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Peptide Modified Mesenchymal Stem Cells as Targeting Delivery System Transfected with MiR-133b for the Treatment of Cerebral Ischemia

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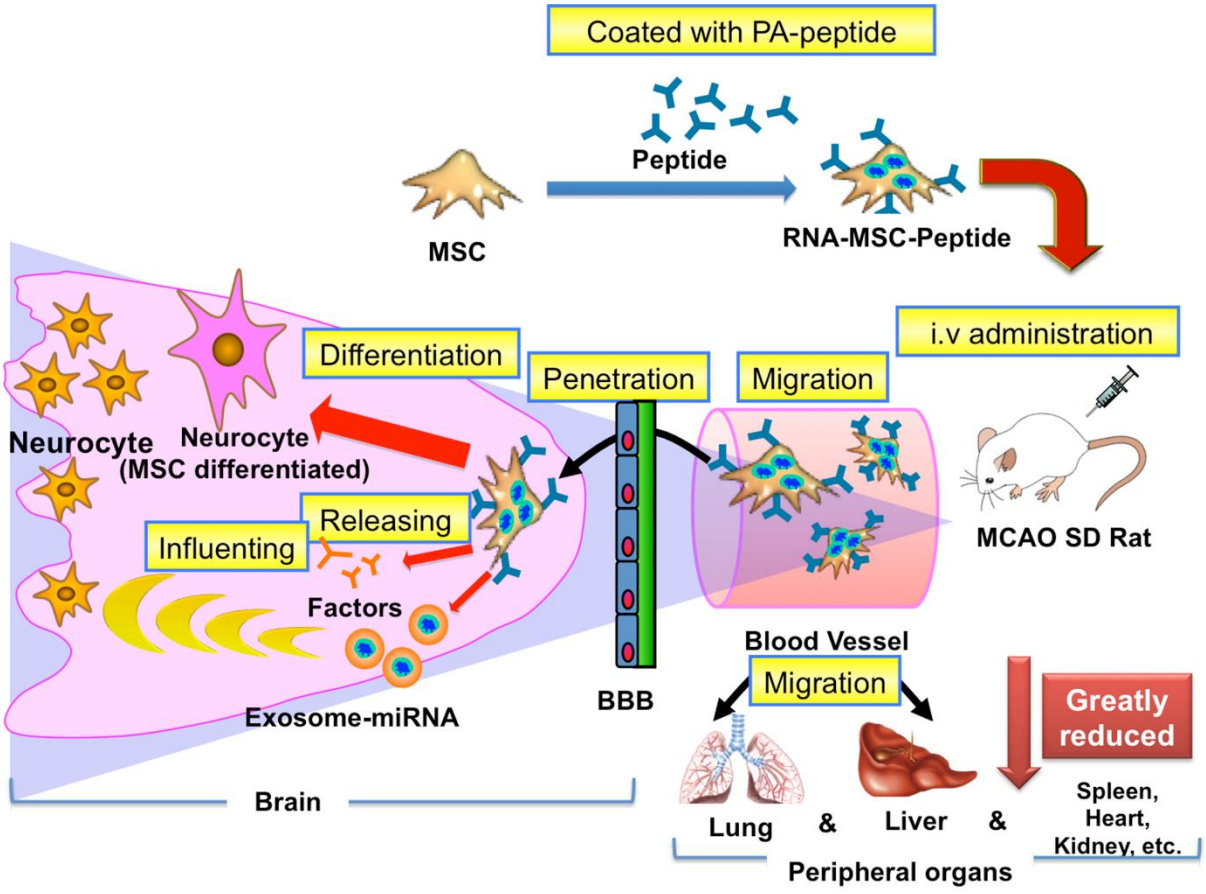

Abstract

Mesenchymal stem cells (MSCs) have been regarded as potential targeting vehicles and demonstrated to exert therapeutic benefits for brain diseases. Direct homing to diseased tissue is crucial for stem cell-based therapy. In this study, a peptide-based targeting approach was established to enhance cell homing to cerebral ischemic lesion. Palmitic acid–peptide painted onto the cell membrane was able to direct MSCs to ischemic tissues without any observed cell cytotoxicity and influence on differentiation, thus reducing accumulation of cells in peripheral organs and increasing engraftment of cells in the targeted tissues. With enhanced cell homing,

MSCs were used to deliver miR-133b to increase the expression level of miR-133b in an ischemic lesion and further improve therapeutic effects. This study is the first to develop MSCs co-modified with targeting peptide and microRNAs as potential targeting therapeutic agents. This targeting delivery system is expected to be applicable to other cell types and other diseases aside from stroke.

Keywords: mesenchymal stem cells, targeting delivery system cell, cerebral ischemia, membrane coating, peptide-based cell targeting, miR-133b transfection, stem cell-based targeting therapy

1. Introduction

Ischemic stroke is one of the most common causes of death and disability worldwide; it disturbs neuronal circuitry and disrupts the blood-brain barrier(BBB), leading to functional disability(Van and Dennis, 1998). Efforts have been devoted to relieve ischemic injury through the administration of drugs(Yu et al., 2005a; Yu et al., 2005b), success has been rather limited(Gladstone et al., 2002). To develop the novel targeting drug delivery system that can makes contribution to enhance targeting ability of therapeutic agents while reducing side effects is necessary. And one of the main challenges is to develop vehicles that can target brain across the BBB(Pardridge, 2007).The biomaterial-based vehicles, for example, stem cell has emerged as a potential strategic approach to injured or diseased tissue(Doeppner et al., 2012; Karp and Leng Teo, 2009; Kraitchman et al., 2005; Matsuse et al., 2011).It has been proven that stem cell can not only deliver therapeutic drug, but also enhance functional recovery by replacing lost brain tissues and inducing the release of trophic factors(Bacigaluppi et al., 2008; Bacigaluppi et al., 2009; Brenneman et al., 2010; Joyce et al., 2010).

Bone marrow (BM)-derived mesenchymal stem cells (MSCs) have been considered as promising therapeutic agents because they can be easily obtained and expanded rapidly ex vivo for autologous transplantation without encountering ethical and immunological problems(Hayase et al., 2015). Several experimental studies have demonstrated that MSC transplantation significantly improves neurological function, promotes endogenous

neurogenesis, and reduces apoptosis; a few MSCs can differentiate into neural lineage cells(Bao et al., 2011; Chen et al., 2003a; Chen et al., 2001a). MSCs were reported to migrate through BBB and directly to lesions of stroke or trauma(Chen et al., 2003b). Thus MSCs are considered promising targeting vehicles because of their intrinsic capacity to migrate to injured tissues after systemic transplantation(Chavakis et al., 2008; Chen et al., 2001a). However, as previously reported, the percentage of homing cells is relatively low, and only a limited number of MSCs could survive and engraft into the ischemic lesion because some cells die once exposed to unfavorable conditions. Hence, improving the engraftment efficiency of MSCs into ischemic lesions is important. With enhanced homing ability of MSCs, more cells could accumulate in the diseased tissue while reducing their distribution in non-target organs, thus reducing side effects. Moreover, the number of transplanted cells could be decreased. Peptide modified nanoparticles have achieved the goal of brain targeting(Li et al., 2011; Qin et al., 2012). Thus, peptide modification of stem cells was proposed as a methodology for stem cell-based brain targeting therapy in this study. In fact, targeting stem cells to injured or diseased tissue is decisive in posing therapeutic effect(Doeppner et al., 2012; Karp and Leng, 2009; Kraitchman et al., 2005; Matsuse et al., 2011).This study aims at conducting a prospective study on mesenchymal stem cells (MSCs) as brain targeting drug delivery system, with specific emphasis on the use of peptide coating as a novel method to enhance the homing capability of MSC.

Cell surface modification, especially targeting peptide painting is a transient cell coating

approach that can be used to label cells efficiently and increase cell localization in myocardial ischemia cells in a nontoxic manner(Kean et al., 2012). This transient modification approach would not pose long lasting influence on cells, and dissociated peptides from the membrane are biodegradable and even beneficial for ischemic tissue, thus paving the way for brain treatment study(Huang et al., 2012). Briefly, in the present study, the brain targeting CLEVSRKNC peptide(Hong et al., 2008) was selected and coated onto the cell surface via a lipid raft to induce the migration of MSCs to the ischemic lesion and to trigger a synergistic effect. This study investigated overall effect of targeting peptide coating onto MSCs，including whether or not membrane modification could result in cytotoxicity, cell behavior changes, and cell differentiation and the ability of peptides to induce cell homing.

Moreover, gene modification is always a promising method of enhancing therapeutic benefits. In a previous experiment, rats treated with fibroblast growth factor-2 (FGF-2)-modified MSCs or brain-derived neurotrophic factor (BDNF)-modified MSCs and subjected to middle cerebral artery occlusion (MCAO) exhibited significantly reduced infarction volume 14 d after MCAO(Ikeda et al., 2005; Kurozumi et al., 2004). In this study, MicroRNAs (miRNAs) have been introduced because of their significant functions in many regulatory mechanisms. It has been demonstrated that miR-133b promotes functional recovery from Parkinson's disease(Dreyer, 2010; Kim et al., 2007; Sanchezsimon et al., 2010) and spinal cord injury(Moulton, 2011). Collective studies have shown that exosomes from MSCs mediate miR-133b transfer, which promotes functional recovery from cerebral

ischemia(Xin et al., 2013). This phenomenon can be attributed to the increase in miR-133b expression in MSC-generated exosomes under ischemic conditions and the transfer of such exosomes into neural cells that promote neurite outgrowth(Xin et al., 2012). In our previous research, we obtained a promising non-viral carrier called spermine-pullulan (SP) that can engineer BMSCs with TGF-β1 or CMV-TK successfully(He et al., 2012; Jo et al., 2010; Zhang et al., 2014).Therefore, we performed miR-133b transfection by SP to increase the level of miR-133b in ischemic brain via the secretion of miR-133b-enriched exosomes.

In our previous work, we have successfully constructed a three-dimensional reverse transfection system(He et al., 2011) and demonstrated enhanced migration of MSCs to tumors(Hu et al., 2012; Zhang et al., 2014). These findings strongly support the feasibility of using MSCs as targeting vehicles. In the current study, we used MSCs as multi-functional therapeutic agents for miR-133b transfection and cell surface modification. The results indicated that peptide-based targeting paints can enhance the homing and engraftment of MSCs, thereby increasing the expression of miR-133b and the number of cells that differentiate into neural cells.

2. Materials and Methods

2.1Materials

Male Sprague-Dawley (SD) rats (body weight of 250–280 g) were purchased from SLAC Laboratory Animal Co. Ltd. (Shanghai, China). All procedures performed were in accordance

with Zhejiang University's guidelines for the welfare of experimental animals. The study was approved by the animal ethics committee of Zhejiang University (animal experimentation ethics approval no. Zju2010-1-02-015).

2.2Methods

2.2.1Middle Cerebral Artery Occlusion (MCAO) Model

SD rats were subjected to MCAO using the same procedures described in reported papers(Chen et al., 2001b; Longa et al., 1989). Briefly, the rats were anesthetized(Zhang et al., 2008), upon which the left common carotid artery, the external carotid artery (ECA) and the internal carotid artery (ICA) were exposed. A length of 4-0 monofilament nylon suture (18.5–19.5 mm) was advanced from the ECA into the lumen of the internal carotid artery until it blocked the origin of the MCA. 90 min later, reperfusion was performed by withdrawal of the line until the tip cleared the lumen of the ICA.

2.2.2Syngeneic MSCs Culture

Bone marrow mesenchymal stem cells were obtained from the bone of three-week old male SD rats as previously reported(He et al., 2011). Briefly, the bone marrow of the femurs were collected and cultured in modified isolation medium including low-sugar Dulbecco's modified Eagle's medium (DMEM), 15 % fetal bovine serum (FBS), L-glutamine, penicillin(50U/mL), and streptomycin (50U/mL) under the condition of 37℃in a 95%air, 5%

CO_2 atmosphere. Second to fifth passage cells that were at sub-confluence were applied for experiments.

2.2.3Palmitic acid (PA)–peptide Coating

The PA–CLEVSRKNC and PA–CLEVSRK(FITC)NC peptides were manufactured by Sangon Biotech (Shanghai, China). Liquid chromatography/mass spectroscopy confirmed that these peptides have >98% purity. Cell coating with lipid-modified peptides was assessed using PA–CLEVSRK(FITC)NC coated onto MSCs. The peptides (50 μg/mL) were applied to cells (1×10^6 cells/mL, DMEM without serum) at 37 ℃ with shaking for 10 min. Afterward, the cell suspension was centrifuged at 1500 rpm for 5 min at room temperature, washed twice with phosphate-buffered saline (PBS), and then analyzed by flow cytometry. Meanwhile, MSCs were dyed with 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate (Dil) (Invitrogen, USA) following a procedure of incubation under the condition of 10 min at 37 ℃ then 20 min at 4℃.A series of cross-sections was placed in a horizontal direction by 1.2mm to obtain horizontal sectional images of Dil-labeled MSCs as well as FITC-peptide coated MSCs respectively. Dil-labeled MSCs were modified with the PA–CLEVSRK(FITC)NC peptide and then observed under a fluorescence microscope or confocal microscope. For stability assessment, MSCs were coated with PA–CLEVSRK(FITC)NC as described above, washed, stored in DMEM at 4 ℃ or 37 ℃, and then analyzed by flow cytometry at each time point (1, 2, 3, and 4 h).

The cytotoxicity of the PA–peptides was evaluated by MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MSCs were seeded on a 96-well plate (5×10^3 cells/well) and then cultured for 24 h. The PA–peptides were added (0 mg/mL to 0.1 mg/mL) with the replacement of DMEM medium without serum. After 10 min of shaking, the MTT assay was performed immediately to evaluate the acute cytotoxicity. Long-term effects were also evaluated by allowing 24 h recovery after either 10 min exposure or 1 h exposure to the PA–peptides.

2.2.4In vivo cell Homing Analysis

MSCs were labeled with Vybrant™orange viable cell dye (Invitrogen, USA) or Dil before in vivo tracking. Unincorporated fluorescent dye was removed by washing the DMEM and centrifugation at 1200 rpm for 5 min. The labeled MSCs were then coated with 50 μg/mL of the PA–CLEVSRK(FITC)NC or control peptide PA-CRPPR peptide for 10 min with shaking at 37 °C. Afterward, the cells were washed, re-suspended in PBS, and immediately intravenously injected into the rats at 3 d post-MCAO (2×10^6 cells/rat or 3×10^6 cells/rat). Labeled MSCs without PA–peptide coating were injected as the control. Following 2h or 24h of circulation, the rats were sacrificed to collect brain, lung, liver, heart, kidney, and spleen tissues. The tissues were directly embedded in OCT compound as well as frozen in liquid nitrogen, and were further made frozen slices (Leica, CM 1850). The process was carried out with protection from light. The collected organs were immediately investigated using an in

vivo imaging system (Maestro, CRI, USA) and slices were observed under confocal microscope.

2.2.5Preparation of SP and SP/RNA Complexes

SP was synthesized in a laboratory according to a protocol previously reported(Jo et al., 2007). All RNA products were purchased from GenePharma (Shanghai, China). For miRNA transfection, MSCs were seeded on a 24-well plate (5×10^4 cells/well) and cultured for 24 h at 37 ℃ in 5% CO_2. The RNA was mixed with SP or Lipofectamine2000 for 30 min, and the resulting mixture was added to the cells (100 μl/well), with the replacement of DMEM without serum. After 6 h of incubation, the medium was changed to fresh DMEM supplemented with 15% FBS, and the cells were handled differently for specific purposes.

2.2.6Evaluation of Transfection Efficiency and Cytotoxicity Test

To evaluate transfection efficiency, FAN-RNA with fluorescence was utilized to prepare complexes with SP at different N/P ratios (N/P = 5, 10, 20), where N stands for the nitrogen of the non-viral vector and P stands for the phosphorus of RNA. Lipo/RNA was utilized as the control. After 6 h of transfection, the cells were washed and observed directly under a fluorescence microscope. The transfected cells were dissociated from the plates and suspended in PBS for flow cytometry assessment.

A cytotoxicity test was performed with MTT assay. The expression of miR-133b was

evaluated through real-time reverse transcription polymerase chain reaction (RT-PCR) (Applied Biosystems, USA) by using kits purchased from GenePharma (Shanghai, China) according to the manufacturer's protocol.

2.2.7Influence of Modified MSCs on Neural Cells after Oxygen-glucose Deprivation (OGD) in vitro

Enzyme digestion and mechanical isolation were adopted for the primary culture of astrocytes. Briefly, the cortices of 1 d old SD rats were isolated and cut to small slices for trypsin digestion. After digestion, the cells were cultured in DMEM medium supplemented with 10% FBS at 37 ℃ in 95% air and 5% CO_2 atmosphere. After 9 d to 11 d of incubation, the flasks containing cells were shaken for 12 h to separate oligodendrocytes from astrocytes. Neuro-2A was purchased from China Academy of Science.

The cultured neural cells were seeded in a Transwell plate and exposed to an OGD system, wherein the cells were cultured in a glucose-deprived medium at 37 ℃ in <1%O_2 and 5% CO_2 atmosphere. After 60 min or 120 min of exposure to OGD, the medium was changed to DMEM with FBS, and MSCs with or without peptide coating were added to the upper chambers. After 6 h of co-culture, the upper chambers were stained with crystal violet to assess the migration of MSCs. Furthermore, MSCs with or without PA-peptide modification were added onto the upper chambers for another 3 d or 5d of co-culture. Afterward, the cells were fixed for immunohistochemistry.

2.2.8Transplantation and Behavioral Test

The rats were subjected to cell transplantation at 3 d post-MCAO (i.v.). We divided the rats into five groups (n=4 rats each) as follows: control (MCAO rats without treatment), MSC (MCAO rats given naïve MSCs), peptide (MCAO rats given PA–peptide-coated MSCs), miR-133b (MCAO rats given miR-133b-transfected MSCs), and pep-133b (MCAO rats given MSCs co-modified with miR-133b and PA–peptide). The dose of cells transplanted was 2×10^6.

A behavioral test was performed 1, 7, and 14 d after cell transplantation. The modified neurological severity score (NSS)(Borlongan et al., 1995; Chen et al., 1996; Schallert et al., 1997; Shohami et al., 1995), a composite of motor, sensory, reflex, and balance tests, was utilized to assess the neurological function(Germanò et al., 1994). A high severity score of injury indicates a severe injury(Chen et al., 2001a). If a rat is unable to perform the test or lacks a tested reflex, it obtains a score of 1.

2.2.9Histological Analysis

At 14 d after cell transplantation, perfuse the rats with formalin, then removed and embedded their brains in paraffin. The cerebral tissues were cut at 2 mm intervals to obtain six brain slices and then cut into 10 μm-thick coronal sections. Glial fibrillary acidic protein (GFAP) immunohistochemical staining was performed to evaluate the thickness of glial scar.

2.2.10Assessment of Differentiation

At 3 d post-MCAO, brain extracts were obtained from the rats following previously described methods to assess the differentiation of MSCs into neural cells(Chen et al., 2003c; Xin et al., 2012). Briefly, dissected the brains on ice and measured their wet weights rapidly, followed by homogenization in neurobasal medium (GIBCO, USA), and add B27. The homogenate was centrifuged and the supernatant was collected. Then, MSCs with or without peptide coating were seeded onto a 24-well plate under the culture of brain extract medium. Immunohistochemisty staining (neuron-specific nuclear protein (NeuN, Millipore, USA), βIII-tubulin (Abcam, USA) and GFAP) was performed to detect differentiation. Briefly, fixe the cells with 4% paraformaldehyde and treat them with primary antibody. Afterward, add FITC-conjugated antibody for coloration. 4',6-Diamidino-2-phenylindole staining was performed for nuclear identification.

Fourteen days after cell administration, the rats were perfused with saline to evaluate differentiation in vivo. The brains were obtained, and the frozen sections were prepared. Immunohistochemical staining (NeuN) was applied to localize neurons derived from transplanted MSCs. The procedures were performed as indicated above.

2.2.11Statistics

Data were expressed as the mean ± SD. Statistical evaluation of differences between

experimental group means was done by performing multiple Student t-tests and one-way ANOVA. Statistical significance was considered at $P<0.05$.

3. Results

3.1In Vitro Assessment of PA–peptide Coating

The CLEVSRKNC peptide can selectively home to ischemic brain tissue and detect apoptosis(Hong et al., 2008). The PA–peptide is supposed to be able to coat the cell because palmitate serves as an anchor to integrate into the cell membrane(Kean et al., 2012). Our initial work demonstrated the appropriate conditions for coating by considering the modification efficiency and stability, cell cytotoxicity, and extent at which the peptide can remain on the cell surface instead of being internalized into the cell. Concentration of peptides and duration of incubation are two basic factors while considering balance of efficient modification and avoidance of internalization. In this study, PA–CLEVSRK(FITC)NC was synthesized to indicate the presence of the peptide. At low concentration of PA-peptides (25 μg/mL), modification efficiency and fluorescence intensity were under satisfaction (Fig 1). As the concentration increased, modification efficiency was increased accordingly (Fig 1a). Peptides coating was clearly observed immediately after 10 min's shaking, and they were remaining on the cell surface rather than being internalized after 24 h when the concentration was 50 μg/mL (Fig 1b). At high concentration (100 μg/mL), cell loading of PA-peptide was observed (Fig 1b). Meanwhile, MTT assay was performed to assess cell cytotoxicity

immediately after 10 min of exposure, after which 24 h was allowed for cell recovery. After 10 min ofco-culture with the PA-peptide, MSCs exhibited no obvious cytotoxicity (Fig 1c),and no evident apoptotic cells were observed. By contrast, high concentrations of the peptide promoted cell growth. Meanwhile, the number of cells increased both in the conditions of 10 min of exposure and 1 h of extended exposure after 24 h of recovery (Fig 1c). Thus, the optimal condition was proposed as 50 μg/mL PA-peptide with shaking for 10 min at 37 ℃, which was in consistent with the results obtained by Kean et al.(Kean et al., 2012).

When treated with 50μg/mL PA-peptide, positive cells were demonstrated to be over 60% that had been successfully modified with the peptide (Fig 1a and 2a).FITC labeling showed distribution of peptides (Fig 2b) on the cell surface. Moreover, co-localization of the peptide (FITC labeled) and MSCs (Dil cell membrane labeled) indicated that the peptide surrounded the cells and attached to the cell surface (Fig 2b). Further evidence was showed by the co-localization efficiency calculated by the computer, which reached over 70% (Fig 2c). To evaluate the stability of cell surface modification, MSCs painted with PA–CLEVSRK(FITC)NC were cultured under two conditions for up to 4 h. Flow cytometry results showed that most cells (over 95%)cultured at 37 ℃ or 4 ℃ remained positive within 4 h (Fig 2d).The culture condition of 37 ℃ was better than 4 ℃, although no significant difference was observed. The capability of MSCs to differentiate into neural cells was also determined to further investigate the potential effect of the peptide coating on cells. When cultured in the medium of ischemic brain extracts, both naïve MSCs and peptide-coated

MSCs differentiated into neural cells (Fig 2e). This result demonstrated that the peptide modification posed no potential cytotoxicity or side effects on MSCs.

3.2In Vivo Distribution of PA–peptide and MSCs

The TTC-stained brain slices in Fig 3c confirm the establishment of MCAO model animals. The CLEVSRKNC peptide can preferentially home to the ischemic hemisphere after circulation for either 15 min or 2 h in vivo(Hong et al., 2008). In the present study, we examined whether or not the PA–CLEVSRKNC peptide can successfully tract MSCs to ischemic brain tissue with the co-localization of peptide and MSCs. After the PA–CLEVSRK(FITC)NC peptide was coated onto MSCs that were dyed with nuclear staining without the influence of cell surface modification, the cells were allowed to circulate for 2 h in the rat body at 3 d post-MCAO before observation using in vivo fluorescence imaging. The PA–peptide selectively migrated to the ischemic brain, whereas the MSCs accumulated in the ischemic tissue and lungs (Fig 3a). The frozen slices also evidenced the co-localization of MSCs and PA-peptide (Fig 3b). Based on our previous study, MSCs intrinsically migrate to the lungs(Zhang et al., 2014).Therefore, some uncoated cells or cells that separate from the PA–peptide could potentially migrate to the lungs. However, localization of the PA–peptide and MSCs showed a high degree of overlap, suggesting that the modified peptide could direct the cells to the target tissue.

3.3Cell Homing Induced by PA–peptide Coating

The enhanced capability of the PA–peptide-coated MSCs to accumulate into the damaged brain after systemic cell injection was determined in an OGD model in vitro to mimic the ischemic microenvironment, which was supposed to facilitate cell migration. Neuro-2a (neuron cell line) and primary cultured astrocytes were exposed to OGD for either 1 h or 2 h, MSCs and PA-peptide modified MSCs were directly co-cultured with the damaged cells. Allowing for 6 h incubation, migrated MSCs were detected by crystal violet staining. The migration of MSCs were enhanced with increasing exposure to OGD, moreover, the migration ability was significantly enhanced when coating with PA-peptide in astrocytes co-culturing group (Fig 4a). Although no significant differences in migration ability were observed between the naïve MSCs and PA–peptide-coated MSCs when co-culturing with Neuro-2a, however, the integrity of the cell morphology can be detected in the peptide-coating group but not in the naïve MSC group (Fig 4b). These results suggested that the peptide can induce the migration of intact MSCs.

Further investigation of enhanced targeting capability was also studied through in vivo localization after a series of tests that showed successful peptide coating and good retention. The timing of transplantation is an important factor; therefore, we performed screening in the present work. A previous study demonstrated that cell transplantation at 3 d after injury can increase cell engraftment and survival(Rosenblum et al., 2012). Hence, 3 d post-MCAO was chosen as the appropriate time for transplantation in the present study. The findings of the in

vivo imaging system showed that systemically injected MSCs, with or without coating, accumulate in the ischemic brain hemisphere. The inherent capability of naïve MSCs to migrate to the ischemic hemisphere has been previously demonstrated(Chen et al., 2001a). However, compared with naïve MSCs, those with peptide paints increased the number of cells that engraft into the ischemic hemisphere at 24h post-MCAO, the total signal of MSCs increased to 2.33 times as much as naïve MSCs (Fig 5), while control peptide can't (Fig S1). A large amount of cells were distributed in the ischemic core and border. The results demonstrated that the targeting peptides successfully guided MSCs to the ischemic brain. The peptide coating reduced the accumulation of cells in the lungs compared with naïve MSCs, which showed higher tendencies to get trapped into the lungs (Fig 5). The MSCs were distributed in the main organs, such as liver and kidney, which may indicate metabolism (Fig 5a). When we reduced the amount of transplanted cells (2×10^6 cells), the trend of homing to ischemic area wasn't changed while the PA-peptide modified MSCs also showed enhanced homing ability compared to naïve MSCs (Fig 5b). These results suggested that the peptides can direct the MSCs to ischemic lesion and retain MSCs in the brain.

3.4Protection of Injured Neural Cells

The functional behavior of PA-peptide modified MSCs was also investigated to determine whether peptide modification would be beneficial for the recovery of injured neural cells. Neuro-2a and primary cultured astrocytes were subjected to OGD for 2 h, whereas treatment

was performed immediately by adding MSCs and PA-peptide modified MSCs without cell contact to injured cells. The cells that survived under the protection of PA-peptide coated MSCs showed morphological integrity (Fig 6). Statistical analysis revealed that naïve MSCs significantly increased the number of surviving neural cells, while PA-peptide modified MSCs didn't show statistical significance (Fig 6). However, PA-peptide modification doesn't change the protective effect exhibited by MSCs. It was clear from Fig 6 that Neuro-2A and Astrocytes have better morphology in the Pep-MSCs group. Compared to control group, either naïve MSCs or PA-peptide modified MSCs increase cell survival and enhance the recovery of ischemic injured cells. Moreover, PA-peptide can enhance the ability of targeting of MSCs to the ischemic site so that more MSCs can play a protective role on neural cells in ischemic regions.

3.5 MiR-133b Transfection

In our previous report, we established a non-viral gene transfection system by screening synthesized non-viral vectors(Hu et al., 2010). SP was demonstrated to be the most suitable non-viral vector because of its high transfection efficiency and low cytotoxicity(He et al., 2011; Zhang et al., 2014). It was the first time that SP was used to deliver microRNAs. Therefore, adequate transfection conditions, including N/P ratio and period of transfection, were screened in the current study. When the N/P ratio varied from 5 to 20, quantitative analysis performed using flow cytometry revealed that over 95% of cells contained

FAN-RNA, thereby confirming the successful delivery of microRNA into MSCs (Fig 7a). In addition, strong fluorescence signals indicated the high efficiency of RNA delivery (Fig 7b). The proportion of positive cells reached 99.9% when the N/P ratio was 20. Considering that no obvious cytotoxicity was observed (Fig 7c), we selected the N/P ratio of 20 and the transfection period of 4 h as appropriate conditions.

The selected transfection conditions were utilized to further evaluate miR-133b expression. SP was used to deliver rno-miR-133b mimics to MSCs, and real-time RT-PCR was performed 24 h after transfection. The expression level of miR-133b in the transfected MSCs was 18,000-fold higher than that in the naïve cells (Fig 7d). This result confirms the feasibility of the proposed of miR-133b transfection method.

The functional behavior of miR-133b-transfected MSCs was also investigated to determine whether increased miR-133b levels in MSCs are beneficial to the recovery of injured neural cells. Primary cultured astrocytes were subjected to OGD for 1 h, and treatment was performed immediately by adding MSCs and miR-133b-transfected MSCs without cell contact to the injured cells. The cells that survived under the protection of miR-133b-transfected MSCs showed morphological integrity (Fig 7e). Statistical analysis revealed that miR-133b-transfected MSCs significantly increased the number of surviving astrocytes (Fig 7f). Although the mechanism behind the obtained results remains to be elucidated, the results clearly suggest that the delivery of miR-133b to neural cells through exosomes can promote neurite outgrowth(Xin et al., 2012). The data suggest that

miR-133b-transfected MSCs increase cell survival and enhance the recovery of ischemic injured cells.

3.7Interaction of MSCs and Ischemic Brain

The effects of MSCs on the ischemic brain and the influence of the ischemic microenvironment on MSCs were investigated after successfully modifying MSCs via targeting peptides and miRNA transfection. The presently established method of cell modification resulted in a beneficial therapy for stroke because an increased amount of stem cells were engrafted in the ischemic tissue with increased miRNA-133b secretion. In the therapeutic experiments, neurological deficits were significantly reduced 7 d after the administration of both peptide coatings and miR-133b-transfected MSCs (Fig 8a). After 14 d of treatment, either the peptide-coated MSC group or the pep-133b group obtained lower scores than the MCAO groups (Fig 8a). The pep-133b treatment significantly reduced neurological deficits compared with the MSC group, although naïve MSCs also showed therapeutic effects. Meanwhile, immunohistochemistry staining was performed to observe imperceptible changes within the ischemic brain. The proliferation of GFAP-positive glial cells and fibrils indicated the process of scar formation. After 14 d of treatment with peptide-coated MSCs, a significantly thinner scar wall was observed compared with the non-treatment group (Fig 8b).

Co-localization images show that exogenous MSCs survived and differentiated to neurons

in all the treatment groups but there is no significant differences (Fig 8c). The results indicate that the differentiation potential of MSCs is unaffected when co-modified with targeting peptides and miR-133b.

4. Discussion

Biomaterial-based brain targeting is a promising field in research of targeting drug delivery system. Nano-carriers, which were made of lipids or polymeric materials, were promising candidates for brain targeting since they could cross the BBB(Garcia-Garcia et al., 2005). Conjugation of targeting moieties onto nanoparticles through covalent or non-covalent bonding enabled nanoparticles to enhance targeting ability(Celia et al., 2011). The modification of ligands, including small peptide or protein, transferrin receptor or antibody, has been investigated and achieved the goal of targeting(Boado et al., 1998; Pardridge, 2003; WM, 1999). Compared to nanoparticles, MSCs-based brain targeting therapy was more promising since MSCs not only can act as therapeutic agents but also can serve as potential targeting vehicles. Studies have showed MSCs have great therapeutic potential for neurological diseases(Chen et al., 2001a; Dharmasaroja, 2009). Collective studies have demonstrated the inherent propensity of MSCs to localize to injured or inflamed tissues, especially lesions in the brain(Azizi et al., 1998; Nakamura et al., 2004; Yano et al., 2006), suggesting that MSCs are potential targeting vehicles. However, the homing capability of MSCs has been debated since transplanted cells have been found to be widely distributed in

systemic tissues or become entrapped in non-targeted organs after intravenous injection(Kraitchman et al., 2005). Hence, efforts have been devoted to developing techniques and methods that would improve the targeting ability of MSCs. Genetic engineering and cell surface modifications were proposed as efficient methods for utilization(Huang et al., 2012). Inflammatory chemokines and factors have been widely recognized to be mainly responsible for cell homing behavior; stromal cell-derived factor-1 and its receptor chemokine receptor four (CXCR4) have been regarded as most effective inflammatory chemokines and factors for recruiting cells(Peled et al., 1999; Yu et al., 2012). The overexpression of CXCR4 or other similar ligands in MSCs often requires genetic modification, which poses certain limitations in transferring therapeutic genes/drugs using MSCs. By contrast, cell surface coatings may provide opportunities for combining drug delivery with targeting agents to employ MSCs as multi-functional vehicles.

With regard to cell surface modification, the proposed methodology could introduce specific molecules on cell membranes to direct them to desired target regions. The chemical modification strategy was proposed very first, such as covalently coupling the sialyl Lewis X (SLeX) moiety on the surface of MSCs through biotin streptavidin because SLeX was considered as a homing receptor(Kang et al., 2011). In addition, the effectiveness of a two-step cell surface antibody coating approach was investigated to deliver stem cells to sites of inflammation(Chen et al., 2000; Ko et al., 2009). Although the abovementioned methods were successfully performed without compromising MSC viability, adhesion, proliferation,

and multi-differentiation potential, a simpler palmitated-peptide-based cell painting approach was highlighted because the effects this transient modification on cells are less than the effects of covalent modification(Kean et al., 2012). The results of the present study demonstrated successful modification and enhanced stability of targeting peptide on BM MSCs. Moreover, no significant cytotoxicity was observed both in vitro and in vivo. The co-localization of the PA–peptide and MSCs at 2 h after systemic transplantation elucidated the ability of this peptide to target and guide the cells to ischemic regions. The selected targeting peptide (CLEVSRKNC) can be used to successfully detect neuronal cells undergoing apoptosis at the penumbra region of stroke lesions(Hong et al., 2008), after which accumulated MSCs could rescue the apoptotic cells by releasing trophic factors or replacements after differentiation. The large increase in cell localization was detected based on fluorescence intensity, which indicated enhanced homing capability after peptide modification. In addition, the increase in cell accumulation prolonged the time of residence, suggesting the enhanced engraftment of MSCs in the ischemic region. With regard to cell accumulation in peripheral organs, a large amount of cells injected at one time (3×10^6 cells and above) often led to the entrapment of more cells in the non-targeted organs, such as lung and liver (data not shown). With enhanced cell homing, the dose of cells transplanted could be reduced (2×10^6 cells in this study) to decrease massive distribution while simultaneously ensuring that enough cells are provided for therapy. Furthermore, the peptide coating still reduced the distribution in non-targeted organs compared with naïve MSCs even under lower dose of cells injected.

The modification via lipid raft anchoring onto the cell membrane is supposed to be transient, after which the peptides would be separated from the cells after a certain period. Hence, the issue of long-term effect on cells could be eliminated. Another issue is the potential internalization of peptides, which was not clearly clarified, although this phenomenon was not observed under controlled concentration. However, this issue should not be a concern because no deleterious effects were observed during the entire period of culturing. The process of PA–peptide separation from the cell membrane in vivo and whether or not a small amount of PA–peptide could still affect the host cells remain to be elucidated. Complete evaluation must be conducted to fully optimize the peptide modification process.

Choosing the surface modification strategy increases the chance of combined therapeutic drug delivery. Previous studies demonstrated that miR-133b can substantially down-regulate myocardial infarction(Bo et al., 2009; Bostjancic et al., 2010), ischemia perfusion(Bo et al., 2009), and rat brain at post-MCAO(Xin et al., 2012), indicating the important function of miR-133b in modulating ischemic injury(Ye et al., 2011). The reported work also showed that the expression level of miR-133b significantly increased after MSC administration via MSC-secreted exosomes. The exosomes containing miR-133b that was transferred into astrocytes may downregulate expression of connective tissue growth factor, reduce the glia scar wall, and induce neurite outgrowth(Xin et al., 2012). Based on the above data, the PA–peptide modified-MSCs were used to deliver miR-133b to increase the expression level of miR-133b in the ischemic lesion and further improve therapeutic effects. In addition,

miR-133b was successfully transfected with high efficiency via the delivery of SP, an efficient non-viral vector. The MSCs transfected with miR-133b showed better protection and promotion of proliferation of ischemic astrocytes in vitro. The results from the small-scale experiment of in vivo therapy also demonstrated significant improvement of functional recovery after treatment with the MSCs co-modified with the PA–peptide and miR-133b while simultaneously thinning the glial scar. The mechanism underlying therapeutic benefits was not explicit; paracrine action and replacement by stem cells were supposed to be crucial for stem cell therapy. In the present study, the MSCs differentiated into neuronal cells after cerebral ischemia, suggesting that the replacement of lost host cells can elicit therapeutic benefits. The differentiation of MSCs was mainly detected in neuronal cells other than astrocytes. This result can be attributed to the ability of the targeting peptide to detect apoptotic neuronal cells. The in vivo therapeutic experiment was not completed because it was only performed to demonstrate the therapeutic potential of MSCs co-modified with PA–peptide and miR-133b. The present study aims to establish a universal methodology that could combine cell surface modification with RNAi/gene transfection, thus making MSCs excellent targeting agents. The proposed method is expected to be applicable to other cell types and diseases aside from stroke. However, the mechanism and underlying processes still need to be thoroughly investigated.

5. Conclusions

The present research investigates developing mesenchmal stem cells (MSCs) modified with targeting peptides as a means of establishing a universal method for MSC targeting. In this study, a peptide-based targeting approach was established to enhance cell homing to cerebral ischemic lesions and miR-133b was transfected with high efficiency via the delivery of SP to showed better protection and promoted the proliferation of ischemic astrocytes in vitro. The results of in vivo therapy showed the palmitic acid–peptide painted onto the cell membrane was able to direct MSCs to ischemic tissues while the chosen peptide could detect apoptotic neurons. Targeting peptides coating reduced the accumulation of cells in peripheral organs, while increasing the engraftment of cells in the targeted tissues. With improvement in cell homing and functional recovery, pep-133b-MSCs exhibited therapeutic effect on ischemic stroke.

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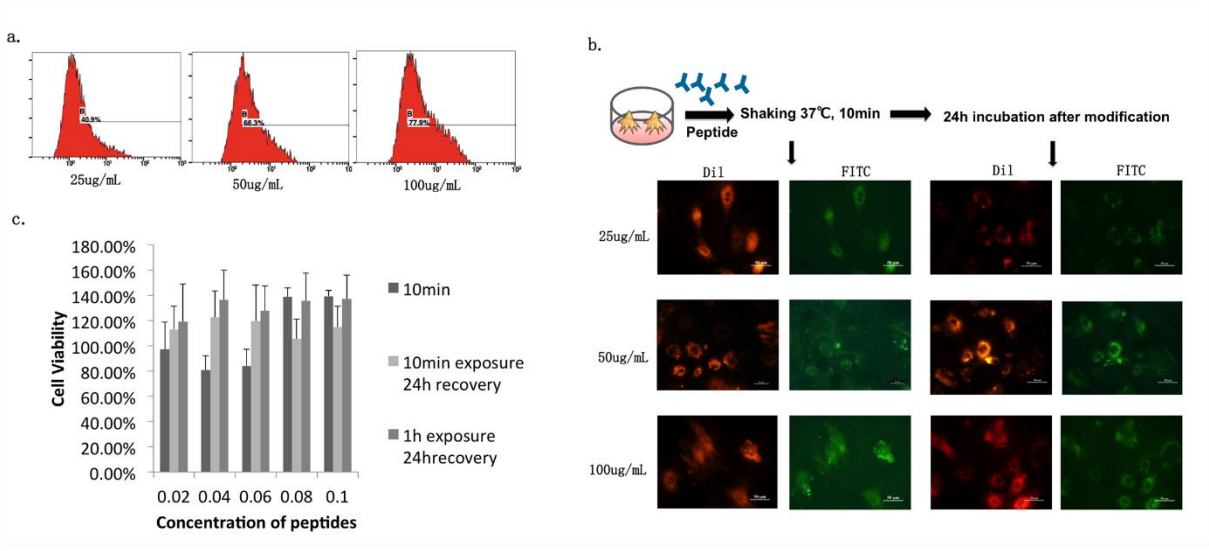

Fig 1. In vitro screening of palmitic acid–peptide coating. (a) PA–CLEVSRK(FITC)NC peptide (25μg/mL, 50μg/mL and 100μg/mL) coating efficiency assessed using flow cytometry. (b) Co-localization of peptide (FITC labeled) and MSCs (Dil labeled). The images were taken either after initial incubation or post-24 incubation. (c) Cell viability analyzed using MTT assay. MSCs exposed to PA–peptide (0 mg/mL to 0.1 mg/mL) were assessed using MTT after 10 min exposure, 10 min exposure with 24 h recovery, and 1 h exposure with 24 h recovery. The values represent the mean ± SD.

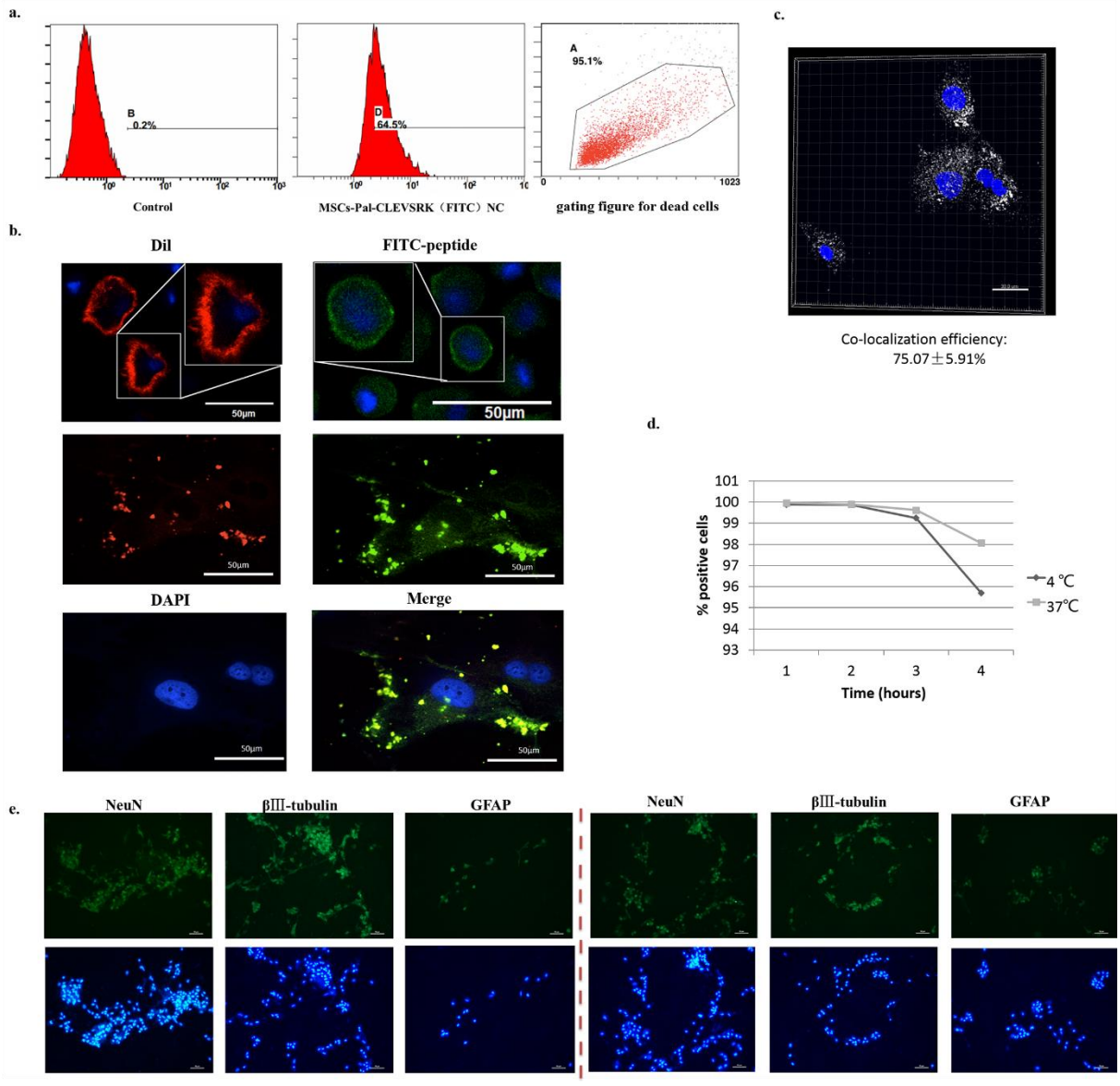

Fig 2. In vitro assessment of palmitic acid–peptide coating. (a) PA–CLEVSRK(FITC)NC peptide coating efficiency assessed using flow cytometry. (b) Confocal fluorescence images of Dil labeled MSCs (left) and FITC-peptide coated MSCs (right) in horizontal direction. Co-localization of peptide (FITC labeled) and MSCs (Dil labeled). (c) Co-localization efficiency analyzed by Metamorph offline (software 7.7.6). (d) Time course evaluation of the % positive at 4 °C and 37 °C, as analyzed by flow cytometry.(e)

Immunofluorescence staining of NeuN, β Ⅲ-tubulin, and GFAP evaluated differentiation of MSCs (left part) and PA-peptide-coated MSCs (right part) under the culture of brain extracts. FITC-positive cells are green, whereas DAPI-positive cells are blue.

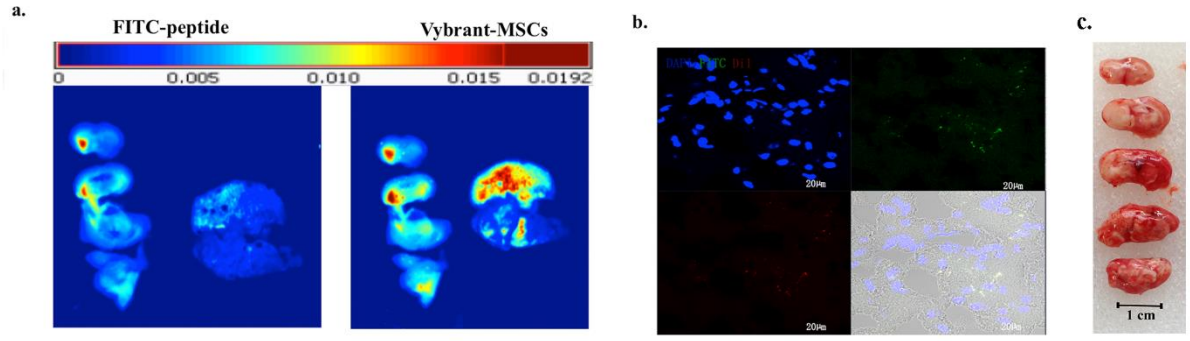

Fig 3. In vivo distribution of palmitic acid–peptide and MSCs. (a) MSCs (stained with Vybrant^{TM} orange viable cell dye) coated with FITC-conjugated PA–peptide [PA–CLEVSRK(FITC)NC] were allowed to circulate for 2 h in MCAO rats. Brain slices and lungs were detected using in vivo imaging systems to determine the localization of the peptides (left) and MSCs (right). The fluorescence bar indicates the fluorescence intensity and is shown in different colors. (b) Frozen slices were made after 2h circulation of PA-peptide (FITC labeled) modified MSCs (Dil labeled) in MCAO rats. DPAI were used to detect nuclear. (c) TTC-stained brain slice of MCAO rat.

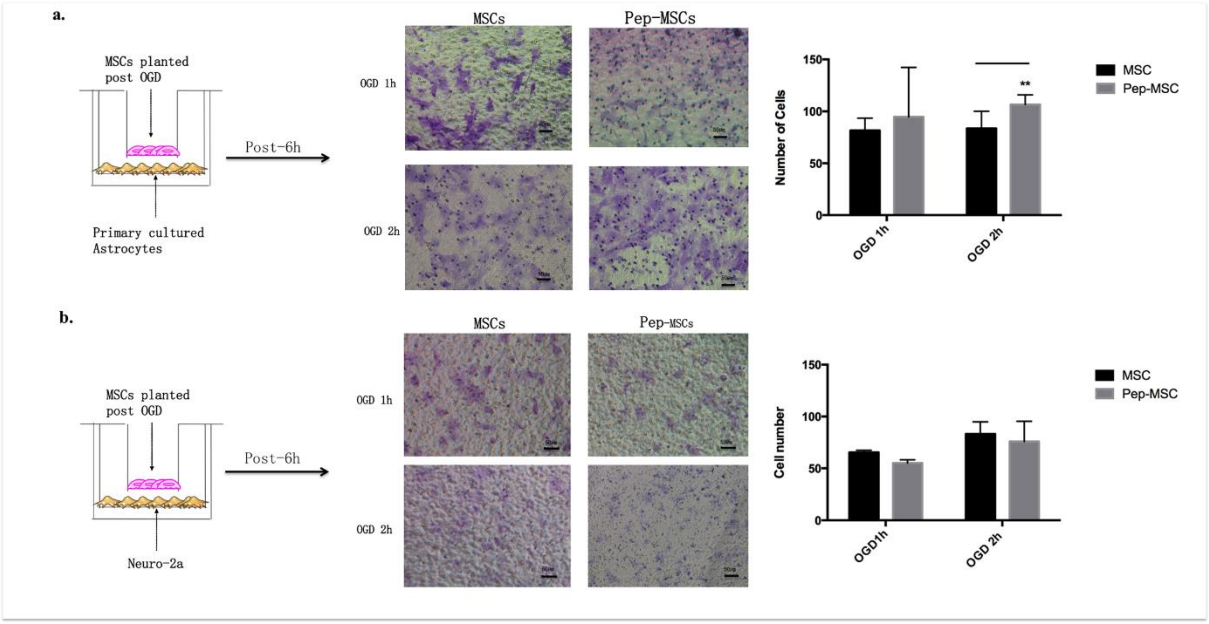

Fig 4. In vitro migration of MSCs. In vitro migration of MSCs in response to (a) astrocytes (b) Neuro-2a at 60 min or 120 min post-OGD using a Transwell plate (8 μm pores). Crystal violet staining was performed to indicate migrated MSCs. *p<0.05,**p<0.01.

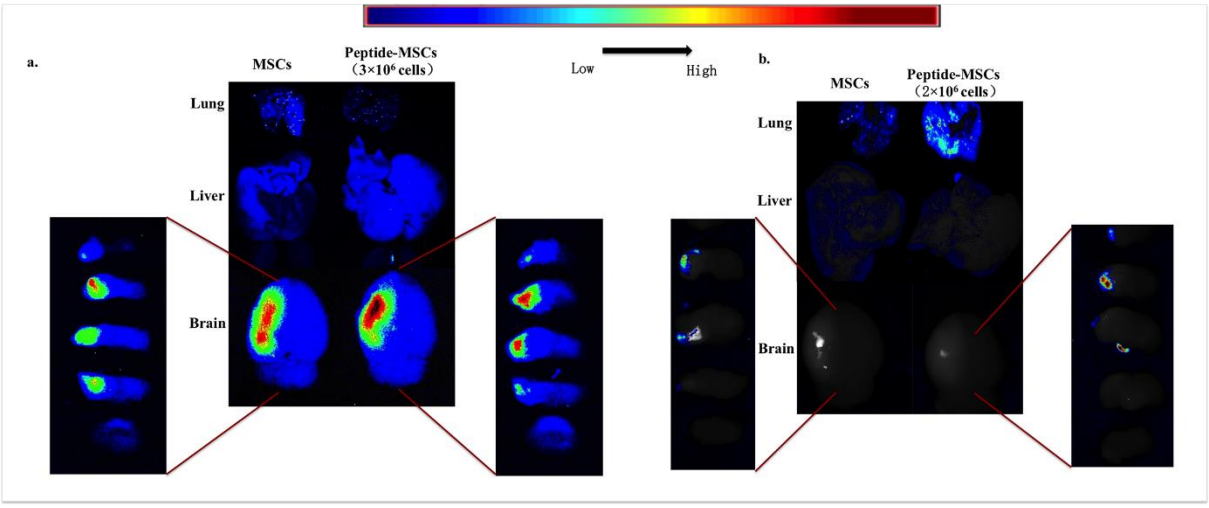

Fig 5. In vivo cell homing assessment. MSCs (stained with Dil) with or without PA–peptide coating were intravenously injected into rats at 3 d post-MCAO. Distribution of MSCs either(a) 3×10^6 cells or (b)

2×10^6cells in the brain at 24 h after cell administration. Lung, liver and brain tissues and brain slices were analyzed using in vivo imaging systems. The fluorescence bar indicates the fluorescence intensity and is shown in different colors.

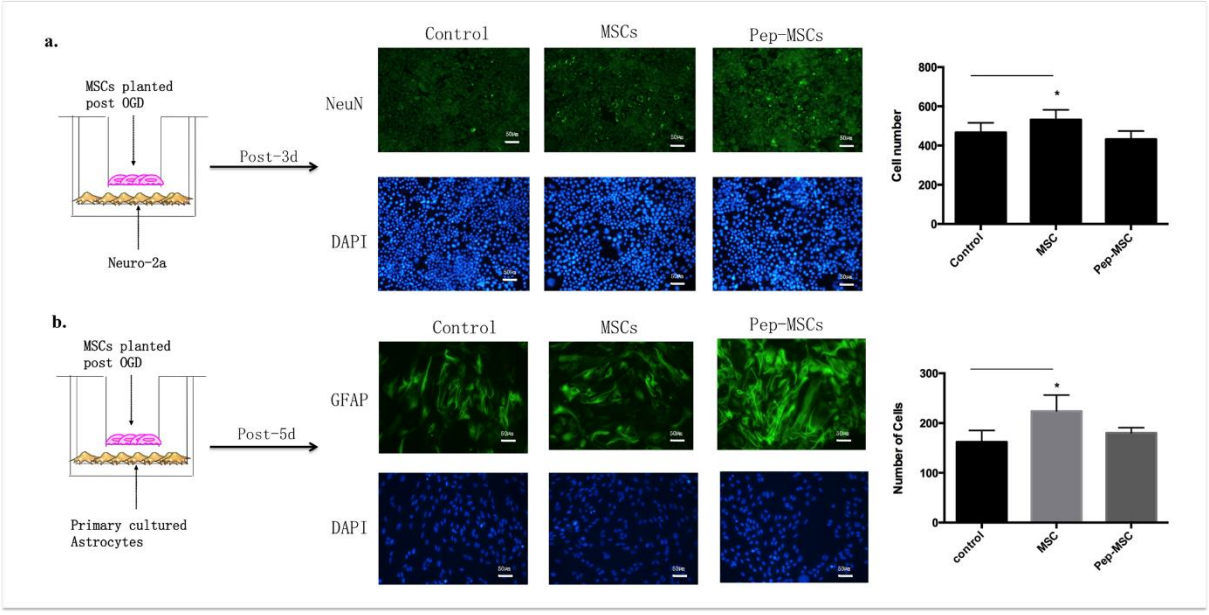

Fig 6. MSCs protect injured neural cells. Naïve MSCs or MSCs modified with PA-peptide were immediately added to the upper chamber to co-culture with (a) Neuro-2a or (b) astrocytes at 120 min post-OGD using a Transwell plate (8 μm pores). NeuN or GFAP immunofluorescence staining was performed to determine the survival of Neuro-2a or astrocytes (green), and DAPI staining was performed to detect the nuclei (blue). The number of survived neural cells counted from four random fields is shown, *$p<0.05$.

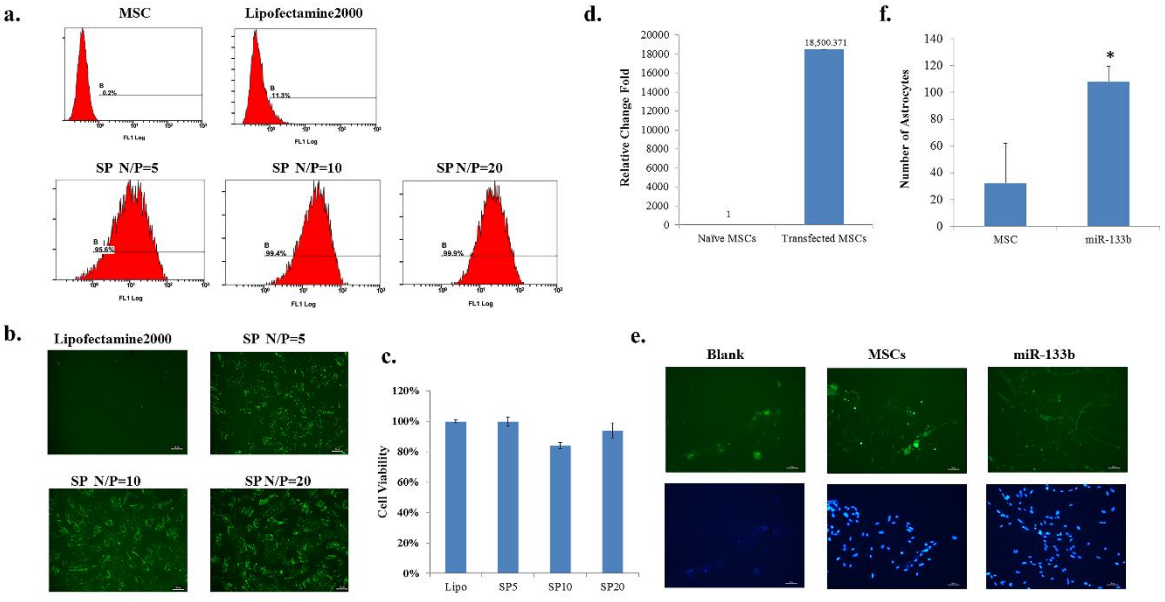

Fig 7. MSCs transfected with miR-133b. Transfection efficiency of spermine-pullulan (SP) was analyzed via (a) flow cytometry and (b) fluorescence microscopy using FAN-RNA (green fluorescence). Lipofetamine2000 was used as the control. (c) Cytotoxicity of SP/miR-133b with different N/P ratios was evaluated using MTT assay. The values represent the mean ± SD. (d) miR-133b expression was analyzed using real-time RT-PCR.(e)Naïve MSCs or MSCs transfected with miR-133b were immediately added to the upper chamber to co-culture with astrocytes at 60 min post-OGD using a Transwell plate (8 μm pores). GFAP immunofluorescence staining was performed to determine the survival of astrocytes (green), and DAPI staining was performed to detect the nuclei (blue). (f) The number of survived astrocytes counted from four random fields is shown, *$p<0.05$.

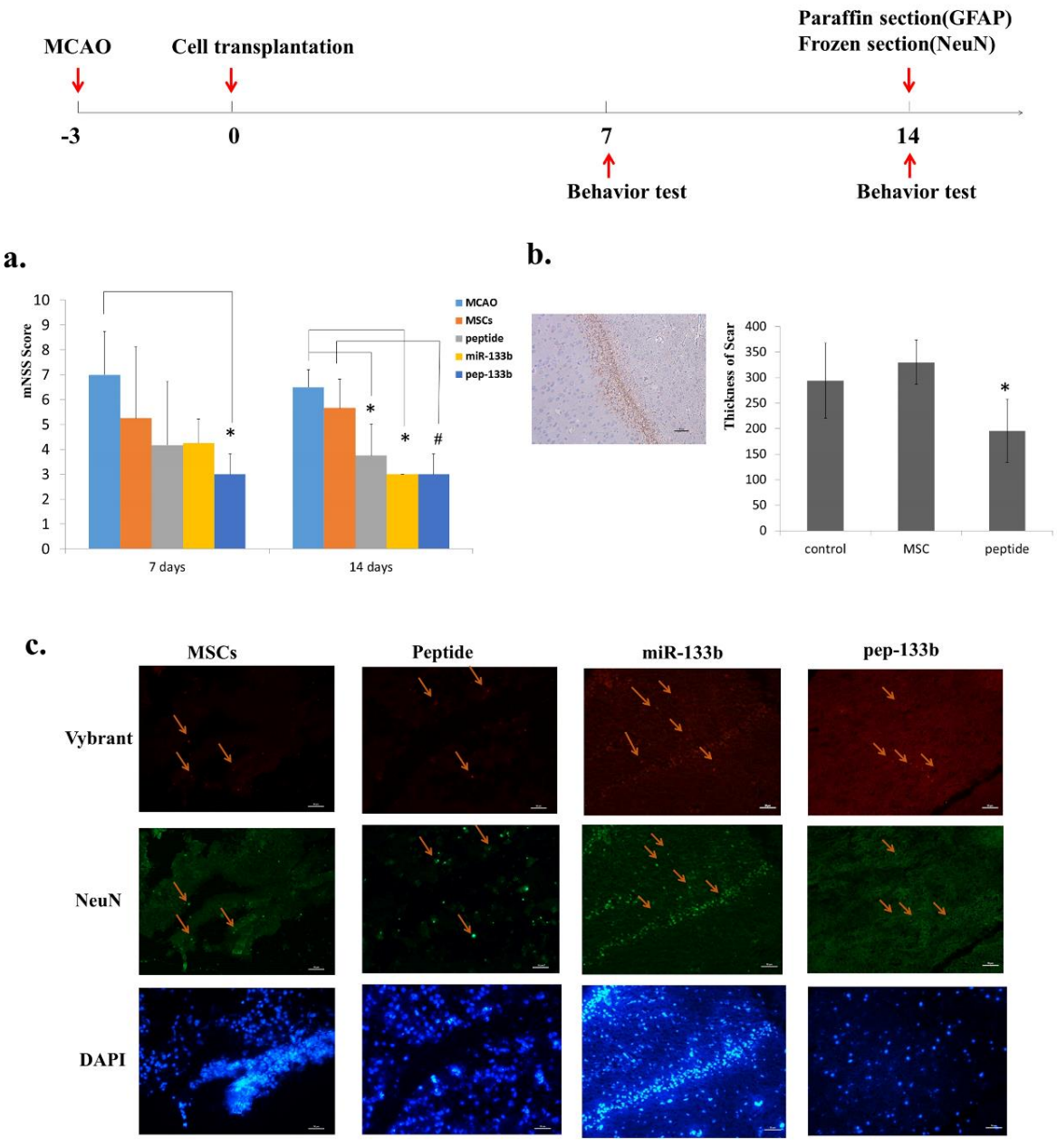

Fig 8. Interaction between MSCs and ischemic brain. The therapeutic experiment is shown above. (a) mNSS assessment was performed at 7 and 14 d after cell transplantation (n=4). (b) GFAP

immunohistochemical staining was performed to indicate scar thickness at 14 d after cell administration. Micrographs were analyzed using image J. (c) Double-staining was performed to evaluate in vivo differentiation of MSCs into neural cells. MSCs stained with Vybrant^{TM} orange viable cell dye were colored red, cells subjected to NeuN immunofluorescence staining were colored green, and cells subjected to DAPI staining were colored blue. The arrows indicate differentiated MSCs. The values represent the mean ± SD,*$p<0.05$ compared with the control, and #$p<0.05$ compared with naïve MSCs. The following groups were included: MCAO (MCAO rats without treatment), MSCs (MCAO rats given naïve MSCs), peptide (MCAO rats given PA–peptide-coated MSCs), miR-133b (MCAO rats given miR-133b-transfected MSCs), and pep-133b (MCAO rats given MSCs co-modified with miR-133b and PA–peptide). The dose of cells transplanted was 2×10^6, and the cells were all intravenously injected.