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Differentiation of reprogrammed human adipose mesenchymal stem cells toward neural cells with defined transcription factors

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ABSTRACT

Somatic cell reprogramming may become a powerful approach to generate specific human cell types for cell-fate determination studies and potential transplantation therapies of neurological diseases. Here we report a reprogramming methodology with which human adipose stem cells (*h*ADSCs) can be differentiated into neural cells. After being reprogrammed with polycistronic plasmid carrying defined factor OCT3/4, SOX2, KLF4 and c-MYC, and further treated with neural induce medium, the *h*ADSCs switched to differentiate toward neural cell lineages. The generated cells had normal karyotypes and exogenous vector sequences were not inserted in the genomes. Therefore, this cell lineage conversion methodology bypasses the risk of mutation and gene instability, and provides a novel strategy to obtain patient-specific neural cells for basic research and therapeutic application.

1. Introduction

Adipose-derived mesenchymal stem cells (ADSCs) are multipotent somatic cells that hold promise for stem cell-based therapies. It has been demonstrated that ADSCs can be obtained relatively easily by a less invasive method can self-renew and be induced to differentiate along several cell lineages such as adipocytes, osteoblasts and chondrocytes. Thus, ADSCs has become an ideal cell resource for regenerative medicine, since the ethical and immunological rejection limitations can be overcome [1].

Therefore, lots of differentiation investigations from ADSCs to neural cells were tried [2]. Several in vitro studies described the conditions under which ADSCs can be differentiated into neural-like cells [3]. However, controversy persists regarding the differentiation potentials of ADSCs to neural lineages [4]. Some protocols were reversible and transiently since ADSCs might revert to normal morphologies after passaging with these methods [5]. Therefore, it is critical to stress the prior technology that can induce ADSCs into rigorous quality neural cells for accurately modeling human diseases from genetically neurogenetic disorders and for the development of drug therapies.

The induced pluripotent stem cells (iPSCs) technology represents a significant advance in the field of regenerative medicine and provides a powerful tool for the study of cell-fate transitions [6]. A cocktail of key pluripotency genes (Oct3/4, Sox2, Klf4 and c-Myc) were sufficient to reprogram human somatic cells into iPSCs that are capable of differentiation into all three germ layers during embryoid body (EB) formation and in vivo teratoma analysis in immune-compromised mice. It has also been demonstrated that neuronal subtypes can be successfully generated from patient-specific iPSCs derived from an amyotrophic lateral sclerosis patient, whose motoneurons were affected by the diseases [7]. Bi Song's group has generated functional neural cells from relapsing-remitting multiple sclerosis patient-specific iPSCs [8]. More recently, Yong-Hee Rhee's group identified sets of protein-based human iPSCs and efficiently generated functional dopamine neurons, which were used to treat a rat model of Parkinson disease [9]. In all these experiments, the EB formation method was adopted to generate the neural subtypes after induced the fibroblasts were induced into iPSCs. The efficacy of differentiation and function of these iPSCs derived neural cells will undoubtedly be an intense area of future investigation [3,10].

Although the production of iPSCs has been achieved in mouse and human, a major impediment of iPSCs in regenerative medicine has been the quality control procedures related to the culture of iPSCs that is particularly important [11]. Moreover, viral-based delivery pluripotent factors methods increase the risk of insertional mutagenesis in target cell genome [12]. Thus, it is crucial to perform karyotype analysis on iPSCs upon generation and regularly throughout maintenance in culture because the cells are subject to genetic instability, especially after prolonged serial passage [13].

A non-viral polycistronic plasmid was used in our previous studies to reprogram ADSCs into iPSCs under feeder-free conditions [14]. Here we applied the similar protocol firstly to induce

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ADSCs to a plastic intermediate states, then to induce them to differentiate toward neural cells. In this way, the efficient differentiation of ADSCs toward neural cells and maintenance of differentiated cells can be achieved.

2. Materials and methods

2.1. Isolation and culture of ADSCs

The adipose tissue was aseptically collected from subcutaneous fat pads of the patient under anesthesia, detail of the work conformed to the provisions of the Declaration of Helsinki (as revised in Edinburgh 2000). Fat pad tissues were excised, finely minced, and digested with 0.1% collagenase type 1 (Gibco, USA) at 37 °C for 40 min. An equal volume of basic medium (DMEM, HyClone, USA) supplemented with 10% fetal bovine serum (FBS, HyClone, USA) was added to neutralize the collagenase. Then, samples were centrifuged at 1500 rmp for 10 min at room temperature to obtain pellets. The pellets were washed with basic medium once, resuspended in basic medium, and filtered through a 0.075-mm cell strainer to remove undigested debris. The resulting cells were planted in a 25-cm^2 culture flask in DMEM medium supplemented with 10% fetal FBS, 2 mmol/L L-glutamine, 50 U/ml penicillin, 50 mg streptomycin (Invitrogen, Australia). Medium was changed every 3 days. Cells were passaged until reaching approximately 70–80% confluence.

2.2. Transfection and cell culture

Human ADSCs of the third passage were used for the transgenes with a polycistronic that encoding *OCT4*, *SOX2*, *KLF4* and *c-MYC*. Briefly. 24 hours before transfection, exponentially growing cells were harvested by trypsin and replated at a density of 1.5×10^5 in each well of 6-well plates in the appropriate complete DMEM medium. Cells were first transduced with individual polycistronic plasmid on day 0. Transduction was repeated on day 4 using the same volume of plasmid DNA, and cultured in complete DMEM medium. The polycistronic plasmid consisting of a neomycin-resistance cassette (Neo^r) allows stably transfected eukaryotic cells to be selected using G418 (Invitrogen, Australia). On day 7 after the first transduction, culture medium switched from the *h*ADSCs growth medium to G418-containing (200 μg/ml) growth medium for selection. A week later, the medium was replaced with growth medium without drug selection for another week culturing. The growth medium was DMEM/F12(1:1) containing 20% FBS, 2 mM L-glutamine, 1% nonessential amino acids, 1 mmol/L sodium pyruvate, 0.1% mM β-mercaptoethanol (HyClone, USA), 20 ng/ml basic fibroblast growth factor (bFGF, Chemicon, USA), 50 U/ml penicillin, and 50 μg/ml streptomycin. Then, the pluripotent marker expression of the transfected cells was evaluated.

2.3. In vitro differentiation of human iPSCs into neuronal cells

After the cells proliferated in growth medium for a week, the culture medium was switched to neural induced medium (neurobasal medium supplemented with 20 ng/ml bFGF, 20 ng/ml epidermal growth factor (Peptrotech, USA), 2% B27 supplement (NeuroCult, Canada) and 0.5 μg/ml retinoic acid (Invitrogen, Australia). The cells were visualized with a phase-contrast microscope. When the numbers of cell masses reached 15–20, the cells were passaged (1:2) and seeded onto the six-well adherent plates coated with poly-L-ornithine/laminin (Sigma, USA) at 2.5×10^4 cells/cm^2 in neural induced medium. The medium was changed every 3–4 days. The cells were cultured for 4 weeks.

2.3.1. Southern blotting

Cells were suspended in cell lysis buffer (50 mM Tris–HCl, pH 8.0, 400 mM NaCl, 100 mM EDTA, 0.5% sodium dodecylsulfate) supplemented with Proteinase K (50 μg/ml, Takara). After overnight incubation at 55 °C, high molecular weight DNA was extracted with a Genomic DNA Prep Kit (TaKaRa, Japan). Fragments resulting from overnight 100 Unit/μl BamI (TaKaRa, Japan) digestion of 20 μg of genomic DNA were separated on a 0.7% agarose gel, transferred to a positive charged BrightSter-Plus nylon membrane (Ambion) in alkaline solution(0.4 M NaOH and 2 M NaCl), and hybridized with synthesized biotin-labeled probes of GFP and then detected with the Biostar-BioDetected Kit (Ambion). The sequences of the primers used to amplify the probe are supplied in follows. GFP: 5′-ACGTAAACGGCCACAAGTTCAGC-3′, 5′-TAGTGGTTGTCGGGCAGCAGCAC-3′.

2.4. Fluorescence immunostaining

Cells were fixed in 4% paraformaldehyde for 20 min at 25 °C, washed three times with PBS, and blocked for 15 min with 5% FBS in PBS containing 0.1% Triton-X, After incubation with primary antibodies against OCT4 (#886906, Abcam); Nanog (#885871, Abcam); Tra-1-60 (#sc-21705, Santa Cruz); SSEA4 (#sc-21704, Santa Cruz); nestin (#250764, Abbiotec); GFAP (#sc-9065, Santa Cruz); RIP (#sc-7781, Santa Cruz); Nurr1 (#sc-5568, Santa Cruz) and TUJ1 (bs-2670R, Bioss) for 1 h in 1% PBS containing 0.1% Triton-X, cells were washed three times with PBS and incubated with fluorophore-labeled appropriate secondary antibodies at room temperature for 1 h and 50 μg/mL Hoechst for 5 min. Secondary antibodies: Goat anti-mouse lgM-PE (#sc-3768, Santa Cruz) or Goat anti-rabbit lgG-PE (#sc-3739, Santa Cruz). Fluorescence imaging was performed using Olympus AS-70 microscope and Image-Pro Plus Software.

Cell number of the neural lineages was determined by counting the number of positive cells per field at 40× magnification, under a fluorescent microscope (Olympus AS-70 microscope). Six visual fields were randomly selected and 221–284 cells were counted for each sample.

2.5. Reverse-transcription polymerase chain reaction (RT-PCR) and quantitative real-time PCR

Total RNA was extracted using TRizol Reagent (Invitrogen, CA, USA) and treated with RNase-Free DNase Set (QIAGEN, Germany) to remove any contaminating genomic DNA. For RT-PCR, cDNA was synthesized using a PrimeScript RT reagent Kit (Takara Bio Inc., Japan), after which RT-PCR was run using ExTaq (Takara Bio Inc.). The primers used are listed in Table 1.

2.6. Statistical analysis

Each experiment was independently performed more than three times ($n \geqslant 3$). Relative data were expressed as mean ± SEM.

3. Results

3.1. hADSC transfection with the polycistronic plasmid

The reprogramming factors (Oct3/4, Sox2, Klf4 and cMyc) were cloned into the pIRES2-EGFP plasmid under the transcriptional control of the CMV promoter. In order to minimize the number of plasmids, we used a polycistronic expression cassette where the four factors were separated by T2A, P2A and E2A peptides (4F2A). The backbone of the pIRES2-EGFP plasmid carried a

Table 1
Primers for reverse-transcription polymerase chain reaction.

Gene		Sequence
OCT3/4(NM_002701)	Sense	ACTCCTGCTTCGCCCTCA
	Antisense	TCGGATTTCGCCTTCTCG
SOX2(NM_003106)	Sense	TCGCAGCCGCTTAGCCTCGT
	Antisense	AACAGCCCGGACCGCGTCAA
KLF4(NM_004235)	Sense	GACTCACCAAGCACCATC
	Antisense	CAGCCAGAAAGCACTACAA
c-Myc(NM_002467)	Sense	CCTCATCTTCTTGTTCCTCCT
	Antisense	ACAGCGTCTGCTCCACCT
TERT(NM_001193376)	Sense	GGAACCATAGCGTCAGGGAGG
	Antisense	AAGTGCTTGGTCTCGGCGTA
Nanog(NM_024865)	Sense	GTCTGGACACTGGCTGAA
	Antisense	GACCATCCTGGCTAACAC
nestin(NM_006617)	Sense	ACCCTTGCCTGCTACCCT
	Antisense	AGCCTGTTTCCTCCCACC
REX1(NG_009317)	Sense	TGAAAGCCCACATCCTAACG
	Antisense	CAAGCTATCCTCCTGCTTTGG
Nurr1(AB017586)	Sense	GTTTCCAGGCTCCTCATTG
	Antisense	CAACACCACCCTTACTCAACA
β-Actin(E00829.1)	Sense	CGCTGCTCAGGGCTTCTTGT
	Antisense	TGTCCATGTCGTCCCAGTTGGT
GAPDH(NM_017008)	Sense	CTCTGCTCCTCCCTGTTC
	Antisense	CCAGTAGACTCCACGACATAC

neomycin-resistance cassette (Neo^r) and a GFP gene that can select and monitor the transfected cells (Fig. 1A).

In our reprogramming experiments, *h*ADSCs were transfected with polycistronic plasmids containing pluripotency genes on day 0 without feeder cell layers. Transfection was repeated on day 4 using the same volume plasmid. It could be detected that the cells expressed GFP after transfected 48 h. After the first transfection 7 days, culture medium was switched from the *h*ADSCs growth medium to G418-containing growth medium for selection of transfected *h*ADSCs. A week later, the medium was replaced with growth medium without drug G418 for cells proliferation (Fig. 1B). During the period, these cells firstly changed their morphology from typical fibroblast-like to round shape (Fig. 1C and D), then into compactly assembled state with abundant nucleus / cytoplasm (Fig. 1E). And they expressed positive GFP fluorescence (Fig. 1F–H).

3.2. Reprogrammed *h*ADSCs expresings pluripotency markers

Reprogrammed *h*ADSCs (R-*h*ADSC) were expanded and characterized for pluripotency. As revealed by immunofluorescence, the R-*h*ADSCs expressed undifferentiated pluripotency markers such as Oct3/4 (Fig. 2A), Nanog (Fig. 2B), tumor recognition antigens 1-60 (TRA-1-60) (Fig. 2C) and SSEA4 (Fig. 2D). To further evaluate the expression of pluripotency markers, we performed RT-PCR analysis and found that R-*h*ADSCs expressed not only pluripotency genes Oct4, Sox2, Klf4 and c-Myc, but also ESCs specific pluripotency genes REX1 and TERT. Comparatively, *h*ADSCs only expressed Klf4 and c-Myc genes (Fig. 2E). Therefore, based on the morphology and pluripotency marker expression, the R-*h*ADSCs at least displayed partly typical characteristics of pluripotent stem cells. Additionally, karyotyping was normal in R-*h*ADSCs after transfected with plasmids 4 weeks later.

To rule out genomic integration of pIRES-GFP sequences other than cDNAs, we performed Southern blot analysis using the BamHI-digested cells genomic DNA. GFP probe shows hybridization similar molecular weight band(s) indicating no exogenous vector sequences in the genomes of R-*h*ADSCs. Although highly unlikely, we could not rule out the possibility that small pieces of polycistronic plasmid vector had inserted into the genome of cell line but were not observed because of the detection limits of Southern blot analysis.

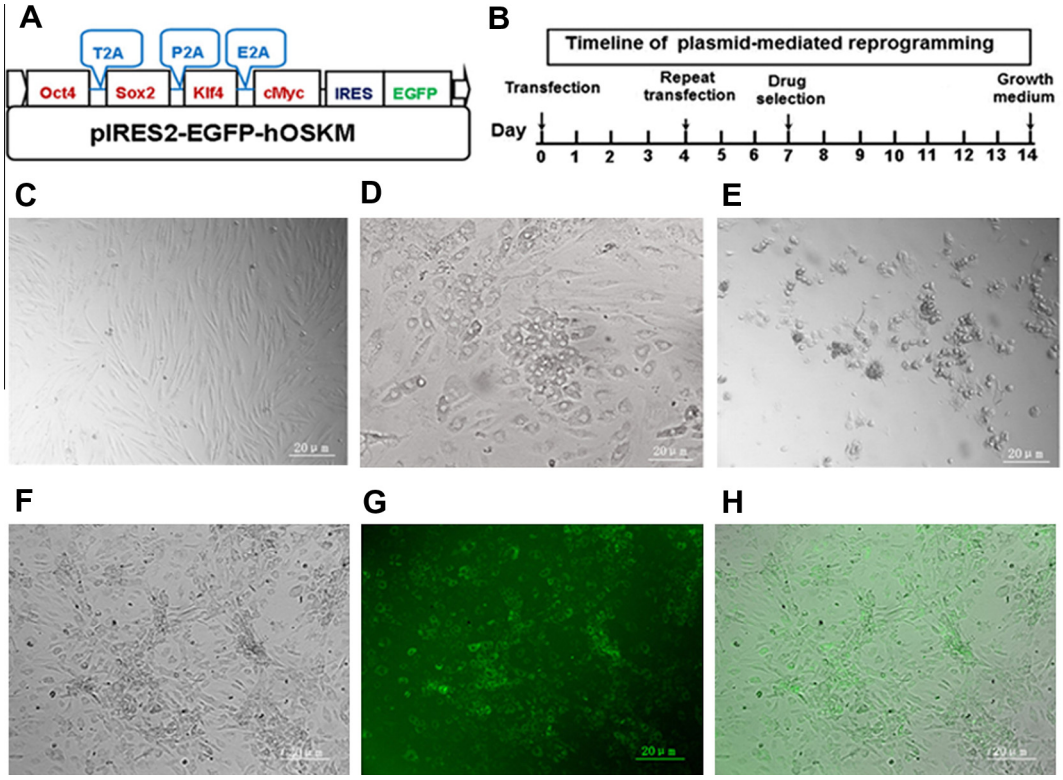

Fig. 1. Reprogramming *h*ADSCs with a polycistronic plasmid. (A) Schematic of the polycistronic plasmid containing four transcriptional factors (Oct3/4, Sox2, Klf4 and c-Myc) sequence fused in-frame via three 2A (T2A, P2A and E2A) elements. (B) Schematic diagram for reprogramming *h*ADSCs. (C) Morphology of *h*ADSCs before transfected. (D) Morphology of reprogrammed *h*ADSCs after transfected 14 days. (E) Morphology of reprogrammed *h*ADSCs after transfected 21 days. (F) Comparative representation of GFP expression after transfected 14 days. Phase contrast. (G) Drug selected cells expressed GFP positive fluorescence. (H) The merger of (F) and (G).

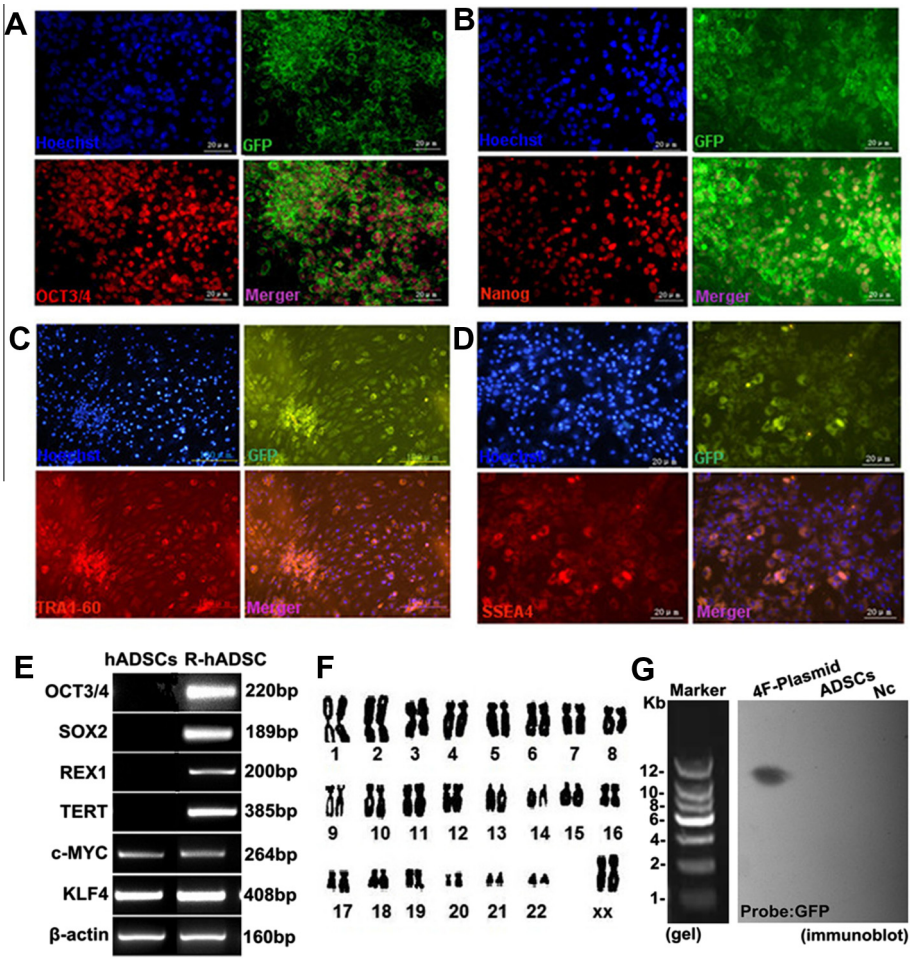

Fig. 2. R-*h*ADSC expressed the pluripotency markers. (A–D) Immunofluorescent staining with OCT3/4, Nanog, TRA1-60, SSEA4. (E) RT-PCR demonstrated that R-*h*ADSCs expressed pluripotency related genes Oct3/4, Sox2, REX1, TERT, c-Myc, and Klf4, while the *h*ADSCs expressed c-Myc and Klf4 only. (F) Karyotype of R-*h*ADSC was normal 46XX. (G) Southern blot analyses indicated R-*h*ADSCs had no plasmid sequence insertion.

3.3. R-hADSCs were able to differentiate into neural-like cells

In order to assess the differentiation potential of R-*h*ADSC toward neural lineages, the cells were subjected to neural induction culture. As control, *h*ADSCs did not change their morphology towards neural cells (Fig. 3A). However, R-*h*ADSCs displayed a profile suggestive of neural induction. Indeed, five days after culturing in induction medium, the cell clusters were dissociated and resulted in morphologically uniform neuronal precursor cells (Fig. 3B). Upon continually induced differentiation, it was observed that the majority of cells extended neurosynaptic states at 10 days (Fig. 3C), 15 days (Fig. 3D), 21 days (Fig. 3E). As shown by RT-PCR (Fig. 3F), the stem cell markers Sox2, Klf4, and c-Myc were detected in the induction process at 5 days (N-5), 10 days (N-10) and 20 days (N-20). Then, the expression of the neural progenitor cell markers nestin at N-5, N-10 and N20, mature neuronal marker Nurr1 appeared at N10 and N20, further supporting the neural differential potential of R-*h*ADSCs.

We next assessed if, with time in culture, these induced R-*h*ADSCs could be differentiated into mature neurons, astrocytes and oligodendrocytes. In all of our experiments, the control *h*ADSCs were cultured in the same conditions as R-*h*ADSCs but detected the neural cells related markers expression negatively (Fig. 4A–E). However, five days after culturing in induction medium, the R-*h*ADSCs were detected with nestin expression (Fig. 4A′). By day 10, these neurosphere-like cells started to express neural markers, TUJ1 (Fig. 4B′) and some cells adopted astrocytic marker, GFAP (Fig. 4C′). At the next 5 days, some cells expressed oligodendrocytes marker RIP (Fig. 4D′) [18]. As determined by counting the number of positive cells per field under the fluorescence microscope, the number of TUJ1, GFAP and RIP-positive cells were 74.6 ± 4.8% (n = 264), 64.3 ± 5.4% (n = 284) and 47.3 ± 6.4% (n = 221), respectively.

Meanwhile, after 3 weeks culturing in neural induce medium, the neurosynaptics were found to grow out from the cells and the expression of mature neural marker nurr1 was also detected (Fig. 4E′). Our results showed that the differentiated R-*h*ADSCs comprised a mixed population of cells expressing one or more of the following markers: TUJ1, 82.7 ± 7.3% (n = 266), GFAP, 70.5 ± 8.0% (n = 258), RIP, 60.4 ± 5.2% (n = 212) and Nurr1, 73.6 ± 9.2%, (n = 244) respectively.

4. Discussion

Neural tissue has historically been regarded as having poor regenerative capacity but recent advances in the growing fields of regenerative medicine have opened new hopes for the treatment of nerve injuries and neurodegenerative disorders. The generation of patient-specific ADSCs derived functional neural cells is of great therapeutic attractive approach with regard to the potency of neurodegenerative disorders [15,16]. In the previous studies, *h*ADSCs have been reported to trans-differentiate towards neurons and glial cells [3]. However these findings are not universally accepted

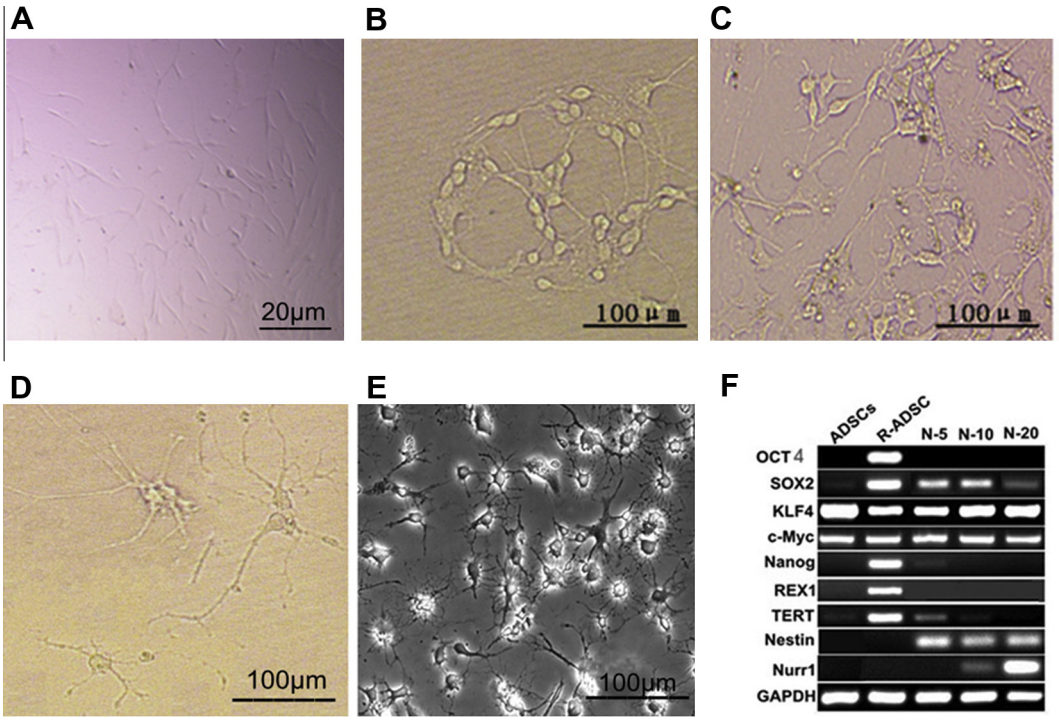

Fig. 3. Induction of R-ADSCs into neural lineage differentiation. (A) ADSCs morphology cultured in neural differentiated culture medium agter 21 days. (B–D) The R-*h*ADSCs adopted morphology following 5, 10, 15 days respectively in neural differentiated culture medium. (E) The cells morphology after 21 days induction. (F) RT-PCR analysis performed on *h*ADSCs, R-*h*ADSC, N-5, N-10 and N-20 showing that the expression of the neural progenitor cell markers and the neural markers.

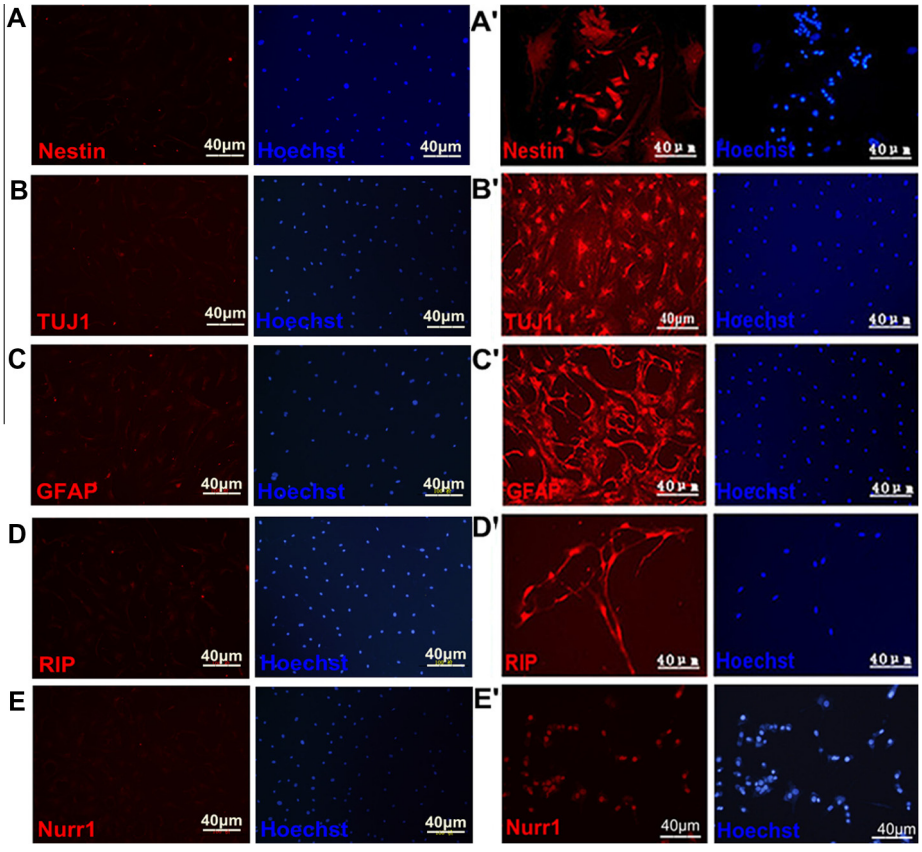

Fig. 4. Immunofluorescent staining to analyze whether ADSCs and R-ADSCs express neural lineage specific markers. (A–E) for ADSCs, as control. (A′) 5 days after induction, the R-ADSCs showed expressing nestin marker. (B), (C) After 10 days differentiated culture, the R-ADSCs had been detected expressing the neuronal marker TUJ1 and the astrocyte marker GFAP. (D) After15 days, the R-ADSCs expressed the oligodendrocytes marker RIP. (E) At 21 days after induction, the R-ADSCs expressed mature neuronal marker Nurr1.

and have been disputed in the literatures. Whilst ADSCs have been studied as a potential source of neurons, their role will most likely be restricted to cell-based neuroprotective strategies rather than for neuronal replacement [17].

Currently, further advances have been reported in which adult cells can be directly converted from other cell types into neural cell lineages [18]. Researchers have identified a set of transcription factors that induce the conversion of mouse fibroblasts into neurons by the activation of the neural factors Ascl1, Brn2, and Myt1l [19], and the conversion of human fibroblasts into neural stem cells by the factors Brn4/Pou3f4, Sox2, Klf4, c-Myc, plus E47/Tcf3 [20]. Although it has been shown that fibroblasts can be directly converted into dopaminergic neurons, this work is still in its infancy, and further tests will be needed to confirm the karyotype of these cells, and to determine whether the cells have been completely reprogrammed [10]. More importantly, the question then arises whether, these cocktail of the neural factors will be possible to directly convert any easily obtainable cell types into neural cells for regenerative, including ADSCs.

Comparatively, the iPSCs-derived EB models and the reprogramming methods have some advantages over the method of directly inducing ADSCs into neural cells especially in neurodegenerative diseases models. Neurodevelopmental disorders are particularly relevant to the reprogramming method and the iPSCs-derived EB model because patient-specific ADSCs can be reprogrammed to an early or pluripotent state. Therefore, neural tissue generated from these cells could represent the early stages of disease [21]. Indeed, the neuronal immaturity means that adult-onset diseases might be more difficult to model than earlier-onset diseases [22].

Traditionally, EB formation method was used to derive neural cell types from iPSCs [23]. However, EB formation needs specific culturing conditions and manipulations, and EB has the potential to develop into multiple different tissue lineages spontaneously [24]. Therefore, the EB formation method was a complicated process that increases not only the risk of neoplasia and undesired cell types, but also the time span and the probability of genomic instability.

For transplant therapy, it is particularly important to consider the advantages and disadvantages of factor delivery method for reprogramming. The use of integrating viruses for transgenes has represented a major roadblock in the pursuit of clinically relevant applications, as genomic insertion has been shown to alter gene function, and residual activity of reactivation of viral transgenes in reprogrammed somatic cells can interfere with their developmental potential and frequently leads to the formation of tumors in chimeric animals [25]. Comparatively, the constructed polycistronic plasmid in this experiment can be transfected into somatic cells without the need for viral packaging and can be subsequently removed from cells by culturing in the absence of drug selection. More importantly, the plasmid vector cannot replicate in *h*ADSCs because its replication needs cells expression of the SV40T antigen [14]. Therefore, the nonintegraing plasmid approaches were more suitable for clinical translational application albeit the reprogramming efficiencies with current plasmid methods (0.01%) are several orders of magnitude lower than those achieved with integrating vectors (0.1–1%) [25].

Because of the reduced discomfort and stress associated with cell sample collection, the optimal cell type must be considered in a given reprogramming method for clinical application. In previous studies, human fibroblasts and *h*ADSCs had been successfully reprogrammed to iPSCs [14]. Likewise, both of them are easily available and they can be expansion of primary cells to an adequate number in vitro. Nevertheless, ADSCs are unique human cell types that have supporting proliferation and self-renewal properties. By RT-PCR analysis, ADSCs express the factors Klf4 and c-Myc endogenously which play critical role in the reprogramming pluripotency. Another property of ADSCs was that it not only intrinsically secret high levels of pluripotency-sustaining factors including basic FGF and LIF, but also express endogenous factors in self-renewal such as TGFβ1, fibronection-1,vitronectin, and activin A [26]. While fibroblast obtained from skin tissues were more easily to be acquired DNA damage by accumulated UV-induced mutations. Therefore, ADSCs might be more suitable for a given application [27].

In summary, a reprogramming approach that can convert human ADSCs to patient-specific neural cell types by non-integrative plasmid method has been developed in this study. This method may be an ideal option for avoiding immunological rejection during cell transplantation. The abundant availability of fat biopsies would make it relatively easy to establish an adipose-derived "cell library" from individuals.

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