

Comparison of intracerebral transplantation effects of different stem cells on rodent stroke models

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In the present study, induced pluripotent stem cells (iPSCs), induced neural stem cells (iNSCs), mesenchymal stem cells (MSCs) and an immortalized cell line (RMNE6), representing different characteristics of stem cells, were transplanted into normal and/or injured brain areas of rodent stroke models, and their effects were compared to select suitable stem cells for cell replacement stroke therapy. The rat and mice ischaemic models were constructed using the middle cerebral artery occlusion technique. Both electrocoagulation of the artery and the intraluminal filament technique were used. The behaviour changes and fates of grafted stem cells were determined mainly by behaviour testing and immunocytochemistry. Following iPSC transplantation into the corpora striata of normal mice, a tumour developed in the brain. The iNSCs survived well and migrated towards the injured area without differentiation. Although there was no tumourigenesis in the brain of normal or ischaemic mice after the iNSCs were transplanted in the cortices, the behaviour in ischaemic mice was not improved. Upon transplanting MSC and RMNE6 cells into ischaemic rat brains, results similar to iNSCs in mice were seen. However, transplantation of RMNE6 caused a brain tumour. Thus, tumourigenesis and indeterminate improvement of behaviour are challenging problems encountered in stem cell therapy for stroke, and the intrinsic characteristics of stem cells should be remodelled before transplantation. Copyright © 2015 John Wiley & Sons, Ltd.

KEY WORDS—iPSCs; iNSCs; MSCs; immortalized GABAergic neuronal progenitor cell line; cerebral ischaemia; cell transplantation

INTRODUCTION

It is well known that injuries in the central nervous system caused by many different disorders, including stroke, are difficult to recover from, and to date, there have been no effective therapies for stroke recovery. Using surgical decompression or thrombolytic therapy in clinical practice may have some benefit but only in the early stages after stroke because the time window for these interventions' efficacy is very short. As for the chronic stage of stroke, only some supportive and symptomatic treatments are applied solely for functional rehabilitation.¹

Recently, intracerebral transplantation of stem cells has been considered a promising approach for treating stroke. The stem cells can continuously self-renew and differentiate into multiple lineages, which may replace the damaged brain areas.^{2,3} As a result, quite a few different stem cells, including neural stem cells (NSCs),^{4,5} embryonic stem cells (ESCs),^{6,7}

mesenchymal stem cells (MSCs)^{8,9} and the induced pluripotent stem cells (iPSCs),^{10–12} are already used for treatment of stroke lesions in animal models. The results reported from these studies seem to be promising, showing that the grafted cells could survive, proliferate, migrate and differentiate into functional neurons, some of which even established synaptic connections to the host cells. The sensorimotor function of the model animals was also shown to be partly improved.^{13,14}

However, several problems are associated with using stem cell therapy for brain injury repair, including poor availability of the cells, immune rejection from the host and uncertain fate in the brain after grafting, which present obstacles for clinical use of stem cell therapy. To solve these problems, the selection of specific and suitable stem cells should be a primary approach for stroke treatment because different stem cells have different characteristics.

Among stem cells that have already been tested for stroke treatment, MSCs seem to be promising candidates. After transplantation into the ischaemic brain of animal models, the MSCs appeared to survive well, migrate to the injured area and improve animal behaviour,^{15,16} although differentiation in the brain was very rare. Recently, other novel stem

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cells have emerged and been reported to have good prospects for neural restoration. These include iPSCs,^{17,18} the induced neural stem/progenitor cells (iNSCs)^{19,20} and a thermally controlled immortalized γ -aminobutyric acid (GABA)ergic neuronal progenitor cell line (RMNE6).²¹ All types were found to be, to a certain extent, capable of overcoming the limitations and challenges in the practical application of stem cells. It is worthwhile, therefore, to explore and compare the effects of these newly created stem cells in conjunction with MSCs on the improvement of behaviour and neural repair of rodent stroke models following intracerebral transplantation of the cells. Both mice and rats were chosen as models because the cells have different origins and the xenogenic immune rejection should be avoided. Middle cerebral artery occlusion (MCAO) was performed in these animals to observe therapeutic effects. The present study's results, however, have not demonstrated striking curative effects in these models, indicating that the approach of stem cell therapy for stroke needs further refinement.

MATERIALS AND METHODS

Animals and grouping

The animal studies were approved by the guidelines of the Institutional Animal Care and Use Committee of the Capital Medical University. Adult male C57BL/6J mice weighing 25–30 g and adult male Sprague–Dawley rats weighing 230–250 g were used for the study. The animals were housed under a 12 h light–12-h-dark cycle with free access to food and water.

The mice were divided into three experimental groups: sham operation, vehicle and iNSC groups ($n=8$ each). The rats were also divided into three experimental groups: vehicle, MSC and RMNE6 groups ($n=8$ each). The transplantation or injection was performed on the third day after MCAO.

Cell preparation

The iPSCs were from the Institute of Zoology, Chinese Academy of Sciences, and were cultured according to the methods provided by the literature.²² The iNSCs labelled with green fluorescent protein (GFP) were prepared by the Xuanwu Hospital Capital Medical University.²⁰ The MSCs were isolated from male Sprague–Dawley rats by adherent culture. A retrovirus plasmid, pLXSN-enhanced GFP (eGFP), was transduced into the PT67 packaging cell line, and the MSCs were then transfected with the conditioned medium collected from the retrovirus-producing cell line PT67/eGFP. After being selected with G418, the GFP-marked MSCs were cultured in α -MEM medium (Invitrogen USA) containing 10% foetal bovine serum (FBS) (Invitrogen USA) at 37 °C in a humidified 5% CO₂ atmosphere, passaged every 3–4 days. The medium was changed every alternate day. The immortalized GABAergic neuronal progenitor cell line (RMNE6)²¹ was created in the Beijing Institute of Neuroscience and was treated using the following methods. The RMNE6 line grew in the

DMEM/F-12 (Invitrogen USA) containing 10% FBS, was incubated in the 37 °C and 5% CO₂ incubator (Heraeus Germany) and was passaged every 3 days without changing the medium.

Focal ischaemic models

All animals were anaesthetized with 6% chloral hydrate (6 ml kg⁻¹, i.p.). Body temperature was maintained at approximately 37 °C using a heating bed during the surgical procedures. MCAO in the mice was performed by electrocoagulation. An approximately 1-cm incision was made on the right face between the outer canthus and the ear. The temporal fascia and temporal muscle were bluntly separated. The skull was opened with a dental drill and bitten away with microforceps to expand the operation field. The cerebral dura mater was torn off before the MCA was fulgurized with an electrocoagulation pen. In the sham-operation group, the skulls were only opened and the middle cerebral arteries were not coagulated.

Focal brain ischaemia in the rats was induced by the intraluminal filament technique. A midline skin incision was made in the neck, exposing the left common carotid artery (CCA), external carotid artery and internal carotid artery. A monofilament nylon thread (40 mm) with a 0.34-mm-diameter tip was advanced from the left CCA bifurcation until it blocked the origin of the MCA. Following the operation, the animals were kept warm on an electric blanket until wake.

Transplantation procedure

The cells were dissociated with trypsin and washed with phosphate buffer solution (PBS) for three times. Any mouse embryo fibroblasts (MEF) were removed from iPSCs. The cell density was adjusted to 1×10^5 – 1×10^6 μl^{-1} and placed in ice to prepare for transplantation. All animals were anaesthetized with 6% chloral hydrate (6 ml kg⁻¹, i.p.) and fixed in a stereotaxic instrument (David Kopf, USA) on the third day after MCAO. A midline skin incision was made in the skull with subsequent drilling for a burr hole. Cells were then stereotaxically injected into the corresponding positions of the normal and ischaemic mice and rats using a Hamilton syringe (Table 1).

The injection speed was 1 $\mu\text{l min}^{-1}$ and controlled by a syringe pump. The needle was retained in place for an additional 10 min before slowly retracting 1 mm every 3 min. The vehicle group received 0.01 M PBS using the same method.

Behavioural tests

Each animal was subjected to a series of behavioural tests at different time points. These tests included the corner test and cylinder test in the mice and the rotarod test in the rats. Behavioural tests were started the day after MCAO and were then performed on days 7, 14, 21 and 28 after transplantation or injection of PBS in both cell transplantation and vehicle groups and on days 1, 10, 17, 24 and 31 after MCAO in the sham-operation group.

Table 1. The dose and transplantation site of different cells

Cells	Animal	Cell number	Position	Coordinate		
				AP (mm)	L (mm)	DV (mm)
iPSCs	Normal mouse	2×10^5	Corpus striatum	-1.5	2	4.5
iNSCs	Normal mouse	3×10^5	Cortex	-0.5	2	3
	MCAO mouse	3×10^5	Right cortex	-0.5	2	3
MSCs	MCAO rat	1×10^6	Left corpus striatum	0	3	5.5
RMNE6	MCAO rat	1×10^6	Left corpus striatum	0	3	5.5

AP, anteroposterior to the bregma; L, lateral to the bregma; DV, dorsoventral from the skull.

Corner test

This test was carried out as described by Zhang *et al.*²³ with a slight modification. The mouse was placed between two pieces of 30×20-cm cardboard. The two boards were gradually moved in from both sides to encourage the mouse to enter a 30° corner with a small opening along the joint between the two boards. When the mouse entered the deep part of the corner, both sides of the vibrissae were simultaneously stimulated by the two boards. The mouse reared forwards and upwards, then turned back to face the open end. Twenty trials were performed for each mouse, and the percentage of right turns was calculated. Only turns involving full rearing along either board were recorded. This test detected the integrated sensorimotor function, as it involves both stimulation of the vibrissae (sensory/neglect) and rearing (motor response).

Cylinder test

The cylinder test¹¹ was adapted for assessing forelimb usage and rotation asymmetry in the mouse. The mouse was placed in a transparent cylinder 9 cm in diameter and 15 cm in height and was videotaped during the test. A mirror was placed behind the cylinder at an angle to enable the rater to record forelimb movements when the mouse was turned away from the camera. After the mouse was put into the cylinder, the first forelimb contact against the wall after rearing and during lateral exploration was recorded using the following criteria: (1) When the forelimb first contacted the wall during a full rear, it was recorded as an independent wall placement for that limb, (2) simultaneous use of both the left and right forelimbs by contacting the wall of the cylinder during a full rear and lateral movements along the wall were recorded as 'both' movement, (3) when one forelimb (right forelimb for example) placed first and kept contact with the wall followed by the other forelimb (the left in this instance), a 'right forelimb independent' movement and a 'both' movement were recorded; however, if the other (left forelimb) made several contacting movements on the wall, a 'right forelimb independent' movement and only one 'both' movement were recorded, and (4) when the mouse explored the wall laterally, alternating both forelimbs, it was recorded as a 'both' movement. A total of 20 movements were recorded during the 10-min test. The final score was determined according to a previous formula

described in the rat.¹⁰ That is, $\text{score} = (A - B)/(A + B + C)$ (A, unimpaired forelimb movements; B, impaired forelimb movements; C, both movements). This test evaluated forelimb use asymmetry for weight shifting during vertical exploration.

Rotarod test

The rotarod test was performed to evaluate the degree of hemiparesis and coordinated movements in rats. Before MCAO, all rats were trained to stay on an accelerating rotarod (slowly increasing from 4 to 40 rpm within 5 min) until they could remain on the rotarod for ≥ 200 s. After MCAO, the time that they could remain on the same accelerating rotarod was measured at different time points. The data were presented as a percentage of the mean time (three trials) on the rotarod compared with the baseline time obtained before the MCAO.

Drawing materials and sectioning

The cell-transplanted rodents were perfused with 0.9% physiological saline to rinse the blood and were subsequently perfused with 4% paraformaldehyde (PFA) to fix the brain tissue on day 28 after transplantation. The iPSC-transplanted mice underwent the same procedures on days 14 and 28 after transplantation. The brains were removed and fixed in 4% PFA again for 4–6 h and then sunk to the bottom of 30% sucrose.

The coronal sections with a thickness of 20–30 μm from the whole brain were cut in series with a freezing microtome set (Leica Germany). Sections were mounted on slides and prepared for staining with haematoxylin and eosin (HE) or an immunohistochemical method.

HE staining

The mice's slides were routinely stained with haematoxylin (0.2%) and eosin (0.5%) 2 weeks after iPSC transplantation.

Triphenyltetrazolium chloride (TTC) staining

To determine whether the MCAO model was successfully established, as well as to determine infarction location and size, TTC staining was performed in the mice and rats that underwent MCAO. The additional mice and rats that underwent MCAO were anaesthetized and sacrificed the

day following ischaemia. The brains were removed immediately and cut into a series of 1-mm coronal slices. These slices were stained in 2% TTC (Sigma USA) in PBS at 37 °C for 15–30 min and protected from light.

Immunohistochemical investigation

Immunohistochemical staining of neuron-specific nuclear protein (NeuN) or glial fibrillary acidic protein (GFAP) was performed to reveal whether the transplanted iNSCs and MSCs predominantly differentiated into neurons or astrocytes. Hoechst 33258 staining was used to evaluate cell viability. Double staining of Simian virus 40 (SV40)/GABA was used to trace the immortalized GABAergic neuronal progenitor cells (RMNE6).

From each brain, a set of 12 slices were washed with 0.3% phosphate buffered saline with Triton X-100 (PBST) and then incubated in 3% donkey serum or 10% goat serum in 0.3% PBST at room temperature for 1 h and then respectively incubated in mouse anti-NeuN antibody-conjugated biotin (Chemicon Millipore USA, 1:200), rabbit anti-GFAP antibody (Abcam UK, 1:5000), mouse anti-SV40 antibody (Santa Cruz USA, 1:50) or rabbit anti-GABA antibody (Sigma USA, 1:1000) with 1% donkey serum or 10% normal goat serum at 4 °C overnight. The sections were then washed for three times in PBS with subsequent incubation in streptavidin–cyanine 3 (Cy3; Jackson USA, 1:400), donkey anti-rabbit Cy3 (Jackson USA, 1:400), goat anti-rabbit Alexa Fluor 594 (Invitrogen USA, 1:500), goat anti-mouse Alexa Fluor 594 (Invitrogen USA, 1:500) or goat anti-rabbit

Alexa Fluor 488 (Invitrogen USA, 1:500) for 2–3 h. After the sections had been washed in PBS, the nuclei were stained by Hoechst 33258 for 10 min. Finally, the slides were cover-slipped using mounting medium. The primary antibody was substituted for 3% donkey serum or 10% normal goat serum in PBS in the control studies.

Statistical analysis

All behavioural data were expressed as mean \pm SEM and analysed using two-way (time and treatment) ANOVA of repeated measures. $P < 0.05$ was considered statistically significant.

RESULTS

MCAO model

An ischaemic area was clearly seen in the cerebral cortex of mice and in the cerebral cortex and corpus striatum of rats after (Figure 1A and 1B) using TTC staining. The behaviour experiments also proved that the MCAO model construction was successful in the mice and the rats. Both corner and cylinder tests showed that the MCAO mice could develop movement disorders. The scores in the vehicle group, in which MCAO with injection of PBS was successful, were higher than those in the sham group (Figure 1C and 1D). The rotarod test showed a lack of motor coordination, indicated by a $<100\%$ for the duration of motor testing in all groups (Figure 2A).

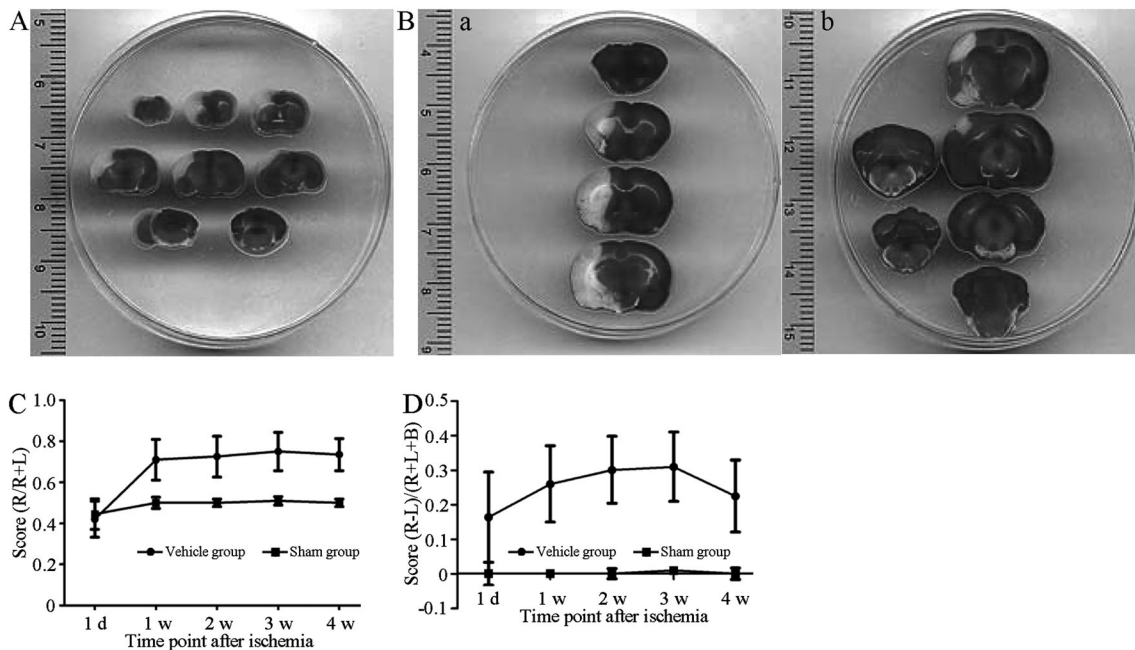


Figure 1. TTC staining: For the MCAO mouse by electrocoagulation, the white ischaemic area was located in the cerebral cortex (Figure 1A). Moreover, for the MCAO rat by the intraluminal filament technique, the white ischaemic area was located in the cerebral cortex and the corpus striatum (Figure 1B). The results of statistical analysis of behavioural tests indicated that the scores of the vehicle-group animals were higher than of the sham-group ones, having significant difference in the corner test ($F = 15.849$, $P < 0.001$) and the cylinder test ($F = 25.483$, $P < 0.001$; Figure 1C, 1D)

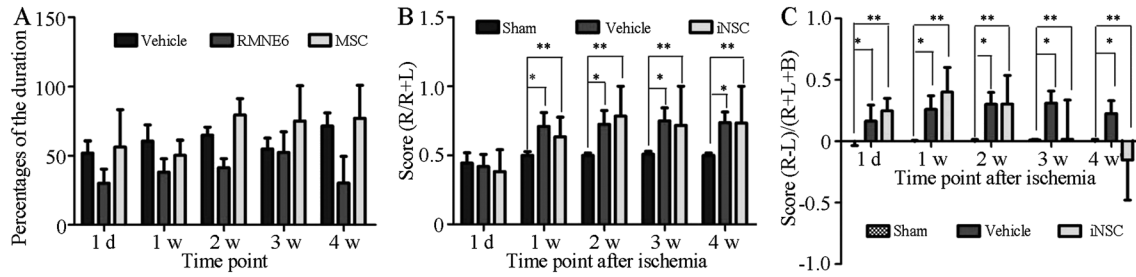


Figure 2. Changes of behaviour of MCAO animals at different time points. (A) Rotarod test, (B) corner test and (C) cylinder test. The scores of the corner test and the cylinder test had significant difference between the sham-operation group and the vehicle and iNSC groups, but there was no statistical difference between the vehicle group and the iNSC group. The percentages of rotational durations relative to the pre-MCAO level had no statistical difference between the vehicle group, MSC group and RMNE6 group in the rotarod test. $*P < 0.05$ versus the vehicle group. $**P < 0.05$ versus the iNSC group

Animal survival after transplantation

In the MCAO model by electrocoagulation, the rate of survival of the mice is almost 100% 3 days after cerebral ischaemia. Moreover, the rate of survival of the normal and the model mice is also almost 100% after cell transplantation. However, the ischaemic mice by intraluminal filament technique can hardly survive until the third day on which cell transplantation is performed.

In the model rats by intraluminal filament technique, the rate of survival can reach to the above 50% on the third day after cerebral ischaemia. However, 0.5–1% rats can survive till the fourth week after cell transplantation.

Behaviour alternation

A significant difference ($P < 0.05$) in behavioural alternations was observed between the sham-operation group and both the vehicle group and the iNSC group. There was no statistical difference, however, between the vehicle group and the iNSC group ($P > 0.05$) in either the corner or cylinder tests (Figure 2B and 2C) nor was any difference noted among the vehicle, MSC and RMNE6 groups in the rotarod test ($P > 0.05$; Figure 2A). Therefore, cell transplantation in the present study seemed to have no significant effects on behavioural improvement in the MCAO animals.

Cell survival and migration

The three cell types (iNSCs, MSCs and RMNE6) survived well in the ischaemic brain after being implanted at the boundary zone adjacent to the ischaemic core (Figures 3A–F, 4A–C and 5A–C). The implanted iNSCs were densely distributed in the injected area, showing fusiform, triangular or quadrilateral shapes without obvious slender processes. The MSCs appeared spherical, ovoid and fusiform and had no developed processes. The RMNE6 cells were mainly fusiform, some of which tapered at both ends. Certain iNSCs and MSCs could clearly be seen migrating to the ischaemic area (Figures 3A and 4A), and the MSCs were especially found in the necrotic cavity and the necrosis area (Figure 4C). The iPSCs also survived in the corpus striatum of the normal mice. These cells with deeply stained nuclei were circular or elliptical with pale cytoplasm. The whole cell was strongly basophilic, and some cells were in the mitotic phase

(Figure 6A–C). The iNSCs also survived in the cortex of the normal mice (Figure 3G–I and 3J–L), although their number was much greater in the ischaemic cortex (Figure 3A and 3D) than in the normal cortex.

Cell differentiation

After transplantation of the GFP-marked iNSCs in the cerebral cortex of the normal mouse, few GFP/NeuN double-positive cells were seen in the normal cortex. A large part of the whole green cell was occupied by the oval, red, NeuN-positive nuclei marked with blue Hoechst 33258 so that the entire cell was yellow or nearly white (Figure 3G–I). There were no GFP/GFAP double-positive cells in the cerebral cortex of the normal mouse (Figure 3J–L). For those cells transplanted into the ischaemic brain, neither GFP/NeuN nor GFP/GFAP double-positive cells were found in the grafted area (Figure 3A–C and 3D–F).

Cellular differentiation of GFP-marked MSCs transplanted in the infarct area of the rats, reflected by the presence of double-labelled NeuN or GFAP cells, was not seen either (Figure 4A–C).

Tumourigenesis

Neoplasm growth was clearly seen in the brain of normal mice after iPSC transplantation. The growing neoplasm was prominent on the surface of the brain 4 weeks after the iPSCs were injected in the corpus striatum (Figure 6A). The cells continuously proliferated 2 weeks after transplantation but showed limited growth *in situ* (Figure 6C). They dyed deeply (Figure 6D and 6E), and some were in mitosis (Figure 6E). Similarly, neoplasm was also seen in the MCAO rats into which RMNE6 cells were transplanted (Figure 6B). No macroscopic tumour cells were found in either rodent's brains when iNSCs or MSCs were used for the transplantation.

DISCUSSION

In this study, four different kinds of stem cells (iPSCs, iNSCs, MSCs and RMNE6) were selected for transplantation into normal and ischaemic rodent models to explore and compare their efficacy in the treatment of cerebral

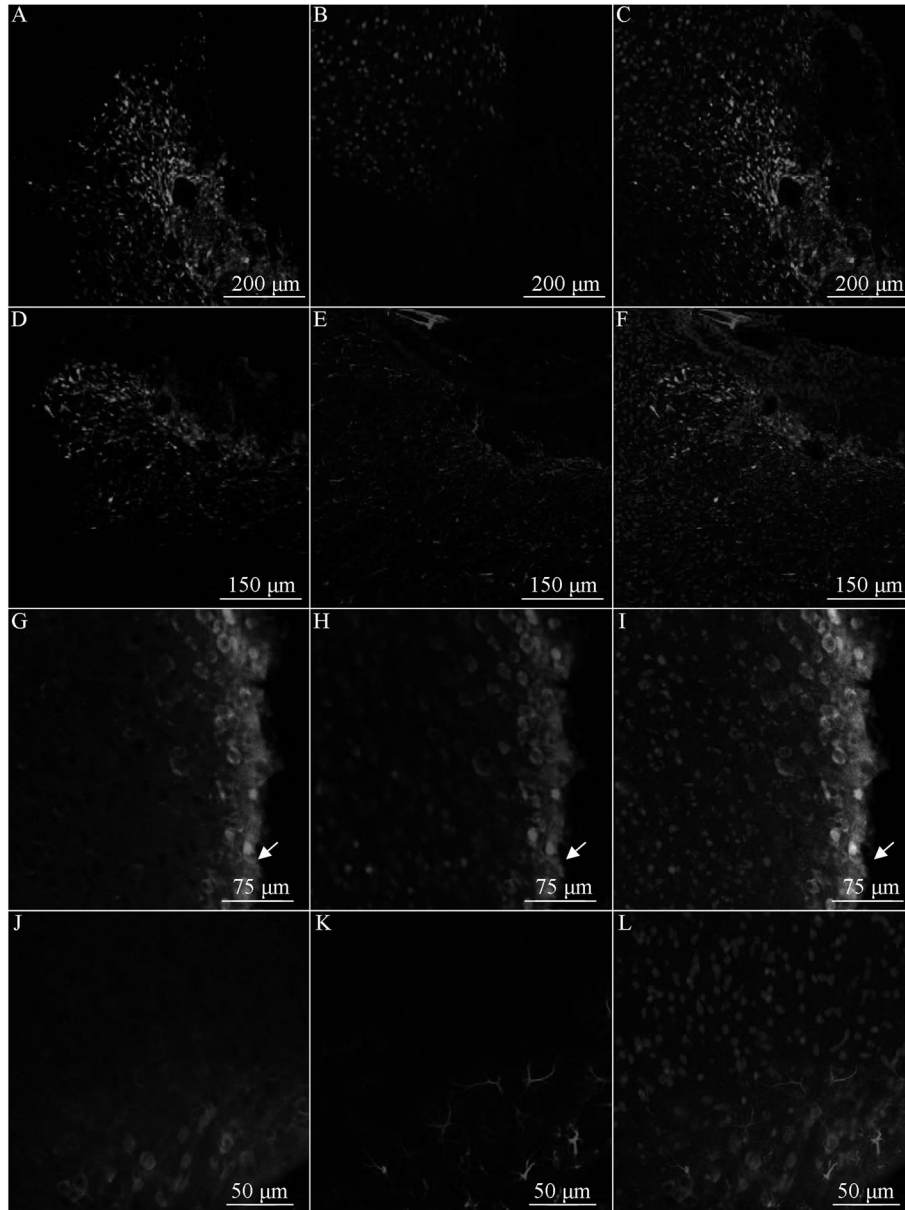


Figure 3. Survival, migration and differentiation of iNSCs. In the ischaemic cortex (A–F), the green GFP-labelled iNSCs were found near the necrotic area, but no GFP/NeuN (A–C) and GFP/GFAP (D–F) double-positive cells were found. NeuN (G–I) and GFAP (J–L) immunofluorescence staining demonstrated that there were a few of GFP and NeuN double-positive cells (arrow) and no GFP and GFAP double-positive cells in the normal cortex (G–L). [A, D, G and J, GFP (green); B and H, NeuN (red); E and K, GFAP (red); C, F, I and L, merged images; the blue represented nuclei stained by Hoechst 33258]

ischaemia because stem cells have long been proven to have favourable effects in treating conditions caused by stroke.^{2,3} These four cell types were considered the most representative for general use in cerebral grafting.

The intraluminal filament technique is more widely used; however, the method is not applicable in the mice of this experiment because of the high mortality rate. So, for the MCAO model, the electrocoagulation was used in the mice and the intraluminal filament technique was used in the rats. From the results of TTC, cerebral ischaemia injury is successful, although the size of ischaemia was different.

Although the two methods cannot be completely equal, the size of ischaemia has few effects on this experiment from the point of view of the fate of the transplanted cells in the brain. Because of the difference of the origin of these cells, the stem cells from the same source were for allogeneic transplantation in order to avoid immune rejection between species.

Since iPSCs were created by Takahashi and Yamanaka in 2006/2007, there have been several studies on their use in ischaemic brain injury therapy.^{10–12,17,18} The advantages of iPSCs are their easy availability, autologous transplantation

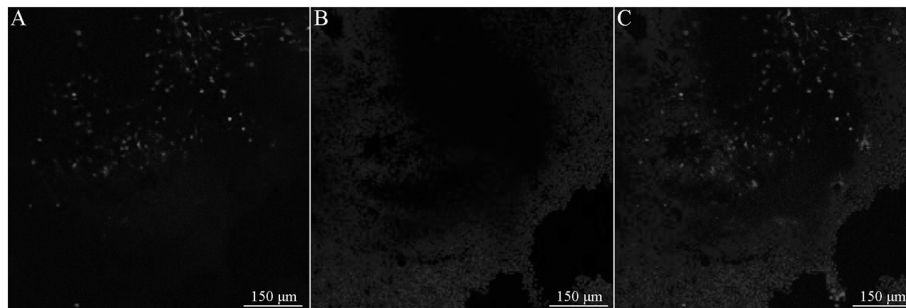


Figure 4. Survival and migration of MSCs in the ischaemic brain. The GFP-labelled MSCs (A) were located in and around the necrotic cavity (B). [A, GFP (green); B, Hoechst 33258 (blue); C, merged image]

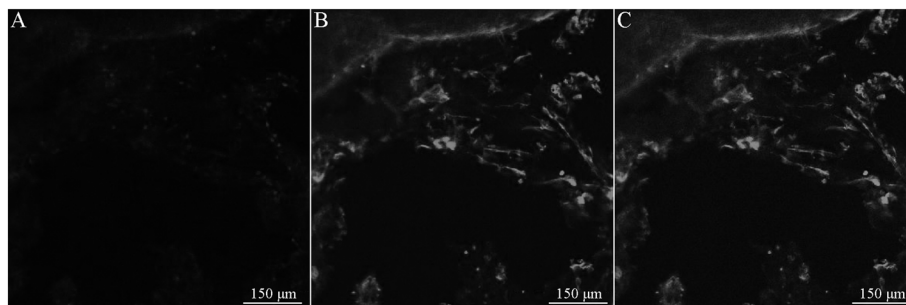


Figure 5. Survival of SV40/GABA double-positive RMNE6 cells in the ischaemic brain. The SV40 was located in the cell nuclei and stained with red, while the GABA was located in the cytoplasm and coloured by green. The whole cells were mainly fusiform, some of which tapered at both ends [A, SV40 (red); B, GABA (green); C, merged image]

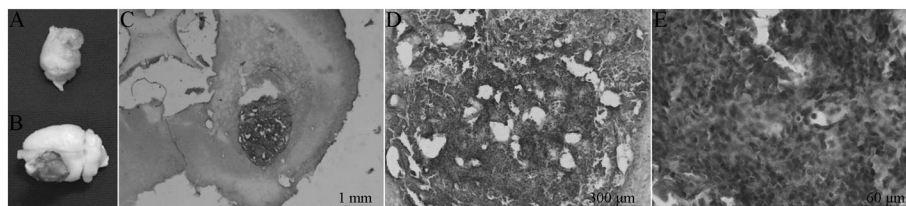


Figure 6. Tumourigenesis after cell transplantation. Panel A shows that a tumour appeared 4 weeks after iPSCs were injected into the corpus striatum of the normal mouse. Panel B shows that a neoplasm was raised 4 weeks after RMNE6 cells were implanted into the brain of the MCAO rat. Panels C, D and E are HE staining pictures and indicate tumour formation 2 weeks after iPSCs were injected into the corpus striatum of the normal mouse

without immune rejection and lack of ethical concerns compared with ESCs and NSCs.²⁴ Among individual reports, however, there has been no definitive answer on whether or not transplantation of iPSCs results in tumourigenesis.^{10–12} This may be due to factors differing between studies, including differences in transplant approaches and recipients' brain conditions.^{10–12} In a study reporting the neural differentiation of iPSCs without tumourigenesis in the ischaemic brain, the author suggested that microenvironments in the brain might be a potential factor for neural differentiation.¹² Regardless, iPSCs have the same high incidence of tumours as ESCs injected in the brain^{25,26} because iPSCs have similar morphological, functional and biological characteristics.^{17,18} That may explain why no consistent reports about the effects of iPSCs on the behaviour of animal models with cerebral ischaemia are currently available.^{10–12} In terms of cell behaviour, however, it is unsurprising that in the present

study, the iPSCs continued to proliferate after transplantation without exiting the cell cycle, as is consistent with other experimental results,^{10,11} and that tumourigenicity should remain a significant obstacle for using iPSCs as the donor cells. In fact, the original intention of this experiment is to explore iPSCs' effects on the stroke. However, iPSCs were found to form tumours in the normal brain of mice. Therefore, the iPSCs have not continued to be implanted in the ischaemic brain.

The iNSCs were similar to iPSCs, into which terminally differentiated somatic cells were reprogrammed and converted by several defined transcription factors.^{19,20} Unlike iPSCs, however, the iNSCs realized direct transdifferentiation between different germ layers without a pluripotent stage. In a sense, these cells were more advanced than the iPSCs, especially considering their lack of tumourigenicity.^{19,20} After injection into the dentate gyrus of the mouse, the iNSCs

could survive and differentiate into mature neurons and establish synaptic connections with host neurons.^{19,20} The iNSCs were not oncogenic in the brain, although they were transduced with the protooncogene *c-Myc*, which was also transduced in the iPSCs.^{17,18} These results indicated that the iNSCs could be a new source for cell replacement therapy. Unfortunately, in this study, only few iNSCs successfully differentiated into NeuN-positive cells or into GFAP-positive astrocytes in the normal cortex. Similar findings were obtained in the ischaemic cortex. It is possible that the neocortex does not provide a suitable environment for differentiation of the cells compared with the dentate gyrus, which has vigorous neurogenesis in the adult brain. It is therefore understandable that the behaviour of the model animals was not improved. Further study into the iNSC differentiation is needed, both *in vitro* and *in vivo*, because this study was the first to use iNSCs to treat ischaemic cerebral injury.

The RMNE6 cell line is an immortalized GABAergic neuronal progenitor cell line transduced with the SV40 T tsA58 gene.²¹ The RMNE6 cells can be considered neuronal precursor cells because they express several characteristic markers such as nestin, β -Tub-III, Microtubule-associated protein 2 (MAP-2) and GABA *in vitro* and cannot differentiate into the GFAP-positive astrocytes *in vivo*.²¹ Instead of integrating into the host brain and replacing the lost neurons, RMNE6 cells were expected to play a neuroprotective role in cerebral injury, as they can spontaneously express and release many neuroprotective factors such as nerve growth factor, brain-derived neurotrophic factor, neurotrophin-3, neurotrophin-4/5, and glial-cell-derived neurotrophic factor.²¹ In the present study, however, the cells proliferated and formed a neoplasm after being grafted in the ischaemic brain, although they failed to form tumours in the normal rat brain.²¹ This could be due both to the existence of tsA58 SV40 large tumour antigen in the RMNE6 cells and to the difference of the ischaemic microenvironment from the normal state in the brain.

The MSCs have historically been used to treat ischaemic brain injury and have the most experimental data of all cells assessed in this study. Many experiments have demonstrated that model animal behaviour is more or less improved after transplantation with MSCs, although cell differentiation is rare.^{15,16} It has also been shown that the well-survived MSCs and more marrow-derived cells can be seen in the ischaemic brain area even after intravenous administration.²⁷ The reason was ascribed to the penumbras, the area adjacent to the ischaemic core and robust expression of developmental proteins and genes, which simulates an embryonic tissue.^{28,29} Certain researchers discovered that the majority of grafted bromodeoxyuridine-labelled MSCs were located in the ischaemic boundary zone, and some of them could move into the adjacent striatum and cortex, double labelled with the neuronal markers NeuN, MAP-2 and the astrocytic marker GFAP.^{15,16} A possible explanation for improved behaviour following MSC transplantation is that these cells may play an important role in neural protection and regeneration by releasing neurotrophic factors,³⁰

inhibiting inflammation,^{31,32} decreasing apoptosis,³³ promoting and strengthening endogenous neurogenesis of the subventricular zone and subgranular zone³⁴ and facilitating vascular regeneration.³⁵ Many other factors, including the timing, administration route and cell dose of transplanted MSCs, should be also considered. The grafted MSCs in the present experiment unexpectedly did not show differentiation, and animal behaviour was not improved, but there was also no tumour formation. This may be a result of a different administration route compared with that used in other experiments in which the MSC transplantation contributed to the functional recovery of model animals. Regardless, the MSCs remain the most secure donor cells and the easiest to harvest.

Although the results were not ideal, our study was the first to compare the outcomes of four kinds of stem cells considered representative for general use in normal and ischaemic murine animals. To date, there has seemed to be no more promising measure for stroke recovery than stem cell transplantation. It is conceivable, however, that there remains much progress to be made for stem cell transplantation to satisfactorily treat cerebral ischaemia. To improve the effect of cell therapy, the selection of suitable stem cells needs to be focused on.

CONFLICT OF INTEREST

All authors have no conflict of interest regarding this paper.

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