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Reprogramming MSCs Through In Vitro Differentiation and Dedifferentiation for Enhancing Therapeutic Potential In Vivo

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The overall effectiveness and clinical use of stem cells is largely limited by low levels of cell survival and desired differentiation in vivo (*1*). Our recent studies show that mesenchymal stem cells (MSCs) can be reprogrammed to enhance their in vivo therapeutic potential through in vitro differentiation and dedifferentiation.

Recent studies have shown that terminally differentiated mammalian cells can be manipulated in vitro to undergo dedifferentiation into induced pluripotent stem cells (iPSCs) through genetic reprogramming (*2*). While these reprogrammed cells offer promise for patient-specific regenerative therapy, the therapeutic potential of iPSCs is limited by low efficiency, immunogenicity, reprogramming errors, and genomic instability leading to cancer (*3*).

We investigated whether it was possible to induce dedifferentiation in culture without gene manipulation and obtain reprogrammed stem cells with improved therapeutic potential. We observed dedifferentiation in vitro when we studied the plasticity of adult rat bone marrow MSCs (*4*). After in vitro induction and differentiation into 5 hydroxytryptamine (5-HT)-sensitive neurons, these cells could revert back to a morphological and phenotypical state similar to that of MSCs upon withdrawal of neuronal induction medium. The dedifferentiated MSCs (DeMSCs) could undergo further expansion in vitro and could be induced to redifferentiate into 5-HT sensitive neurons again, indicating that these adult stem cells are more plastic than previously thought. This notion is further supported by later studies demonstrating that monoclonally derived MSCs could cross the oligo-lineage boundary and become cells of unrelated lineages (*5, 6*). While direct transdifferentiation has been proposed to be the major route of MSC plasticity, we have demonstrated that dedifferentiation is a prerequisite for changing cell fate and redifferentiating into a different lineage, i.e. from neurons to epithelial cells or vice versa (*6*). In manipulating cell fates of MSCs in vitro, we made an interesting observation that cells that had undergone dedifferentiation exhibited stronger differentiation potency, i.e. had higher neuronal differentiation rates and neuronal marker expression levels when induced to redifferentiate. This suggests that DeMSCs may not be identical to their original MSCs counterparts.

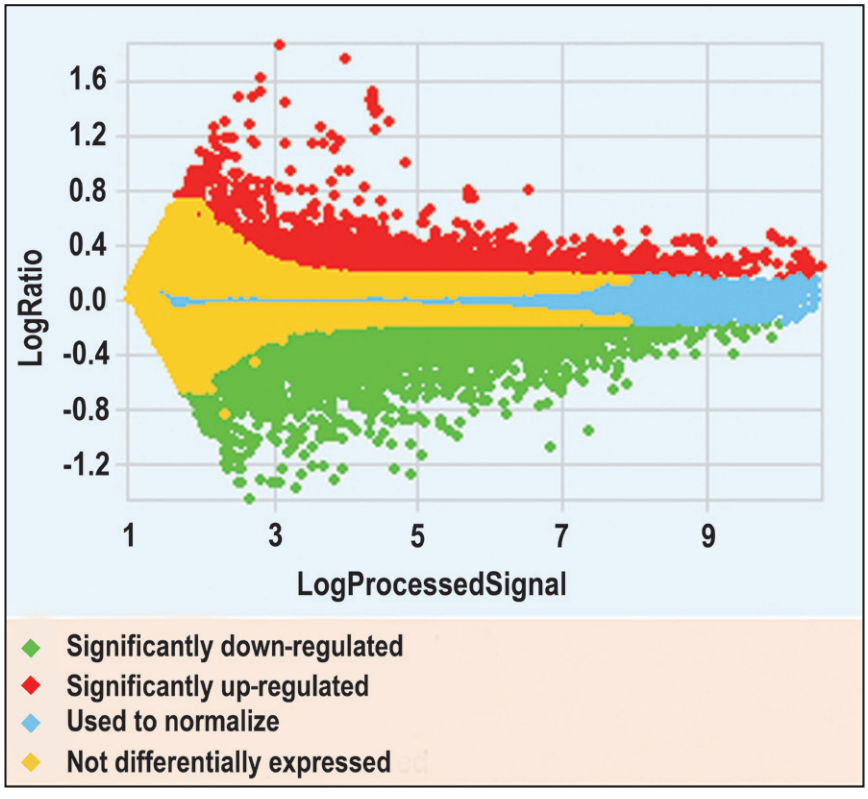

Figure 1. Scatterplot showing differential genes expressed in DeMSCs vs. MSCs as assessed by a rat whole genome microarray. Red: up-regulated genes (650/41012, 1.5%) in DeMSCs; Green: down-regulated genes (1240/41012, 3%); Yellow: genes not differentially expressed.

In a recent study, we set out to characterize DeMSCs and compare them to the undifferentiated MSCs (*7*). While DeMSCs were found to retain their immunophenotypic characteristics and mesodermal potential, detailed analysis of gene expression profiling showed 650 (1.5%) genes up-regulated and 1,240 (3.0%) genes down-regulated by more than twofold in DeMSCs compared to undifferentiated MSCs (Figure 1), indicating that DeMSCs had been reprogrammed. Further analysis showed that 13 out of 84 apoptosis-related genes examined were differentially expressed between MSCs and DeMSCs, with enhanced expression of bcl-2 family proteins in DeMSCs. Enhanced survival

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in DeMSCs was also demonstrated with a greater number of viable cells, compared to MSCs, observed under oxidative stress. We also noticed that the expression of neuronal markers in DeMSCs was much higher than that in undifferentiated MSCs, indicating that DeMSCs maintained some neuronal traits, and therefore possibly greater potential for redifferentiation into neurons. The enhanced survival and neuronal potency of DeMSCs, which can persist up to three to four passages, prompted us to investigate the therapeutic potential of DeMSCs in vivo using a rat model of neonatal hypoxic-ischemic brain damage (HIBD), a common cause of long-term neurological disability in children with no effective treatments currently available (*8*). Our results showed that while both transplanted GFP-tagged MSCs or DeMSCs were readily found near the injection sites three days after transplantation, only DeMSCs could be detected by day seven (Figure 2A, 2B), with some co-localization with neuronal markers NF-M and MAP2, indicating enhanced survival and neuronal differentiation in vivo. Importantly, a shuttle box test to evaluate cognitive function (*9*) showed significantly greater improvement in DeMSC-treated animals compared to MSC-treated rats one to two months after cell transplantation. Taken together, our results suggest that MSCs can be reprogrammed through culture manipulations with enhanced therapeutic potential in vivo.

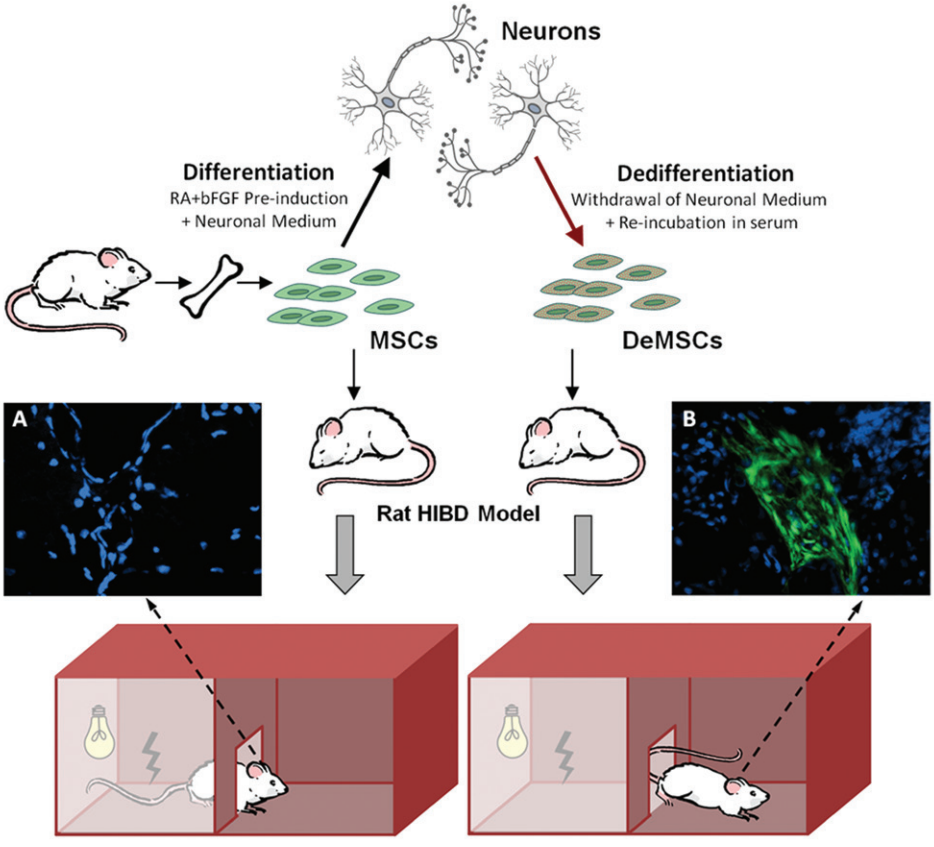

Figure 2. Schematic illustration of the experimental design and beneficial effects of DeMSCs in HIBD model (from reference *7*). To initiate neuronal differentiation, rat bone marrow derived monoclonal MSCs were first pre-induced with media consisting of DMEM/F12/10% FBS/10^{-7} M all-*trans*-retinoic acid (ATRA) and 10 ng/mL basic fibroblast growth factor (bFGF). Cells were washed with PBS and transferred to neuronal induction media composed of modified neuronal medium (MNM, DMEM/2% DMSO/200 µM butylated hydroxyanisole (BHA)/25 mM KCl/2 mM valproic acid/10 µM forskolin/1 µM hydrocortisone/5 µg/mL insulin) for 24–48 hours. Dedifferentiation was achieved by withdrawal of neuronal induction medium, and then reincubating cells in DMEM/F12 with 10% FBS for 24–48 hours. To determine the therapeutic effect of DeMSC in vivo, GFP-MSCs or GFP-DeMSCs were injected into the right lateral cerebral ventricle five days after the induction of hypoxic-ischemic brain damage (HIBD) rat model. Representative brain sections obtained seven days after transplantation show no GFP-expressing MSCs (**A**) but strong GFP signal from DeMSCs (**B**), indicating enhanced survival. Shuttle box test evaluating learning and memory ability was performed, demonstrating significantly greater improvement in DeMSC-treated animals as compared to the MSC-treated rats one to two months after cell transplantation.

Our findings provide a novel and clinically practical method to overcome the hurdles faced by current MSC-based therapy. The dedifferentiation-driven reprogramming in culture may prove to be a simpler, safer, and more effective approach in regenerative medicine compared to iPSCs. Interestingly, the genes known to be responsible for inducing iPSCs are not significantly altered in DeMSCs, suggesting different mechanism(s) involved. In particular, significant upregulation of miR-34a was observed in DeMSCs and was linked to enhanced cell survival and neural potentiality, hinting at the possible involvement of an epigenetic mechanism in the reprogramming. It remains unclear whether the dedifferentiation-driven reprogramming is limited to MSCs committed to neuronal lineage differentiation. Further investigation will undoubtedly improve in vitro reprogramming of MSCs to maximize the therapeutic efficacy of DeMSCs as a novel and practical treatment strategy in regenerative medicine.

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