Dedifferentiation-reprogrammed mesenchymal stem cells for neonates with hypoxic-ischaemic brain injury

FY Yang, XH Zhang, LL Tsang, HC Chan, XH Jiang *

KEY MESSAGES

1. Human umbilical cord–derived mesenchymal stem cells (hUC-MSCs) can be manipulated via neuronal differentiation and dedifferentiation *in vitro*.
2. Compared with naïve hUC-MSCs, dedifferentiated hUC-MSCs reveal distinguished stem cell phenotype such as enhanced cell survival, neuronal differentiation potential, and cell migration.
3. Local administration of hUC-MSCs or dedifferentiated hUC-MSCs significantly improves brain functional recovery in hypoxic-ischaemic encephalopathy rat model.
4. Compared with hUC-MSC, dedifferentiated hUC-MSCs exhibit stronger repair function, as demonstrated by more improved motor, learning, and memory abilities.
5. The enhanced therapeutic effects of dedifferentiated hUC-MSCs are attributed to enhanced neural protection and promotion of endogenous repair.

Hong Kong Med J 2019;25(Suppl 5):S12-6
HMRF project number: 01120056

[1,2] **FY Yang,** [1,2,3] **XH Zhang,** [1,2] **LL Tsang,** [1,2,3,4] **HC Chan,** [1,2,4] **XH Jiang**

[1] *Epithelial Cell Biology Research Centre, The Chinese University of Hong Kong*
[2] *School of Biomedical Sciences, Faculty of Medicine, The Chinese University of Hong Kong*
[3] *Sichuan University-The Chinese University of Hong Kong Joint Laboratory for Reproductive Medicine, West China Second University Hospital, Chengdu, China*
[4] *The Chinese University of Hong Kong, Shenzhen Research Institute, Shenzhen, China*

* Principal applicant and corresponding author: xjiang@cuhk.edu.hk

Introduction

Traditional treatments for hypoxic-ischaemic encephalopathy (HIE) involve prevention of apoptosis, necrosis, and inflammation. Stem cell–based therapy has shown many benefits in animal models. However, low levels of mesenchymal stem cells (MSC) survival and differentiation *in vivo* greatly limit their therapeutic effects and clinical use. We have demonstrated that rat bone marrow–derived MSCs can be reprogrammed via neuronal differentiation and dedifferentiation with enhanced cell survival and differentiation in treating HIE in neonatal rats.[1] In this study, we expand dedifferentiation platform to human umbilical cord–derived MSCs (hUC-MSCs), which are promising cell source for HIE treatment, because many neonates experience an HIE insult around the time of birth. Hypoxia has been shown to stimulate MSC survival, proliferation, and stem cell potential.[2] Thus, hUC-MSCs derived from the hypoxic neonate may have a better therapeutic potential.[3]

Methods

We tested our hypothesis in both cell culture and neonatal HIE rat model. This study included two parts: (1) to characterise dedifferentiated hUC-MSCs and compare the phenotypic properties (proliferation, cell survival, differentiation, migrative abilities, neurotrophic effects) of dedifferentiated hUC-MSCs (De-hUCMSCs) with unmanipulated hUC-MSCs *in vitro*, and (2) to evaluate the therapeutic efficacy of De-hUCMSCs and determine the mechanisms underlying the beneficial effects of De-hUCMSCs in a HIE rat model.

Results

Part 1

When subjected to the pre-induction medium followed by modified neural medium, hUC-MSCs rapidly underwent dramatic morphological changes. After 6 hours of neural induction, >95% of cells presented with neuron-like morphology. In contrast, withdrawal of modified neural medium rapidly reverted MSC-derived neuron-like cells back to mesenchymal morphology. We then used various cell functional analyses to characterise the De-hUCMSCs, which exhibited a survival advantage over undifferentiated hUC-MSCs when challenged with hydrogen peroxide (Fig 1a). Next, we examined the migratory ability of hUC-MSCs and De-hUCMSCs toward different kinds of growth factors. Both were seeded in the upper chamber of transwells while different growth factors were added to the lower chambers. The migratory ability

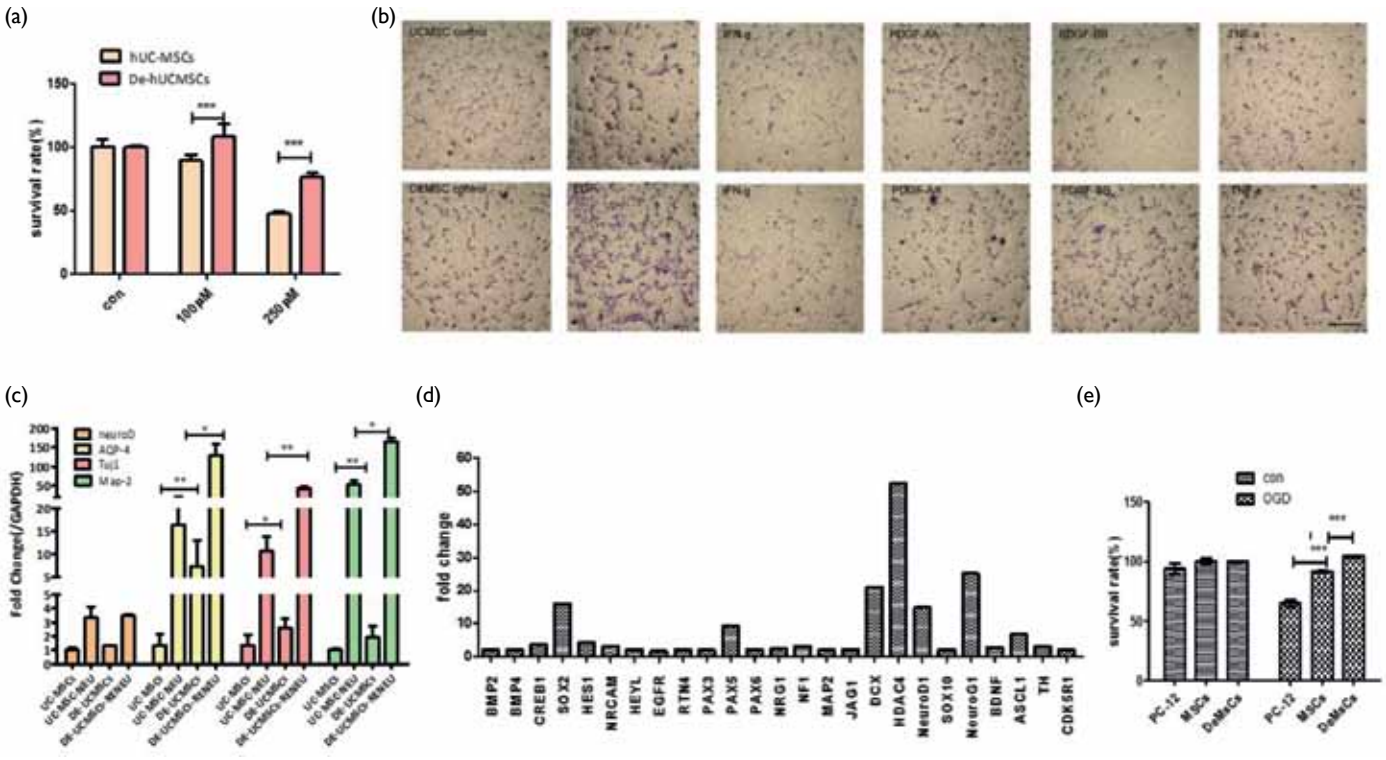

FIG. 1 Characterisation of dedifferentiated human umbilical cord–derived mesenchymal stem cells (hUC-MSCs): (a) Dedifferentiated hUC-MSCs exhibited survival advantage over hUC-MSCs. Untreated hUC-MSCs or dedifferentiated hUC-MSCs were plated in 96-well plates and challenged with 0-250 μM hydrogen peroxide for 24 hours. (b) Transwell assay showed dedifferentiated hUC-MSCs exhibited enhanced chemotaxis toward EGF and PDGF-BB. (c) Real time RT-PCR analysis of Aqp4, Sox-2, Tuj1, and NeuroD during neuronal differentiation, dedifferentiation and redifferentiation of hUC-MSCs. (d) Differentially expressed genes involved in neurogenesis in dedifferentiated hUC-MSCs and hUC-MSCs were examined by focused PCR array. (e) Condition media derived from either hUC-MSCs or dedifferentiated hUC-MSCs were added into serum-deprived and low glucose treated PC-12 cells for 24 hours. Cell proliferation was assessed using MTT assay.

of hUC-MSCs was mildly enhanced in the presence of all growth factors, whereas De-hUCMSCs exhibited much enhanced chemotaxis to EGF and PDGFBB (Fig 1b). These findings suggest that the enhanced chemotaxis exhibited by De-hUCMSCs were specific to certain growth factors. In addition, we determined the neuronal differentiation and dedifferentiation at the molecular level. As shown by real time PCR analysis, the expression levels of Sox-2, NeuroD, Aqp4, Masashi-1, Tuj-1, and Map2 were markedly increased in differentiated cultures compared with undifferentiated hUC-MSCs. Dedifferentiation from the neuronal to the stem cell phenotype was associated with a marked reduction in the expression of neuronal proteins. However, the expression of Mashashi-1, Tuj-1, and Map2 in De-hUCMSCs was higher than that in undifferentiated hUC-MSCs, suggesting that De-hUCMSCs retained some neuronal traits. Indeed, De-hUCMSCs could undergo redifferentiation with full expression of the neuronal markers (Fig 1c). Next, various genes involved in neurogenesis were compared between hUC-MSCs and De-hUCMSCs using focused PCR array profiling. The expression of multiple genes involved in neurogenesis were dramatically increased in De-hUCMSCs (Fig 1d), indicating a more potentiality of De-hUCMSCs towards neural fate. Furthermore, co-culture with either hUC-MSCs or De-hUCMSCs dramatically increased the number of viable cells after OGD, with significantly larger number of cells observed in co-culture with De-hUCMSCs as compared to that with hUC-MSCs (Fig 1e), indicating enhanced neuroprotective effects on PC-12.

Part 2

Treatment groups

A total of 134 adult Sprague Dawley rats were used. 24 rats were used in the preliminary experiment to establish the conditions of the rat HIE model. Ten rats were used to establish intracranial stem cell delivery method.

In the first experiment, 45 rats were divided into three groups (15 per group): phosphate-buffered saline (PBS), UC-MSCs, and De-hUCMSCs groups. 60-70% neonatal rats survived after HIE procedure. Three days after, stem cell treatment was

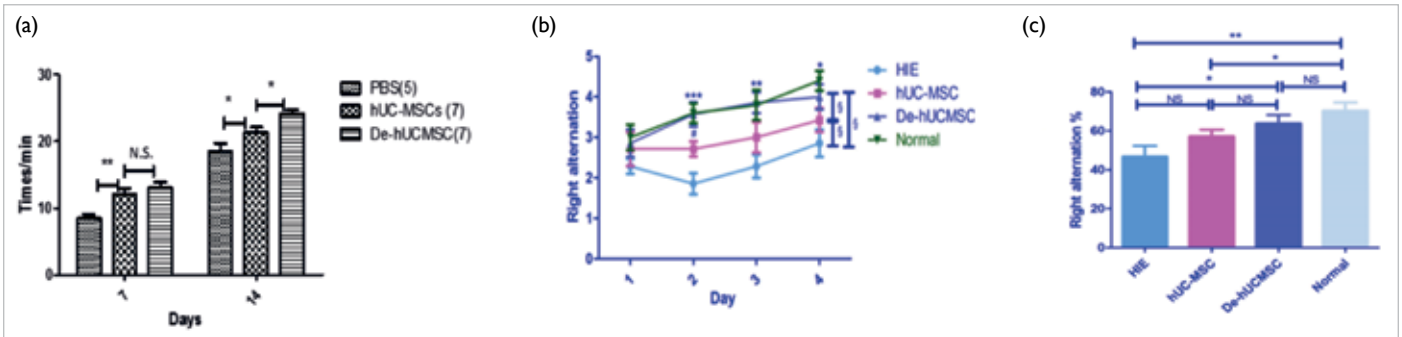

FIG. 2 Dedifferentiated human umbilical cord–derived mesenchymal stem cells (hUC-MSCs) significantly improves brain function recovery in hypoxic-ischaemic encephalopathy (HIE) rats. (a) hUC-MSCs and dedifferentiated hUC-MSCs transplantation improved holding time of neonatal rats. (b and c) Y-maze assay tested on postnatal day 42. Forced alternation was recorded in 4 consecutive days.

given intracranially to the survived neonatal rats. At day 6 after stem cell transplantation, rats were deeply anaesthetised, and the brain was fixed by trans-cardiac perfusion of 4% PFA. Fixed brain was embedded in OCT and 5-μm coronal sections were cut by cryostat. In the second experiment, 45 rats were used for behaviour tests (rotarod and shuttle box test) before and after stem cell transplantation. In the third batch of experiment, 10 neonatal rats were used for testing the feasibility of systemic injection of stem cells.

Functional outcomes

The sensorimotor cortex, striatum, and hippocampus of rats were predominantly damaged after HIE insult. Therefore, two corresponding behavioural tests were chosen; Y maze test was used for assessing spatial learning and memory for function of the hippocampus, and rotarod test for a motor cortical dysfunction.

In the rotarod test, the rats were given two attempts (5 minutes each) daily for 3 consecutive days of training. During the training period, the rotarod was set on an accelerating mode (from 4 to 20 rpm over 5 minutes), and this rotational speed was increased by 5 rpm each day. On day 3 of training, the rotational speed had reached 30 rpm, representing the speed used for the subsequent day's challenge. At day 7 (11 days after stem cell treatment), rats were given two attempts, and the summed duration of on rod-holding was recorded. In comparison with PBS-treated animals, both hUC-MSC- and hDe-UCMSC-treated groups significantly increased holding time at day 7 (11 days after stem cell treatment), while transplantation of either hUC-MSCs or De-hUCMSCs appeared to increase the holding time, this effect was more pronounced in rats receiving De-hUCMSCs than hUC-MSCs. In addition, at day 14, De-hUCMSC-treated rats showed a better recovery compared to hUCMSC-treated rats. (Fig 2a).

We then performed Y-maze with spontaneous and forced alternation in stem cell-transplanted HIE rats to measure their spatial working memory after they grew up (post-natal day 42). The percentage of alternation behaviours (both forced and spontaneous alteration) significantly decreased in HIE rats. This indicated that HIE mice developed working memory impairment. While hUC-MSC treatment slightly improved forced alternation behaviour, the alternation behaviour in De-hUCMSC-treated group was more significantly improved, in which almost recovered to a level equivalent to the normal group (Fig 2b, c). Altogether, these results indicated that De-hUCMSCs more effectively improved brain functional recovery in HIE.

Mechanistic studies

To determine the molecular mechanisms underlying the therapeutic effects of stem cells, rats were sacrificed and the brains were fixed by transcardiac perfusion of 4% PFA to perform immunofluorescent staining. The brain sections were stained with haematoxylin and eosin for evaluation of injured area. First, we sacrificed the rats 3 days after stem cell transplantation to determine whether hUC-MSCs or De-hUCMSCs could engraft into the injured area. Both hUC-MSCs (n=4) and De-hUCMSCs (n=5) engrafted into the injured area of the brain, there were no differences in the engraftment ability between two groups. Strikingly, both hUC-MSCs or De-hUCMSCs transplantation significantly decreased the injured lesion in the brain. However, there were no significant change between hUC-MSCs group or De-hUCMSCs group. We performed further immunofluorescent staining in the lesion boundary zone compared to the contra-side using neuron markers, such as MAP2, NeuN, and Nestin to evaluate the effect of stem cell treatment on neuronal protection in different groups

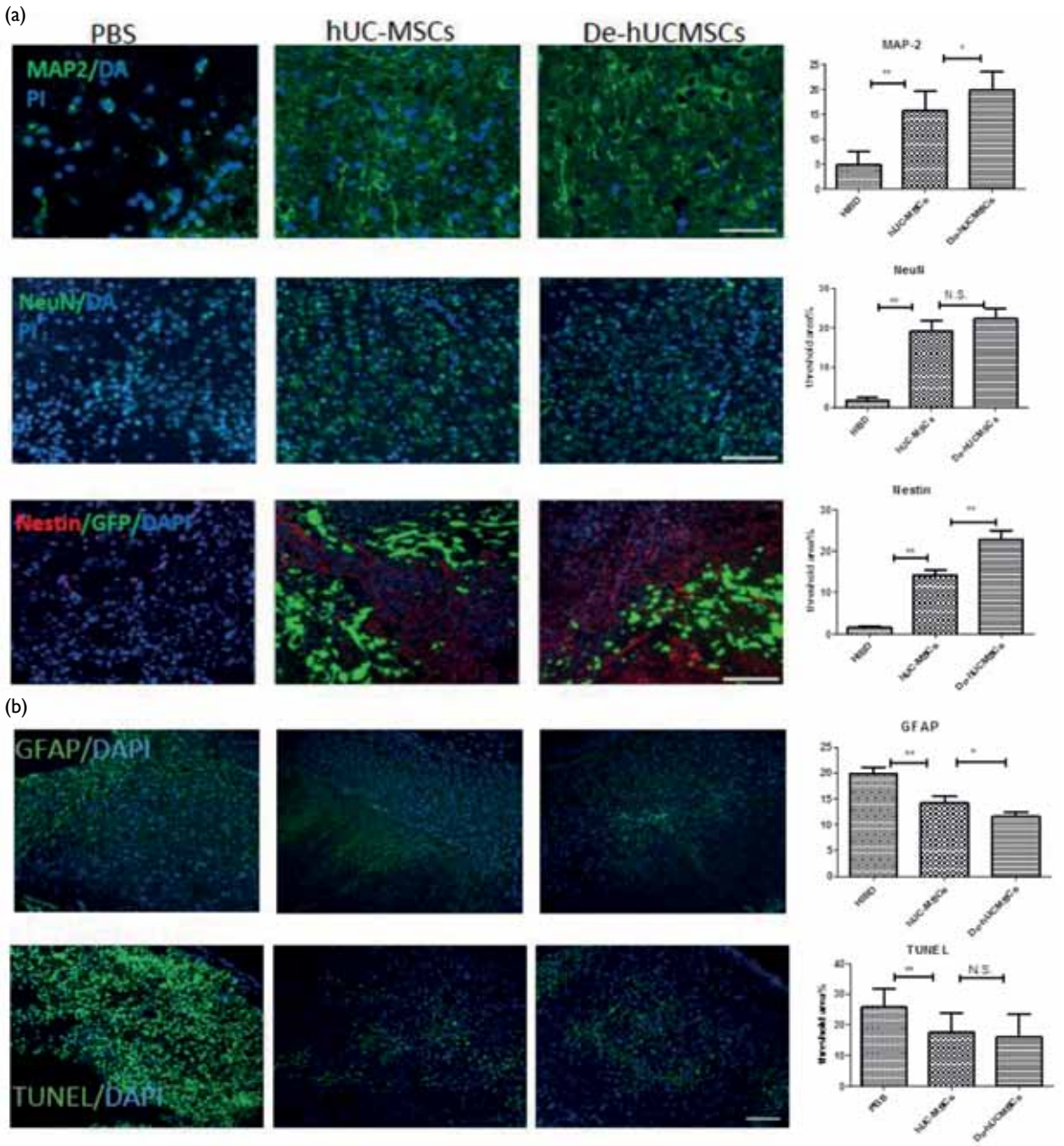

FIG. 3 Dedifferentiated human umbilical cord–derived mesenchymal stem cells (hUC-MSCs) exhibits enhanced neural-protective roles in hypoxic-ischaemic encephalopathy (HIE) rats. (a) Immunofluorescent staining showing dedifferentiated hUC-MSCs had better neural protective roles compared to hUC-MSCs (n=8 for each group). (b) GFAP staining showing less damage in dedifferentiated hUC-MSC–treated HIE rats; TUNEL staining showing both hUC-MSCs and dedifferentiated hUC-MSCs decrease the apoptosis-induced neural damage in HIE.

(Fig 3a). The expression of neuronal markers was dramatically increased with stem cell treatment. Of note, the expression of MAP2 and Nestin was more significantly increased in the De-hUCMSC-treated rats than in the hUC-MSC-treated rats, indicating De-hUCMSC with enhanced protective role in neuronal damage.

GFAP-staining was performed to identify reactive astrocytes in the brain after HIE. When neurons are damaged by ischaemia, astrocytes will generate to fill the space of dead neurons. Thus, the expression level of GFAP indicates the degree of brain damage. HIE alone exhibited significantly increased percentage of $GFAP^+$ cells in the lesion boundary zone of the injured hemisphere compared to contra-side. Stem cell treatment significantly reduced the $GFAP^+$ astrocyte in the injured brain compared to the PBS treatment. However, the effect

of De-hUCMSC on reducing the GFAP$^+$ astrocytes in the injured was more profound (Fig 3b). We then stained the brain tissues with TUNEL to evaluate the apoptotic response in untreated or stem cell–treated HIE models. Both hUC-MSCs and De-hUCMSCs treatment significantly decreased TUNEL positive cells compared to the PBS-treated group, indicating that stem cell treatment alleviated apoptosis-induced damage in HIE model (Fig 3b). However, there was no difference between hUC-MSCs and De-hUCMSCs treatment.

Conclusions

Local administration of hUC-MSCs or De-hUCMSCs significantly improved HIE recovery. Nonetheless, De-hUCMSCs exhibited stronger repair function than hUC-MSCs. Better functional recovery after treatment with De-hUCMSCs is associated with better neuronal protective roles. Although neither hUC-MSCs nor De-hUCMSCs express Nestin after engraftment, cells around them express extremely high levels of Nestin, suggesting that hUC-MSCs or De-hUCMSCs probably establish a niche benefit for endogenous stem cell regeneration in HIE brains. This effect is much stronger in De-hUCMSC-treated HIE brain. Altogether, De-hUCMSCs may elicit better therapeutic efficacy by paracrine effects on neuro-protection and neuro-regeneration.

With easy culture manipulation and low tendency of tumour formation, dedifferentiation strategy provides a feasible approach to enhance the therapeutic efficacy of stem cell therapy. De-hUCMSCs could be a superior source of stem cells to treat HIE.

Acknowledgement

This study was supported by the Health and Medical Research Fund, Food and Health Bureau, Hong Kong SAR Government (#01120056).

Results from this study have been published in: (1) Chen R, Lee WY, Zhang XH, et al. Epigenetic modification of the CCL5/CCR1/ERK axis enhances glioma targeting in dedifferentiation-reprogrammed BMSCs. Stem Cell Reports 2017;8:743-57. (2) Zhang J, Weng ZH, Tsang KS, Tsang LL, Chan HC, Jiang XH. MycN is critical for the maintenance of human embryonic stem cell-derived neural crest stem cells. PLoS One 2016;11:e0148062. (3) Ke C, Biao H, Qiangian L, Yunwei S, Xiaohua J. Mesenchymal stem cell therapy for inflammatory bowel diseases: promise and challenge. Curr Stem Cell Res Ther 2015;10:499-508.

References

1. Liu Y, Jiang X, Zhang X, et al. Dedifferentiation-reprogrammed mesenchymal stem cells with improved therapeutic potential. Stem Cells 2011;29:2077-89.
2. Dos Santos F, Andrade PZ, Boura JS, Abecasis MM, da Silva CL, Cabral JM. Ex vivo expansion of human mesenchymal stem cells: a more effective cell proliferation kinetics and metabolism under hypoxia. J Cell Physiol 2010;223:27-35.
3. van Velthoven CT, Kavelaars A, van Bel F, Heijnen CJ. Mesenchymal stem cell transplantation changes the gene expression profile of the neonatal ischemic brain. Brain Behav Immun 2011;25:1342-8.