Resveratrol (RES) plays a critical role in the fate of cells and longevity of animals via activation of the sirtuins1 (SIRT1) gene. In the present study, we intend to investigate whether RES could promote the self-renewal and neural-lineage differentiation in human umbilical cord derived MSCs (hUC-MSCs) in vitro at concentrations ranging from 0.1 to 10 μM, and whether it exerts the effects by modulating the SIRT1 signaling. Herein, we demonstrated that RES at the concentrations of 0.1, 1 and 2.5 μM could promote cell viability and proliferation, mitigate senescence and induce expression of SIRT1 and Proliferating Cell Nuclear Antigen (PCNA) while inhibit the expression of p53 and p16. However, the effects were reversed by 5 and 10 μM of RES. Furthermore, RES could promote neural differentiation in a dose-dependent manner as evidenced by morphological changes and expression of neural markers (Nestin, βIII-tubulin and NSE), as well as pro-neural transcription factors Neurogenin (Ngn)1, Ngn2 and Mash1. Taken together, RES exerts a dosage-dependent effect on the self-renewal and neural differentiation of hUC-MSCs via SIRT1 signaling. The current study provides a new strategy to regulate the fate of hUC-MSCs and suggests a more favorable in vitro cell culture conditions for hUC-MSCs-based therapies for some intractable neurological disorders.

INTRODUCTION

Mesenchymal stem cells (MSCs) have been spotlighted in the neural regenerative medicine (Can and Karahuseyinoglu, 2007). Since the controversial effects of RES on different cells retard the research progress and limit its clinical application, this study aims to investigate the role of RES in the fate of human umbilical cord derived MSCs (hUC-MSCs) in vitro. We will examine the viability, proliferation, cell cycle, apoptosis, senescence, neural differentiation, as well as the SIRT1 signaling of hUC-MSCs exposed to different concentrations of RES. This study will enlighten the anti-aging effect of RES and promote the application of RES modified hUC-MSCs in neural regeneration, contributing to efficient stem-cell based therapy for neural injury and neural degenerative disorders.

MATERIALS AND METHODS

Isolation and culture of hUC-MSCs

hUC-MSCs were isolated as previously described (Ma et al., 2007).
Resveratrol Regulates the Fate of hUC-MSCs
Xinxin Wang et al.

The cultured cells were harvested at passage 4 (P4) for a typical experiment. The study was approved by the Ethics Committees of the First Affiliated Hospital of Zhengzhou University and consented by the donors of umbilical cord.

**Identification of hUC-MSCs by flow cytometry**

hUC-MSCs were harvested and washed with ice-cold PBS. Cells were labeled with the following antibodies: CD29-PE, CD44-FITC, HLA-ABC-FITC, HLA-DR-FITC, CD34-PE, CD45-PE, CD51-FITC, and CD105-PE (BD Bioscience, USA) before analyzed by the FACS Calibur flow cytometer (Becton-Dickinson, USA).

**Administration of RES**

RES 50 μM stock solution (trans-3,4,5-trihydroxystilbene; R5010; Sigma, USA) was suspended in DMSO (Sigma, USA) and diluted to specific concentration before use. The final working concentration of DMSO was less than 0.1% for the in vitro experiment. hUC-MSCs were subjected to different doses of RES in the following experiments.

**CCK-8 assay**

Cell viability was quantitatively determined by a CCK-8 kit (Dojindo Molecular Technologies, Japan) according to the manufacture’s protocol. Briefly, hUC-MSCs at P4 were plated in 96-well plate at a density of 1000 cells/well and cultured in 100 μM DMEM/F12 with RES (0.1, 1, 2.5, 5, 10, 20, 50 and 100 μM) for 1, 3, 6, 8, 10 and 12 days, rinsed with PBS and incubated in DMEM/F12 with 10 μl CCK-8 for each well for 2 h at 37°C and the absorbance (OD) of the solution was measured by a microplate reader (Bio-Rad, USA) at a wavelength of 450 nm.

**EdU labeling**

To examine the effect of RES on the proliferation rate of hUC-MSCs, cells at P4 were seeded in 24-well plate (8000 cells/well) for 24 hours to allow for stabilization and then exposed to RES (0, 0.1, 1, 2.5, 5 and 10 μM) for 6 days before EdU (RiboBio, China) was added. The EdU labeling duration was determined at 24h according to the average cell-doubling time for hUC-MSCs. Images were visualized using fluorescent microscope (Olympus, Japan). The red (EdU-labeled) and blue (Hoechst-labeled) cells were counted.

**Senescence associated β-galactosidase staining**

hUC-MSCs at P4 were plated at similar density in their respective media and cultured for 6 days before senescence-associated β-galactosidase (SA-β-gal) staining (Beyotime, China). Briefly, cells were fixed in 4% (v/v) formaldehyde for 10 min before PBS and incubated in DMEM/F12 with 10 μl CCK-8 for each well for 2 h at 37°C and the absorbance (OD) of the solution was measured by a microplate reader (Bio-Rad, USA) at a wavelength of 450 nm.

**Cell cycle detection**

hUC-MSCs at P4 were incubated with RES (0, 0.1, 1, 2.5, 5 and 10 μM) for 6 days before propidium iodide (PI) staining performed as previously described (Ma et al., 2014).

**Cell apoptosis detection**

Cell apoptosis was analyzed by the FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, USA) following the manufacturer’s instructions. Briefly, hUC-MSCs at P4 were incubated with RES (0, 0.1, 1, 2.5, 5 and 10 μM) for 6 days and then collected for FITC- Annexin V and PI staining as previously reported (Zhu et al., 2016).

**Neural differentiation**

hUC-MSCs at P4 were incubated with RES (0, 0.1, 1, 2.5, 5 and 10 μM) for 6 days before neural differentiation induction, which was modified on the basis of the protocol previously described (Karahayenoglu et al., 2007). hUC-MSCs were pre-induced for 24 h in DMEM-LG containing 20% fetal bovine serum (FBS), 10 ng/ml basic fibroblast growth factor (bFGF, Peprotech, USA) was added for an additional 24 h, then incubated in the induction medium for another 24 h: DMEM-LG with 2% DMSO (Sigma, USA), 100 μM butylated hydroxyanisole (Sigma, USA), 25 mM KCl, 10 μM forskolin, 5 μg/ml insulin and 1 μM hydrocortisone, followed by neurobasal medium, supplemented with 10% FBS, 10 ng/ml epidermal growth factor (Sigma, USA), 10 ng/ml B27 supplement (Gibco, USA), 1 x B-27 supplement (Gibco, USA), and 2 mM L-glutamine (Sigma, USA) for the maintenance of differentiation.

**Immunofluorescence staining**

The 1 x 10^5 cells were plated on 24-well plate and after rinsing with PBS, cells were fixed with 4% paraformaldehyde for 10 min at room temperature. After blocking with 10% normal goat serum, cells were incubated overnight at 4°C with specific antibodies against Nestin (1:50, SantaCruz, USA), βIII-tubulin (1:100, Cell Signaling Technology, USA) or Neuron Specific Enolase (NSE, 1:3,000, Abcam, USA). After rinsing in PBS three times, cells were incubated for 1 hour at room temperature in Cy3-conjugated anti-mouse/rabbit anti IgG (1:1,000, Molecular Probes, USA) for visualization, followed by DAPI staining (Biotec, China). Immunofluorescent images were visualized with fluorescence microscopy. The negative control was incubated with PBS instead of primary antibody, and no immunoreactivity was observed.

**Quantitative real-time polymerase chain reaction (qRT-PCR)**

Total RNA was isolated using Trizol reagent (Invitrogen) according to the instructions, and mRNA was reverse transcribed using PrimeScript™ RT-PCR Kit (TaKaRa, Japan). qRT-PCR were performed on an ABI 7500 real-time PCR system (Thermo Fisher Scientific, USA) using SYBR Premix Ex Taq™ (Perfect Real Time, Takara, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was employed as the internal standard. Relative expression levels of different genes were calculated using the 2 -ΔΔCt. The sequences of primers for qRT-PCR were shown in Table 1.

**Western blotting**

Cells were incubated with RES of the indicated concentrations above before Western Blotting, which was performed as previously described (Ma et al., 2014). Primary antibodies (SIRT1, 1:1000; PCNA, 1:1000; p53,1:1000; p21,1:500; p16,1:500, cell signaling technology, USA) were employed.

**Statistical analysis**

Data were expressed as mean ± standard deviation (SD). Analysis of Variance (ANOVA) followed by LSD test were employed to determine the significance between different groups. p < 0.05 was considered statistically significant. Data were representative of three independent experiments. All data analyses were using SPSS 18.0 statistical software.
RESULTS

Morphologic and phenotypic characteristics of cultured hUC-MSCs

hUC-MSCs grew out from the Wharton’s jelly 7-10 days after isolation, and displayed a monolayer of bipolar spindle-like or fibroblast-like morphology with a whiskpool-like array. However, the cells underwent morphological changes and became flat, swollen and irregular as the passage number increased (Figs. 1A-F). The morphologic characteristics of hUC-MSC. (A) primary cells (P0); (B) P1; (C) P3 (D) P9; (E) P11; (F) P15. Arrow: Wharton jelly. Scale bars = 50 µm.

Effects of RES on the viability of hUC-MSCs

We found that RES exhibited a dose-dependent promoting effect on the viability of hUC-MSCs at a concentration of 0.1, 1 and 2.5 µM after incubating for 6 days (p < 0.05, Fig. 2). However, cell viability was inhibited after 6 days incubation of 5 and 10 µM RES (p < 0.05), and in the presence of 20, 50 and 100 µM RES, the OD 450 values of the CCK-8 test assay indicates in each treatment condition. Compared with the CON, the OD 450 is significantly increased in the 0.1, 1 and 2.5 µM RES groups 6 days later, in contrast, the OD 450 significantly decreased 6 days later in the 5, 10 µM RES groups, and 1 day later in the 20, 50 and 100 µM RES groups. CON: cells without RES incubation. *p < 0.05 vs. CON.

Effects of RES on the senescence of hUC-MSCs

As shown in Figs. 3C and 3D, there were significantly less SA-β-gal positive cells in the 0.1, 1 and 2.5 µM RES treated groups (p < 0.05), whereas, significantly more cells stained positive for SA-β-gal activity in the 5 or 10 µM RES groups (p < 0.05). These results indicated that 0.1, 1 and 2.5 µM RES inhibited senescence, whereas 5 and 10 µM RES accelerated senescence, with the most effective anti-senescence detected at 2.5 µM RES.

Effects of RES on the cell cycle of hUC-MSCs

Flow cytometry was used to detect the increased percentages of cells in S and G2/M phases (Fig. 3E). There was a reduction in the percentage in G0/G1 phase (p < 0.05) in the 5, 10, 20, 50 and 100 µM RES groups, and 1 day later in the 20, 50 and 100 µM RES groups. CON: cells without RES incubation. *p < 0.05 vs. CON.

Effects of RES on the apoptosis of hUC-MSCs

Flow cytometric detection of Annexin-V-FITC in / Propidium

Table 1. Sequences of primers for qRT-PCR

| Gene   | Sequence                  | bp   |
|--------|---------------------------|------|
| Ngn1   | 5′-CCAAAGACTTGCTCCACACA-3′ (F) | 164  |
|        | 5′-CTTTAAGCTCGGTTCCTCTC-3′ (R) |      |
| Ngn2   | 5′-CCTGGAAACATCCTCCTCAA-3′ (F) | 81   |
|        | 5′-TACCCAAAGCCAAGAATTGC-3′ (R) |      |
| Mash1  | 5′-CCAGTTGTACCTGACACC-3′ (F)  | 73   |
|        | 5′-TGCCACTTTGAGTTGGAC-3′ (R)  |      |
| SIRT1  | 5′-GAGATAACCTTCTGGTCGGTAGA-3′ (F) | 194  |
|        | 5′-CGGCAATATCTTTGAAGGTTG-3′ (R) |      |
| PCNA   | 5′-GTAGTAGAAGGCTCCTGTTG-3′ (F)  | 190  |
|        | 5′-TCTCTATGTAACAGCTTCCTC-3′ (R) |      |
| p53    | 5′-CCGCAATCGATCTCGCTG-3′ (F)   | 118  |
|        | 5′-ATCATCCATTGCCCTGAC-3′ (R)   |      |
| p21    | 5′-CCGTGCTACGTTCCTGACCC-3′ (F)  | 130  |
|        | 5′-GCGTTTGGAGTGGAGTTGAG-3′ (R)  |      |
| p16    | 5′-CTTGCGTGAAGTACCCG-3′ (F)    | 94   |
|        | 5′-CCCTCTCTTTTCTGCCCGC-3′ (R)  |      |
| GAPDH  | 5′-ACCCACTCTCCACCTTTCG-3′ (F)  | 125  |
|        | 5′-CTGTTGCTGTAGCAGAAATTGTTCG-3′ (R) |      |
Resveratrol Regulates the Fate of hUC-MSCs

Xinxin Wang et al.

**Fig. 3.** RSE exerts concentration-dependent effect on the proliferation, senescence, cell cycle and apoptosis rate of hUC-MSCs. hUC-MSCs were pre-incubated with RES (0.1, 1, 2.5, 5 and 10 μM) for 6 days before the detections. (A) Cells were exposed to EdU for 24 h and then fixed and stained. Images were taken using UV/Blue dual filter, and cells labeled with EdU were Red. Scale bar = 100 μm. (B) The percentage of EdU-labeled (proliferating) cells vs. total cells. (C) The SA-β-gal-positive cells exhibited blue color (indicated by arrows) under phase-contact microscope. Scale bar = 50 μm. (D) Column represents percentage of senescent cells vs. total cells based on the photos taken in each group. (E) RES increases the percentage of S-phase cells in a dosage-dependent manner. Stacked columns represent the relative distribution of cells in S, G2/M or (S+G2/M) phase detected by Flow cytometry. (F) Annexin V FITC / PI staining of the hUC-MSCs showed no significant change of apoptotic and necrotic cells in the 0.1, 1 and 2.5 μM RES treated group (p > 0.05), while 5 and 10 μM RES treatment resulted in significantly less Annexin V FITC-/PI- cells, with concomitant higher percentages of Annexin V FITC+/PI-, Annexin V FITC+/PI+ and Annexin V FITC-/PI+ cells (p < 0.05). Results were from three independent experiments. Error bars represent SD. CON: cells without RES incubation. *p < 0.05 vs. CON.

Iodide (PI) staining revealed a dose-dependent downward trend in the percentages of apoptotic and necrotic cells among the RES (0.1, 1 and 2.5 μM) treatment groups (Fig. 3F), although there was no significant difference when compared with the control (p > 0.05). 5 and 10 μM RES resulted in significantly lower percentage of Annexin V FITC-/PI- normal cells, with a concomitant higher percentages of Annexin V FITC+/PI-, Annexin V FITC+/PI+ and Annexin V FITC-/PI+ cells (p < 0.05), indicating more apoptosis, necrotic and dead cells than the control group (Fig. 3F).

**Effects of RES on the neural differentiation of hUC-MSCs**

hUC-MSCs underwent neuronal differentiation by a multi-step protocol. Most differentiated hUC-MSCs demonstrated neural
Resveratrol Regulates the Fate of hUC-MSCs
Xinxin Wang et al.

Fig. 4. RES stimulates neuronal lineage differentiation of hUC-MSCs. (A) Representative images of neural differentiation of hUC-MSCs pre-incubated with different doses of RES. Arrow: neuronal-shaped cells. Scale bar = 100 μm. (B) Quantitation of the neuronal differentiated rate by counting the neuronal-shaped cells and total cells. The results were presented as the mean ± SD derived from three samples. (C) Representative immunofluorescence pictures of the expression of Nestin, βIII-tubulin and NSE (Red) in the hUC-MSCs with different RES treatment. Nuclei were counterstained with DAPI (blue). Scale bar = 100 μm. (D) Percentages of cells positive for specific neural markers (Nestin/βIII-tubulin/NSE) vs. Total cells detected by immunofluorescence. (E) The relative gene expression of Ngn1, Ngn2 and Mash1 in the different groups, which normalized by the housekeeping gene GAPDH. Data are presented as the mean ± SD derived from three independent experiments. Abbreviations: MSC: hUC-MSCs without neural induction. d-MSCs: hUC-MSCs underwent general neural differentiation induction without RES pre-incubation. *p < 0.05 vs. CON.

appearance with retracted cell body, refractive karyon, protruded dendrites and axons after neural induction (Fig. 4A), while hUC-MSCs without induction retained long fusiform shape. RES pre-incubation facilitated the neural differentiation of hUC-MSCs in all concentrations (Fig. 4A). There were more neuronal-shaped cells in RES-pretreated hUC-MSCs than that without pretreatment, especially at the concentration of 2.5, 5 and 10 μM (p < 0.05, Fig. 4B). Immunofluorescence of hUC-MSCs without neural induction detected low expression of Nestin, βIII-tubulin and NSE. Pre-treated with 0.1, 1, 2.5, 5 and 10 μM RES before neural induction reduced the expression of Nestin, while up-regulated the expression of βIII-tubulin and NSE in a dose-dependent manner (Figs. 4C and 4D).

RES regulates the expression of proneural transcription factors in hUC-MSCs
The qRT-PCR analysis revealed that after neural differentiation, the expression of Neurogenin1 (Ngn1) was decreased, while
Ngn2 and Mash1 were increased (p < 0.05), and the effect was enhanced by RES in a dose-enhancing manner (Fig. 4E). These data indicate that 2.5, 5 and 10 μM RES significantly promotes the neuronal differentiation of hUC-MSCs by regulating Ngn1, Ngn2 and Mash1 (p < 0.05).

RES regulates SIRT1 signaling of hUC-MSCs

To explore the molecular mechanism of RES on the cell viability, proliferation, cell cycle, apoptosis and senescence of hUC-MSCs, we examined the expression of critical genes involved in those biological processes by qRT-PCR and Western Blot, including SIRT1, PCNA, p53, p21, and p16. As indicated in Fig. 5A, the expression of SIRT1 and PCNA in hUC-MSCs were significantly increased by 0.1, 1 or 2.5 μM RES (p < 0.05), while inhibited by 5 or 10 μM RES (p < 0.05). On the contrary, the expression of p53 and p16 were inhibited by 0.1, 1 or 2.5 μM RES (p < 0.05) and enhanced by 5 or 10 μM RES (p < 0.05). However, p21 underwent no significant change with RES treatment in the present study (p > 0.05). The protein expression detected by Western blot (Fig. 5B) was consistent with those of the qRT-PCR. Since SIRT1 is a critical player in anti-aging, p53, p21 and p16 are involved in apoptosis and cell cycle inhibition (Luo et al., 2001; Zhang et al., 2011) and PCNA is a S-phase cell marker (Mikula-Pietrasik et al., 2012), our results indicated that 0.1, 1 and 2.5 μM RES promoted cell survival, proliferation and suppresses aging by stimulating SIRT and PCNA, while inhibiting p53 and p16 in vitro. Whereas, the S-phase cycle arrest and senescence induced by 5 and 10 μM RES were associated with a decrease of SIRT1 and PCNA while increase of p53 and p16.

DISCUSSION

Regulating the self-renewal and lineage commitment of stem cells is the key factor in regenerative medicine. Studies have shown that RES promotes the survival of adipose-derived mesenchymal stem cells (Pinari et al., 2013), enhance the proliferation and osteoblastic differentiation of human bone marrow-derived mesenchymal stem cells (Dai et al., 2007) and direct the neuronal differentiation of human bone marrow mesenchymal stem cells (Joe et al., 2015). However, some researchers argued that RES inhibited the proliferation of neural progenitor cells (Park et al., 2012), negatively regulated the neurogenic potential of neural precursors (Saharan et al., 2013). These results suggested that RES might play different or even opposite role in different cell type under specific milieu. Since hUC-MSCs are promising candidate for some intractable diseases, it is essential to gain better understanding of the effect of RES on hUC-MSCs development.
treatment was the underlying mechanism for the cell proliferation boost. Moreover, it was revealed that the activity and half-life of p53 could be reduced by SIRT1, and result in increase in cell survival and decrease in cell apoptosis under various DNA-damaging conditions (Hubbard and Sinclair, 2014). Additionally, in line with the report (Chen et al., 2014) that the level of p16 was up-regulated with decreased SIRT1 expression and contribute to cell senescence and cell cycle arrest (da Luz et al., 2012; Vassallo et al., 2014), the impaired expression of SIRT1 in hUC-MSCs by 5 and 10 μM RES, along with the enhanced p53 and p16 result in cell senescence, cell cycle arrest and apoptosis. Taken together, the effect of RES on the fate of hUC-MSCs is mediated, at least in part, by Sirt1 signaling. However, it is also important to note that, gene expression could be regulated by various factors in a complex way, and the specific roles of SIRT1 may depend on the SIRT1 level, activity, subcellular localization, promoter occupancy and predominant substrates of SIRT1 in the specific microenvironment of different cells (da Luz et al., 2012). Therefore, the controversy in different literatures regarding the effects of RES and SIRT1 could be explained by the response of specific tissues and cells (Chen et al., 2014; Kumazaki et al., 2013; Marambaud et al., 2005; Saharan et al., 2013) and caution should be taken before establishing links between SIRT1 activation and neural protection. Moreover, it is provoking to study the cross-talk between SIRT1 signaling and many other signaling pathways, such as the Wnt/β-catenin, PI3K/AKT (Tsai et al., 2013), ER-ERK1/2 (Dai et al., 2014), AKT/mTOR/cGMP (Song et al., 2016), etc.

Herein, we suggest future studies on the electrophysiology of the neural-like cells, which would further confirm the effects of RES on the function of the neural differentiated hUC-MSCs. Effects of prolonged RES exposure also need to be explored in order to analyze the duration-dependent effect of RES on the biology of hUC-MSCs. Moreover, researches on animals models are warranted to facilitate the clinical application of RES pre-modified hUC-MSCs in treating Alzheimer’s disease, Parkinson, stroke, trauma induced brain injury and other neurodegenerative and neural injury disorders.

CONCLUSION

The present study indicates that RES exerts concentration-dependent effects on the fate of hUC-MSCs in vitro by regulating cell viability, proliferation, senescence, cell cycle, apoptosis and neural-lineage differentiation through activation of SIRT1 signaling. It highlights the promising application of RES in MSCs-based therapy in neuro-regenerative medicine and encourages further exploration on the role of RES in the development of stem cells to meet specific purposes in scientific research and clinical application.

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