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# Neuron Regeneration After Intracerebral Haemorrhage by Transplantation of the Oligodendrocyte Progenitors Derived from Neural Stem Cells

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**ABSTRACT**

**Background:** Intracerebral haemorrhage (ICH) as a cerebrovascular accident that carries a high mortality and in survivors, severe disabilities. Efforts put into this devastating illness has not converted into an effective treatment. The severe motor and sensory impairments that follow an ICH could be due to the basal ganglia white matter damage. The maintenance of white matter function is primarily dependent on the integrity of the axonal bundles and myelin sheaths. The cells that form these sheaths are the oligodendrocytes (OLGs). Oligodendrocyte progenitor cells (OPCs), precursors of OLGs, may participate in the secretion of growth factors, resulting in its functional recovery after white matter injury.

**Objectives:** To evaluate treatment effects of Neural Stem Cells (NSC) derived Oligodendrocyte Progenitor cells (OPC) on experimental intracerebral haemorrhage.

**Methods:** 60 adult male C57/BL6 (20-25g) mice were used in this study. ICH was induced and 7days later, Neural Stem Cells (NSC) derived Oligodendrocyte Progenitor cells (OPC) were intracerebral injected. Behavior test and molecular assessments were conducted 1 week after cell injection.

**Results and Conclusions:** In this study, we have immortalized the human neural stem cells

(NSCs) and generated OPCs from the NSCs. We have utilized these OPCs, induced from human NSCs to carry out topical treatment of the murine ICH. These grafted OPCs have been demonstrated to promote its functional recovery from experimental ICH in mice.

**Keywords:** Intracerebral Haemorrhage; Neural Stem Cell; Regeneration

**Abbreviations:** ICH: Intracerebral Haemorrhage; CNS: Central Nerves System; NPCs: Neuroepithelial Progenitor Cells; TGF: Transforming Growth Factor; MAP2: Microtubule-Associated Protein 2

## Introduction

Spontaneous intracerebral haemorrhage (ICH) is a catastrophic disease with a 30-50% mortality, and survivors are often live with long-term neurological deficits [1]. After blood vessels corrupted, blood clot formation could cause the occurrence of brain edema [2]. The hematoma mass effect and tissue disruption result in primary damage, while inflammatory reaction and erythrocyte lysis cause secondary damage [3]. Although it is difficult to reduce the primary injury, treatments could help to suppress the secondary brain damage. For the current treatment methods, no effective treatment has been used on this devastating disease. Some studies indicated that a better understanding of the white matter injury may lead to a new way for treating ICH [4]. White matter contains axons, myelin sheaths that surround them, as well as oligodendrocytes which produce myelin [5]. Integrated axons are important for central nervous system communication, and large abundant of afferent and efferent pathways gathered in white matter. In human, around 80% of ICH patients were reported with white-matter damage, due to its essential role, even

small lesion in white matter may lead to the impairment of neuronal function [6]. It has been known that oligodendrocytes regulate the neuronal function recovery and white matter injury [7]. In the central nerves system (CNS), oligodendrocytes originate from oligodendrocyte progenitor cells (OPCs), which derived from neuroepithelial progenitor cells (NPCs) in the embryonic stage [8]. OPCs secrete a series of growth factor such as transforming growth factor (TGF) to stimulate endothelial cells to express tight junction protein [9]. In addition, OPCs also secrete growth factors that promote the proliferation of endothelial cells and enter regions that lack of oxygen [4].

Evidence showed that adult human brain contains NSCs/NPCs in the periventricular area provides a promising approach to the CNS regeneration after injury by using grafted NSCs through the activation of endogenous regenerative capacity of damaged cells [10]. However, after CNS damage, reactive endogenous astrocytes form a glial scar and are considered to be detrimental to the axonal regeneration [11,12]. Previous studies have found that transplanted NSCs give rise almost exclusively to astrocytes and to relatively few oligodendrocytes and neurons [13,14]. Suppression of astroglial differentiation could effectively reduce the graft-induced aberrant axonal sprouting and promote further sensory and motor recovery [15]. We hypothesized that OPCs transplantation could promote the neuron regeneration after ICH in mice. After transplanting the OPCs into the experimental ICH animals, the correlation between functional and histological recovery were assessed for the control groups and the OPC-treated groups, and our results suggested that OPCs transplantation could help the recovery of ICH mice and promoted the neuron regeneration.

## Materials and Methods

### Immortalization of NSCs

The human neural stem cells (Gibco, N7800-100) were immortalized by transfecting TERT and BMI1 gene which carry GFP gene (SBI, CD513B-1). Firstly, we cultured the neural stem cells with EGF and bFGF in knockout DMEM basic medium in suspension and replaced with new medium every 2 days, thus then the cells will be passaged every 4 days. Plate the neural stem cells in poly-L-lysine and laminin coated dishes for two days, and add lentivirus containing TERT and BMI1 genes into the attachment culture NSCs. Large numbers of GFP positive cells grow rapidly as the cell proliferation.

### Neuron, Astrocyte, and Oligodendrocyte Differentiation from NSCs

For neuron differentiation, the neural stem cells were cultured in Neuron induction medium-1 containing Neurobasal Medium and DMEM/F12 (1:1) supplemented with N2, B27, 10 ng/ml BDNF, and 200 mM ascorbic acid. Four days later, the culture medium was replaced with Neuron induction medium-2 containing Neurobasal Medium and DMEM/F12 (3:1) supplemented with 0.5% N2, B27, 20 ng/ml BDNF, and 200 mM ascorbic acid. 7 days later, the cells were collected and stained with anti-III tubulin antibody. For astrocyte differentiation, the neural stem cells were cultured in Astrocyte induction medium containing neurobasal medium, 10% FBS, 1× NEAA and 2 mM L-glutamine. 7 days later, the cells will be collected and stained with anti-GFAP antibody.

For OPC differentiation from NSCs, the neural stem cells were differentiated into OPCs with OPC induction medium containing DMEM/F12 supplemented with 20 ng/ml FGF2, 20 ng/ml PDGF-AA and 100 ng/ml SHH for 5 days. We used the Oligodendrocyte induction medium containing neural base medium supplemented with 0.4 ng/ml T3, 200 ng/ml SHH, 100 ng/ml noggin, 10 μM dibutyryl cyclic-AMP sodium salt, 100 ng/ml IGF-1 and 10 ng/ml NT3, to culture the OPCs for generating pre-oligodendrocytes and mature oligodendrocytes.

### Animal Preparation and ICH Model Establishment

Our animal experiment protocols were approved by the Animal Ethics Committee of Shenzhen Second People's Hospital. A total of 60 adult male C57/BL6 (20-25g) mice were included in this study. The mouse was anesthetized with intraperitoneal administration of a mixed solution containing 50 mg/kg ketamine and 5 mg/kg xylazine and was placed in a prone position on a stereotaxic frame, then an incision was made over the scalp. A borehole was drilled 2.5 mm lateral to bregma, and 0.5 μl of the collagenase solution (0.075 Units, Sigma, C5138) was injected 3 mm deep into the mouse brain by using a 26-gauge Hamilton syringe. The syringe was left in place for 10 minutes to prevent back-leakage before being withdrawn, than withdraw needle at a rate of 1 mm/min.

### Cell Transplantation

7 days after ICH surgery, mice were anesthetized using same methods of modeling and fixed on stereotaxic frame. Two boreholes were drilled anteriorly and posteriorly with 1mm distance to the ICH borehole respectively. 25,000 OPCs in 1μl of saline were injected 3 mm into the brain at each borehole. The whole procedure was limited to 10 minutes, then wait for 5 minutes to withdraw the syringe at a rate of 1mm/min.

### Behavior Test

Mice gaits were recorded and analyzed by the Cat-Walk XT system (Noldus Information Technology, Wageningen, Netherlands). Cat Walk XT contained hardware part of a glass walkway plate with green light, an inverted high-speed video camera, and a software package for quantitative gait assessment. The footprint area was illuminated and scattered when mice walked through the glass plate and captured by the inverted camera.

Mice were trained to walk fluently through the glass walkway for 5 days before collagenase injection. Each mouse needed to complete at least 3 runs. A typical run contained at least 3 step cycles, and we calculated the average data of the parameters from all step cycles.

### Western-Blot Analysis

The mice were overdose anesthetized and sacrificed via decapitation. Right striatum was collected and well mixed with sufficient volume of cold RIPA lysis buffer that containing protease inhibitor cocktail. Protein concentration was then measured with a BCA kit after the extraction. Proteins (10μg-20μg) were later loaded into 8 to 10% SDS-polyacrylamide gel. The proteins were then transferred to nitrate cellulose membrane, and blocking by 5% non-fat milk, further incubated at 4°C overnight with primary antibody. Washed membrane with TBST for three times with 10 mins each, and then incubated with secondary antibodies for another 2 h at room temperature, finally detected signals by using enhanced chemiluminescent (Thermos Fisher Scientific).

### Statistical Analysis

All data were presented as mean ± SEM. Statistical analyses were conducted by IBM SPSS 22.0 software. For behavior and molecular tests, statistical analyses were processed by one-way ANOVA followed by post hoc tests for between-group comparisons. $P < 0.05$ after Tukey adjustment for multiple comparisons was considered statistically significant.

## Results

### Immortalization of Human Neural Stem Cells

Previous literature described that TERT and BMI1 could be used to immortalize kinds of stem cells [16]. We immortalized the human neural stem cells by transfecting TERT and BMI1 gene through lentivirus which carry GFP gene (Figure 1A). The immortalized neural stem cells sustained a strong proliferation ability and expressed GFP protein (Figure 1B). We use a TRAP assay kit (Millipore Catalog No. S7700) to detect the telomerase activity of the immortalized neural stem cells. In Figure 1C, we know that the telomerase activity of immortalized neural stem cells was significantly increased, and the 293T cell is positive control, while the 293T cell heat treatment is negative control for the activity loss when heat. The two templates are positive control, and the two immortalized NSCs groups have strong signal, while the two groups of heat treatment for the immortalized NSCs are negative (Figure 1C). These results suggested that the neural stem cells were immortalized successfully.

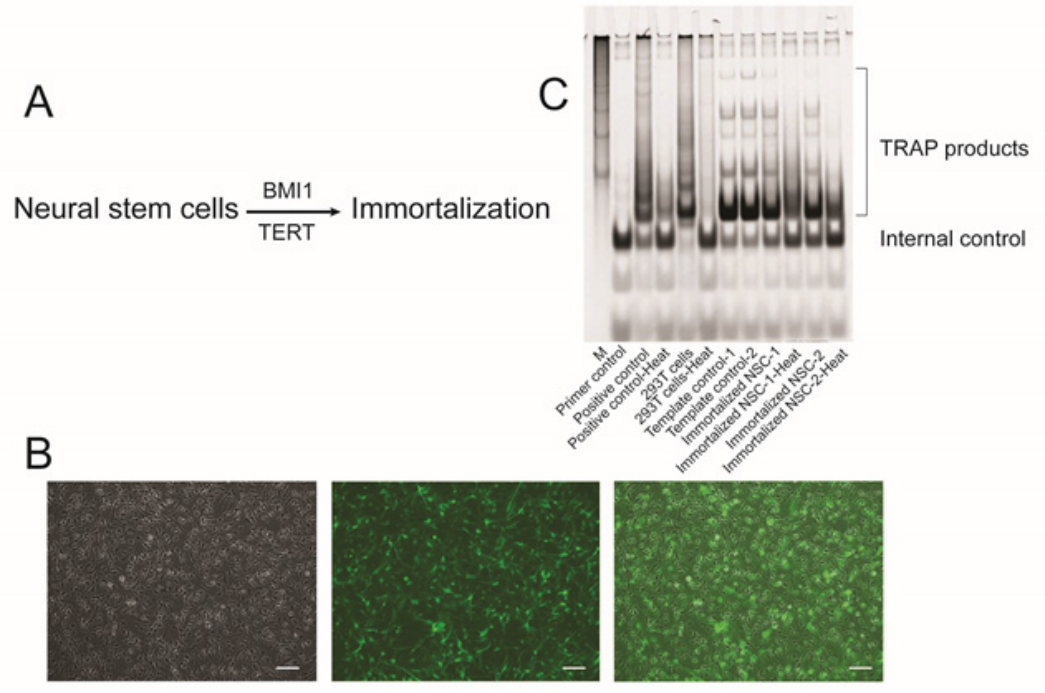


**Figure 1:** Immortalization of the human neural stem cells.

A. Immortalization of the neural stem cells with TERT and BMI1.

B. Images of the attachment culture for the immortalized neural stem cells. Scale bar, 100 μm.

C. The telomerase activity test of the immortalized human neural stem cells.

### Oligodendrocyte Progenitor Cells Were Produced from Human Neural Stem Cells

Neural stem cells could differentiate to several kinds of neural cell types with conditional culture medium [17]. We have a try to differentiate the neural stem cells to neurons, astrocytes, and oligodendrocytes. The neuron differentiation protocols were described in a Figure 2A. We used neuron induction medium to generate neurons from the neural stem cells, and the neuron marker, a-III tubulin, was highly expressed in the differentiated neurons (Figure 2B). Meanwhile, we generated astrocytes with conditional culture medium, and the astrocytes could express the astrocyte specific marker, GFAP (Figures 2C & 2D). We also developed a OPC differentiation protocol to generate OPCs (Figure 3A). We can see the OPC morphology after differentiation (Figure 3B), and a series of OPC markers were expressed in the induced OPCs (Figure 3C).

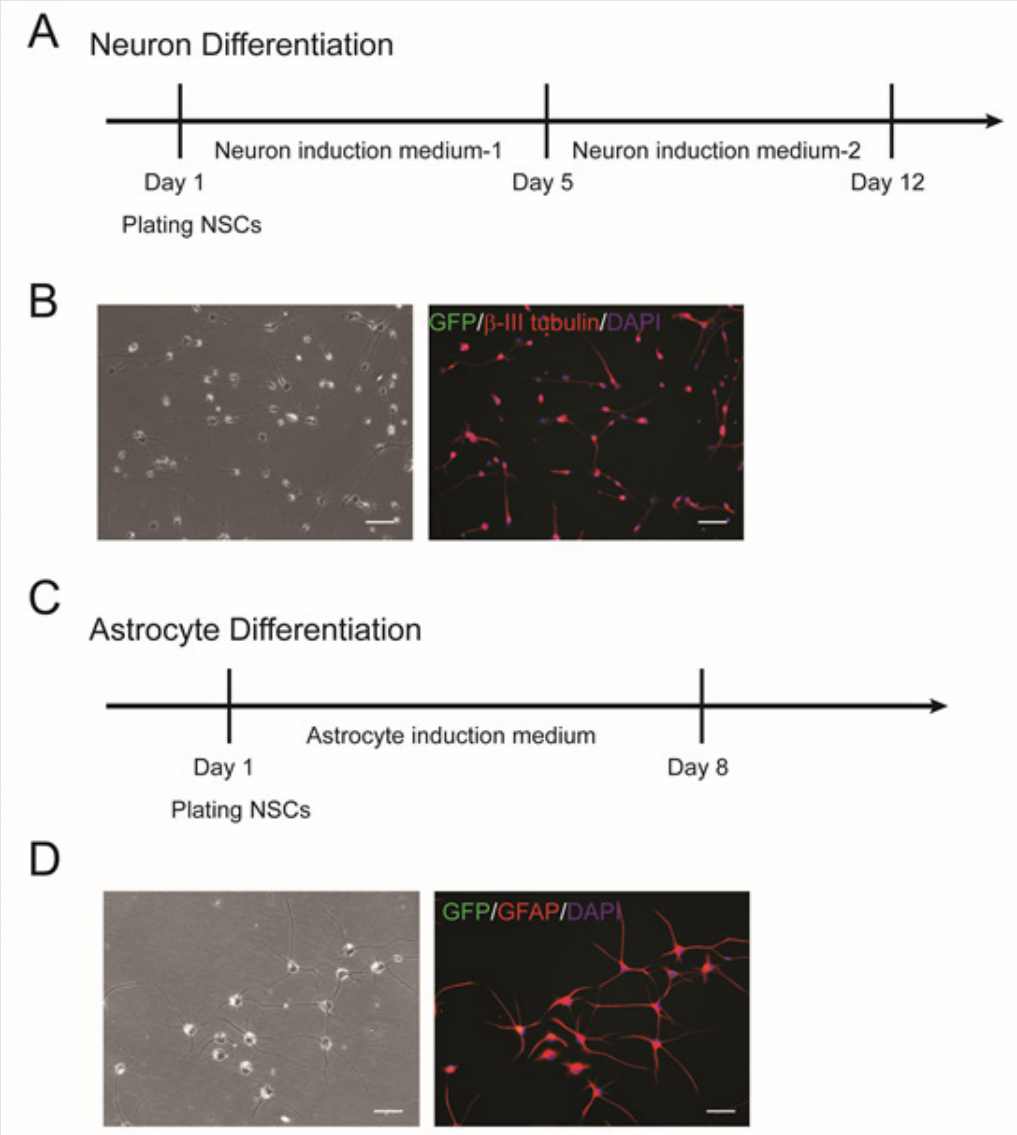


**Figure 2:** Differentiation of neural stem cells into neurons and astrocytes.

A. Schematic of the neuron differentiation protocol from neural stem cells.
B. The differentiated neurons are stained with β-III tubulin antibody. Scale bar, 50 μm.
C. Schematic of the astrocyte differentiation protocol from neural stem cells.
D. The differentiated astrocytes are stained with GFAP antibody. Scale bar, 50 μm.

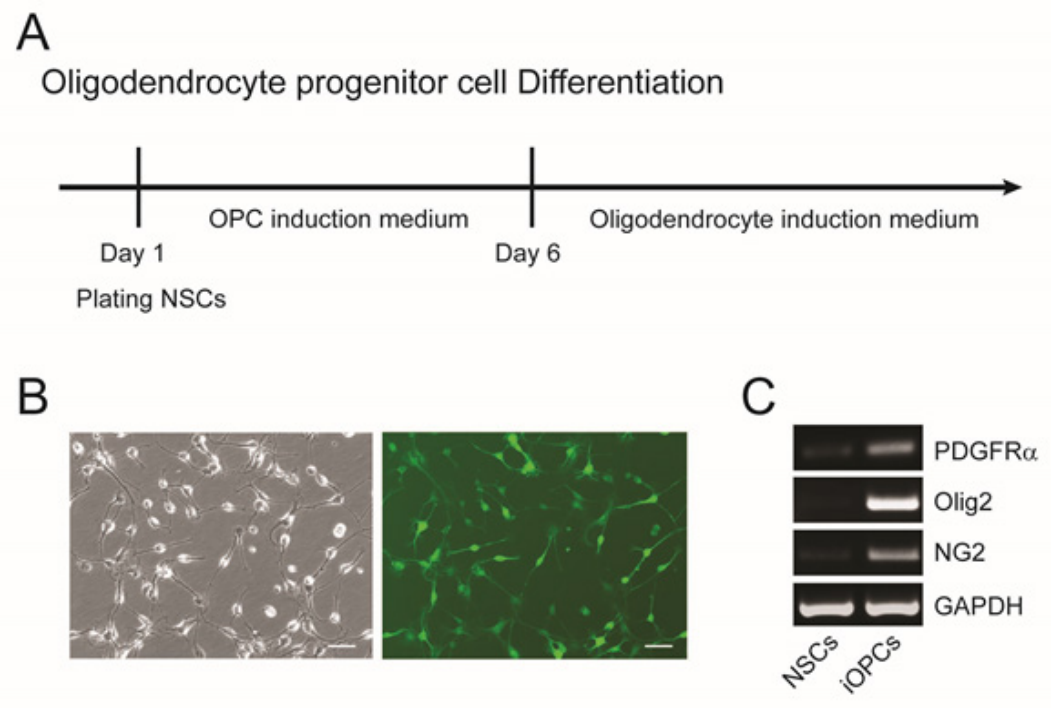


**Figure 3:** Differentiation of neural stem cells into oligodendrocyte progenitor cells (OPCs).
A. Schematic of the oligodendrocyte progenitor cell differentiation from neural stem cells.
B. Images of the differentiated OPCs from neural stem cells. Scale bar, 50 μm.
C. RT-PCR for detecting the expression of OPC markers.

## Locomotors Function Detection for Ich Mice Transplanted with Opcs by Catwalk System

To study the influence of OPCs transplantation on neuron regeneration, we have a try to establish mouse ICH model. Bacteria collagenase was injected in the right side of striatum, and the hematoma formation will be in the right striatum. Three days after ICH surgery, the mice were given an overdose of phenobarbital and perfused. Brain corona dissection showed the different extents of hemorrhage from anterior-posterior coordinates in the right striatum, and it confirmed that we established the ICH mouse model successfully (Figure 4A). Cat Walk gait analysis system is a highly sensitive tool for assessing gait and locomotion [18]. The mouse traverses a glass plate voluntarily (towards a goal box), and its footprints are captured (Figure 4B). CatWalk visualizes the prints and calculates data related to print dimensions and the relationship of the time and distance relationships between footfalls. The parameters we selected to assess the mice locomotor function are Stand time, Swing speed, and Step cycle. Swing Speed is calculated for the normal groups and the OPCs treated groups, and the statistic result showed a similar gait style in the mice treated with OPCs to the Normal group (Figure 4C). Stand time describes the duration of contact of an individual paw with the glass plate. The bar chart illustrates ICH mice treated with OPCs performed the similar locomotor function compared to the normal mice, while ICH mice take a longer duration of stand (Figure 4D). Step cycle describes the duration of two consecutive initial steps by the same paw, and the OPC treated mice take a short time, closed to the level of normal mice (Figure 4E). According to the gait analysis results, we concluded that OPC transplantation can improve the mice locomotor function after ICH.

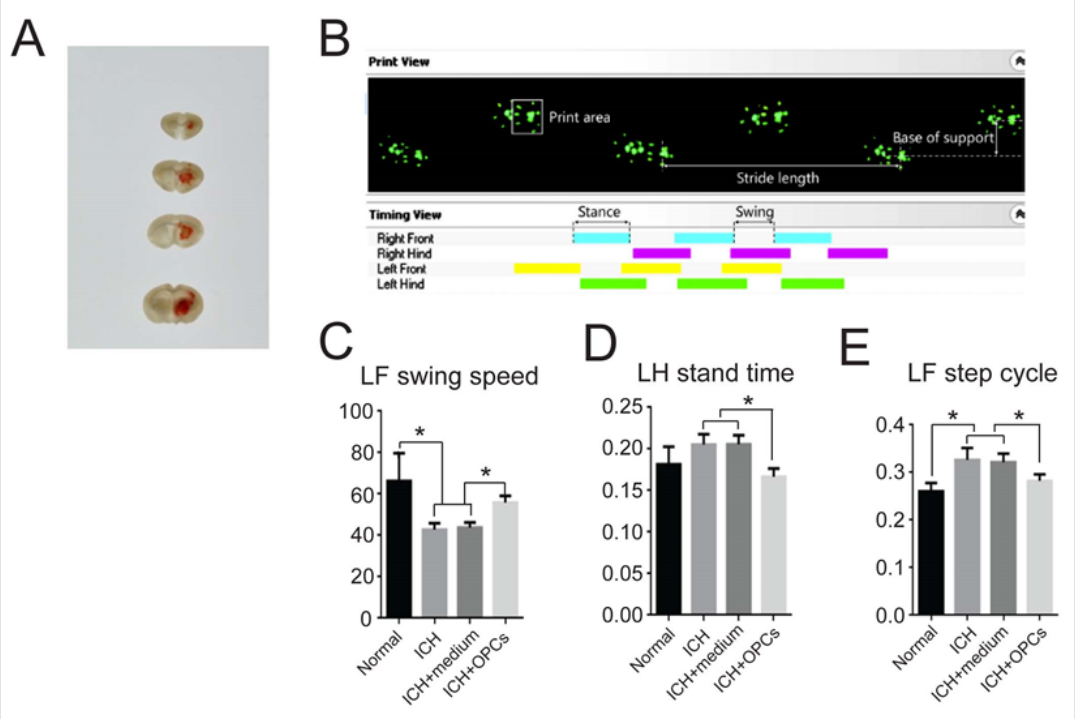


**Figure 4:** Mouse ICH model establishment and Catwalk system.

A. The mice were anaesthetized and perfused, and various amounts of hemorrhage were presented on the brain section.

B. Footprint is captured with green light from the Catwalk system.

C. Swing speed of left forelimb (LF).

D. Stand time of left hindlimb (LH).

E. Step cycle time of the left forelimb. "*" indicates $p<0.05$.

## Transplantation of Opcs Promoted the Expression of Neuron Markers

Striatum Neurofilament-L (NF-L), Neurofilament-M (NF-M), and Microtubule associated protein 2 (MAP2) was investigated by Western blot analysis after behavior test, in order to evaluate the difference on neuron regeneration introduced by the different treatments for the ICH mice. Neurofilaments is necessary for the maturation of regenerating myelinated axons in mice [19]. For evaluating the axon regeneration of ICH model injected with OPCs, we assessed the expression of neurofilament in the corticospinal tract tissue of Pons for the surgical control (ICH group), ICH mice injected with medium (medium groups), and ICH mice injected with OPCs (OPC groups). We found that the expression of neurofilament-L was increased in the OPC-treated groups compared to the ICH groups (Figures 5A & 5B). Microtubule-associated protein 2 (MAP2) is a sensitive marker of seizure-related brain damage [20]. In our result, OPCs transplantation induced the expression of MAP2 compared to the ICH groups (Figures 5C & 5D). Thus, then we concluded that OPCs transplantation could promote the neuron regeneration of ICH mice.

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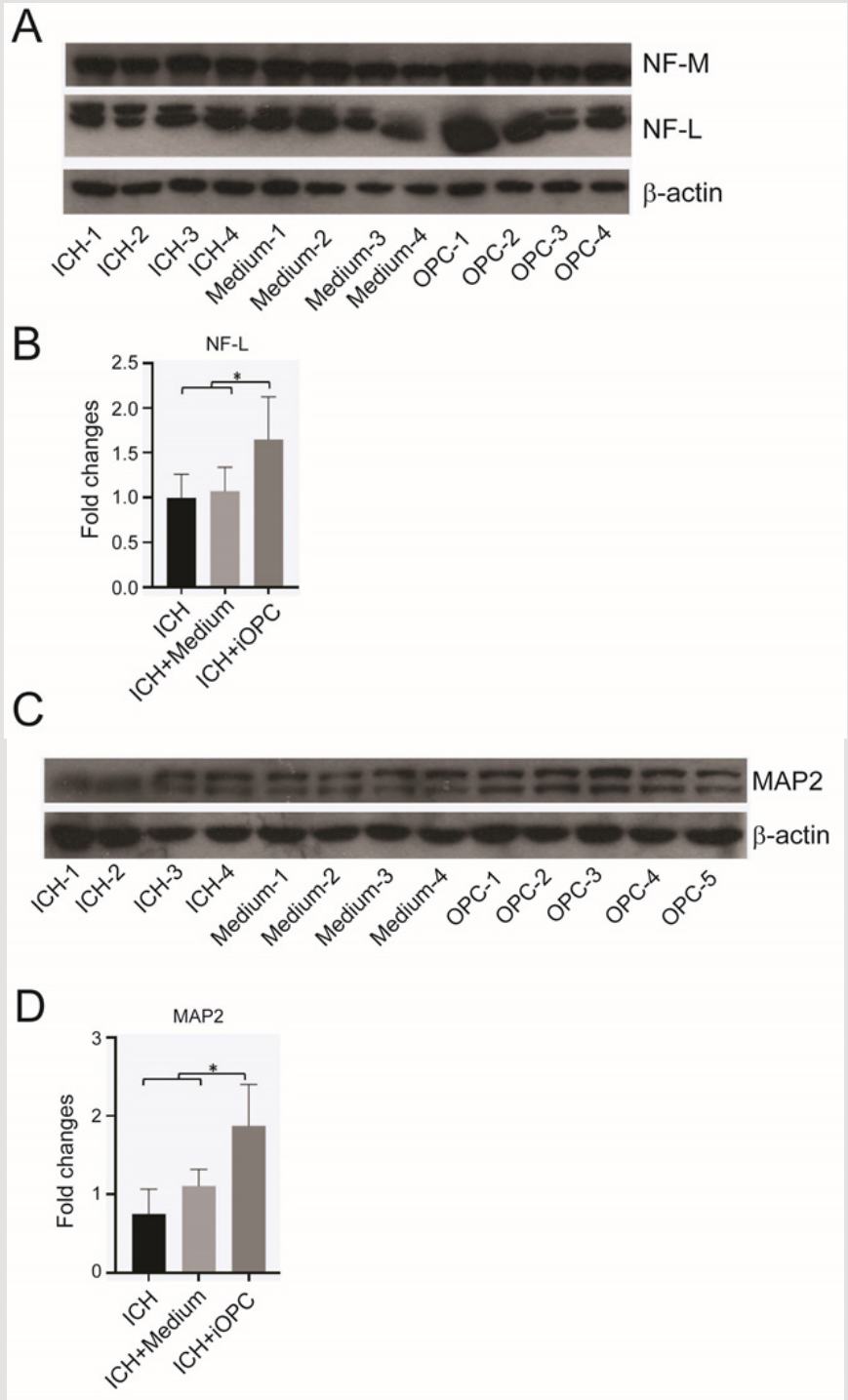


**Figure 5:** The expression of NF-M and NF-L in corticospinal tract of surgical control mice (ICH), ICH mice injected with medium (medium), and ICH mice injected with OPCs (iOPC).

A. The expression of NF-L and NF-M in the three groups.

B. The quantitative statistic for the expression of NL-L. "*" indicates $p<0.05$.

C. The expression of MAP2 in the three groups.

D. The quantitative statistic for the expression of MAP2. "*" indicates $p<0.05$.

## Discussion

Grafting of neural stem cells into experimental ICH in mice has been shown to promote neurological recovery, [13,14] and the mechanism could be that of neuro-regeneration. [21] More recently, oligodendrocyte progenitor cells (OPCs) can be reprogrammed from fibroblasts, using iPSC (induced pluripotent stem cell) technology, by over-expression of several transcription factors, such as Sox 10, Olig02 and Zfp536. [22,23] These OPCs derived from human iPSC has been shown to promote myelination and axon regeneration in a murine model of congenital hypomyelination. [24] This concept of utilizing a specialized OPCs generated from neural stem cells (NSCs) has inspired us to develop the topical OPCs treatment for murine ICH. In our study, we have generated OPCs from immortalized human NSCs and demonstrated that their transplantation in murine ICH improves neurological outcome neuro-regeneration, although the underlying mechanism remains unclear. MicroRNAs networks are dynamically regulated in developing oligodendrocytes, and microRNAs modulates oligodendrocyte progenitor cell development and differentiation. [25] During the induction of OPCs from NSCs, micro RNAsmay play essential roles in regulating the expression of OPC marker and mediate the proliferation and survival, and the regulation mechanism is worth further exploring. In the current study, we have established some preliminary data that the murine ICH model treated with NSCs-induced OPCs could promote axon regeneration after ICH as reflected by the statistically increased levels of Neurofilament-L (NF-L), MAP2, and an improved performance in three Catwalk parameters.

## Ethical Approval

The procedures involving animals and their care were conducted under the approval of the Animal Ethics Committee of the Second People's Hospital of Shenzhen.

## Conflicts of Interest

There are no conflicts of interest.

## Declarations of Interest

NO.

## Ethics Declarations

The procedures involving animals and their care were conducted under the approval of the Animal Ethics Committee of the Second People's Hospital of Shenzhen.

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