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Human embryonic stem cells and their derivates in the treatments of spinal cord injury

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Abstract

Spinal cord injury (SCI) causes myelopathy, damage to white matter, and myelinated fiber tracts that carry sensation and motor signals to and from the brain and is a major cause of paralysis. Currently, there are no effective therapies to reverse this disabling condition. Human embryonic stem cells (hESCs) are pluripotent cells that have the capability to differentiate into nearly all cell types, including neuronal and glial fate cells. Therefore, these cells are a promising source of differentiated oligodendrocytes and motoneurons and could be used to treat neurological disorders and traumas, including SCI. Following SCI, oligodendrocytes were shown to be highly vulnerable to the factors existing in inflamed tissue and may undergo cell death. This loss of myelinating cells will cause abnormal neuronal functionality but hESC-derived oligodendrocyte transplantation can restore the functional outcome. Our findings demonstrate that oligodendrocytes and motoneuron progenitors derived from hESC when transplanted in rat model with completely transected spinal cord restore locomotor function and represent a viable cell-based strategy for restoring neuronal dysfunction in patients with spinal cord damage.

Key words: embryonic stem cells, spinal cord injury

1. Introduction

Spinal cord injury (SCI) causes myelopathy, damage to white matter, and myelinated fiber tracts that carry sensation and motor signals to and from the brain and is a major cause of paralysis. Currently, there are no effective therapies to reverse this disabling condition. Human embryonic stem cells (hESCs) are pluripotent cells that have the capability to differentiate into nearly all cell types, including neuronal and glial fate cells [1]. Therefore, these cells are a promising source of differentiated oligodendrocytes and motoneurons[2, 3] and could be used to treat neurological disorders and traumas, including SCI. Following SCI, oligodendrocytes were shown to be highly vulnerable to the factors existing in inflamed tissue and may undergo cell death. This loss of myelinating cells will cause abnormal neuronal functionality but hESC-derived oligodendrocyte transplantation can restore the functional outcome. Recently, few studies have demonstrated that transplantation of oligodendrocyte progenitor cells (OPC) derived from hESC and motoneuron progenitors (MP) derived from mouse embryonic stem cells (mESC) can provide both trophic support for spared axons and participate in remyelination

of the injured spinal cord [4-7]. The use of hESC derived neural progenitors for treatment of SCI has been described [3, 8] in which authors showed that transplanted hESC-OPC survived, integrated, differentiated and remyelated damaged tissue resulting in a significant improvement of locomotor function of rats with spinal cord contusions. However, there is no study that describes transplantation of hESC-OPC and hESC-MP using an animal model with complete spinal cord transection. Therefore, in this study we sought to evaluate the behaviour and efficiency of grafted hESC-OPC and/or hESC-MP into female rats after spinal cord transection. Our findings demonstrate that oligodendrocytes and motoneuron progenitors derived from human embryonic stem cells represent a viable cell-based strategy for restoring neuronal dysfunction in patients with spinal cord damage.

2. Material and methods

2.1 Cell culture and differentiation

Primary hESC colonies (H9, H9-GFP and H1 lines, WiCell Inc., Madison, WI) were mechanically dispersed into several small clumps, which were cultured on fresh commercially available human foreskin fibroblasts (American Type Culture Collection, Manassas, VA, USA), inactivated by mitomycin C in ES medium containing Knockout-DMEM (Invitrogen), 100 μM ß-mercaptoethanol (Sigma), 1 mM L-glutamine (Invitrogen), 100 mM nonessential amino acids, 20% serum replacement (SR; Invitrogen), 1% penicillin-streptomycin (Invitrogen), and 8 ng/ml basic fibroblast growth factor (bFGF; Invitrogen). ESC medium was changed every second day. Human ESC were passaged by incubation in 1 mg/ml collagenase IV (animal-free, Invitrogen) for 5-8 minutes at 37°C or mechanically dissociated and then moved to freshly prepared human foreskin fibroblast layer.

Cells were differentiated toward OPC according to already published protocols [3, 8]. Briefly, cell clumps were placed for 2 d in 50% hESC growth media and 50% glial restriction media (GRM) [8] in ultra-low attachment 6-well plates (Corning). This medium was then replaced with 100% GRM supplemented with 20 ng/ml EGF (Sigma-Aldrich) and 10 μM/ml all-trans-retinoic acid (RA) in DMSO for an additional 7 days. During 25 days the cells were exposed to GRM supplemented with 20 ng/ml EGF. Then, floating yellow spheres were plated in 6-well plates (BD) 1:30 Matrigel for 1 week. The progenitors were migrated from spheres. Cell cultures were then replated on 1:30 Matrigel substrate, and cultured for 1 week in GRM supplemented with 20 ng/ml EGF. The duration of the protocol was 42 days. At this point the cells were ready for transplantation. For transplantation the cells were disaggregated mechanically using a glass pipette.

For motoneuron differentiation, we used modified protocol of Li and col.[9]. Briefly, the H9 cell line was permanently transfected with plasmid carrying GFP (H9-GFP). Cell clumps were placed in normal hESC medium for 4 days without bFGF to form embryoid bodies (EB) in ultra-low attachment 6-well plates (Corning). The medium was changed daily. Then the EB were placed in normal 6-well plates on 1:30 Matrigel substrate to attach in motor induction medium (MIM) composed of DMEM:F12 with Glutamax, N2 supplement, heparin (2 μg/mL) and bFGF (20 ng/mL). After 1 day EB attached and after 4 days rosettes start to appear. From day 8, 0,1 μM all-trans-RA was applied in MIM. The medium with RA was changed daily. At day 16 the rosettes were mechanically cut and placed in ultra-low attachment 6-well plates as round structure-neuromasses in motor medium (MM) consisting of neurobasal medium, N2 supplement, 1 μM cAMP, 0,1 μM RA and supplemented with 200 ng/mL SHH (R&D) for 7 days. The neuromasses were maintained in MM medium supplemented with SHH (50 ng/mL) until the day of transplantation. At the day of transplantation these neuromass-like structures

were disaggregated mechanically with a glass pipette. This is the stage when the early motoneuron progenitors (ISL1+ and Tuj1) starts to mature[9].

2.2 Experimental groups

To determine whether OPC and/or eGFP-expressing MP are capable to improve motor function when immediately transplanted after SCI, approx 1,5 million cells were transplanted into the spinal cord in the acute phase after a complete transection of the spinal cord at the thoracic level [10, 11]. Fourteen rats per group were used. Three different transplantation experiments were performed: the rats were treated with a single cell type (OPC; n=16, or MP; n=16) or in combination (OPC+MP; n=16) and each was followed after transplantation for immunohistochemical evidence of cell incorporation in the lesion site and their survival. We performed electrophysiological and behavioural studies of functional recovery from hindlimb paralysis. We, therefore, defined five groups of animals, including sham and controls (n=14). Acute transplantation controls included animals that received vehicle-only injections. For more details of surgery procedure and other methods used in this study please see Supplementary data.

2.3 Behavioral Assessment

Functional recovery was assessed by evaluators blinded to treatment groups. Open field locomotor test using the Basso-Beattie-Bresnahan (BBB) Locomotor Rating Scale [12] was performed in a plastic tray (50×80×40). One week before injury, each animal was acclimated to the open-field and scored. The BBB test was performed every week after injury during 4 months when two independent examiners observed and recorded with video digital camera (Sony) the hindlimb movement of the rats, which range from 0 (no hind movement) to 21 (normal gait). The videos were analysed frame by frame using ImageMixer 3SE software and scored independently by two observers blinded to the treatment group.

2.4 Electrophysiology measurements in vivo

The motor potentials were evoked and recorded according to a prior study [13]. The main difference in our study was that the cranial screw was not implanted and a needle electrode was used. According to the anaesthetics study of Oria et al.[14] the protocol was administered intravenously as a bolus dose of 10 mg/kg. For the recording of evoked potential [MEP and compound motor action potential (CMAP)] one needle electrode was placed in the tibialis anterior muscle (cathode) and one needle electrode subcutaneously at the foot pad level (anode). For the induction of CMAP following peripheral nerve stimulation, one electrode was placed in the muscle (cathode) and another subcutaneously (anode), both near the sciatic nerve. For the induction of MEP (after central stimulation) one needle electrode was placed subcutaneously at the level of the lower jaw (anode) and a needle electrode (cranial level) was used for the cathode. For ground, an electrode was placed subcutaneously in the lumbar region. The electrophysiological recordings were performed with an electromyographer (Medtronic Keypoint Portable, Denmark) and the bandpass used was 2Hz to 10KHz. Throughout the experiments, the duration of the pulse was 0.1 ms. The recordings were started by measuring the maximum amplitude of the CMAP. This was achieved by stimulating the sciatic nerve with a single pulse of supramaximal intensity. In order to induce MEP, a stimulation of 25 mA intensity was applied at the needle electrode (cranial level). Results were expressed as Latency (ms) and Amplitude (%) (MEP/CMAP ratio).

2.5 Statistical methods

BBB scores were analysed by repeated measures 2way ANOVA with Bonferroni multiple comparison test at each time point. The differences were significant when $P<0.05$.

3. Results

The experimental procedure of cell transplantation is presented in **Fig. 1.**

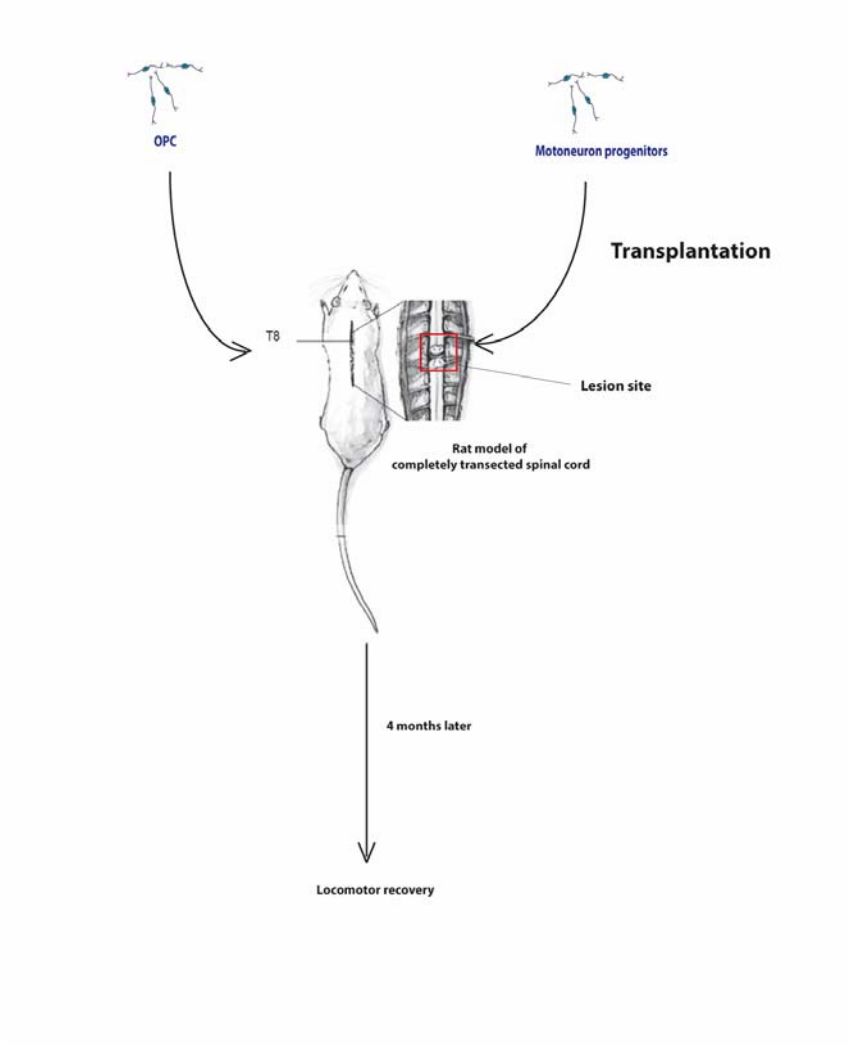

Fig. 1. Surgical and transplantation procedure of OPC and MP in rat model of completely transected spinal cord.

3.1 Animals

The majority (80%) of animals survived following injury. Some animals died due to ulcers, autophagia or considerable weight loss one month after surgery. There was a loss of about 10-20% in animal body weight (data not shown) during the first month but the animals recovered following 4 months post-injury. No formation of teratoma was observed 120 days after cell transplantation. Complete spinal cord transection lesion was characterized by an obvious traverse scar at the T8 lesion epicentres and neuronal necrosis and cavitations rostral and caudal (below and above) the lesion site. The transaction site was characterized by the presence of the white tissue between cord stumps. Reactive gliosis was detected by immunohistochemistry using anti-GFAP. These results confirmed that as a consequence of the transection of spinal cord, abundant loss of oligodendrocytes [15] occurred at considerable distance from the lesion.

3.2 Transplanted cells survived, migrated, and differentiated within the spinal cord

A total of 1,5 million cells were transplanted rostrally and caudally to the lesion site at T8 in 5- to 7-week-old female rats in the acute phase of SCI. We specifically tested three transplantation strategies including control and sham groups. The first group included rats transplanted with GFP-expressed MP (MP group). In the second group of animals OPC previously labelled with Hoechst were transplanted (OPC group). The third group of rats were given equal quantity of OPC, previously labelled with Hoechst and GFP-expressing MP (OPC+MP group). In all animals, including the control animals, we administered subcutaneously the phosphodiesterase type 4 inhibitor (Rolipram) an axonal growth supporter[4]. Four months after lesion, control animals treated with or without Rolipram showed only scattered NF-positive fibres in the central scar. Many NF-immunoreactive fibres were stopped at the host-scar interface. Rolipram did not dramatically increase amounts of NF-positive fibres in control rats compared with the rats without Rolipram, in accordance with behavioural tests (see below). The survival of the transplanted cell was less than 1%**.** The lesion site of control rats without transplants was negative for anti-human nuclei staining, GFP fluorescence and NF70 (data not shown).

Either GFP-expressing MP or MP, previously dissociated, were injected into the rostral and caudal areas 1-2 mm away from the centre of injury to avoid central cavitation. Surviving mature MP-derived GFP cells were observed within the grey matter of the spinal cord (Fig. 2A).

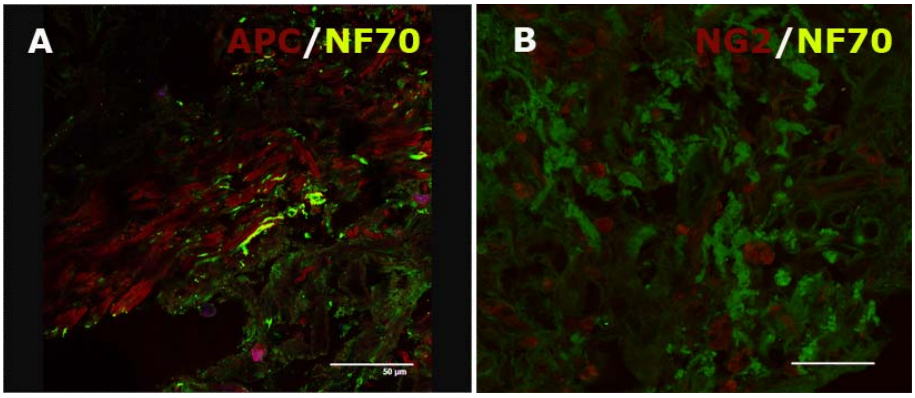

Fig. 2. Acute transplantation of hESC-derived OPC or MP resulted in differentiation toward oligodendrocytes and neurons. (A) The neuronal fibres specific for human grafts ($NF70^+$) surrounded with oligodendrocyte marker APC indicates dual fate of transplanted OPC (B) Neuronal fibres positive for human specific $NF70^+$ (green) were detected in the lesion site surrounded with glial $NG2^+$ cells in MP transplanted animals.

Four months after transplantation, GFP-labelled MP were visualized as a dense mass of elongated, brightly fluorescent cells extending from the lesion site (Fig. 2A). These MP-derived neurons persistently expressed GFP which confirmed that MP were capable of surviving and engrafting for at least 4 months after transplantation (Fig. 3). The MP were gathered in the lesion gap, which was surrounded by an intensively GFAP-positive border of reactive astrocytes reaching 4 mm caudal and 5 mm cranial. NF70 immunostaining (the lesion samples injected with MP) suggests that these cells differentiated toward neurons in the glial scar (Fig. 2). The phenotype of these neurons remains unknown since these GFP neurons were neither interneurons ($Pax2^-$ neurons) nor motoneurons ($HB9^-$) 4 months after transplantation (data not shown). An additional proof of dual terminal differentiation of hESC derived MP is the presence of APC^+ cells surrounding $NF70^+$ filaments (Fig. 2) that suggests that transplanted cells also have the capacity to mature to oligodendrocytes.

Transplanted OPC previously labelled with Hoechst survived and migrated over short distances of the spinal cord during the postimplantation survival period in all treated animals (Fig. 3).

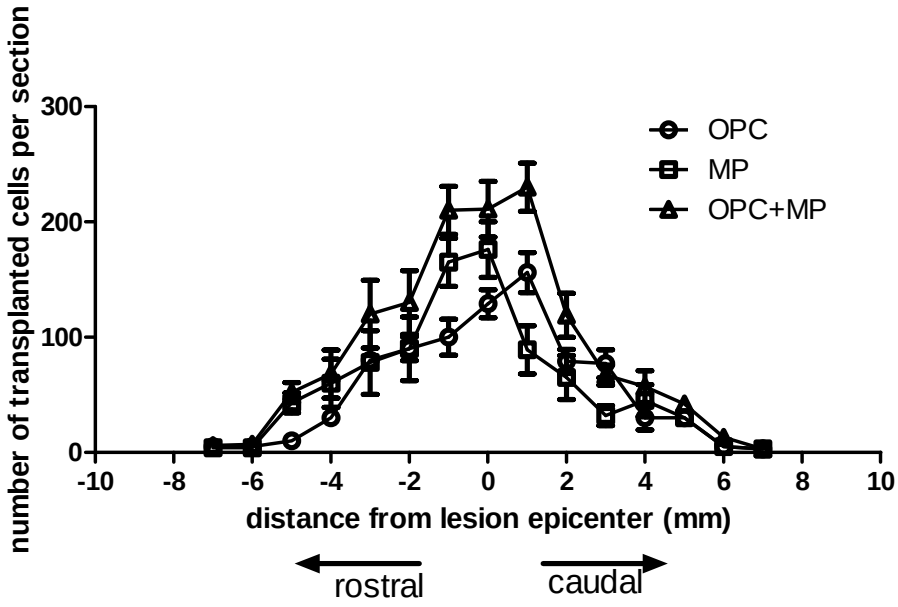

Fig. 3. Distribution of hNU (OPC, OPC+MP) and GFP (MP) immunostained transplanted cells in transverse sections 4 months after transplantation. Error bars illustrate mean±s.e.m.

Most of the human nuclei positive cells were located in the lesion site (Fig. 3). Double staining to human nuclei and GFAP or O4 revealed that the majority of transplanted cells differentiated to astrocytes and oligodendrocytes in the lesion site (data not shown). Interestingly, immunohistochemical analysis revealed $NF70^+$ fibres in the lesion site suggesting that human OPC also differentiated toward neuronal cells (Fig. 2B) often in close association with $NG2^+$ cells (Fig. 2B). Specificity of NF70 antibody to human neurons confirms human origin of these cells suggesting that a considerable number of OPC transplanted cells differentiated toward neurons and oligodendrocytes.

3.3 Behavioural assessment

Hindlimb motor function was assessed using the Basso-Beattie-Bresnahan (BBB) Locomotor Rating Scale [12, 16]. The behavioural results (Fig. 4) were collected by weekly BBB tests during the 4 month monitoring period for each group of animals. Before the injury, all animals showed normal locomotor activity, scored as 21 on the BBB scale, although all injured rats manifested complete hind limb paralysis 7 days after injury, resulting in a score of 0. The BBB scores were in the range of 0-1 or 2 in the control animals during the 4 months after SCI. In contrast, MP, OPC, and OPC+MP groups showed hind limb functional locomotor recovery which increased gradually after 3 weeks of transplantation. Four months after transplantation all 42 transplanted animals displayed BBB scores significantly ($P<0.001$) higher than that achieved by the control group. OPC animals reached a final average BBB score of 6, which is significantly ($P<0.001$) higher than the control group that reached only 1.5. MP rats partially recovered hindlimb movements and after 4 months reached a final average score of 6, significantly higher than control animals. Also, the OPC+MP group regained significantly ($P<0.001$) more functional recovery than the control group and vs. single cell type treatment (Fig. 4) reaching a final average BBB score of 9. Significantly higher ($P<0.001$) BBB score in OPC+MP score was

reached after 5 weeks of transplantation compared with control animals and after 12 weeks compared with single cell treatment (Fig. 4).

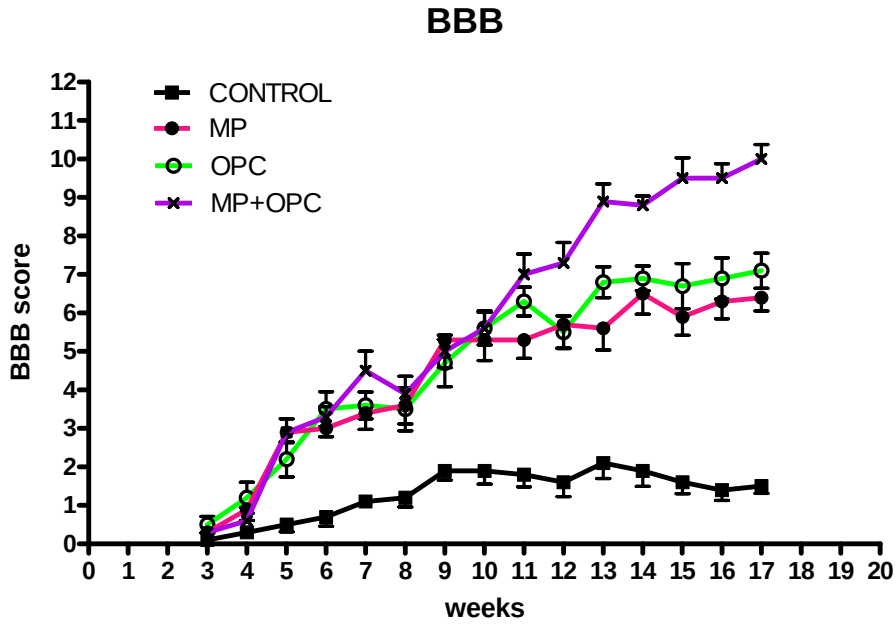

Fig. 4. Locomotor BBB test. Starting 4 weeks after the transplantation, a significant increase ($P<0.001$) in locomotor recovery as determined by the BBB locomotor rating scale was observed in all transplanted animals compared with controls. The values are presented as mean±s.e.m.

3.4 Electrophysiological evaluation

Electrophysiological tests were performed in all rats before and after the surgery and transplantation to ensure the complete transection of the spinal cord. Motor evoked potentials (MEP) were used to evaluate the function of spinal cord descending tracts[17]. Electrophysiological tests were performed for 4 months in all animals at monthly intervals. The electrophysiological tests demonstrated complete interruption of spinal motor pathways after the injury (Fig. 5). In control animals, MEP did not recover over time, indicating that interruption of supraspinal axons was maintained until the end of the observation period (Fig. 5).

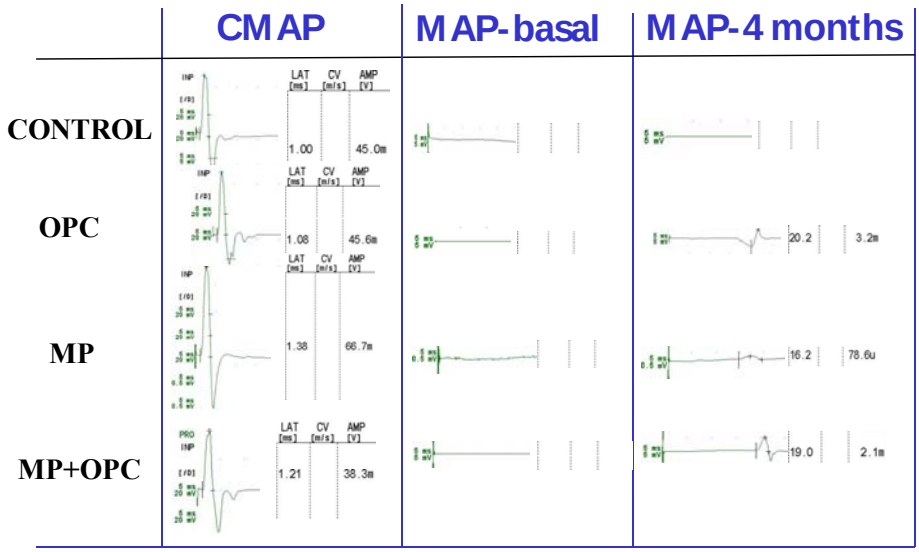

Fig. 5. In vivo electrophysiology. Representative electrophysiological recordings in control and transplanted animals after 4 months of the experiments.

In contrast, in the OPC group and/or MP group, MEP reappeared approximately 40 days after grafting (data not shown). Four months after injury the amplitude of the evoked action potentials showed recovery in OPC and/or MP groups. Comparatively, animals that received OPC and MP tended to show MEP of higher amplitude than those with a single cell progenitor type treatment (**Fig. 6**).

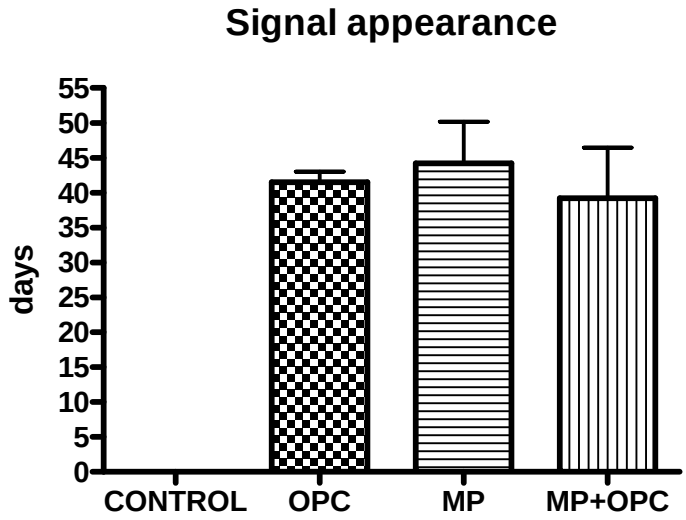

Fig. 6. Signal appearance. After approximately 40 days the electrophysiological signal appeared in the transplanted but not in the control animals.

Although the rats treated with OPC showed higher amplitude after 3 months compared with other treatments, the mean amplitude of evoked potentials after 4 months were higher but not statistically significant (P>0.05) in the OPC+MP group compared with OPC or MP (**Fig. 7**) groups.

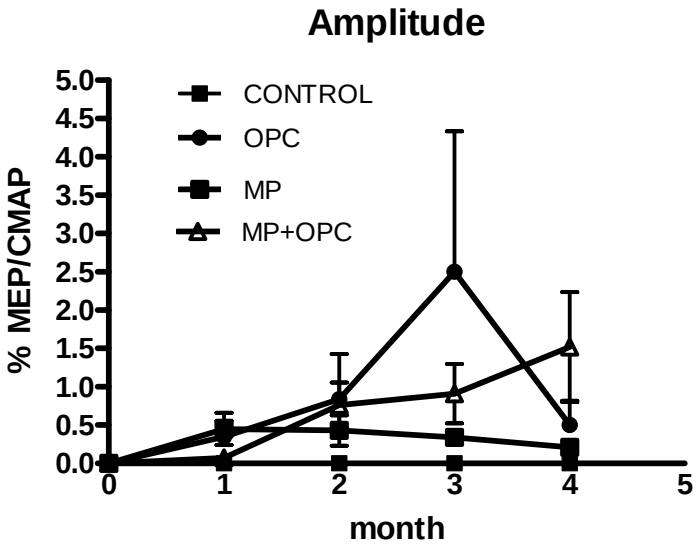

Fig. 7. Plot graphics of the evolution of latency (D) of electrophysiological analysis of control and transplanted animals.

Also the animals transplanted with a single type of cells, OPC or MP, showed shorter, but not statistically significant latency in comparison with OPC+MP (Fig. 8).

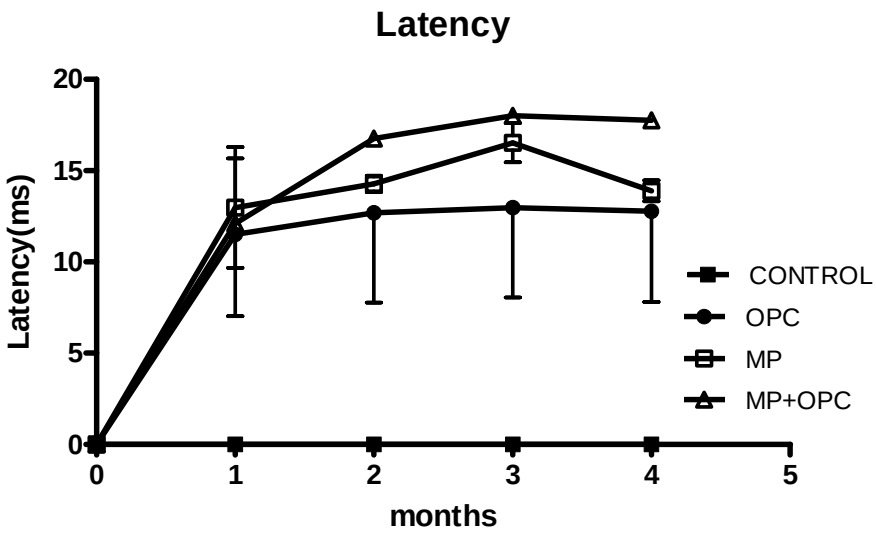

Fig. 8. Plot graphics of the evolution of latency (D) of electrophysiological analysis of control and transplanted animals

4. Discussion

Our results demonstrate that acute transplantation of a single cell progenitor type (OPC or MP) or a combination of thereof into completely transected spinal cord promotes partial recovery of hindlimb movement. As implantation of OPC in the contusion model has been reported to promote partial functional recovery following SCI[8], we focused on upgrading this study to assess the regenerative properties of OPC and MP derived from hESC using an animal model with complete spinal cord transection. To our knowledge this is the first study that describes cell transplantation of hESC-MP followed by a detailed in vivo electrophysiological assay.

A novelty of this study is that after spinal cord transection OPC- and/or MP-transplanted rats showed recovery of MEP. Immediately after complete transaction, MEP disappear due to disruption of all descending tracts. In the control group we did not observe MEP throughout the experiment due to the failure of spontaneous axonal regeneration within non-transplanted spinal cord[18]. The recoveries of MEP can be attributed to the reconnection of the axons above and below the lesion site and the contribution of oligodendrocytes. The time of reappearance of MEP in treated animals is associated with the stabile partial recovery observed by BBB, suggesting that recovery of motor skills was due to the recovery of descending control of hindlimb movements.

The BBB score of the MP group was higher than in the control group and immunohistochemistry analysis confirmed that hESC-MP survive, migrate and engraft for at least 120 days in the lesion site. These data suggest that the application of ex vivo conditioning may allow efficient generation of new neurons in non-neurogenic regions as it is SCI. Interestingly, the immunohistochemistry analysis showed clear evidence that these progenitors have the capacity to finally differentiate to mature oligodendrocytes and neurons in the lesion site. Our strategy did not result in the formation of anatomically, physiologically, and functionally active motor units between transplanted axons and host muscle, but the fact that these cells innervate the lesion site filling the gap between the rostral and caudal stumps as well

as significantly improved locomotor function of lesioned rats suggests that hESC-MP have promising regenerative potential.

Interestingly, transplanted OPC had significant recovering effects too. In a contusion model even after severe contusive SCI, surviving axons persist in the subpial rim of white matter and restoration of the oligodendrocyte population by replacement therapy has been considered as a potentially attractive strategy to promote remyelination after SCI[3, 8, 19]. But in the case of the spinal transection model there is no evidence of the presence of spared host axons. After analysis of control rats, survival of host axons or spontaneous regeneration was not observed. Immunohistochemical analysis showed differentiation of transplanted cells toward neuronal cells, which was confirmed by the presence of human specific $NF70^{+}$ neurofilaments in the lesion site. The functional locomotor recovery analysis of the rats transplanted with OPC and MP showed significantly better hindlimb recovery than the animal groups treated with a single cell type. These results have shown that combination of OPC and MP is superior approach comparing with a single type strategy used in our study or compared with the studies where other cell stem types strategies used in the same rat model of SCI[10, 20-22]. As demonstrated in our results, locomotor improvement after transplantation the OPC and MP is associated with abundant presence of human NF-positive fibers and oligodendrocytes in the lesion. That means that hESC derived OPC and MP were able to differentiate into mature oligodendrocytes and neurons. This was not the case in our previous study where we used ependymal stem cells to treat animals with spinal cord contusion[19].

5. Conclusions and perspectives

Taken together, our study demonstrates that hESC–OPC and hESC-MP, when transplanted into the spinal cord immediately after the injury, survived for at least 4 months; migrated at least 3 mm away from the lesion; differentiated into appropriate cell types without forming teratomas and improved locomotor function. These results open up new possibilities and strategies for future clinical applications in SCI and constitute a promising approach for repairing the damaged spinal cord especially if transplantation is shortly delayed after the lesion.

Извод

Људске ембриоиналне матичне ћелије и њихови деривати у лечењу повреда кичмене мождине

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Резиме

Повреда кичмене мождине (SCI) изазива мијелопатију, оштећење беле масе, и мијелинисане трактове влакана која преносе осећајне и моторне сигнале ка мозгу и из њега, и најчешћи су узрок парализе. Тренутно не постоји ефикасна терапија која би променила ово онеспособљавајуће стање. Људске ембриоиналне матичне ћелије (хЕСЋс) су плурипотентне ћелије које имају способност да се поделе у готово све типове ћелија,

укључујући и неуралне и глија ћелије. Стога, ове ћелије су перспективан извор диференцираних олигодендроцита и мотонеурона и могле би се користити у лечењу неуролошких поремећаја и траума, укључујући SCI. После SCI, олигодендроцити се показују као изузетно рањиви на факторе који постоје у ткиву које је под упалом и могу да доведу до изумирања ћелија. Овај губитак мијелинских ћелија ће изазвати абнормалну функционалност неурона али трансплантација олигонендроцита насталих од hECS може обновити функционални исход. Наши налази показују да олигодендроцити и мотонеуронски прогенитори настали из hESC, пресађени на моделу пацова са потпуно пресеченом кичменом мождином, обнављају локомоторну функцију и представљају одрживу стратегију на бази ћелија за обнављање дисфункције неурона код пацијената са оштећењем кичмене мождине.

Кључне речи: ембрионалне матичне ћелије, повреда кичмене мождине

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