5. (A–D) The ratios of p-JNK/JNK and pP38/p38 were significantly lower in the T group and there was no significance in the ratio of p-ERK/ERK among the three groups (N = 6). Comparison with the Ctrl group, $^{*}P < 0.05$. Comparison with the C group, $^{\#}P < 0.05$.

affected. Neurons labeled with CM-Dil can survive for up to 4 weeks *in vitro* and for up to one year *in vivo* (Honig and Hume, 1989). Along with it, the choroidal plexus epithelial cells we cultured for 20 days were dyed red fluorescence strongly and continuously, and their viability was excellent.

The external cues, such as polypeptides secreted by the surrounding tissue or adhesion molecules, and neuronal activity could modulate axonal growth as well as dendritic shape and complexity (Neuser *et al.*, 2013). The local microenvironment is the key factor to affect the cellular morphology and biological behavior. It was reported that neural precursor cells transplanted on organotypic hippocampal slices had more mature morphology than those transplanted on membrane inserts (Morgan *et al.*, 2012). Consistently, we found that the choroidal plexus epithelial cells transplanted on organotypic spinal cord slices had small cell bodies than which transplanted on membrane inserts.

The formation of functional contacts between the seed cells and the host tissue is a primary issue for cell transplantation. Several studies *in vitro* and *in vivo* have shown that the neural stem cells and brain tissue can influence each other, and functionally integrate into a new system (Jäderstad *et al.*, 2010; Hofer *et al.*, 2012; Shamloo *et al.*, 2015). But the integration of transplanted choroidal plexus epithelial cells with organotypic spinal cord slices was unclear. In our experiment, the synaptophysin positive vesicles were located between neurons and transplanted choroidal plexus epithelial cells. Furthermore, the expression of synaptophysin increased sharply in western blotting. It indicated that there were new synaptogenesis in the new system, and the formation of morphological and functional junctions between transplanted cells and host's cells.

The choroidal plexus epithelial cells possess the peculiarities of stem cells. As grafted cells, they have the excellent ability of survival, proliferation, and differentiation, which is dependent largely on the microenvironment of new system (Huang *et al.*, 2013a). It was demonstrated that the choroidal plexus epithelial cells could differentiate into neurons (Itokazu *et al.*, 2006; Bolos *et al.*, 2013) and astrocytes (Kitada *et al.*, 2001). In our experiment, the choroidal plexus epithelial cells transplanted into organotypic spinal cord slices could survive for a long time. Immunochemical study of GFAP (a marker for astrocyte) and TUB-III (a marker for neuron) showed the process of choroidal plexus epithelial cells differentiating into astrocytes, and possibly differentiating into neurons. Meanwhile, western blotting showed the expressions of GFAP and TUB-III increasing significantly. The results above indicated the differentiations of choroidal plexus epithelial cells into astrocytes and neurons in the new system.

The generic mitogen-activated protein kinases (MAPK) intracellular signaling pathway is associated with the cell inflammation, survival, proliferation, differentiation, and

apoptosis (Sun *et al*., 2015). The MAPK is shared by four distinct cascades, including the extracellular signal-related kinases (ERK1/2), Jun amino-terminal kinases (JNK1/2/3), p38-MAPK and ERK5. ERK can be activated by mitotic factors such as EGF and b-FGF, which is associated with cell proliferation. JNK and P38 can be activated to regulate apoptosis and inflammation. The biological behavior of grafted cells and host tissue in the new system is also modulated by it. We found the MAPK pathway in the new system was different with which in the organotypic spinal cord slices without cells transplantation. In the new system, the phosphorylation level of JNK and P38 decreased, which indicated the decrease of apoptosis and inflammation. But the phosphorylation level of ERK was almost unchanged, which indicated the absence of cellular proliferation. Combined with western blotting results, the overall effect of the changes of MAPK pathway in the new system was the promotion of cells survival and differentiations into neurons and astrocytes.

Conclusion

Transplanted choroidal plexus epithelial cells can integrate with organotypic spinal cord slices into a new system. The cells can differentiate into neurons and astrocytes, and the new synaptic junction can be built in the new system. And there are changes of MAPK signaling pathway, which are related with cells survival, apoptosis, and differentiations. But our observation time for the new system, merely about two weeks, is relatively shorter. The long-term changes need to be fully and completely investigated furthermore. And how the transplanted choroidal plexus epithelial cells proliferate, adhere, spread, and differentiate in the new system also need to be deeply investigated furthermore.

Availability of Data and Materials: The data used to support the findings of this study are available from the corresponding author upon request.

Authors' Contribution: JJL designed the experiments, performed the experiments, and wrote the manuscript. XYD performed the experiments and analyzed the data. LX performed data analysis and interpretation. SLH conceived the project and designed the experiments. All authors read and approved the final manuscript.

Ethics Approval: All experimental procedures were approved and supervised by the Xi'an Jiaotong University Animal Experimentation Committee. Ethical approval for the study was obtained from the Medical Ethics Committee of the Second Affiliated Hospital of Xi'an Jiaotong University (No. 2014-2118, March 08, 2014), Xi'an, 710004, China. All efforts were made to minimize animal suffering.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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