Human Embryonic Stem Cell-derived Cerebral Organoids for Treatment of Mild Traumatic Brain Injury in a Mouse Model

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Abstract

Objective: There are no effective treatments for relieving neuronal dysfunction after mild traumatic brain injury (TBI). Here, we evaluated therapeutic efficacy of human embryonic stem cell-derived cerebral organoids (hCOs) in a mild TBI model, in terms of repair of damaged cortical regions, neurogenesis, and improved cognitive function.

Methods: Male C57BL/6J mice were randomly divided into sham-operated, mild TBI, and mild TBI with hCO groups. hCOs cultured at 8 weeks were used for transplantation. Mice were sacrificed at 7 and 14 days after transplantation followed by immunofluorescence staining, cytokine profile microarray, and novel object recognition test.

Results: 8W-hCOs transplantation significantly reduced neuronal cell death, recovered microvessel density, and promoted neurogenesis in the ipsilateral subventricular zone and dentate gyrus of hippocampus after mild TBI. In addition, increased angiogenesis into the engrafted hCOs was observed. Microarray results of hCOs revealed neuronal differentiation potential and higher expression of early brain development proteins associated with neurogenesis, angiogenesis and extracellular matrix remodeling. Ultimately, 8W-hCO transplantation resulted in reconstruction of damaged cortex and improvement in cognitive function after mild TBI.

Conclusion: hCO transplantation may be feasible for treating mild TBI-related neuronal dysfunction via reconstruction of damaged cortex and neurogenesis in the hippocampus.

Introduction

Neuronal loss after traumatic brain injury (TBI) is a consequence of acute inflammatory responses which persist from weeks to months. Apoptosis and necrosis of neuronal cells along with long-term neuro-inflammation increase the risk of neuronal dysfunction including cognitive impairment and motor dysfunction after TBI.[1] TBI is classified as belonging to one of three levels of severity, termed mild, moderate, and severe TBI based on the results of radiological findings, loss of consciousness, alteration of consciousness level, post-traumatic amnesia, and Glasgow coma scale (GCS) score.[2] Mild TBI accounts for about 75% of TBIs and is believed to be a benign clinical disorder without gross abnormal radiological findings.[3–5] Most mild TBI-related symptoms are resolved within three months of trauma; however, approximately half of mild TBI patients complain of cognitive impairment to their physicians. [6] In addition, poor cognitive outcomes were observed in 13.5% of patients after mild TBI which was significantly higher than seen with controls (4.5%).[7] These findings suggest that mild TBI is likely to have more cellular or tissue damage than is perceived by current radiologic examination.[5] A histological study revealed that hemosiderin-laden macrophages were observed in perivascular spaces and lymphocytes within white matter.[8] Mild TBI also resulted in synapse reduction and extensive dendrite degeneration, although it initially caused only minimal cell death in the cortex.[5]

Currently, there is no effective treatment for neuronal damage after mild TBI, with physicians generally prescribing non-steroidal anti-inflammatory medications and muscle relaxants in the acute period. As well, the neurostimulant methylphenidate has been empirically used for mild TBI-related cognitive impairment.[9] Accordingly, there is a need to develop new types of therapeutics that reduce local neurological damage and increase regeneration of damaged brain tissue to promote neurological recovery. Stem cell transplantation has increasingly been considered as a promising therapeutic option for alleviating neurological damage after TBI.[1,10] However, most stem cell transplantation attempts have exhibited low survival rates and inadequate neurological differentiation, resulting in low therapeutic efficacy for TBI.[11] The survival rates of cells, and overall anti-inflammatory, and angiogenic properties are improved following transplantation if administered in the form of three dimensional tissue rather than cell suspension alone.[12–14] In this respect, cerebral organoids (CO) may have an advantage for TBI treatment due to stable morphology and a more diverse set of neuronal cells in the damaged brain.[15] Here, we performed transplantation of human embryonic stem cell (hESC)-derived cerebral organoids (hCOs) into a mouse model of mild TBI to determine whether hCOs could reconstruct brain tissue and improve neuronal dysfunctions after trauma.

Results

Generation of hESC-derived COs

We established an *in vitro* hESC culture system by feeder independent methods using matrigel, and verified the quality by morphology and by unique pluripotency markers SSEA4, OCT4, SOX2, and TRA-1-60 at every passage (Fig. 1A and 1B). For the generation of COs from hESCs as described in Lancaster's protocol, we adopted serum-free floating cultures of embryoid body-like aggregates with quick reaggregation,[16,17] and our modified protocol provides a convenient system using a horizontal orbital shaker instead of spinning bioreactors (Fig. 1C). Since incomplete morphological differentiation could be prevented by adding a higher concentration of Y-27632 (50 μM from day 0 to day 4),[18] we supplemented the cultures with 50 μM of Y-27632 early in embryonic body aggregation. Embryoid bodies (EBs) were induced to develop into a neuroectoderm at day 7, and were then embedded in a 3D matrix supplemented with matrigel to create a neuroepithelial lineage at day 11. After four days of culture in matrigel droplets, the organoids were transferred to an Erlenmeyer flask and cultured long-term with continuous orbital mixing. Several neuroepithelial-like structures formed and continuous development of the organoid was observed in phase-contrast images (Fig. 1D). Histological analyses revealed COs showing unique characteristics of primate fetal cerebral cortex development involving ventricle-like structures and a cerebral cortex. In the early stages (~ 6 weeks), the organoids expressed Ki67 and SOX2 in the ventricular zone (VZ) and subventricular zone (SVZ). The neural stem cell marker nestin, was also expressed in the VZ and outer layers, while the newborn neural marker of doublecortin (DCX) was expressed in the outer layers (Fig. 1E).

hCO transplantation into mild TBI mice

To enhance survival of hCO in the presence of an inflammatory response, 8W-hCOs were transplanted into injured brains one week after injury (Fig. 2A and 2B).[19] The animals were then observed for 2 weeks to examine whether therapeutic effects manifested. As shown in Fig. 2C, loss of brain tissue was observed in the mTBI group while mTBIs treated with hCOs showed good engraftment and survival in the damaged cortex at both 7 and 14 days post-transplantation (DPT). As well, in the hCO-treated group, human nuclear antigens (hNAs) were located in the dorsal of the hippocampus *in vivo* and cortex tissue could be accurately reconstructed in the damaged area (Fig. 2D).

Neuroprotection and reconstruction of cortical lesions

We compared the neuroprotective effects of transplanted hCOs on mild TBI at 7 and 14 DPT, with FJB staining (Fig. 3A). TBI mice with hCO transplantation revealed neuroprotective effects as there were significantly less FJB + cells in the CA1 and dentate gyrus (DG) of hippocampus, and ipsilateral cortex. We further investigated whether hCO transplantation could protect blood vessels and promote angiogenesis in the lesion periphery. Microvessel density, shown as smooth muscle actin (SMA) + staining, was significantly increased at both 7 and 14 DPT in mild TBI with hCO transplantation compared to without (Fig. 3B). Vascular-like structures were also observed in engrafted hCOs at 14 DPT, and this was confirmed as blood vessel ingrowth by SMA immunostaining (Fig. 3C, 3D and 3E). To investigate the differentiation fate of hCOs *in vivo*, we performed immunostaining for neurons, astrocytes and oligodendrocytes using beta-III tubulin antibody (TUJ1), glial fibrillary acidic protein (GFAP), and oligodendrocyte transcription factor 2 (OLIG2) as markers. Most cells within the transplanted hCOs had differentiated into TUJ1 + immature neurons while astrocyte and oligodendrocyte markers were rarely expressed. The astrocytes and oligodendrocytes of host mice were observed to be closely connected to neurons of engrafted hCOs (Fig. 3F).

Enhanced neurogenesis in SVZ and DG

We evaluated hCO-induced neurogenesis in the SVZ and DG after transplantation, since it has been shown that stem cell transplantation generates new neurons in these two regions of the adult mammalian brain including the border of the lateral ventricles of the brain (SVZ) and the subgranular zone (SGZ) of the DG in the hippocampus.[20] Mild TBI with hCO mice, exhibited significantly increased GFAP + cells around the SVZ with movement to the cortex across the corpus callosum(CC) at 7 and 14 DPT compared to untransplantated (Fig. 4A and 4B). Prominent DCX + cells, (a newborn neuron marker) were observed in DG, indicating endogenous neurogenesis was significantly induced after hCO transplantation (Fig. 4C and 4D). In contrast, mild TBI mice without hCO transplantation did not show obvious neurogenesis in DG within 2 weeks post-transplantation. In the sham-operated group, proliferation and neurogenesis were not clear in either SVZ or DG (Supplementary Fig. S2).

Immune response after hCO transplantation

Since mesenchymal stem cells are generally known to have immunomodulatory capabilities,[21] we evaluated the effect of microglia on cortical lesions after hCO transplantation. Immunofluorescence staining showed that hCO transplantation had no significant effect on alleviating inflammation at 7 and 14 DPT compared to mild TBI without hCO. The reduced numbers of Iba1-positive cells at 14 DPT seems to be a common mechanism observed in inflammatory responses during regeneration in the central nervous system after brain damage (Supplementary Fig. S3).

Cytokine and chemokine profiles of hCOs at 8 weeks

We further examined cytokine and chemokine expression in hCOs at 8 weeks (Fig. 5). Proteins associated with neuronal proliferation (IGFBP2, HGF, IGF, and FGF2), neuroprotection (MIF, ApoA1, and TFF3), angiogenesis (GDF15, VEGF, PCAM1, angiogenin, OPN, endoglin, and DKK1) and extracelluar matrix remodeling (EMMPRIN, DPP4, SERPINE1, uPAR, and MMP9) were highly expressed. As well, there was no expression of proteins related to the immune response *in vitro* which is a unique characteristic of mesoderm lineages.

Restoration of impaired cognitive function

We tested mice by NOR to evaluate improvements in cognitive impairment after hCO transplantation. Representative results showing heat map analysis of mouse tracking in each group is presented in Fig. 6. Mice in each group showed similar behavior in terms of preferential investigation of novel objects before induction of mild TBI (Fig. 6A and 6B). However, the degree of the impaired discrimination index between mild TBI with and without hCO transplantation reached a significant difference at 7 DPT and 14 DPT (Fig. 6C and 6D). hCO transplanted mice exhibited an elevated preference for novel objects, indicating improvement in cognitive functioning. Moreover, the difference in the therapeutic effect was more pronounced at 14 DPT than 7 DPT.

Discussion

Following TBI, apoptosis and necrosis of neuronal cells along with neuro-inflammation, increase the risk of neuronal dysfunction including symptoms of cognitive impairment and motor dysfunction.[1] Thus, even if the initial brain injury treatment is successful, it remains necessary to continue to reduce neuronal cell damage and alleviate neuroinflammation. Treatment studies for the purpose of alleviating neuronal dysfunction are increasingly using stem cells of varying types, such as neural cell types and fetal neural stem/progenitor cells of murine or human origin.[22–24] McGlinley et al.[25] reported that human neural stem cell transplantation targeted to the fimbria fornix significantly improved cognition in two hippocampal-dependent memory tasks at 4 and 16 weeks post-transplantation. Nevertheless, low survival rates and inadequate neuronal differentiation of transplanted stem cells are a concern in stem cell use for TBI.[11] Compared to neurodegenerative conditions in which the disease progresses slowly, TBI is characterized by acute inflammation immediately after trauma, with secondary cerebral edema and subsequent chronic inflammation, making it difficult for transplanted stem cells to survive. In this respect, COs may be

advantageous in TBI treatment due to their stable morphology and neuronal differentiation status with a diverse set of healthy neurons.[15,26] To this point, two studies on the usefulness of hCOs in rodent models of TBI have been published.[26,27] Wang et al.[27] reported that transplanted hCOs resulted in improved neurological function and neurogenesis without aggravating apoptosis and inflammation in a rat model of TBI. Bao et al.[26] then showed that transplanted hCOs exhibited neuronal differentiation, vascularization, and reduced glial scars with improved memory and spatial learning in severe combined immunodeficient (SCID) mice with severe TBI. Unlike these studies,[26,27] we analyzed the effects of hCOs in mild TBI. Mild TBI does not usually cause deterioration of motor function and brain structural damage compared with moderate to severe TBI. However, neuronal loss, synapse reduction and dendrite degeneration can continue in the cortical lesions after the acute phase of TBI.[5] In addition, reduced performance with cognitive impairment has been associated with mild TBI in patients.[28] Nevertheless, the reality is that there is no specific treatment for neuronal dysfunction occurring after mild TBI. Our study shows promise however, as it revealed that hCO transplantation can contribute to reconstruction of damaged cortical regions, provide neuroprotective effects in the CA1 and DG regions of the hippocampus, and alleviate cognitive impairment following mild TBI.

It is still unclear at what stage of culture the hCOs are optimal for transplantation. In the rat model of TBI, hCOs at 55 days showed better transplantation outcomes than at 85 days in terms of enhanced neurogenesis and higher survival rate. Kitahara et al.[29] performed transplantation of hCOs at 6W and 10W in SCID mice and compared graft survival and axonal extension. 6W-hCOs revealed greater axonal extension along the corticospinal track while graft overgrowth with higher numbers of proliferative cells were observed than with 10W-hCOs. In this study, we observed that 6W-hCO formed neural stem cells and ventricle-like structures, while14W-hCO showed neuronal differentiation into TUJ1 + neurons and CTIP2 + neurons in the cortical layer (Supplementary Fig. 1). Moreover, as in the previous study,[30] cellular proliferation in terms of Ki67 + cells were significantly reduced in the 14W-hCO (Supplementary Fig. 1). Therefore, we determined that 8W-hCO transplantation was suitable for treatment of mild TBI taking into account cellular proliferation and axonal differentiation.[29] Nevertheless, the optimal development stage for hCOs may vary depending on TBI severity and the purpose of treatment. It is worth bearing in mind that when using hCOs for targeting mild TBI in the clinic, it will be important not to induce a severe immune compromised state or excessive inflammation in the host. Our results here demonstrate that immune suppression with cyclosporine A is sufficient for hCO transplantation in mild TBI. Contrary to the results of single-cell transcriptomic analysis of vessel organoids,[31,32] inflammation-related factors were not observed via proteome array in the 8W-hCOs used in this study (Fig. 5). Our findings also show that 8W-hCOs have a therapeutic potential with a focus on neuronal cells, which may exclude the possibility of immune responses from microglia, fibroblasts and immune-related cells. Moreover, expression of these proteins indicated that 8W-hCOs strongly require vascularization, which has been described as a tradeoff between vascularization and neurogenesis during brain development. Interestingly, the timing of events in neurogenesis and angiogenesis is such that they occur almost simultaneously. However, Vogenstahl et al.[33] reported that the nervous system precedes the vascular system during embryogenesis. Thus, we

believe that for mild TBI, 8W-hCOs may be effective in facilitating therapeutic effects without causing inflammatory responses in the host.

Even for mild TBI, attention needs to be paid to damage and recovery of the hippocampus as well as the damaged cortical region locally. Mild TBI induces neurophysiological changes in the hippocampus such as in expression of gamma-aminobutyric acid (GABA), tyrosine kinase, and N-methyl-D-aspartate.[34] These changes can be attributed to increases in excitatory post-synaptic currents and neuroinflammation, as well as reductions to inhibitory postsynaptic currents.[34] In this study, we also observed that FJB + degenerative neurons were prevalent in the periphery, including the ipsilateral cortex, and CA1 and DG of hippocampus at both 7 DPT and 14 DPT in untransplanted mice, while hCO transplantation significantly reduced their numbers (Fig. 3A). Thus, hCOs appear to fulfil additional therapeutic functions because they not only repair damage in the cortex but also facilitate recovery of hippocampal damage with neurogenesis.

3D-COs are heterogeneous in that they contain a diverse set of neuronal cells and this differentiates them from 2D-cultured neural stem cells which are relatively homogeneous.[15] Mansour et al.[35] demonstrated blood vessels with functional neuronal networks in COs after transplantation of human pluripotent stem cell-derived COs into the adult mouse brain. In addition, transplanted COs exhibited enhanced survival and multilineage neurodifferentiation.[14] Structural organization with the surrounding normal host tissue can also be expected, with integrated neuronal connectivity. Following direct transplantation of hCOs into the injured cortical regions in our study, engraftment was well maintained and reconstructed. Thus, hCO transplantation can be considered an attractive and likely superior alternative to 2D-cultured neural stem cells.

This study has some limitations. First, we did not evaluate white matter restoration after hCO transplantation. Degradation of integrity in specific white matter tracts was associated with visual tracking deficits in patients with persistent symptoms after mild TBI.[28,36] Diffuse loss of white matter integrity also seemed to be associated with systemic mechanisms of damage involving consecutive inflammatory responses in patients with mild TBI.[37] Otero-Ortega et al.[38] reported that white matter injury was partially restored by adipose-derived mesenchymal stem cell treatment in a rodent model of subcortical stroke. Thus, additional study is required to determine whether hCO transplantation exhibits improvement in white matter integrity after mild TBI. Second, detailed typing of glial cells was not performed, although neuronal differentiation was observed in this study. Transplantation of hCOs at 8 weeks was considered to be an appropriate time point for mild TBI when considering the survival of transplanted cells and proliferation and differentiation status of the hCOs, thus, we did not proceed to culture for longer times. 8W-hCOs are typical of the early dorsal forebrain in humans, which is correlated with the developing human cerebral cortex at 4–10 weeks of conceptional age. In this period, the cellular composition mainly consists of neural stem cells, radial glial cells, neuronal progenitors, and early neurons.[39,40] Sivitilli et al.[41] presented the results of single-cell RNA sequencing on individual organoids cultured at different periods, which indicated that mature astrocytes and oligodendrocytes appeared in

long-term organoid culture. Pasca et al.[42] reported that almost 20% of glial fibrillary acidic protein (GFAP) + cells increased by 6 months in human cortical spheroids 3D-culture. Marton et al.[43] also showed that of myelin basic protein (MBP) + cells constituted ~ 28% of the total at 5 months in human oligodendrocyte spheroid cultures. In addition, other important cell types such as microglia and endothelial cells derived from the mesoderm were not present in the COs in previous researches and these features are common limitations of CO research which need to be overcome.[44] Accordingly, it will be necessary to develop hybrid culture systems with various types of cells to generate more complete COs, using highly controlled protocols.

Conclusion

hCO transplantation may be feasible for the treatment of mild TBI-related neuronal dysfunction as it was shown to facilitate therapeutic reconstruction of damaged cortex and neurogenesis in the hippocampus.

Methods

Maintenance of human embryonic stem cell

Human H9 embryonic stem cells (hESCs) were obtained from WiCell Research Institute (Madison, WI, USA) at passage 26 and cultured under feeder-free condition. hESCs were cultured on 6-well plates coated with hESC-qualified matrigel (Corning Life Sciences, Bedford, MA, USA) in mTesR™1 medium (STEMCELL Technologies, Vancouver, BC, Canada), and routinely passaged every 4–6 days with Versene® (0.02% EDTA; Lonza, Basel, Switzerland), upon reaching 80% confluence. Before passaging, unhealthy hESC clones were picked-up manually. This research was performed in compliance with the guidelines of the Declaration of Helsinki, and approved by the Institutional Ethics Committee of Hallym University Medical Center (IRB No. CHUNCHEON 2021-03-010).

hESC differentiation into cerebral organoids

To differentiate hESCs into cerebral organoids (COs), a modification of the method described by Lancaster et al. was used.[18,45] Briefly, hESCs were detached from the plate by Dispase (STEMCELL Technologies, Vancouver, BC, Canada) and dissociated into single cells using 0.05% Trypsin/EDTA (Sigma-Aldrich, Korea). Cells were plated at 9000/well into 96-well round-bottom ultra-low attachment plates (Corning inc, Corning, NY, USA) to generate embryoid bodies (EBs) with low levels of basic fibroblast growth factor (bFGF) in hESC medium. The low-bFGF medium contained 75% DMEM/F12 (Welgene, Korea), 20% knock out serum replacement (KOSR) (Invitrogen, Carlsbad, CA, USA), 3% embryonic stem cell quality fetal bovine serum (hES-quality FBS) (Gibco, Grand Island, NY, USA), 1× GlutaMAX (Invitrogen, Carlsbad, CA, USA), 1× minimum essential media-nonessential amino acids (MEM-NEAA) (Invitrogen, USA), 0.385 μmol 2-mercaptoethanol (Gibco, USA), 4 ng/mL bFGF (Peprotech, Korea). The low-bFGF medium was exchanged every other day for 6–7 days with medium supplemented with 50

μmol Rho-associated protein kinase (ROCK) inhibitor (Y-27632) (STEMCELL Technologies, Canada) during the first 4 days.

When the diameter of EBs reached 500 μm, they were transferred to a 24-well ultra-low attachment plate (Corning, USA) with neural induction medium to form neuroepithelial tissue. The neural induction medium containing Dulbecco's modified eagle medium (DMEM)/F12 with 1× N2 supplement (Invitrogen), 1× GlutaMAX (Invitrogen), 1× MEM-NEAA and 1 μg/mL heparin (Sigma-Aldrich, Korea) was exchanged every other day for 5 days. After 4 or 5 days, neuroepithelial tissues used to differentiate into COs were transferred one by one to matrigel droplets by pipetting into 20 μL cold matrigel on a sheet of sterilized Parafilm M® (Bemis Company, Inc, Neenah, WI, USA) with 3 mm dimples. These droplets were allowed to polymerize at 37℃ for 30 min and were subsequently removed from the Parafilm M® to a 6-well ultra-low attachment plate containing CO differentiation medium without Vitamin A. The CO differentiation medium without Vitamin A contained 1:1 ratio of DMEM/F12 medium and Neurobasal Medium (Invitrogen, USA), 1:200 (v/v) N2 supplement, 1:100 (v/v) GlutaMAX, 1:200 (v/v) MEM-NEAA, 1:4000 (v/v) human insulin solution (Sigma-Aldrich, Korea), 0.1925 μmol 2-mercaptoethanol and 2% B27 supplement without vitamin A (Invitrogen, USA), and was exchanged every other day. After 4 or 5 days, neuroepithelial tissues with some budding outgrowth in matrigel droplets were transferred into 125 mL Erlenmeyer flask (Corning, USA) containing CO differentiation medium and rotated on an orbital shaker at a speed of 80 rpm for long-term culture. The culture medium was changed every 3–4 days with CO differentiation medium as described above except with added 2% B27 supplement with vitamin A (Invitrogen, USA).

hCO transplantation in mild TBI model

After induction of mild TBI in mice as we previously reported (See Supplemental Method),[46] hCO transplantation was performed 1 week later.[14,35] Mice for each treatment group were performed in a randomized order by an investigator blinded to treatment. The different experimental groups were as follows: (1) mild TBI (mTBI) (1W, n = 7 and 2W, n = 7), (2) mTBI treated with hCO (1W, n = 15 and 2W, n = 14), and sham operation (1W, n = 4 and 2W, n = 4). hCO transplantation was performed as follows: first, mice were anesthetized with 2.5% isofurane in oxygen and fixed in a stereotaxic frame. Second, after exposure of the skull via a midline skin incision, a 2 mm diameter of bone flap was opened to create a cranial window. Third, cortical cavities above the hippocampus were made by 2 mm diameter biopsy punch (Kai Industries, Gifu, Japan), and bleeding was controlled with a spongostan (Ethicon, Somerville, NJ, USA). An hCO from 8 weeks in culture was implanted into the cortex cavity and re-covered with the bone flap. The bone flap was then sealed with fibrin glue (Greenplast®, Green Cross PD, Korea) and spongostan (dimensions 1 cm × 1 cm × 1 mm), followed by skin suturing. The mice were intraperitoneally injected with cyclosporin A (Sandimmune ®, Novartis Pharma Stein AG, Stein, Switzerland) at 10 mg/kg/body weight for immune suppression until sacrifice. Outcome measurements were also performed by investigators who were blinded to the treatment methods. All animal experiments were approved by Institutional Animal Care and Use Committee (IACUC) of Hallym University (approval no. HallymR1 (2021-50) and were carried out according to the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments).

Immunohistochemistry staining

hCOs were fixed in 4% paraformaldehyde (PFA) (BYLABS, Korea) overnight at 4°C, washed twice with phosphate-buffered saline (PBS, Welgene, Korea), and cryoprotected in 30% (w/v) sucrose/PBS overnight at 4°C. The hCOs were embedded in Frozen Section Compound (FSC) (FSC 22 Clear, Leica Biosystems, Lincolnshire, IL, USA) and stored at − 80°C until sectioning. Sections (20 μm) were processed using standard immunostaining procedures.

For immunostaining of brain tissue, animals were anesthetized and transcardially perfused with 1× PBS and 4% PFA. Brains were carefully collected and post-fixed overnight in 4% PFA followed by cryoprotection for 24 has described. For cryosectioning and immunohistochemistry, brains were embedded in FSC. Sections (10 μm) were processed using standard immunostaining procedures. For immunostaining, the sections were washed three times in PBS at room temperature, incubated with blocking buffer (0.1% Triton X-100, 5% bovine serum albumin, 5% normal goat serum in PBS), and then incubated overnight with primary antibody at 4°C. After washing with 0.05% tween-20 in PBS three times, sections were incubated with secondary antibody for 30 min at room temperature, followed by 10 μg/mL of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) treatment for 10 min. The sections were washed once more in PBS and mounted with ProLong Gold antifade reagent (Invitrogen, USA). The following primary antibodies and final concentrations were used: Pluripotent Stem Cell 4-Marker Immunocytochemistry Kit (rat anti-human SOX2 (1:100), rabbit anti- human OCT4 (1:200), mouse anti-SSEA4 (1:100), mouse anti-human TRA-1-60 (1:100)) (Invitrogen, USA), rabbit anti-SOX2 (1:100, Novus Biologicals, Littleton, CO, USA), rabbit anti-Ki67 (1:200, Abcam, Cambridge, UK), mouse anti-DCX (1:50, Santa Cruz, CA, USA), mouse anti-nestin (1:50, Santa Cruz, USA), mouse anti-human nuclei (1:200, Novus Biologicals, USA), rabbit anti-FOXG1 (1:5000, Abcam, UK), rabbit anti-PAX6 (1:200, Abcam, UK), rat anti-CTIP2 (1:5000, Abcam, UK), rabbit anti-NeuN (1:1000, Abcam, UK), mouse anti-GFAP (1:1000, Abcam, UK), mouse anti-TUJ1(1:100 R&D systems Inc., MN, USA), mouse anti-SMA (1:50, Santa Cruz, USA) and rabbit anti-Iba1 (1:50, Abcam, UK). Alexa-488 or Alexa-594 conjugated secondary antibodies for mouse, rat or rabbit (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) were at 1:1000.

FJB staining

Sections were stained with FJB to detect dying cells. Briefly, sections were rehydrated and permeabilized with 1% NaOH in 80% absolute alcohol for 5 min, followed by incubation in 70% ethanol for 2 min. The sections were rinsed with distilled water for 2 min and immersed in 0.06% potassium permanganate (Sigma-Aldrich, Korea) for 15 min and washed for 2 min with distilled water. The sections were stained with 0.001% Fluoro-Jade B solution (Histo-Chem Inc., Jefferson, AR, USA) for 30 min at room temperature. After washing three times in distilled water for 5 min, the sections were then dried at 55 ℃ for at least 10 min in the dark and coverslipped with D.P.X (Sigma-Aldrich, Korea) mounting medium. The stained brain tissues were observed at 450–490 nm by fluorescence microscope (Carl Zeiss, GmBH, Jena, Germany).

Cytokine/chemokine profile assay

hCOs were collected after 8 weeks of culturing then hCO lysates were analyzed with a Proteome Profiler Human XL Cytokine Array Kit (R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer's instructions. For each assay, 200 μg of cell lysate protein was used, and cytokine optical densities were obtained with an ImageQuant LAS 500 (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) and quantified with Image J software.

Novel object recognition test

Behavioral setup and analysis was based on our previous protocols.[46] Detailed information about novel objection recognition (NOR) test is presented in Supplemental data.

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Comparison between two means was done by Student's t-test. A two-way analysis of variance (ANOVA) followed by Tukey's post hoc test was performed for multiple comparisons. P-values less than 0.05 were considered statistically significant. Analyses were conducted using GraphPad Prism 8.0 software (GraphPad Software Inc., San Diego, CA, USA).

Declarations

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None.

Author contributions

JPJ and KJT devised the original study design. KJT, DHY, TYK and SWH performed the data collection. CSM and HEP performed statistical analyses. PCH, LY, JH, LJJ, RJK, PJJ, AJH, KHC, and CYJ interpreted the results. JPJ and KJT wrote the manuscript.

Additional information

Competing interest

All the authors have no financial conflicts of interest.

Data availability statement

Inquiries can be directed to the corresponding author.

Ethics statement

This study was approved by the Institutional Review Board (IRB No: 2021-03-010) of the participating institution. All animal experiments were approved by Institutional Animal Care and Use Committee (IACUC) of Hallym University (approval no. HallymR1 (2021-50)

Supplementary information accompanies this paper

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Figures

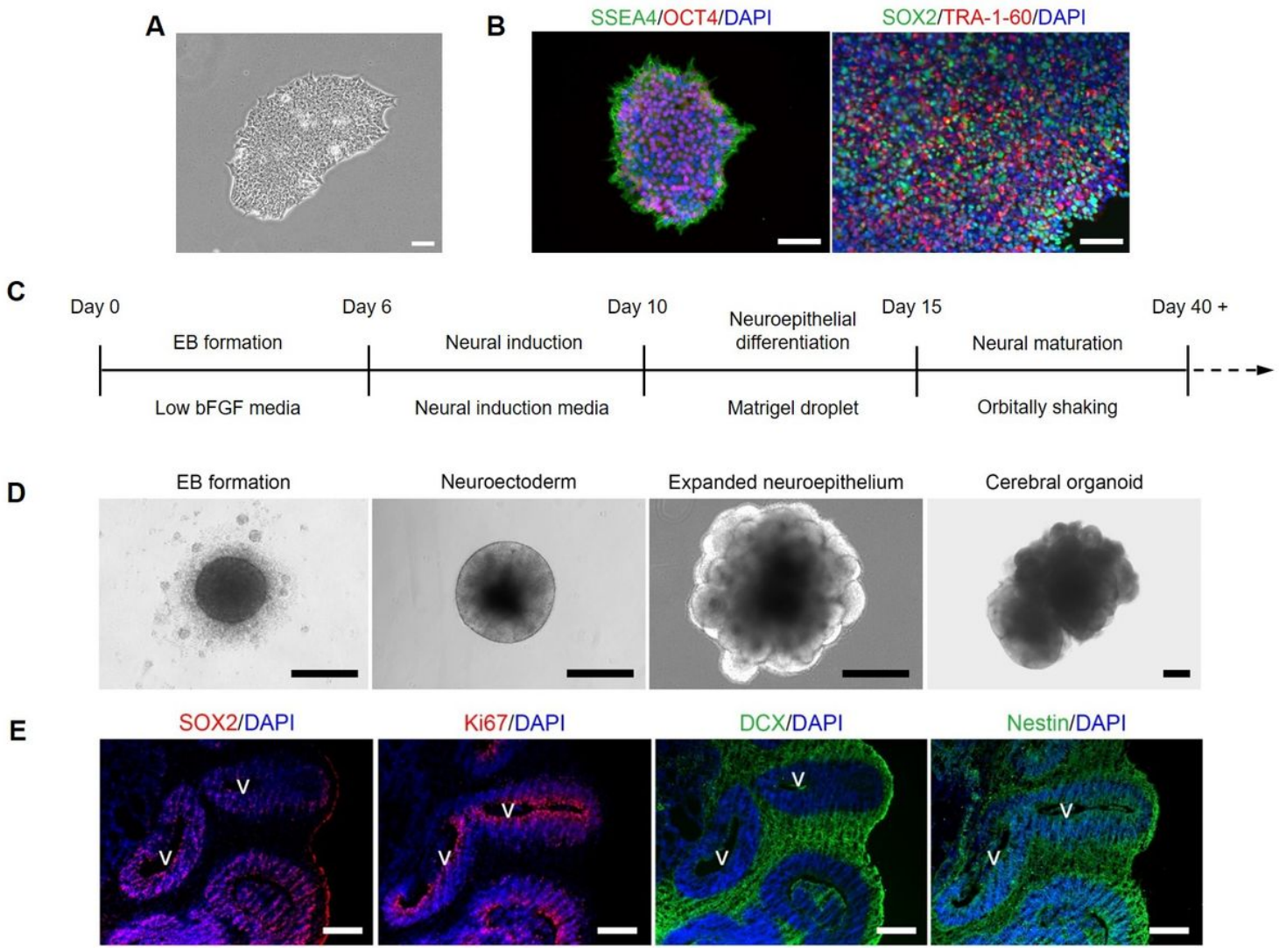

Figure 1

Human embryonic stem cell (hESC) culture and cerebral organoid (CO) culture system. **(A)** Phase contrast image of H9 hESC. Scale bar is 50 μm. (**B)** Immunofluorescence staining for SSEA4, OCT4, SOX2, and

TRA-1-60 in pluripotent hESCs. Scale bars are 200 μm. All cell nuclei were stained by DAPI. **(C)** Schematic diagram of the cerebral organoid culture system. The day embryonic bodies (EBs) were initially generated from hESCs was defined as day 0. EBs gradually showed bright surfaces with relatively dark centers from day 1 to day 6 after induction. Then, the EBs were induced to form neuroectoderm in neural induction media for 4 days. After matrigel embedding for expanding neuroepithelial buds, well-defined polarized neuroepithelium-like structures resembled neural tubes at day 15. **(D)** Representative images of each stage of cerebral organoid formation. **(E)** Sectioning and immunohistochemistry revealed complex morphology with heterogeneous regions containing ventricle-like structures in cerebral organoids at 6 weeks. All cell nuclei were stained by DAPI. Ki67, proliferation marker; doublecortin (DCX), neuronal precursor cell marker; nestin, neuroepithelial stem cells; V, ventricle-like structures. Scale bars are 200 μm. SSEA, Stage-specific embryonic antigen 4; OCT4, Octamer-binding transcription factor 4; SOX2, SRY-Box transcription factor 2; TRA-1-60, podocalyxin; DAPI, 4',6-diamidino-2-phenylindole. bFGF, basic fibroblast growth factor; EB, embryonic bodies.

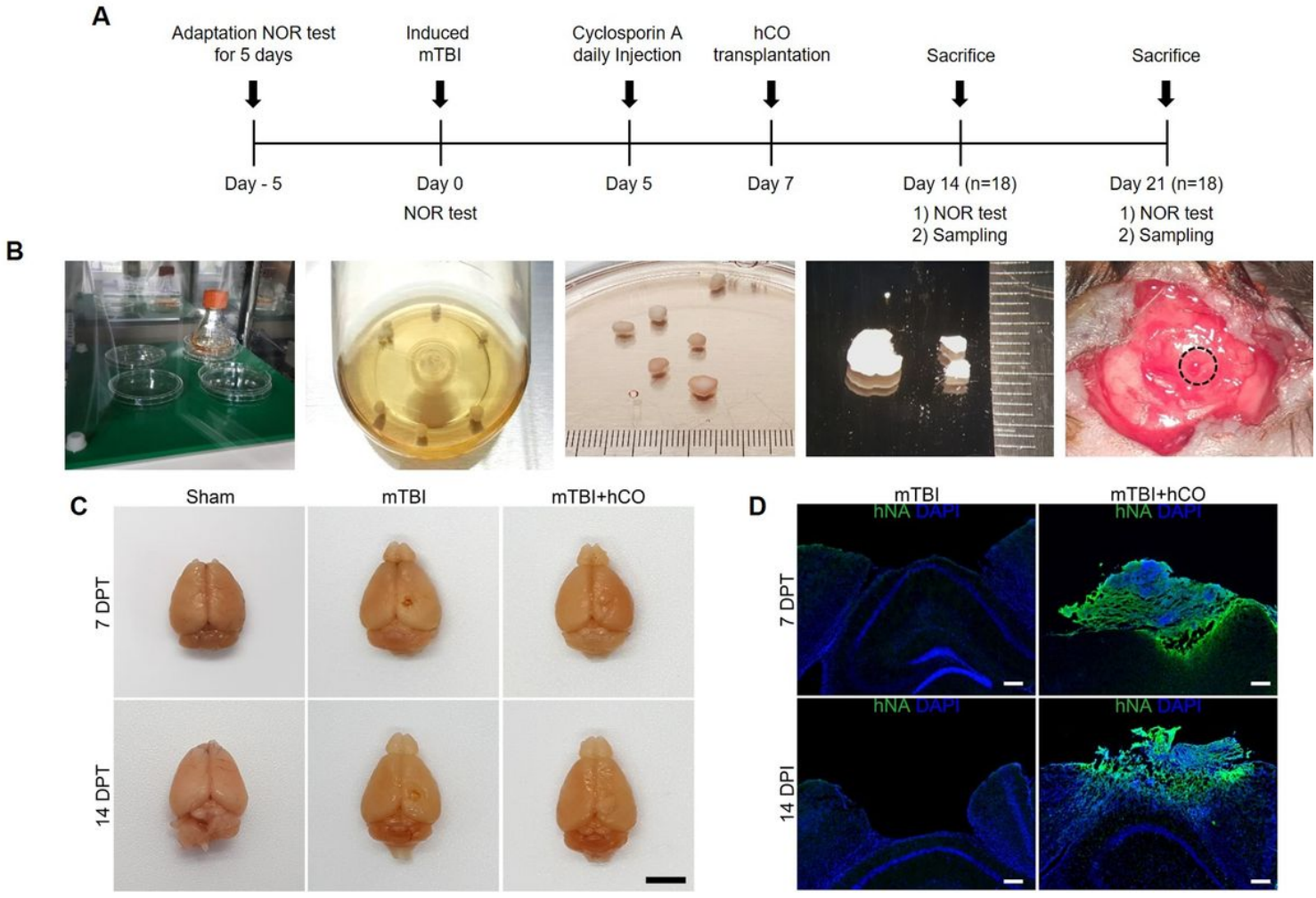

Figure 2

8W-hCO transplantation and engraftment of retrosplenial cortex in the mouse TBI model. **(A)** Schematic showing transplantation of hCOs. **(B)** Transplantation of hCOs into damaged cortex in the mouse TBI

model. The lesion cavity was made at the retrosplenial cortex of 7W old mice by removal of damaged tissue (black dotted line) with insertion of one piece into each cavity. Scale bars are 1 mm. **(C)** Brain tissues collected at 1 week and 2 weeks after transplantation. Scale bars are 5 mm. **(D)** Representative images of engraftment and cell survival in the transplanted hCOs of retrosplenial cortex by immunostaining of human nuclear antigen (colored green). Scale bars are 100 μm.

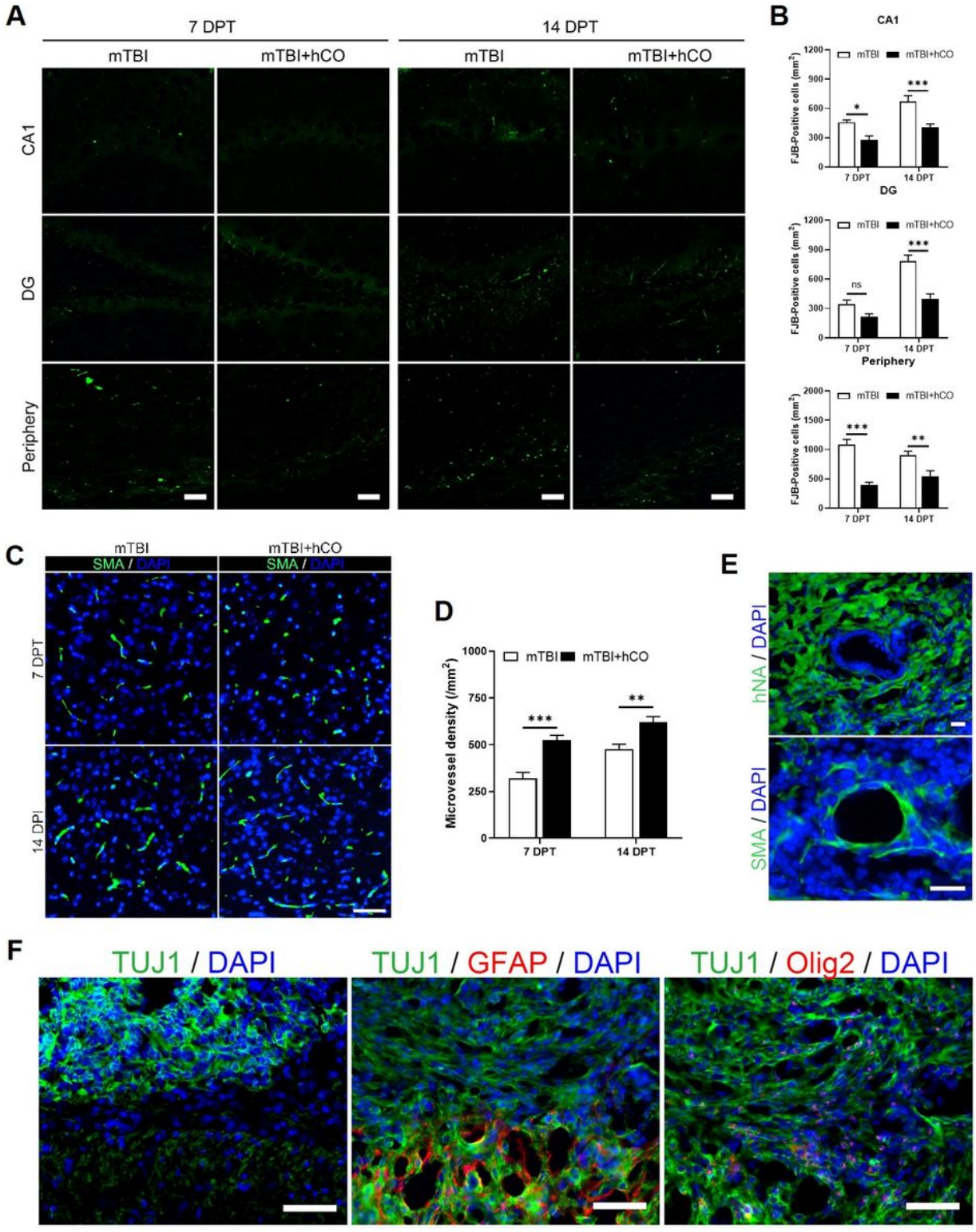

Figure 3

Neuroprotection and reconstruction of damaged cortex after hCO transplantation in a mouse model of mild TBI. **(A)** Representative images of degenerated neurons measured by FJB staining of mouse brain and quantification of the number of FJB positive cells in the three different lesions at 7 DPT and 14 DPT, respectively. Scale bar is 50 μm. **(B)** Quantification of FJB-positive cells in CA1, DG and periphery. **(C)** Immunofluorescence staining for vascular recovery in peripheral lesion of cortex by SMA. Scale bar is 50 μm. **(D)** Quantification of microvessel density in peripheral lesion of cortex. **(E)** Immunofluorescence staining for vascularization in engrafted hCOs with hNA and SMA antibodies. **(F)** Immunohistochemistry for neural differentiation fate in engrafted hCOs using TUJ1 (neuron), GFAP (astrocyte), and Olig2 (oligodendrocyte) antibodies. Scale bar is 50 μm. *P < 0.05, **P < 0.01, ***P < 0.001; n.s., not significant.

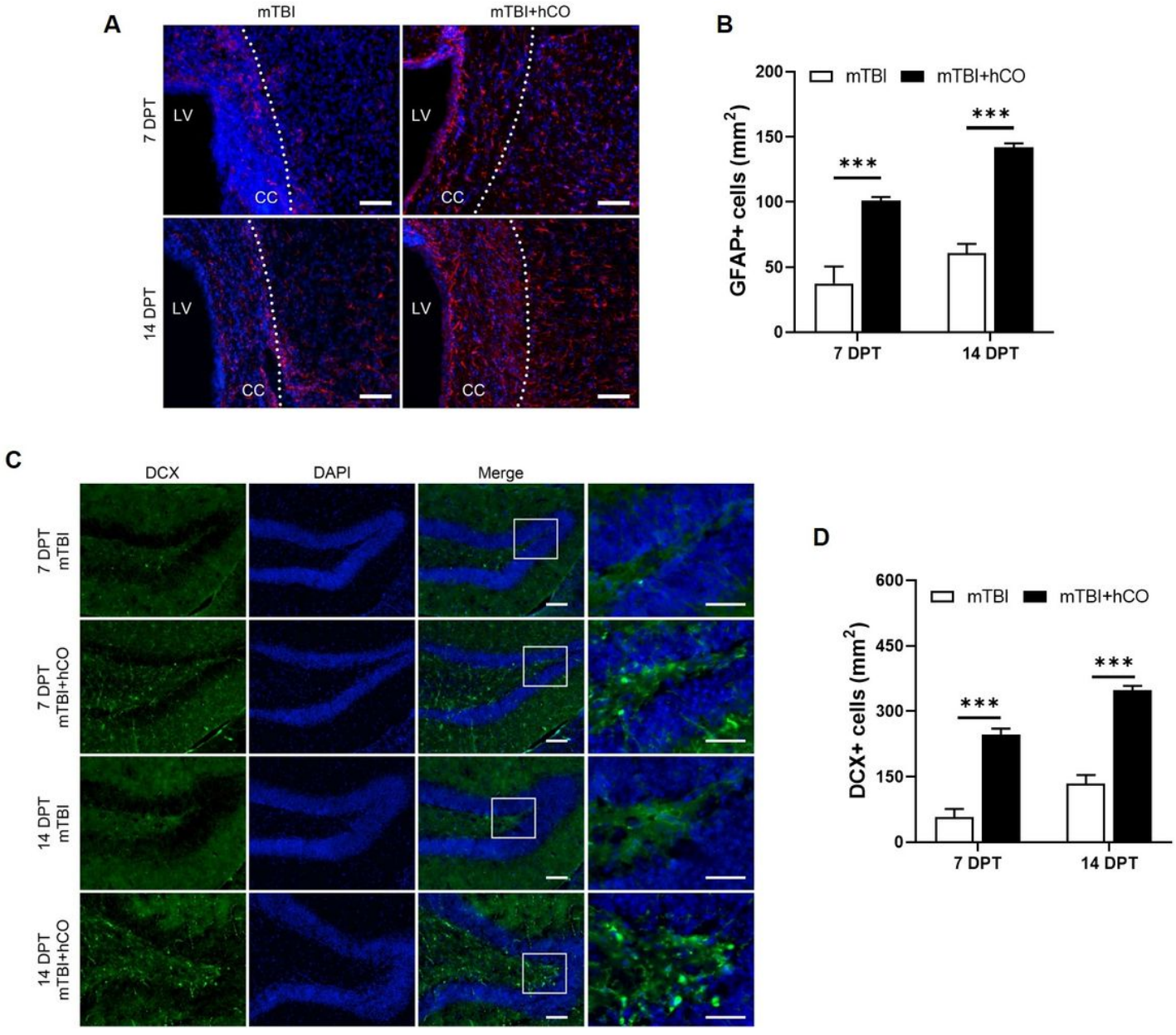

Figure 4

Upregulated activation of endogenous neural stem cells in SVZ and hippocampus after hCO transplantation. **(A)** Representative images of activated GFAP+ cells indicating neural progenitors or

astrocytes in SVZ by immunohistochemistry. Scale bar is 100 μm. **(B)** Quantification of GFAP expression in lateral ventricle and cortex in sections of the lesion. **(C)** Representative images of activated neurons of dentate gyrus in hippocampus by immunohistochemistry for doublecortin (DCX) a marker of new neurons. Merge scale bars are 200 μm. High resolution images of the boxed region in merged image, scale bars are 100 μm. **(D)** Quantification of DCX immunostaining in dentate gyrus and hippocampus in sections of the lesion. LV, lateral ventricle; CC, corpus callosum; Error bars, SEM; ***P < 0.001.

A

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	Pos		Acrp30		ApoA1		Angiogenin		Ang-1		Ang-2		BAFF		BDNF		C5/C5a		CD14		CD30		Pos	
B	-		CD40L		CHI3L1		Adipsin		CRP		Cripto-1		CST3		Dkk-1		DPP4		EGF		EMMPRIN		-	
C	-		CXCL5		Endoglin		CD178		FGF-2		FGF-7		FGF-19		FLT3LG		G-CSF		GDF-15		GM-CSF		-	
D	CXCL1		GH		HGF		ICAM-1		IFN-γ		IGFBP-2		IGFBP-3		IL-1α		IL-1β		IL-1ra		IL-2		IL-3	
E	IL-4		IL-5		IL-6		IL-8		IL-10		IL-11		IL-12 p70		IL-13		IL-15		IL-16		IL-17A		IL-18Bpα	
F	IL-19		IL-22		IL-23		IL-24		IL-27		IL-31		IL-32		IL-33		IL-34		IP-10		I-TAC		KLK3	
G	Leptin		LIF		NGAL		CCL2/ MCP-1		CCL7/ MCP-3		M-CSF		MIF		MIG		CCL3/4 MIP-1α/β		MIP-3α		MIP-3β		MMP-9	
H	MPO		OPN		PDGF-AA		PDGF-AB/BB		PTX3		PF4/ CXCL4		RAGE		CCL5/ RANTES		RBP4		RLN2		Resistin		SDF-1α/ CXCL12	
I	Serpin E1		SHBG		IL-1 R4		CCL17/ TARC		TFF3		TfR/ CD71		TGF-α		TSP-1		TNF-α		uPAR		VEGF		-	
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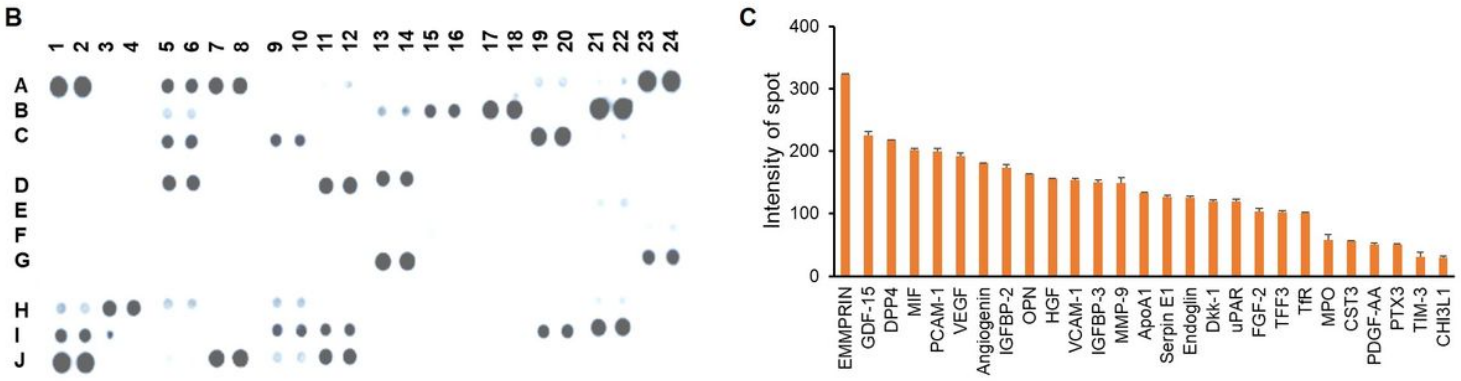

Figure 5

Cytokine and chemokine profile of the hCOs at 8 weeks. **(A)** Template demonstrating the location of spots of various cytokine capture antibodies and positive and negative controls. All spots were performed in duplicate. Rectangles filled with green background represent major expression of cytokines and chemokines at culture. Blank indicates no spot. **(B)** Membrane-based antibody array results in 8W hCOs using a Human XL Cytokine Array Kit. Array images were captured following 5-min exposure to ImageQuant LAS 500. **(C)** Histogram presenting spot intensities indicating cytokine levels. These listed in the order of high expression.

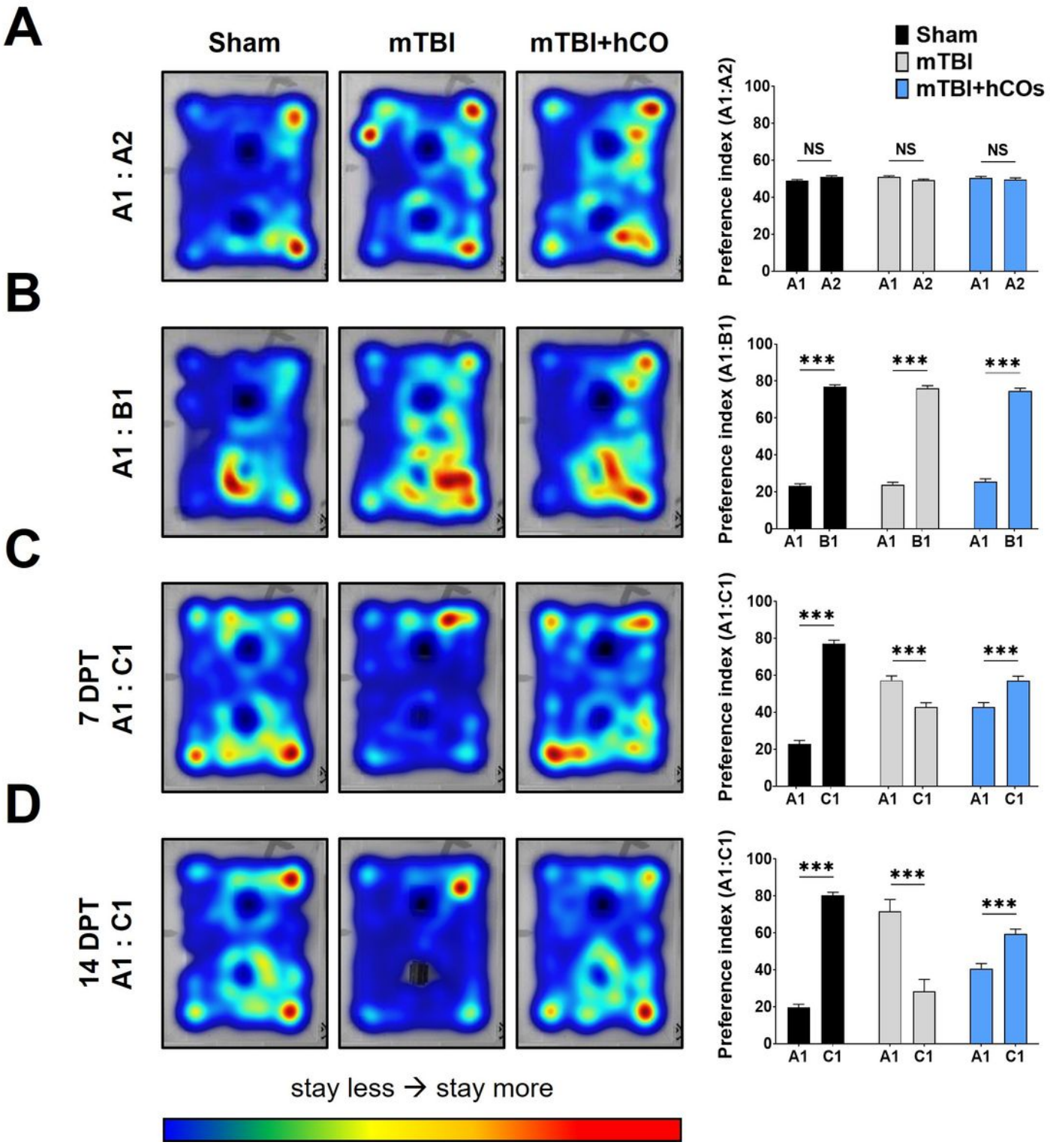

Figure 6

Improvement in cognitive impairment after hCO transplantation in mild TBI model. Heat map analysis of mice tracking in each group via novel object recognition test and quantification of preference before induction of mild TBI **(A and B)** and after TBI **(C and D).** Impaired discrimination index improved after hCO transplantation at 7 DPT and 14 DPT. ***P < 0.001; n.s., not significant.

Supplementary Files

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