Research Article

Caveolin-1 downregulation promotes the dopaminergic neuron-like differentiation of human adipose-derived mesenchymal stem cells

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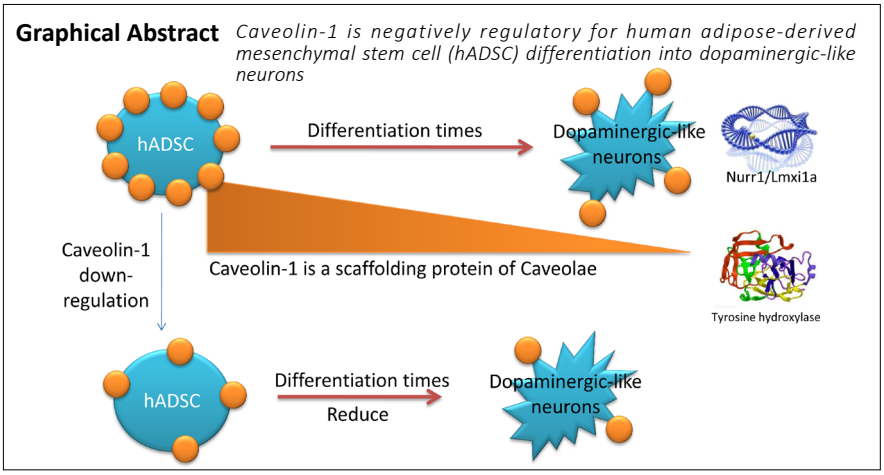
Graphical Abstract *Caveolin-1 is negatively regulatory for human adipose-derived mesenchymal stem cell (hADSC) differentiation into dopaminergic-like neurons*



Abstract

Previous studies have shown that caveolin-1 is involved in regulating the differentiation of mesenchymal stem cells. However, its role in the differentiation of human adipose mesenchymal stem cells into dopaminergic neurons remains unclear. The aim of this study was to investigate whether caveolin-1 regulates the differentiation of human adipose mesenchymal stem cells into dopaminergic-like neurons. We also examined whether the expression of caveolin-1 could be modulated by RNA interference technology to promote the differentiation of human adipose mesenchymal stem cells into dopaminergic-like neurons. The differentiation of human adipose mesenchymal stem cells into dopaminergic neurons was evaluated morphologically and by examining expression of the markers tyrosine hydroxylase, Lmx1a and Nurr1. The analyses revealed that during the differentiation of human adipose mesenchymal stem cells into dopaminergic neurons, the expression of caveolin-1 is decreased. Notably, the downregulation of caveolin-1 promoted the differentiation of human adipose mesenchymal stem cells into dopaminergic-like neurons, and it increased the expression of tyrosine hydroxylase, Lmx1a and Nurr1. Together, our findings suggest that caveolin-1 plays a negative regulatory role in the differentiation of dopaminergic-like neurons from stem cells, and it may therefore be a potential molecular target for strategies for regulating the differentiation of these cells. This study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Dalian Medical University of China (approval No. PJ-KS-KY-2020-54) on March 7, 2017.

Key Words: cells; factor; *in vitro*; neural differentiation; Parkinson's disease; plasticity; protein; stem cells

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Introduction

Mesencephalic dopaminergic neurons, which degenerate in patients with Parkinson's disease (PD), are derived from progenitors located at the ventral midline of the midbrain (Hynes and Rosenthal, 1999; Peterson et al., 2019; Goulding et al., 2020; Tomov et al., 2020). This developmental process is orchestrated by key signaling molecules secreted by neighboring organizers and their intrinsic transcription factors (Tomozawa and Appel, 1986). Characterization of the molecular program that controls the generation of dopaminergic neurons from stem cells may be highly useful for the development of stem cell-based therapies for PD (Veenvliet, 2016).

Human adipose-derived mesenchymal stem cells (hADSCs) are multipotent stem cells found within the adipose tissue, and have been propounded as an ideal source for regenerative medicine (Andersson et al., 2006). hADSCs offer several advantages over other multipotent cells, including rapid

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proliferation and high abundance. In addition to their capacity to differentiate into osteogenic, chondrogenic and adipogenic cell lineages, depending on the specific environment (Prockop, 1997; Pittenger et al., 1999), recent studies have demonstrated that hADSCs can be induced to undergo neuron-like differentiation *in vitro* (Ashjian et al., 2003; Wang et al., 2019). As a potential alternative source of neurons, hADSCs have been demonstrated to have neuroprotective effects in models of PD. Their therapeutic effectiveness may be attributed to the secretion of growth factors and cytokines that exert immunomodulatory, anti-inflammatory and neurotrophic effects (Ahmed et al., 2014; Berg et al., 2015; Lo Furno et al., 2018). However, it remains unknown whether hADSCs can directly differentiate into dopaminergic neurons.

Caveolin-1 (Cav-1) is a scaffolding protein associated with caveolae in the plasma membrane. As well as regulating cell proliferation and senescence, Cav-1 can bind a diverse array of cell signaling molecules and regulate cell signal transduction in caveolae. Cav-1-deficient hematopoietic stem cells exhibit impaired quiescence and induced environmental alterations, which limit their self-renewal and function (Bai et al., 2014). Cav-1 has been implicated in cell migration, tumorigenesis, neurogenesis and embryogenesis (Bhattachan et al., 2020). Volonte et al. (2002) demonstrated that overexpression of Cav-1 arrests mouse embryonic fibroblasts in the G0/G1 phase of the cell cycle through activation of a p53/p21-dependent pathway, indicating a role of the protein in growth arrest. Notably, Cav-1 protein was recently found to regulate the neuronal differentiation of stem cells. A number of studies have investigated the role of Cav-1 in the differentiation of stem cells. In these studies, Cav-1 downregulation was shown to enhance the osteogenic differentiation of human mesenchymal stem cells (MSCs) (Baker et al., 2012) and the neuronal differentiation of neural progenitor cells (Li et al., 2011b) and bone marrow mesenchymal stem cells (BMSCs) (Wang et al., 2013). Given these findings, we hypothesized that Cav-1 may be involved in the dopaminergic-like neuronal differentiation of hADSCs. Here, we study the role of Cav-1 in dopaminergic-like neuronal differentiation of hADSCs *in vitro* by examining the effects of Cav-1 downregulation.

Materials and Methods

Primary culture of hADSCs

Human adipose tissue was obtained from the First Affiliated Hospital of Dalian Medical University, China. This study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Dalian Medical University (approval No. PJ-KS-KY-2020-54) on March 7, 2017. All participants who agreed to donate adipose tissue signed informed consent. hADSCs were isolated as previously described (Han et al., 2014a). Cells were maintained in basal medium (Cyagen, Guangzhou, China) supplemented with 10% fetal bovine serum (Cyagen), 1% L-glutamine (Cyagen) and 1% penicillin/streptomycin solution (Cyagen), and cultured at 37°C in a humidified incubator containing 5% CO_2. hADSCs adhered to the plastic and proliferated. The medium was changed every 2 days unless indicated otherwise. The morphology of purified cells was examined by microscopy. Cells at passage 5 were used for the experiments.

Human adipose tissue collection

Adipose tissue was collected from healthy disease-free liposuction patients. A total of 5 mL of waste adipose tissue was obtained from three adult female patients undergoing medial thigh liposuction. Patients with infectious diseases, tumors or other chronic diseases were excluded.

Flow cytometric analysis

Flow cytometry (BD Pharmingen, San Diego, CA, USA) was performed on hADSCs at passage 5. The cells were washed three times with phosphate-buffered saline (PBS) and counted under a microscope. Cells were analyzed using conjugated mouse monoclonal antibodies. The cells were washed once in flow wash buffer (1× Dulbecco's PBS, 0.5% bovine serum albumin and 0.1% sodium azide), resuspended in blocking buffer (wash buffer with 25 μg/mL mouse IgG), and incubated for 10 minutes at room temperature. A total of 100 μL of cell suspension (1×10^5 cells) was added to each tube, and labeled monoclonal antibodies were added for analysis (FITC and PE). PE isotype control was performed for CD13 PE, CD34 PE and CD106 PE. FITC isotype control was performed for CD45 FITC and CD90 FITC. Antibodies were purchased from BD Pharmingen, and CD106 PE was purchased from BioLegend (San Diego, CA, USA). All tubes were incubated and protected from light for 30 minutes. Following incubation, the cells were washed twice with wash buffer and fixed in 200 μL 4% paraformaldehyde. Results were analyzed using Diva analytical software (BD Pharmingen).

Dopaminergic-like neuronal differentiation

Passage 5 cells were seeded into six-well plates at 1×10^4 cells per well. To generate dopaminergic-like neurons, we followed the three-stage *in vitro* differentiation procedure described in the human/mouse dopaminergic neuron differentiation kit (R&D systems, Emeryville, CA, USA). The human/mouse dopaminergic neuron differentiation kit contains ITS and N-2 MAX media supplements, which are used to select and enrich neural stem cell populations. Bovine fibronectin is included to support cell attachment and spreading. A growth factor panel, consisting of recombinant human basic fibroblast growth factor (bFGF), recombinant mouse fibroblast growth factor 8b (FGF-8b) and recombinant mouse Sonic hedgehog amino-terminal peptide (Shh-N), is included for effective dopaminergic differentiation.

The quantity of each component provided in the kit was estimated to be sufficient for the differentiation of 3×10^7 embryonic stem cells. The cells were exposed to induction medium containing specially formulated ITS and N-2MAX media supplements, which are used to select and enrich neural stem cell populations. Bovine fibronectin is also included to provide support and promote cell attachment and spreading. A growth factor panel, consisting of human bFGF, mouse FGF-8b and mouse Shh-N was also added, which has previously been reported to serve as extrinsic signals required for dopaminergic differentiation. The differentiation procedure was conducted according to instructions in the reference kit (R&D systems).

Lentivirus infection

During the Nestin-positive cell amplification stage, lentivirus infection technology was used to deliver exogenous genes. In brief, polybrene was diluted to 50 g/mL using ADSC culture medium (Cyagen), and Nurr1 and Lmx1a (GENECHEM, Shanghai, China) virus stock was diluted with ENi.S (GENECHEM) to 1×10^8 TU/mL. According to the instructions, we used a multiplicity of infection of 100. The cells were incubated in a 37°C/5% CO_2 environment for 8 hours, and then, the medium was replaced with fresh ADSC normal culture medium (replaced every 2 days). The expression of the viral GFP reporter was observed by fluorescence microscopy (Leica, Solms, Germany) after 72 hours.

Cav-1 gene silencing with RNA interference

Cav-1 shRNA (AM16708; Thermo Fisher, Waltham, MA, USA) was transfected into cells during the Nestin-positive cell amplification stage using Dharmafect 1 (Dharmacon, Waltham, MA, USA, Cat# T-2001-02) transfection reagent in accordance with the manufacturer's instructions. Briefly, cells were transferred to a 12-well plate and cultured until 50% confluent. Transfection was then performed using 2 μL of transfection reagent, 2 μL of 20 mM shRNA solution and 4×10^4 cells in 1 mL of culture medium. The efficiency of gene silencing was assessed with western blot assay and found to be optimal at 72 hours.

Immunofluorescence

hADSCs were differentiated in 24-well chambers at 29 days, rinsed three times with PBS, and incubated in 4% paraformaldehyde overnight at 4°C. Bovine serum albumin (1%) in PBS was used for blocking. Afterwards, the cells were incubated overnight at 4°C with the following primary antibodies: mouse anti-human Cav-1 (1:100; Cell Signaling Technology), rabbit anti-human tyrosine hydroxylase (TH) (1:100; Abcam), rabbit anti-human Lmx1a (1:100; Abcam) or rabbit anti-human Nurr1 (1:100; Santa Cruz Biotechnology). The samples were then rinsed three times thoroughly with PBS and incubated with the following secondary antibodies: anti-rabbit IgG-FITC (Sigma-Aldrich), anti-mouse IgG-FITC (Abcam), anti-mouse IgG-TRITC (Sigma-Aldrich) or anti-rabbit IgG-TRITC (Abcam) for 1.5 hours at room temperature. The cells were thereafter rinsed three times in PBS and incubated for 5 minutes with Hoechst 33258. The samples were then washed twice with PBS and once in deionized water. Stained cells were observed under a confocal laser scanning microscope (SP8, Leica).

Western blot assay

Cells were collected at different times. Cells were seeded in 10 cm dishes and then transfected and induced as described above. The cells were then lysed in a lysis buffer containing RIPA and an inhibitor cocktail (1:10). Cell lysates containing 30 μg of protein were resolved by SDS-PAGE and then transferred onto a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). The membrane was then blocked with 5% skim milk, followed by incubation with the following primary antibodies overnight at 4°C: rabbit anti-human Cav-1 (1:2000; Abcam), rabbit anti-human Lmx1a (2 μg/mL; Abcam), rabbit anti-human Nurr1 (1:100; Santa Cruz Biotechnology), rabbit anti-human TH (1:1000; Abcam) or rabbit anti-human β-actin (1:2000; Abcam). After four washes with Tris-buffered saline/Tween, the membrane was incubated with secondary antibody for 1.5 hours at room temperature. Protein signals were detected with the ECL kit (Beyotime, Shanghai, China) and quantitatively analyzed with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Dopamine release assay

Passage 5 doubling-phase cells were used for the differentiation experiment. Cells were induced to differentiate into dopaminergic neurons at the 3rd stage, and the culture supernatants were collected to analyze basal dopamine release. Dopamine release was measured with the Dopamine Research Elisa assay kit (LDN Labor Diagnostika Nord GmbH & Co. KG), according to the manufacturer's instructions. The absorbance at 450 nm was measured, and dopamine was quantified using a reference curve prepared from known concentrations of dopamine.

Real-time PCR analysis

Cav-1 and key markers of dopaminergic neurons (e.g., TH, Nurr1 and Lmx1a) were assessed by PCR. GAPDH was used as the housekeeping gene. Total RNA was extracted from cells 29 days after differentiation using RNAiso Plus (Takara), and 1 μg mRNA was reverse-transcribed into cDNA in a total volume of 20 μL, as per the manufacturer's instructions (PrimeScript RT reagent Kit, Takara) (**Table 1**). The thermocycling conditions were as follows: initial denaturation at 94°C for 5 minutes, followed by 30 cycles of 94°C for 30 seconds, 56°C for 30 seconds and 72°C for 1 minute, and a final extension at 72°C for 10 minutes. Real-time PCR was performed with SYBR Premix Ex Taq™ II (Takara, Tokyo, Japan). Quantitative calculations of the gene of interest *versus* GAPDH were carried out using the ΔΔCT method.

Table 1 | **Primer sequences**

Gene name	Primer sequences (5'–3')	Product size (bp)
GAPDH	Forward: GCA CCG TCA AGG CTG AGA AC	138
	Reverse: TGG TGA AGA CGC CAG TGG A	
TH	Forward: GTA AGC AGA ACG GGG AGG TG	147
	Reverse: GGT ACG TCT GGT CTT GGT AGG G	
Nurr1	Forward: AAA CCG AAG AGC CCA CAG	474
	Reverse: GCA GGA GAA GGC AGA AAT G	
Lmx1a	Forward: CTC AAC AGA GGC GAG CAT TCA	110
	Reverse: CTG GAC GAC ACG GAC ACT CA	

GADPH: Glyceraldehyde-3-phosphate dehydrogenase; Lmx1a: LIM homeobox transcription factor 1 alpha (Lmx1a); TH: tyrosine hydroxylase.

Statistical analysis

Graphpad Prism 5 for Windows (Graphpad, San Diego, CA, USA) was used for statistical analysis. All results are expressed as the mean ± SEM. The data for each group were consistent with a normal distribution (Shapiro-Wilk test). Student's *t*-test was used to compare differences between groups. One-way analysis of variance followed by the least significant difference *post hoc* test was used for comparisons among the three groups. Dopamine release assay was analyzed using a nonlinear multiple regression method with GraphPad Prism software. $P < 0.05$ was considered statistically significant; $P < 0.01$ was considered strongly statistically significant.

Results

hADSCs can differentiate into dopaminergic-like neurons

After five passages, hADSCs displayed a fibroblast-like morphology when attached to the plastic plate (**Figure 1A**). To exclude the possibility of hematopoietic contamination, we examined the phenotype of the hADSCs. Flow cytometric analysis demonstrated that hADSCs expressed CD13 and CD90, but not CD34, CD45 or CD106 (**Figure 1B**). CD13 and CD90 are generally accepted as specific surface markers of hADSCs. Additionally, ADSC adipogenic, osteogenic and chondrogenic differentiation capacity was assessed in our previous study.

To direct hADSCs towards the dopaminergic fate, *in vitro* differentiation protocols were used to induce the differentiation of hADSCs into dopaminergic neurons (**Figure 2A**). Following differentiation, hADSCs exhibited morphological change; some cells retracted their cytoplasm into globular or spindle-shaped bodies and extended cellular protrusions. Twenty-nine days after the induction, the majority of cells possessed typical neuronal perikarya (**Figure 2B**).

The expression of the neuronal marker TH and the neuronal signaling factors Nurr1 and Lmx1a in differentiated hADSCs was examined to characterize dopaminergic neuron-like differentiation. Levels of TH, Lmx1a and Nurr1 protein and mRNA were low in hADSCs that did not differentiate into the dopaminergic phenotype. However, both protein and mRNA levels of TH, Lmx1a and Nurr1 were significantly increased after induction (**Figure 2C**–**E**). Simultaneously, dopamine release was also detected on day 29. Induction of the dopaminergic-like neuronal phenotype resulted in a dramatic increase in the release of dopamine (**Figure 2F**). Together, these results demonstrate that we effectively induced the differentiation of hADSCs into dopaminergic-like neurons.

Nurr1 and Lmx1a play key roles in the differentiation of hADSCs into dopaminergic-like neurons

The preceding results show that hADSCs can successfully differentiate into dopaminergic-like neurons, and that dopaminergic neuronal signaling molecules are upregulated, indicating that the dopaminergic signaling pathway is activated. To further examine the role of key signaling molecules, lentivirus infection was used to transfect the key molecules. The infection efficiencies of the *Nurr1* and *Lmx1a* viruses were

80.85% and 86.06%, respectively (**Figure 3A** and **B**).

To evaluate the effect of gene targeting on hADSC differentiation into dopaminergic-like neurons, the expression of Nestin, the dopaminergic neuron marker TH, and the secretion of dopamine neurotransmitters were analyzed at 14 and 29 days after differentiation. We examined the effects of *Nurr1* and *Lmx1a* single infection as well as combined infection on the differentiation of hADSCs into dopaminergic-like neurons. Confocal microscopy showed that the combined infection of *Nurr1* and *Lmx1a* viruses resulted in a higher number of $Nestin^+$ cells and TH^+ cells compared with single infection alone (**Figure 3C**).

Enzyme-linked immunosorbent assay was also used to assess dopaminergic-like neuronal differentiation of ADSCs. The released levels of dopamine were 12.36 pg/mL and 13.34 pg/mL, respectively. The released dopamine was 106.47 pg/mL after combined infection with *Nurr1* and *Lmx1a* viruses, which was 10-fold that of individual infection. These results suggest that Nurr1 and Lmx1a overexpression in ADSCs upregulates TH, which in turn increases the release of dopamine by dopaminergic-like neurons (**Figure 3D**). Thus, combined *Nurr1* and *Lmx1a* overexpression can significantly promote the differentiation of hADSCs into mature dopaminergic-like neurons.

Cav-1 is involved in dopaminergic-like neuronal differentiation of hADSCs

To evaluate whether Cav-1 is involved in the dopaminergic-like neuronal differentiation of hADSCs, changes in the expression of Cav-1 during mesencephalic dopaminergic neuron development were analyzed using immunofluorescence staining (**Figure 4A**), RT-PCR and western blot assay. Cav-1 expression decreased during ADSC differentiation into dopaminergic-like neurons. The expression and subcellular localization of Cav-1 in hADSCs were investigated using immunofluorescence. Bright red fluorescent labeling for Cav-1 is detected within the cytosol (**Figure 4A**), indicating that Cav-1 is expressed in hADSCs.

Western blot assay showed that Cav-1 expression diminishes over time (**Figure 4B**). Compared with hADSCs, Cav-1 expression in dopaminergic cells was 26.45% and 39.48% lower at 14 and 29 days, respectively (**Figure 4C**). In support of the western blot results, RNA levels of Cav-1 decreased dramatically during dopaminergic-like neuronal differentiation (**Figure 4D** and **E**). These results indicate that Cav-1 is involved in the differentiation of hADSCs into dopaminergic-like neurons.

Downregulation of Cav-1 promotes dopaminergic-like neuronal differentiation

Small-interfering RNA (siRNA) gene silencing technology was used to further confirm the key role of Cav-1 in dopaminergic-like neuronal differentiation of hADSCs. Cav-1 expression was suppressed using a retrovirus at the differentiation stage known as the Nestin-positive cell amplification stage. As shown in **Figure 5A** and **B**, siRNA transfection resulted in a 35.41% downregulation of Cav-1 in induced dopaminergic-like neuronal cells. This change was associated with a large increase in TH^+ cells, as well as an increase in Nurr1 and Lmx1a expression after induction (**Figure 5C**). The protein and mRNA levels of TH, Lmx1a and Nurr1 increased dramatically during the differentiation of hADSCs following Cav-1 siRNA transfection (Cav-1 KD 29^{th} day) (**Figure 5D**–**F**). Downregulation of Cav-1 also increased dopamine levels (**Figure 5E** and **G**).

Discussion

Cav-1 is a component of caveolae, which are pits in the plasma membrane implicated in endocytosis, calcium signaling, and other signal transduction events (Kurzchalia and Parton, 1999). Members of the Ras/Raf/MEK/ERK kinase cascade are present in caveolae, which modulate pathway activation (Strippoli et al., 2015). The KSR1–cav-1 interaction is required for H-RasV12-induced cellular senescence, transformation, and tumorigenesis (Kortum et al., 2014). Cav-1 has been primarily described as an inhibitor. Moreover, overexpression of Cav-1 attenuates Raf/MEK/ERK activation (Fiucci et al., 2002). The Cav-1-mediated activation of p53 in combination with the Cav-1-mediated modulation of EGF, focal adhesion and small Rho GTPase-dependent signaling promote cellular senescence. Cav-1 is a signaling molecule whose function may control the fine balance between the positive and negative effects of cellular senescence.

Cav-1 functions as a regulator of differentiation in various tissues and cell types, where it has varied biological effects (Samarakoon et al., 2019). A previous study showed that Cav-1 inhibits neuronal differentiation by downregulating the VEGF, p44/42 MAPK, Akt, Stat3 and Wnt/beta-catenin signaling pathways, and that it modulates Notch1/NICD and Hes1 expression during astroglial differentiation in neural progenitor cells (Li et al., 2011a; Peffer et al., 2014; Bandara et al., 2016). Suppressing expression of Cav-1 with siRNA in human mesenchymal stem cells enhances their osteogenic differentiation (Baker et al., 2012). In human BMSCs, downregulation of Cav-1 promotes neuronal differentiation by modulating the Notch signaling pathway (Wang et al., 2013). Overexpression of Cav-1 upregulates various signaling molecules to promote the arborization of primary neurons, and neuron-specific Cav-1 regulates the ultrastructure and functional plasticity of hippocampal synapses (Head et al., 2011; Egawa et al., 2017, 2018; Mandyam et al., 2017).

Here, we studied for the first time the role of Cav-1 in the differentiation of hADSCs into dopaminergic-like neurons. We found that the expression of Cav-1 decreased during the dopaminergic neuronal differentiation of hADSCs, and downregulation of Cav-1 upregulated dopaminergic-like neuronal markers, including TH, Lmx1a and Nurr1. Cav-1 knockdown also promoted the release of dopamine. Our study shows that Cav-1 has a critical regulatory role in the dopaminergic-like neuronal differentiation of hADSCs. Cav-1 is therefore a candidate drug target for PD therapy using dopaminergic neurons derived from hADSCs.

Human BMSCs are adult stem cells that are used in an increasing number of studies. Adult stem cells provide an ideal resource for cell therapy; however, many unresolved issues remain regarding the clinical application of BMSCs, including the fact that cell numbers and their multi-directional differentiation potential are influenced by age (Mueller and Glowacki, 2001). hADSCs may represent a valid alternative to BMSCs because of their pluripotency and ability to differentiate into mesenchymal and non-mesenchymal lineages. Moreover, they are readily accessible and quickly proliferate *in vitro*, with a lower senescence rate than BMSCs (Omar et al., 2019). Furthermore, the number of cells obtained by liposuction aspirates is usually sufficient for some clinical applications, (Kern et al., 2006) and their use is not fraught with ethical concerns as is the case for embryonic stem cells (Lam and Longaker, 2012). Indeed, in our hands, hADSCs have a neuronal differentiation potential similar to that of hBMSCs, but with a higher proliferative capacity. We have reported that hADSCs are able to differentiate into neuron-like cells based on their production of neuron-specific proteins, including β-tubulin-III and neuron-specific enolase, the presence of Nissl bodies, and on their ability to secrete brain-derived neurotrophic factor and nerve growth factor (Han et al., 2014b).

In this study, the dopaminergic differentiation of hADSCs was evaluated by morphological, immunofluorescence, western blot, real-time PCR and enzyme-linked immunosorbent assay

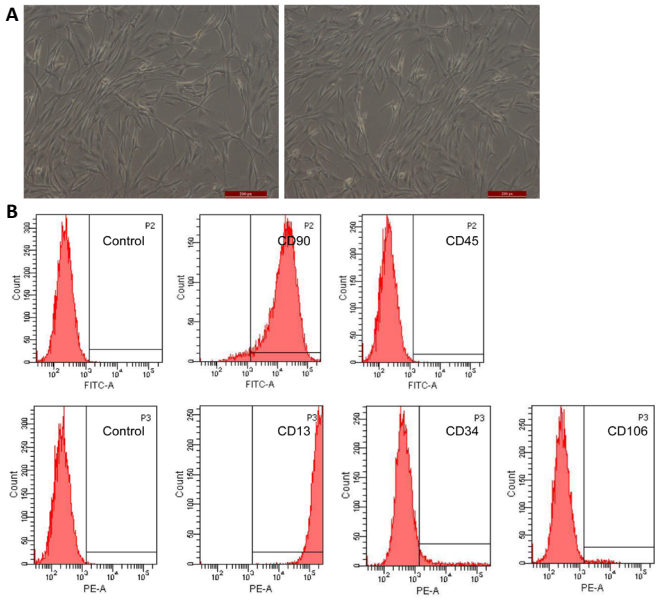

Figure 1 | **Characteristic confirmation of hADSCs.**
(A) Morphology of hADSCs. Scale bars: 200 μm. (B) Flow cytometry for hADSC surface markers. The samples were incubated with anti-human antibodies against CD13, CD90, CD34, CD45 and CD106, respectively. The expression levels of CD markers were tested by flow cytometry. hADSCs showed positive expression of stem cell surface markers CD13 and CD90, but negative expression of CD34, CD45 and CD106. hADSCs: Human adipose-derived stem cells.

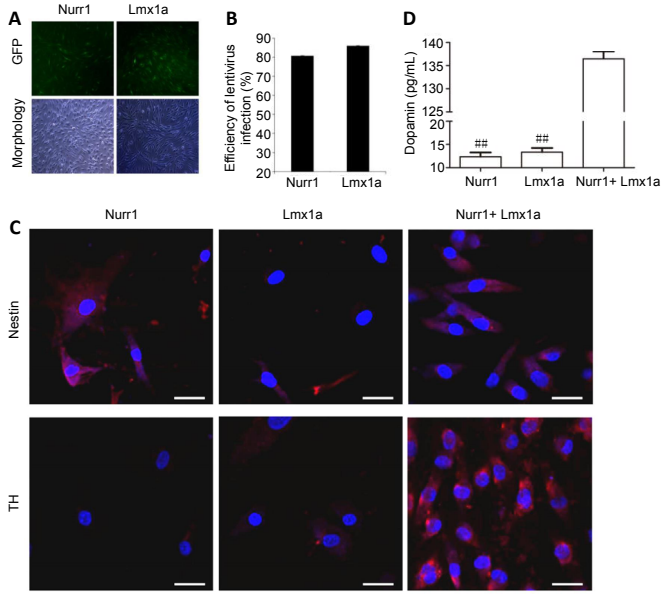

Figure 3 | **Efficiency of lentivirus infection of Nurr1 and Lmx1a in hADSCs.**
(A) GFP was detected to assess the efficiency of lentivirus infection. (B) Quantitative analysis of the expression of GFP. (C) Effect of Nurr1 and Lmx1a on the expression of Nestin and TH. The expression of Nestin and TH observed by confocal microscope was used to evaluate the effects of Nurr1 and Lmx1a on the differentiation of hADSCs. Anti-rabbit Nestin body (1:100, red) and anti-rabbit TH antibody (1:100, red) were employed. Nuclei were counterstained with Hoechst 33258 (1:1000, blue). (D) Effects of Nurr1 and Lmx1a on the release of dopamine. Quantitative analysis of the release of dopamine. ##P < 0.01, *vs*. Nurr1 + Lmx1a group. Data are presented as the mean ± SD (n = 3; one-way analysis of variance followed by least significant difference *post hoc* test). Average of three independent *in vitro* differentiation experiments was calculated. GFP: Green fluorescent protein; hADSCs: human adipose-derived stem cells; Lmx1a: LIM homeobox transcription factor 1 alpha.

analyses. hADSC-derived dopaminergic neurons exhibit a classic neuronal morphology (Yang et al., 2017) and express the dopaminergic neuronal markers TH, Lmx1a (Nefzger et

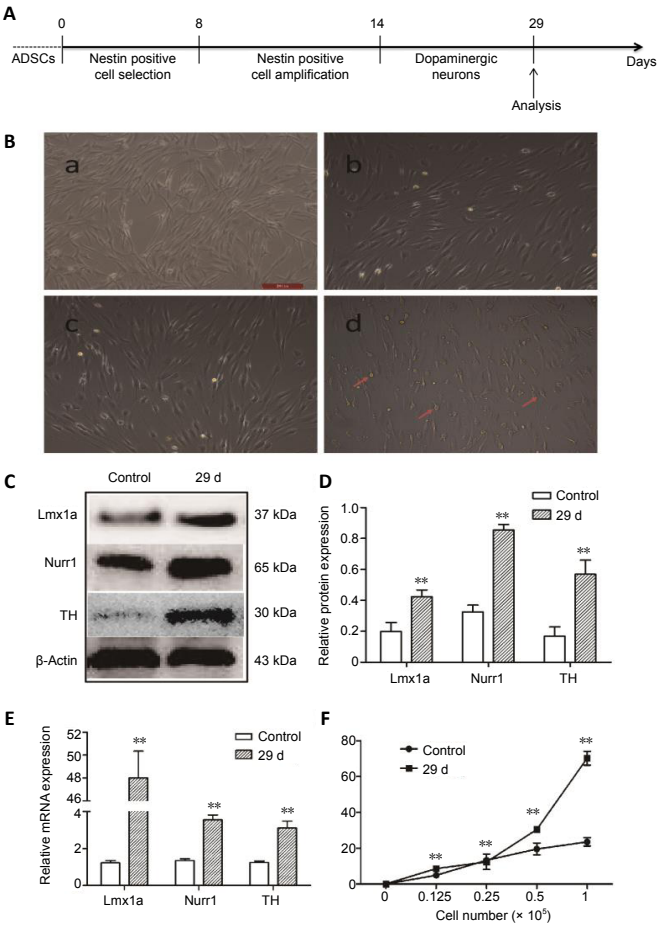

Figure 2 | **hADSCs differentiate into dopaminergic-like neurons.**
(A) Experimental design for the generation of *in vitro* dopaminergic neurons from hADSCs. (B) a: Morphology of undifferentiated hADSCs; b: morphology of hADSCs on the 8th day of induction; c. morphology of hADSCs on the 14th day of induction; d: morphology of hADSCs on the 29th day of induction. Arrows indicate hADSCs. Scale bar: 200 μm. (C) Protein levels of TH, Nurr1 and Lmx1a after dopaminergic-like neuronal induction of hADSCs. β-Actin was used as a control. (D) Quantification of TH, Nurr1 and Lmx1a protein expression normalized to actin. (E) mRNA levels of TH, Nurr1 and Lmx1a after dopaminergic-like neuronal induction of hADSCs. GAPDH was used as a control. (F) Quantitative analysis of the release of dopamine during induction in the control group and differentiated cells on the 29th day. **P < 0.01, *vs*. control group. Data are presented as the mean ± SD (Student's t-test). Average of three independent *in vitro* differentiation experiments (n = 3). hADSCs: Human adipose-derived stem cells; Lmx1a: LIM homeobox transcription factor 1 alpha; TH: tyrosine hydroxylase.

al., 2012; de Luzy et al., 2019) and Nurr1 (Yuan et al., 2015), as well as dopaminergic neuronal proteins that produce dopamine (Trujillo et al., 2009). The evidence for hADSC differentiation into dopaminergic neuron-like cells was based on morphological changes and neuronal marker expression *in vitro*, similar to several other studies (Smits et al., 2006; Zavan et al., 2010).

Dopaminergic neurons in the substantia nigra play key roles in adult brain functions. In diseases like PD, neurodegenerative dopaminergic loss gives rise to profound disturbances in voluntary movement, cognition and emotional behavior (Chakrabarty et al., 2012). In recent years, a number of studies have focused on identifying the molecular mechanisms that regulate the development of midbrain dopaminergic neurons (Wang et al., 2017). Notably, dopaminergic neurons derived from stem cells are valuable for studying the molecular mechanisms of dopaminergic neuron development, for screening pharmaceutical compounds that target dopaminergic disorders, and for cell replacement therapy in

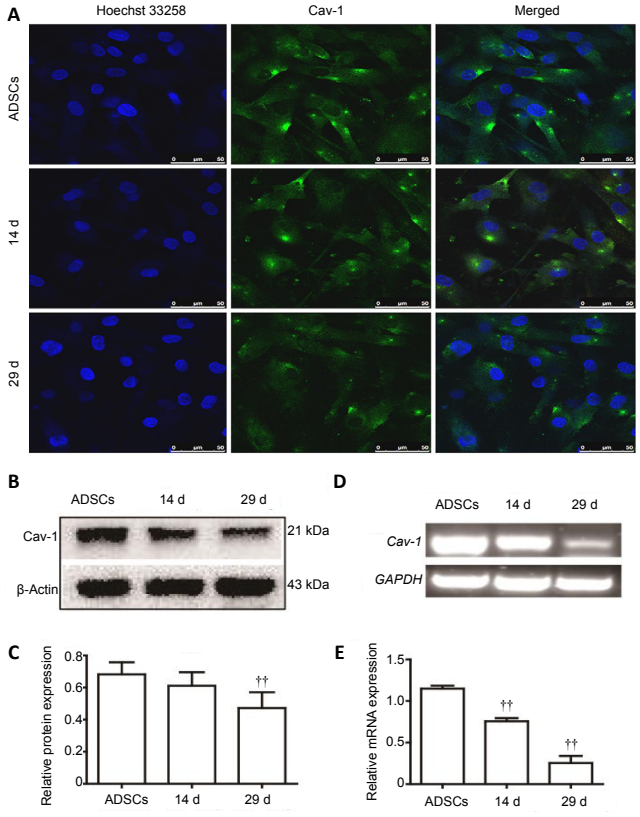

Figure 4 | **Caveolin-1 is involved in dopaminergic-like neuronal differentiation of hADSCs.**

(A) Confocal laser scanning microscopy of the change in Cav-1 expression during the differentiation of hADSCs into dopaminergic-like neurons. The nuclei are stained with Hoechst 33258 (blue), and Cav-1 (green) is stained with antibody to show its localization. (B, C) Western blot assay and quantitative analysis of the expression of Cav-1. (D, E) Polymerase chain reaction and quantitative analysis of the expression of Cav-1 during the dopaminergic-like neuronal differentiation of hADSCs at 14 and 29 days. ††P < 0.01, *vs.* ADSCs group. Data are presented as the mean ± SD (n = 3; one-way analysis of variance followed by least-significant difference *post hoc* test). Average of three independent *in vitro* differentiation experiments was calculated. Cav-1: Caveolin-1; hADSCs: human adipose-derived stem cells.

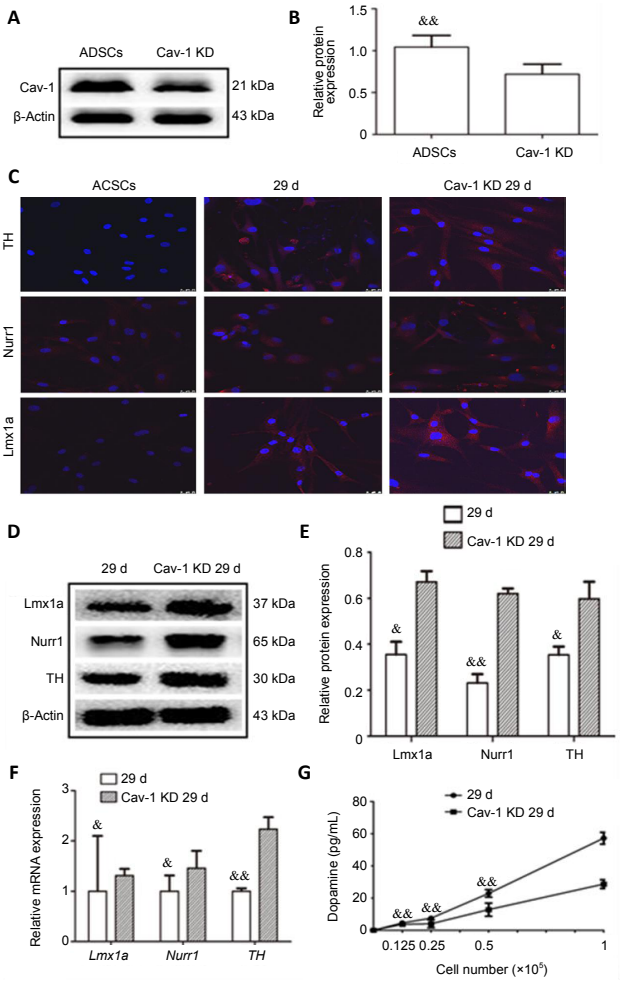

Figure 5 | **Downregulation of Cav-1 enhances the differentiation of hADSCs into dopaminergic-like neurons.**

(A) Protein levels of Cav-1. hADSCs were transfected with Cav-1 siRNA. After siRNA transfection for 72 hours, the expression of Cav-1 was assessed by western blot assay. (B) Quantification of Cav-1 protein expression. (C) Expression and subcellular distribution of TH, Lmx1a and Nurr1 by immunofluorescence: The red fluorescence indicates cells that are positive for TH, Lmx1a and Nurr1. The blue fluorescence indicates nuclei stained with hoechst 33258. Scale bar: 50 μm. (D, E) Western blot assay and quantitative analysis of the expression of TH, Lmx1a and Nurr1. (F) Quantitative analysis of mRNA expression after dopaminergic neuronal induction of hADSCs. (G) Quantitative analysis of the release of dopamine by the differentiated cells at 29 days and by Cav-1 KD cells at 29 days. &P < 0.05, &&P < 0.01, *vs.* Cav-1 KD 29 day. Data are presented as the mean ± SD (Student's t-test; n = 3). Average of three independent *in vitro* differentiation experiments was calculated. Cav-1: Caveolin-1; hADSCs: human adipose-derived stem cells; Lmx1a: LIM homeobox transcription factor 1 alpha; TH: tyrosine hydroxylase; KD: knock down.

PD (Kim et al., 2007). The development of dopaminergy is a complex multi-step process (Nguyen Nguyen et al., 2019). Each step is regulated in a complex but coordinated fashion by intrinsic and extrinsic soluble factors. Various soluble factors promote the differentiation of dopaminergic precursors into TH^+ dopaminergic neurons during different stages of development (Song et al., 2015). These soluble factors include Shh, FGF8, bFGF and Wnt1, followed by activation of several transcription factors, including Lmx1a/b, Foxa1/2, En1/2, Nurr1 and Lamx1a during the different stages of development (Joksimovic and Awatramani, 2014). Thus, we used soluble extrinsic factors, such as Shh, FGF8 and bFGF, to induce the differentiation of hADSCs into dopaminergic neurons, and we measured the expression of the dopaminergic neuronal markers TH, Lmx1a and Nurr1 (Gao et al., 2019). Our study shows that hADSCs can differentiate into TH^+ dopaminergic neurons expressing Nurr1 and Lmx1a. Moreover, we found that dopaminergic neuron development-related signaling molecules play a key role in ADSC differentiation into dopaminergic neurons. Overexpression of Nurr1 and Lmx1a can improve the efficiency of differentiation.

Cav-1 is localized to the cell membrane and participates in multiple signaling pathways. We hypothesized that downregulation of Cav-1 may increase the efficiency of differentiation by activating dopaminergic pathway-related gene expression. The expression of Cav-1 gradually decreased as the differentiation process proceeded. Notably, we found that downregulation of Cav-1 promotes hADSC differentiation into dopaminergic neurons. Our findings suggest that Cav-1 may be a promising drug target for PD therapy. Further study is needed to elucidate the molecular mechanisms underpinning the regulatory role of Cav-1 in dopaminergic neuronal differentiation.

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