

# Potential of resveratrol in enrichment of neural progenitor-like cells induction of human stem cells from apical papilla

**Anupong Songsaad**

Department of Anatomy, Faculty of Science, Mahidol University

**Thanasup Gonmanee**

Chakri Naruebodindra Medical Institute, Faculty of Medicine Ramathibodi Hospital, Mahidol University

**Nisarath Ruangsawasdi**

Department of Pharmacology, Faculty of Dentistry, Mahidol University

**Chareerut Phruksaniyom**

Department of Pharmacology, Faculty of Dentistry, Mahidol University

**Charoensri Thonabulsombat** (✉ [Charoensri.tho@mahidol.ac.th](mailto:Charoensri.tho@mahidol.ac.th))

"Mahidol University , Faculty of Sceince,Phayathai Campus" <https://orcid.org/0000-0002-0549-9037>

---

## Research

**Keywords:** resveratrol, neural progenitor-like cells, human stem cells from apical papilla

**Posted Date:** May 21st, 2020

**DOI:** <https://doi.org/10.21203/rs.3.rs-29139/v1>

**License:**  This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

---

**Version of Record:** A version of this preprint was published on December 14th, 2020. See the published version at <https://doi.org/10.1186/s13287-020-02069-9>.

# Abstract

**Introduction:** Stem cell transplantation of exogenous neural progenitor cells (NPCs) derived from mesenchymal stem cells (MSCs) has emerged as a promising approach of neurodegenerative disease. Human stem cells from apical papilla (hSCAPs) are derived from migratory neural crest stem cells, and exhibit a potential of neuronal differentiation. However, their neuronal differentiation is low and unpredictable. Resveratrol has been described as a sirtuin 1 ( SIRT1 ) activator which plays an important role in enhancing neuronal differentiation. In this study, we investigate the potential of resveratrol as an enhancer on neuronal differentiation through NPCs induction of hSCAPs.

**Methods:** Stem cells were isolated from human apical papilla, and characterized as MSCs. The cellular toxicity of resveratrol treatment to the characterized hSCAPs was investigated by MTT assay. The non-cellular toxicity concentrations of resveratrol were assessed with various pre-treatment times to select the optimal condition that highly expressed the neural progenitor gene, NES . Consequently, the optimal condition of resveratrol pre-treatment was synergistically performed with a neuronal induction medium to trigger neuronal differentiation. The differentiated cells were visualized, the genes profiling was quantified, and the percentage of neuronal differentiation was calculated.

**Results:** The cellular toxicity of resveratrol was not observed for up to 50  $\mu$ M for 12 hours. Interestingly, hSCAPs pre-treated with 10  $\mu$ M resveratrol for 12 hours (RSV-hSCAPs) significantly expressed NES , which is determined as the optimal condition. Under neuronal induction, both of hSCAPs and RSV-hSCAPs were differentiated (d-hSCAPs, and RSV-d-hSCAPs) as they exhibited neuronal-like appearances. Additionally, the neuronal induction medium actively promotes neural progenitor marker expression in both d-hSCAPs, and RSV-d-hSCAPs . The highest expression of NES was observed in RSV-d-hSCAPs. Importantly, the neuronal differentiation of RSV-d-hSCAPs was significantly increased for 4 times.

**Conclusion:** This study demonstrated that pre-treatment of resveratrol strongly induces neural progenitor marker gene expression which synergistically enhances neural progenitor-like cells' induction with neuronal induction medium.

## Introduction

The neurological disorders of the central nervous system (CNS) account for more than 10% of death, and new causes of permanent disability. The most common neurological disorders of CNS consist of Parkinson's disease, Alzheimer's disease, stroke, and traumatic brain injuries [1]. The cardinal characteristic that these neurological disorders have been defined by is loss of neurons, and the corresponding loss of function and disabilities [2]. Adult neurogenesis is the process of generating new neurons from NPCs [3]. Unfortunately, endogenous repairing of affected CNS via the NPCs is restricted, and limited [4]. To improve the quality of life of patients who are suffering from neurological disorders, a replacement of degenerated neurons with exogenous NPCs could be a potential treatment to regenerate the damaged CNS.

Human stem cells from apical papilla were discovered by Sonoyama W. in 2008 [5]. As the name implies, the hSCAPs are localized at the apex part (apical papilla) of the developing tooth which contains the stem cells, and have characterized as MSCs [6]. According to the origin, the hSCAPs represent early stem cell populations that exhibit superior stem cell properties, including self-renewal and differentiation potency, to the other dental-derived stem cells (DSCs), which are isolated from a mature tissue [7]. MSCs can be characterized by differentiation into at least 3 specialized lineages: adipocytes, osteocytes, and chondrocytes [8]. Moreover, neuronal cells can be generated from MSCs by administration of extrinsic factors in the neuronal induction medium as demonstrated in several MSCs-derived tissues, including adipose tissue [9], bone marrow [10], umbilical cord [11], cord blood [12], periodontal ligament [13], and both deciduous and permanent teeth [14]. Therefore, MSCs are an efficient stem cell source for neuronal differentiation. However, the ability of neuronal differentiation of MSCs has some limitations involving a low percentage of differentiation and unpredictability of differentiated cell type [15]. Moreover, most of the engrafted cells die within a week of transplantation, and only a few engrafted cells successfully integrated into the injured area [16].

Recently, medicinal plant-derived natural compounds have become of interest as alternative sources of new therapeutic agents for neurodegenerative disease. Moreover, they exert their potential effects by enhancing neuronal differentiation and adult neurogenesis [17]. Resveratrol (3,4',5-trihydroxy-trans-stilbene) is defined as a natural non-flavonoid polyphenol compound with a stilbene structure obtained from various plants including grapes, peanuts, pine trees, and berry plants [18]. Remarkably, resveratrol has been identified as a *SIRT1* activator playing an important role in enhancing neuronal differentiation and neuroprotection [19]. Previous studies have demonstrated the potential of *SIRT1* activator resveratrol in inducing neuronal differentiation and structural morphological change of MSCs derived from bone marrow [20], umbilical cord [21], cord blood [12], and dental pulp [15] into neuronal cells. Also, pre-treatment of resveratrol to MSCs at an optimal condition significantly promotes NPCs gene expression [20]. Despite recent progress, enhancing NPCs induction of hSCAPs by resveratrol has not yet been investigated.

In this study, we demonstrated the potential effect of resveratrol on neuronal differentiation using the optimal condition that directly drives neuronal differentiation into neural progenitor-like cells of hSCAPs.

## Methods

### Tooth sample collection

Human impacted third molars (n=7) were collected from Thai patients (15-20 years) at the Faculty of Dentistry, Mahidol University, Thailand. The ethical consideration and research protocol were approved by the Ethics Committee on Human Rights Related to Human Experimentation of Faculty of Dentistry/Faculty of Pharmacy, Mahidol University (COE. No. MU-DT/PY-IRB 2019/027.2405). The

inclusion criteria of the teeth consists of the presence of apical papilla tissue, caries-free, and no sign of pulp necrosis, trauma, or periodontal disease.

## **Cell isolation and culture**

The isolation of hSCAPs was performed by the enzymatic-disaggregation method as previously described [6]. Briefly, the teeth were collected in a proliferating medium consisting of Alpha Minimum Essential Medium (αMEM, Gibco, Life Technologies, Grand Island, NY, USA), 10% fetal bovine serum (FBS, Gibco, Life Technologies), 100 U/mL Penicillin, and 100 μM/mL Streptomycin (Gibco, Life Technologies), and washed with 0.1 M phosphate buffer saline (PBS, Sigma-Aldrich, St. Louis, MO, USA). Following a tooth extraction, the apical papilla tissue was separated, dissected into smaller pieces, and digested with a cocktail of 3 mg/mL collagenase type I (Worthington, Lakewood, NJ, USA), and 4 mg/mL dispase II (Sigma-Aldrich) at 37 °C for 1 hour. The digested tissue was filtered through 70 μm cell strainer (Falcon™, Fisher Scientific, Waltham, MA, USA), seeded into a cell culture vessel (T-75 cm<sup>2</sup> flask, Nunc™, Thermo Scientific, Waltham, MA, USA), and cultured in the proliferating medium at 37 °C, 5% CO<sub>2</sub>, and 95% humidity incubator. The medium was changed every 2 days until confluence was achieved. Then, the cells were subculture to expand the cell population. Cells at passages 2-6 were used in this study.

## **Cell surface marker analysis**

The uncharacterized cells (1 x 10<sup>6</sup> cells) were harvested and the cell surface antigen molecules were analyzed by BD FACS Canto Flow cytometer (BD Biosciences, San Jose, CA, USA). The cells were detected for MSCs markers using antibodies as follows: anti-human CD73 (APC/Cy7) (Biolegend, San Diego, CA, USA), anti-human CD90 (Thy1) (Biolegend), anti-human CD105 (Alexa Flour® 488) (Biolegend), and anti-human CD146 (PerCP/Cy5.5) (Biolegend). An antibody against hematopoietic stem cell marker, anti-human CD34 (APC) (Biolegend), was used as a negative control.

## **Colony-forming unit fibroblast**

The uncharacterized cells were seeded in triplicate into 6-well plates (Nunc™, Thermo Scientific) at a density of 500 cells/well, and cultured in the proliferating medium for 12 days. The medium was changed every 2 days. The colonies of these cells were visualized by Giemsa staining.

## **Osteogenic differentiation**

The uncharacterized cells were seeded in 24-well plates (Nunc™, Thermo Scientific) at a density of 2 x 10<sup>4</sup> cells/well, and cultured in the proliferating medium until reaching 80% confluence. Osteogenic differentiation was induced by culturing for 4 weeks in an osteogenic induction medium consisting of 0.1 μM dexamethasone (Sigma-Aldrich), 50 μg/mL ascorbate-2-phosphate (Sigma-Aldrich), 10 mM β-glycerophosphate (Sigma-Aldrich) in αMEM, 10% FBS, 100 U/mL Penicillin, and 100 μM/mL

Streptomycin. The medium was changed every 2 days. The calcification of an extracellular matrix was observed with Alizarin red staining.

### **Adipogenic differentiation**

The uncharacterized cells were seeded in 24-well plates (Nunc™, Thermo Scientific) at a density of  $2 \times 10^4$  cells/well, and cultured in the proliferating medium until reaching 100% confluence. Adipogenic differentiation was induced by culturing for 6 weeks in an adipogenic induction medium consisting of the proliferating medium supplemented with 1  $\mu$ M dexamethasone (Sigma-Aldrich), 50  $\mu$ M Indomethacin (Sigma-Aldrich), 1  $\mu$ g/mL Insulin (Sigma-Aldrich), and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma-Aldrich). Oil Red O was stained to visualize lipid droplets.

### **Cell viability of resveratrol treated hSCAPs**

The cell viability of resveratrol treated hSCAPs was performed by the methylthiazolyldiphenyl-tetrazolium bromide (MTT, Sigma-Aldrich) assay. Resveratrol (trans-3, 4', 5-trihydroxystibene; Sigma-Aldrich) was freshly prepared as a 100  $\mu$ M stock solution by diluting with  $\alpha$ MEM, 100 U/mL Penicillin, 100  $\mu$ M/mL Streptomycin, and maintained in dark condition. The characterized hSCAPs were seeded in 96-well plates (Nunc™, Thermo Scientific) at a density of  $1 \times 10^4$  cells/well. After 24 hours, the hSCAPs were treated with different concentrations of resveratrol (0, 5, 10, 15, 25, 50, and 100  $\mu$ M) for 6, 12, and 24 hours. Then, the MTT assay was performed. The MTT working solution (0.5 mg/mL) was added, and the plates were incubated for an additional 2 hours at 37 °C. After centrifugation, the solution was replaced with dimethyl sulfoxide (DMSO, Fisher Scientific). The absorbance of each well at 570 nm and 690 nm was measured with a micro-plate reader (Epoch, Fisher Scientific, Waltham, MA, USA). The percentage of cell viability of hSCAPs in resveratrol treatments ( $A_{570}$ - $A_{690}$ ) and the 50% inhibitory concentration ( $IC_{50}$ ) were reported.

### **Optimal condition of resveratrol pre-treatment**

The hSCAPs were seeded in 6-well plates at a density  $1 \times 10^5$  cells/well. After 24 hours, the cells were incubated with different non-cellular toxicity concentrations of resveratrol (0, 5, 10, 15, 25, and 50  $\mu$ M) for 12 hours, and qRT-PCR was performed to select the concentration of resveratrol that induced the highest *NES* expression of hSCAPs. Then, the concentration was used to assess *NES* expression at various incubation times (1, 6, 12, and 24 hours). The treatments were also investigated for morphological change and immunocytochemistry with  $\beta$ -III tubulin staining. The hSCAPs treated with resveratrol at the concentration and incubation time that brought the highest *NES* expression will be termed "RSV-hSCAPs", and will be further used for a neuronal induction.

## Neuronal induction

The hSCAPs were seeded on poly-d-lysine (Sigma-Aldrich) coated cover slips (Electron Microscopy Sciences, Hatfield, PA, USA) in 6-well plates at a density  $1 \times 10^5$  cells/well, and pre-incubated with the optimal condition of resveratrol (RSV-hSCAPs) or without resveratrol (hSCAPs). Then, both hSCAPs and RSV-hSCAPs were exposed to 2 phases of neuronal induction medium. First, the cells were incubated with Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (Ham) (DMEM/F-12, Gibco, Life Technologies) supplemented with 10% FBS, 100 U/mL Penicillin, 100  $\mu$ M/mL Streptomycin, 10 ng/mL basic fibroblast growth factor (bFGF, Gibco Life Technologies), and 500  $\mu$ M  $\beta$ -mercaptoethanol (Sigma-Aldrich) for 24 hours. After that, the cells were induced into a phase II neuronal induction medium which consisted of DMEM/F-12, 100 U/mL Penicillin, 100  $\mu$ M/mL Streptomycin, 2% DMSO, and 100  $\mu$ M butylated hydroxyanisole (BHA, Sigma-Aldrich) for 6 hours. The negative control hSCAPs (ctrl-hSCAPs) was pre-incubated for 12 hours with  $\alpha$ MEM, 100 U/mL Penicillin, 100  $\mu$ M/mL Streptomycin, and then cultured with DMEM/F-12, 10% FBS, 100 U/mL Penicillin, and 100  $\mu$ M/mL Streptomycin for 24 hours. The medium was then replaced with DMEM/F-12, 100 U/mL Penicillin, and 100  $\mu$ M/mL Streptomycin for 6 hours.

## Immunocytochemistry

The specimens were fixed in 4% paraformaldehyde (Sigma-Aldrich) in PBS at room temperature for 1 hour, followed by 20% ice-cold methanol (Sigma-Aldrich) in PBS for 5 minutes, then washed with PBS. Subsequently, the specimens were permeabilized with 0.5% Triton X-100 (Sigma-Aldrich) in PBS overnight at 4 °C, and blocked with 15% bovine serum albumin (BSA, Sigma-Aldrich) at 4 °C for 12 hours. The specimens were incubated overnight at 4 °C with anti-mouse Nestin antibody (Biolegend) at a dilution of 1: 500, and anti-mouse  $\beta$ -III tubulin antibody (Biolegend) at a dilution of 1: 1,000 which diluted with 5% BSA in PBS with 0.05% Tween-20 (Sigma-Aldrich). Then, the specimens were conjugated with goat anti-mouse IgG highly cross-adsorbed secondary antibody, Alexa Fluor plus 488 (Invitrogen, New York, NY, USA) at a dilution of 1: 1,000 at room temperature for 4 hours. Nuclei were counterstained, and mounted with ProLong™ Diamond Antifade Mountant with DAPI (Invitrogen). The samples were visualized, and captured by the Digital Fluorescence Microscope, BX53 (Olympus, Hamburg, Germany). The percentage of neuronal differentiation (the number of differentiated cells x 100/total cells) was quantified using the ImageJ program (NIH, Bethesda, MD, USA) by random counting (n=5).

## Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted using the Nucleospin RNA plus kit (Macherey-Nagel, Bethlehem, PA, USA), and converted into cDNA using iScript RT Supermix (Bio-Rad, Hercules, CA, USA). The qRT-PCR was performed using KAPA SYBR® FAST qPCR kits (Sigma-Aldrich) with CFX96™ real-time PCR detection system (Bio-Rad). The qRT-PCR reaction conditions were 95 °C for 3 minutes, followed by 40 cycles of 95 °C for 3 seconds, 60 °C for 20 seconds. The interesting primers (Integrated DNA Technologies, Gemini Singapore Science Park II, Singapore) used in this study are listed in Table 1. The glyceraldehyde 3-

phosphate dehydrogenase (*GAPDH*) was used as an internal control, and the expression of interested genes was measured by  $\Delta\Delta Cq$  method.

## Statistical analysis

The individual experiment was repeated 3 times. The data were expressed as the mean  $\pm$  standard error of mean (SEM), the difference between the experimental groups and the control group were compared using Tukey's Multiple Comparison Test via GraphPad Prism version 5.00.288 (San Diego, CA, USA). The differences with <sup>\*\*\*</sup>*p-value* < 0.001 were considered significant.

# Results

## Characterization of hSCAPs

Firstly, the isolated cells from human apical papilla tissue presented the typical fibroblast and spindle-like shape morphology in plastic adherent culture (Figure 1A). Secondly, the cell surface antigen molecule analysis via flow cytometry demonstrated the cells highly expressed MSCs markers, CD73 (99.8%), CD90 (99.8%), CD105 (83.8%), CD146 (31.3%), and negatively expressed CD34 (0.2%). The isolated cells that co-expressed CD73+, CD90+, CD105+, CD146+, and CD34- were a major population (70.1%) (Figure 1B). The cells effectively formed colonies indicating the self-renewal ability (Figure 1C). In evaluating the neural crest derivative origin, the immunofluorescence reveals the Nestin expression of the isolated cells (Figure 1D). Multipotential differentiation properties were shown by osteogenic and adipogenic induction. The cells produced calcified nodules (Figure 1E) and lipid droplets (Figure 1F) as revealed by Alizarin red and Oil Red O staining, respectively. These indicated that the cells were able to differentiate into osteocytes and adipocytes. Taken together, the isolated cells exhibited MSCs properties, verified as hSCAPs.

## The cellular toxicity of resveratrol on hSCAPs

To evaluate the toxicity of resveratrol on hSCAP viability, the hSCAPs were incubated with 0-100  $\mu\text{M}$  of resveratrol for 6, 12, and 24 hours. The viability of the cells did not observe cytotoxicity at any concentrations of resveratrol treatment for 6 hours (Figure 2A). In contrast, the cellular viability significantly decreased at 12 hours in 100  $\mu\text{M}$  (Figure 2B), and 24 hours in 25  $\mu\text{M}$  resveratrol treatment (Figure 2C), compared to the control group. Additionally, the  $\text{IC}_{50}$  of resveratrol treatment on hSCAPs were shown as 3,380  $\mu\text{M}$  (6 hours), 1,501  $\mu\text{M}$  (12 hours), and 73.33  $\mu\text{M}$  (24 hours) (Figure 2D). As a result, the concentration of resveratrol ranging to 0-50  $\mu\text{M}$  for 12 hours pre-treatment was chosen for use in the next experiment.

## Optimal condition of resveratrol pre-treatment

To evaluate the effect of resveratrol on enhancing neuronal differentiation of hSCAPs, the cells were incubated with 0-50  $\mu\text{M}$  of resveratrol for 12 hours. We hypothesized that resveratrol pre-treatment

would promote the expression of NPCs gene, *NES*. The qRT-PCR demonstrated that the *NES* expression significantly increased at 10  $\mu$ M resveratrol pre-treatment, compared to control. However, the expression significantly decreased at 50  $\mu$ M. (Figure 3A). To determine optimal pre-treatment time, the hSCAPs treated with 10  $\mu$ M resveratrol were investigated for *NES* expression at various incubation times: 1, 6, 12, and 24 hours. The *NES* expression was significantly highest at 12 hours, and dropped at 24 hours of pre-treatment time (Figure 3B). Therefore, the pre-treatment of resveratrol at 10  $\mu$ M for 12 hours was determined as the optimal condition, and further used in the neuronal induction experiment. Moreover, the  $\beta$ -III tubulin immunofluorescence staining revealed that all of the resveratrol treated hSCAPs ranging from 0 to 50  $\mu$ M for 12 hours (Figure 3C) and 10  $\mu$ M for 1, 6, 12, 24 hours of pre-treatment times (Figure 3D) exhibited morphology as the typical fibroblast and spindle-like shape, which was similar to the control and the primary hSCAPs (Figure 1A).

## Neuronal induction

It was demonstrated that pre-treatment with 10  $\mu$ M of resveratrol for 12 hours actively promoted neural progenitor gene expression. To elucidate the effect of resveratrol on neuronal differentiation of hSCAPs, the cells were pre-treated with/without 10  $\mu$ M of resveratrol for 12 hours. We hypothesized that RSV-hSCAPs would differentiate into NPCs more than the hSCAPs. The hSCAPs and RSV-hSCAPs were consequently induced with 2 phases of neuronal induction medium.

The hSCAPs and RSV-hSCAPs were differentiated cells (d-hSCAPs; Figure 4A, and RSV-d-hSCAPs; Figure 4B, respectively) as they showed neuronal-like appearances. Both d-hSCAPs' and RSV-d-hSCAPs' presented several types of neuronal-like morphology, such as round shape, unipolar shape, bipolar shape, multipolar shape, pyramidal shape, and irregular shape. On the other hand, the crt-hSCAPs, which were cultured in the medium without neuronal induction supplements, presented the typical fibroblast and spindle-like shape morphology (Figure 4C), similar to the primary hSCAPs (Figure 1A).

The percentage of neuronal differentiation of d-hSCAPs was  $12.11 \pm 5.08$  %. Interestingly, the neuronal differentiation of RSV-d-hSCAPs was significantly increased to  $54.71 \pm 10.39$  % (Figure 4D). Further, the neuronal induction medium effectively induced NPCs marker expression in d-hSCAPs and RSV-d-hSCAPs. The highest expression of *NES* was distinctly observed in RSV-d-hSCAPs, as compared to crt-hSCAPs and d-hSCAPs. However, expressions of *MAP-2* and *TUBB3* genes, which represent late neurogenic and immature postmitotic neuron, were not significantly different between crt-hSCAPs, d-hSCAPs, and RSV-d-hSCAPs (Figure 4E-G).

## Discussion

Mesenchymal stem cells of dental origin are being considered as a promising source for neurodegenerative therapies due to their self-renewal properties and multipotential differentiation [22]. The hSCAPs derived from a developing root represent a population of early stem cells [7]. Moreover, the hSCAPs have shown the ectomesenchyme origin with migratory neural crest stem cells derivative [23]. Recent studies have demonstrated the characteristics and capacity for multilineage differentiation of

hSCAPs [24]. Among the various dental-origin stem cell populations, hSCAPs exhibited superior profiling, including multipotential differentiation, secretion of neurotrophic factors, and neurite outgrowth stimulation [25]. We characterized hSCAPs according to the minimal criteria of MSCs from the International Society for Cellular Therapy (ISCT) [8]. The isolated cells successfully grew on plastic culture vessels, exhibited the typical fibroblast and spindle shaped morphologies, which represented plastic adherent abilities and the morphology of MSCs. The isolated cells were analyzed for the markers of MSCs. Flow cytometry demonstrated that the cells highly expressed CD73, CD90, CD105, and CD146 but negatively expressed CD34. The cells were able to form colonies that were visualized by Giemsa staining indicating their self-renewal ability. Moreover, to demonstrate multipotency properties, the isolated cells were induced with osteogenic and adipogenic induction media. The calcified nodule was visualized with Alizarin red, and lipid droplets were clearly stained with Oil Red O. Under neuronal induction, the isolated cells were efficiently differentiated into neuronal-like cells (Figure 4A). Finally, the isolated cells were positively stained with Nestin to reveal a migratory neural crest derivative of ectomesenchymal origin (Figure 1D). Taken together, the results verified that the isolated cells established from human apical papilla tissue were MSCs, namely as hSCAPs.

Resveratrol is a non-flavonoid polyphenol compound with a stilbene structure obtained from various plants [18]. Resveratrol has 2 isomeric forms: *cis*-resveratrol and *trans*-resveratrol. Importantly, *trans*-isoform is a stable isoform and is the more predominant common active compound [26], while *cis*-isoform is unstable and the less common compound. However, the *trans*-resveratrol can be converted into *cis*-resveratrol after exposure with heat, UV radiation, or sunlight [27]. Therefore, we freshly prepared the resveratrol solution and maintained it in dark conditions for maximal efficiency. Previous studies have found that resveratrol did not have cytotoxicity on human dental pulp stem cells (hDPSCs) [15] or human bone marrow mesenchymal stem cells (hBM-MSCs) [20] at 0-50  $\mu$ M concentrations for 12 hours. Some studies reported that the cell viabilities of resveratrol treated human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) did not change at 0-50  $\mu$ M for 24 hours [21]. Despite present progress, there are no studies on the cell viability of hSCAPs with different concentrations of resveratrol. In this study, we first demonstrated the cellular toxicity effect of resveratrol on hSCAPs' viability in order to select non-cellular toxic concentrations. The hSCAPs were treated with different concentrations of resveratrol (0, 5, 10, 15, 25, 50, and 100  $\mu$ M) for 6, 12, and 24 hours, and then the MTT assay was performed. We have clearly shown that the resveratrol treatment did not have a cytotoxic effect on the hSCAPs from 0 to 100  $\mu$ M for 6 hours, 0-50  $\mu$ M for 12 hours, and 0-15  $\mu$ M for 24 hours. Additionally, we were firstly reported the  $IC_{50}$  of resveratrol treatment on hSCAPs as 3,380  $\mu$ M (6 hours), 1,501  $\mu$ M (12 hours), and 73.33  $\mu$ M (24 hours). The cellular toxicity of resveratrol as explained by the hormetic dose-response effect lead to positive responses (associated with beneficial effects) at low concentrations, and negative responses (associated with toxic effects) at high concentrations [28]. High concentrations of resveratrol promoted insufficient anti-oxidant defense system activities, induced disruption of mitochondrial membrane potential, and increased reactive oxygen species (ROS) production [29]. Furthermore, it was demonstrated that cell viability is dependent upon the duration of resveratrol supplementation. At longer exposure times increased ROS secretions were observed, which lead to cell

death [30]. Therefore, consistent with previous reports, we selected a non-cellular toxicity concentration of resveratrol from 0-50  $\mu\text{M}$  for 12 hours for further studies.

The application of resveratrol to mesenchymal stem cell-based regenerative medicine has been demonstrated in various *in vitro* bioactivities, including self-renewal, multipotency [31], senescence [21], cell aging [32], osteogenic differentiation [33], adipogenic differentiation [34], and neuronal differentiation [12]. Moreover, resveratrol has an efficiently therapeutic effect on *in vivo* models, including enhancing liver regeneration [35], cardiogenic differentiation in cardiomyopathy [36], and neurogenesis in the hippocampus of Alzheimer's disease [37]. The potential effects of resveratrol are triggered by SIRT1 activation. The SIRT1 acts as the central modulator of bioactivities signaling pathways [38]. Resveratrol indirectly activates SIRT1 by increasing intracellular cAMP following the inhibition of cAMP-dependent phosphodiesterase (PDE) [39]. SIRT1 strongly promotes neuronal differentiation through PKA/GSK3- $\beta$ / $\beta$ -catenin and PKA/ERK1/2 axis [12]. SIRT1 also plays important roles in controlling microtubule dynamics and neurite outgrowth stimulation during axon elongation by deacetylating AKT [40]. Taken together, previous studies confirm the potential effect of resveratrol, through SIRT1 activation, on neuronal differentiation by changing the structural features of the cell into neuronal phenotype.

Previous studies have demonstrated the optimal condition of resveratrol to effectively induce neuronal-associated gene expression and neuronal differentiation. The expression of *NES* and *Musashi* of resveratrol-treated hBM-MSCs were highest at 1  $\mu\text{M}$  for 12 hours [20]. The treatment of the hDPSCs in 15  $\mu\text{M}$  of resveratrol for 12 hours was examined as the optimal condition to induce neural progenitor gene expression [15]. Moreover, the treatment of the hUC-MSCs in 30.0 mg/L of resveratrol for 6 hours successfully induced cell morphology differentiation into neuronal-like cells by up to 90 % [11]. In this study, we investigated resveratrol-induced neuronal differentiation of the hSCAPs. The characterized hSCAPs were incubated in the range of non-cellular toxicity concentrations of resveratrol (0, 5, 10, 15, 25, and 50  $\mu\text{M}$ ) for 12 hours to achieve the highest expression of *NES*. Then, the concentration of resveratrol that drives the most *NES* expression was assessed at 1, 6, 12, and 24 hours to determine the optimal pre-treatment time. We found that hSCAPs treated with 10  $\mu\text{M}$  resveratrol for 12 hours were the optimal condition to enhance the neural progenitor gene, as revealed by qRT-PCR. The resveratrol pre-treatment exerts concentration-specific biphasic responses involving stimulatory and inhibitory dual effects on neuronal progenitor gene expression. Recent studies show that a low concentration ( $\geq 10 \mu\text{M}$ ) of resveratrol triggers the TrkA receptors, and consequently phosphorylated SIRT1 and MAPK axis, and cascading down regulation increased CREB-TF (cAMP response element-binding protein transcription factor) involving the neuroprogenitor gene. On the other hand, higher concentrations ( $\geq 20 \mu\text{M}$ ) of resveratrol inhibited the phosphorylation of TrkA and MAPK, signaling with a result low expression of SIRT1, and neuroprogenitor gene. Moreover, it also decreased the expression of the anti-apoptotic protein Bcl-2 with parallel increases of activated caspase-3 (hallmark of apoptosis) and p75<sup>NTR</sup> (death receptor) [41]. Therefore, consistent with previous reports, experiments suggest that the maximal efficiency expression of *NES* depends on the optimal binding ability between resveratrol ligand and the TrkA receptor, which regrading to high level of MAPK and SIRT1 downregulation. Immunocytochemistry with  $\beta$ -

III tubulin showed that the cell morphology of RSV-hSCAPs were similar to primary hSCAPs, suggesting that resveratrol pre-treatment stimulates the expression of neural progenitor gene markers of the hSCAPs but does not drive differentiation of neuronal cells. We hypothesized that the activated *NES* at optimal conditions for RSV-hSCAPs could be more effectively differentiated into neuronal cells than the hSCAPs.

To further trigger neuronal differentiation, the hSCAPs and RSV-hSCAPs were synergistically cultured with a neuronal induction medium composed of specific chemical compounds and neurotrophic factors, including  $\beta$ -mercaptoethanol, DMSO, BHA, and bFGF, which served as extrinsic signaling factors for promoting morphological change and neuronal differentiation [42]. In previous studies, the hBM-MSCs and RSV-hBM-MSCs were differentiated into neuronal-like cells, which exhibited 2 dendrites with longer than 60  $\mu\text{m}$  under a neuronal induction medium. We observed a high rate of neuronal differentiation derived from the RSV-d-hBM-MSCs [20]. Moreover, the differentiated cells derived from hDPSCs and RSV-hDPSCs (d-hDPSCs and RSV-d-hDPSCs) had significantly increased neuronal-specific marker genes, including *NES*, *Musashi*, and *NF-M*, which indicated that the RSV-d-hDPSCs were superior for neuronal differentiation profiling [15]. In this study, we have demonstrated that neuronal induction media successfully trigger hSCAPs and RSV-hSCAPs into differentiated cells (d-hSCAPs, and RSV-d-hSCAPs), which exhibit neuronal-like morphology in several types, including round shaped, unipolar shaped, bipolar shaped, multipolar shaped, irregular shaped, and pyramid shaped (Figure 4A, and Figure 4B), while the crt-hSCAPs, which were cultured with a basal medium, presented a flattened shaped (Figure 4C) as did the primary hSCAPs. Our study demonstrated that resveratrol pre-treatment effectively enhances neuronal differentiation. The percentage of differentiated cells were significantly increased from  $12.11 \pm 5.08$  (d-hSCAPs) to up to  $54.71 \pm 10.39$  (RSV-d-hSCAPs).

Neuronal-specific marker genes expression was evaluated to confirm the differentiated cells. *NES*, encoded for Nestin protein, is highly expressed in neural progenitor cells in the subventricular zone, and is used widely as an early stage marker of neuronal differentiation [43]. Previous studies provided the neuronal-specific marker genes profiling of neuronal cells derived from RSV-hDPSCs. Both of the early stage markers (*NES* and *Musashi*) and the mature neuron-specific markers (*NF-M*) were significantly increased in RSV-d-hDPSCs and defined the differentiated cells as neuronal cells [15]. Recent studies have demonstrated the neuronal differentiation potential of hSCAPs. The differentiated cells derived from hSCAPs exhibited neuronal-like cell morphology under long term neuronal induction for 5 weeks. However, the qRT-PCR has revealed that the differentiated cells were highly expressed in *NSE*, and weakly detected in *TUBB3* and *NF-M* [44]. This evidence suggests that the hSCAPs are more restricted and committed in their neuronal differentiation at an early stage. Our results provided 2 sequential phases of neuronal induction for 24 hours and 6 hours, and the differentiated cells presented neuronal-like cells in several morphologies. However, the increasing expression was only observed in *NES*, but not in *MAP-2* or *TUBB3* in d-hSCAPs, as revealed by qRT-PCR. According to this outcome, the differentiated cells derived hSCAPs actively expressed the neural progenitor gene, and might characterize as neural progenitor-like cells. We have found that the hSCAPs treated with 10  $\mu\text{M}$  resveratrol for 12 hours created the optimal condition for enhancing the neural progenitor gene, and that the synergistic pre-treatment with resveratrol, and within the optimal condition, triggered differentiation into neural progenitor-like cells

with specific expressions of *NES*, and showed a higher percentage of neuronal differentiation than d-hSCAPs by 4 times. However, the molecular signaling mechanisms, fully terminal neuronal differentiation, and the functional electrophysiological tests need to be confirmed in future studies.

Taken together, we have demonstrated that resveratrol serves as an effective enhancer of neuronal differentiation by promoting neural progenitor gene expression in the hSCAPs, and that the RSV-hSCAPs are more differentiated into neuronal-like cells at the early stage than at the late stage in a neuronal induction medium. These results suggest that a resveratrol pre-treatment of MSCs may be an effective alternative approach for neurodegenerative disease.

## Conclusion

This study demonstrated the capacity of hSCAPs for neuronal differentiation, and that pre-treatment with resveratrol efficiently induces neural progenitor marker gene expression, which synergistically enhances neural progenitor-like cell induction within a neuronal induction medium. Thus, these findings suggest the alternative of using hSCAPs, and the potential of resveratrol treatment as a stem cell-based therapy, for further transplantation in the treatment of neurodegenerative disease.

## Abbreviations

A570-A690: absorbance at 570 nm minus 690 nm; AKT: protein kinase B; bFGF: basic fibroblast growth factor; BHA: butylated hydroxyanisole; cAMP: cyclic adenosine monophosphate; CD: cluster of differentiation; CNS: central nervous system; Crt-hSCAPs: control hSCAPs; d-hBM-MSCs: differentiated cell derived human bone marrow mesenchymal stem cells; d-hDPSCs: differentiated cell derived human dental pulp stem cells; d-hSCAPs: differentiated cell derived human stem cells from apical papilla; DMEM/F-12: Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (Ham); DMSO: dimethyl sulfoxide; DSCs: dental-derived stem cells; ERK1/2: extracellular signaling regulated kinase1/2; FBS: fetal bovine serum; *GAPDH*: glyceraldehyde 3-phosphate dehydrogenase; GFAP: glial fibrillary acid protein; GSK3- $\beta$ : glycogen synthase kinase 3- $\beta$ ; hBM-MSCs: human bone marrow-derived mesenchymal stem cells; hDPSCs: human dental pulp stem cells; hSCAPs: human stem cells from apical papilla; hUC-MSCs: human umbilical cord-derived mesenchymal stem cells; IBMX: 3-isobutyl-1-methylxanthine; IC<sub>50</sub>: 50% inhibitory concentration; ISCT: International Society for Cellular Therapy; *MAP-2*: microtubule associated protein-2; MSCs: mesenchymal stem cells; MTT: methylthiazolyldiphenyl-tetrazolium bromide; *NF-M*: neurofilament medium-type; NPCs: neural progenitor cells; *NES*: human gene encoded for nestin protein; PDE: phosphodiesterase; PKA: protein kinase A; qRT-PCR: quantitative real-time polymerase chain reaction; RSV: resveratrol; RSV-d-hBM-MSCs: differentiated cells derived resveratrol pre-treated hBM-MSCs; RSV-d-hDPSCs: differentiated cells derived resveratrol pre-treated hDPSCs; RSV-d-hSCAPs: differentiated cells derived resveratrol pre-treated hSCAPs; RSV-hSCAPs: resveratrol pre-treated hSCAPs at optimal condition; SEM: standard error of mean; *SIRT1*: sirtuin 1; *TrkA*: tyrosine kinase receptor type 1;  $\alpha$ MEM: Alpha Minimum Essential Medium.

# Declarations

## Ethics approval and consent to participate

This study was approved by the Ethics Committee on Human Rights Related to Human Experimentation of Faculty of Dentistry/Faculty of Pharmacy, Mahidol University, Thailand. (COE. No. MU-DT/PY-IRB 2019/027.2405); project number: 2019/DT068; Principle investigator: Dr. Nisarut Ruangsawasdi, D.D.S, Ph.D.

## Consent for publication

Not applicable

## Availability of data and materials

Not applicable

## Competing interests

The authors declare that they have no competing interests.

## Funding

This work was supported by grants obtained from Science Achievement Scholarship of Thailand (SAST), Central Instrument Facilities (CIF) Faculty of Science, Mahidol University, Thailand.

## Authors' contributions

AS contributed to experimental conceptualization and design, performed all experiments, analyzed and interpreted data, and wrote the manuscript. TG analyzed and interpreted data, and provided considerable manuscript review. NR contributed to experimental conceptualization and design, provided collection and isolation of hSCAPs, contributed human ethical considerations, revision of work, and provided considerable manuscript review. CP assisted in the characterization of hSCAPs, and gene expression analysis. CT contributed to experimental conceptualization and design, considerable manuscript review, supervision of work, and manuscript development. All authors read and approved the final manuscript.

## Acknowledgements

The authors would like to thank Miss. Ratchaporn Srichan and Miss. Supaporn Mala at Oral Tissue, Cells and Molecular Biology Analysis and Research Center, Faculty of Dentistry, Mahidol University for technical assistance, Olympus Bioimaging Center (OBC), Faculty of Science, Mahidol University, Thailand for cell imaging, and Michael Helgeson for the English language review.

## Author details

<sup>1</sup> Department of Anatomy, Faculty of Science, Mahidol University, 272 RAMA VI road, Ratchathewi, Bangkok, 10400, Thailand.

<sup>2</sup> Chakri Naruebodindra Medical Institute, Faculty of Medicine Ramathibodi Hospital, Mahidol University, 111 Bang Pla, Bang Phli, Samut Prakan, 10540, Thailand.

<sup>3</sup> Department of Pharmacology, Faculty of Dentistry, Mahidol University, 6 Yothi road, Ratchathewi, Bangkok, 10400, Thailand.

## References

1. Organization, W.H., *Neurological disorders: public health challenges*. 2006: World Health Organization.
2. Björklund, A. and O. Lindvall, *Cell replacement therapies for central nervous system disorders*. *Nature neuroscience*, 2000. **3**(6): p. 537-544.
3. Eriksson, P.S., et al., *Neurogenesis in the adult human hippocampus*. *Nature medicine*, 1998. **4**(11): p. 1313-1317.
4. Arvidsson, A., et al., *Neuronal replacement from endogenous precursors in the adult brain after stroke*. 2002. **8**(9): p. 963.
5. Sonoyama, W., et al., *Characterization of the apical papilla and its residing stem cells from human immature permanent teeth: a pilot study*. 2008. **34**(2): p. 166-171.
6. Huang, G.T.-J., et al., *The hidden treasure in apical papilla: the potential role in pulp/dentin regeneration and bioroot engineering*. 2008. **34**(6): p. 645-651.
7. Huang, G.-J., S. Gronthos, and S.J.J.o.d.r. Shi, *Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine*. 2009. **88**(9): p. 792-806.
8. Dominici, M., et al., *Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement*. 2006. **8**(4): p. 315-317.
9. Anghileri, E., et al., *Neuronal differentiation potential of human adipose-derived mesenchymal stem cells*. 2008. **17**(5): p. 909-916.
10. Jeong, S.-G., et al., *Valproic acid promotes neuronal differentiation by induction of neuroprogenitors in human bone-marrow mesenchymal stromal cells*. *Neuroscience letters*, 2013. **554**: p. 22-27.
11. Guo, L., et al., *Resveratrol induces differentiation of human umbilical cord mesenchymal stem cells into neuron-like cells*. 2017. **2017**.

12. Jahan, S., et al., *PKA-GSK3 $\beta$  and  $\beta$ -catenin signaling play a critical role in trans-resveratrol mediated neuronal differentiation in human cord blood stem cells*. 2018. **55**(4): p. 2828-2839.
13. Bueno, C., M. Martínez-Morga, and S. Martínez, *Non-proliferative neurogenesis in human periodontal ligament stem cells*. Scientific Reports, 2019. **9**(1): p. 1-17.
14. Gonmanee, T., et al., *Differentiation of stem cells from human deciduous and permanent teeth into spiral ganglion neuron-like cells*. 2018. **88**: p. 34-41.
15. Geng, Y.W., et al., *Differentiation of human dental pulp stem cells into neuronal by resveratrol*. 2017. **41**(12): p. 1391-1398.
16. Fan, W., et al., *mTORC1 and mTORC2 play different roles in the functional survival of transplanted adipose-derived stromal cells in hind limb ischemic mice via regulating inflammation in vivo*. Stem cells, 2013. **31**(1): p. 203-214.
17. An, J., et al., *Modulatory Effects of Natural Products on Neuronal Differentiation*. 2018.
18. Ramprasath, V. and P.J.E.J.o.C.N. Jones, *Anti-atherogenic effects of resveratrol*. 2010. **64**(7): p. 660.
19. Pallàs, M., et al., *Resveratrol and neurodegenerative diseases: activation of SIRT1 as the potential pathway towards neuroprotection*. 2009. **6**(1): p. 70-81.
20. Joe, I.-S., S.-G. Jeong, and G.-W.J.N.I. Cho, *Resveratrol-induced SIRT1 activation promotes neuronal differentiation of human bone marrow mesenchymal stem cells*. 2015. **584**: p. 97-102.
21. Wang, X., et al., *Resveratrol exerts dosage-dependent effects on the self-renewal and neural differentiation of hUC-MSCs*. Molecules and cells, 2016. **39**(5): p. 418.
22. Varga, G. and G. Gerber, *Mesenchymal stem cells of dental origin as promising tools for neuroregeneration*. Stem cell research & therapy, 2014. **5**(2): p. 61.
23. Green, S.A., M. Simoes-Costa, and M.E. Bronner, *Evolution of vertebrates as viewed from the crest*. Nature, 2015. **520**(7548): p. 474-482.
24. Simonovic, J., et al., *Differentiation of stem cells from apical papilla into neural lineage using graphene dispersion and single walled carbon nanotubes*. Journal of Biomedical Materials Research Part A, 2018. **106**(10): p. 2653-2661.
25. Kolar, M.K., et al., *The neurotrophic effects of different human dental mesenchymal stem cells*. 2017. **7**(1): p. 12605.
26. Weiskirchen, S. and R. Weiskirchen, *Resveratrol: how much wine do you have to drink to stay healthy?* Advances in Nutrition, 2016. **7**(4): p. 706-718.
27. Camont, L., et al., *Simple spectrophotometric assessment of the trans-/cis-resveratrol ratio in aqueous solutions*. Analytica chimica acta, 2009. **634**(1): p. 121-128.
28. Calabrese, E.J., M.P. Mattson, and V. Calabrese, *Resveratrol commonly displays hormesis: occurrence and biomedical significance*. Human & experimental toxicology, 2010. **29**(12): p. 980-1015.
29. Posadino, A.M., et al., *Flavin oxidase-induced ROS generation modulates PKC biphasic effect of resveratrol on endothelial cell survival*. Biomolecules, 2019. **9**(6): p. 209.

30. Martins, L.A.M., et al., *Resveratrol induces pro-oxidant effects and time-dependent resistance to cytotoxicity in activated hepatic stellate cells*. Cell biochemistry and biophysics, 2014. **68**(2): p. 247-257.
31. Yoon, D.S., et al., *Different effects of resveratrol on early and late passage mesenchymal stem cells through  $\beta$ -catenin regulation*. Biochemical and biophysical research communications, 2015. **467**(4): p. 1026-1032.
32. Kornienko, J.S., et al., *High doses of synthetic antioxidants induce premature senescence in cultivated mesenchymal stem cells*. Scientific reports, 2019. **9**(1): p. 1-13.
33. Song, L.H., et al., *Resveratrol prevents CsA inhibition of proliferation and osteoblastic differentiation of mouse bone marrow-derived mesenchymal stem cells through an ER/NO/cGMP pathway*. Toxicology in vitro, 2006. **20**(6): p. 915-922.
34. Caldarelli, I., et al., *Resveratrol mimics insulin activity in the adipogenic commitment of human bone marrow mesenchymal stromal cells*. The international journal of biochemistry & cell biology, 2015. **60**: p. 60-72.
35. Okay, E., et al., *Cross effects of resveratrol and mesenchymal stem cells on liver regeneration and homing in partially hepatectomized rats*. Stem Cell Reviews and Reports, 2015. **11**(2): p. 322-331.
36. Pinarli, F.A., et al., *Resveratrol and adipose-derived mesenchymal stem cells are effective in the prevention and treatment of doxorubicin cardiotoxicity in rats*. Pediatric hematology and oncology, 2013. **30**(3): p. 226-238.
37. Wang, X., et al., *Resveratrol promotes hUC-MSCs engraftment and neural repair in a mouse model of Alzheimer's disease*. Behavioural brain research, 2018. **339**: p. 297-304.
38. Donmez, G.J.T.i.p.s., *The neurobiology of sirtuins and their role in neurodegeneration*. 2012. **33**(9): p. 494-501.
39. Park, S.-J., et al., *Resveratrol ameliorates aging-related metabolic phenotypes by inhibiting cAMP phosphodiesterases*. 2012. **148**(3): p. 421-433.
40. Li, X.-h., et al., *Sirt1 promotes axonogenesis by deacetylation of Akt and inactivation of GSK3*. 2013. **48**(3): p. 490-499.
41. Kumar, V., et al., *Differential responses of Trans-Resveratrol on proliferation of neural progenitor cells and aged rat hippocampal neurogenesis*. Scientific reports, 2016. **6**: p. 28142.
42. Sanchez-Ramos, J., et al., *Adult bone marrow stromal cells differentiate into neural cells in vitro*. Experimental neurology, 2000. **164**(2): p. 247-256.
43. Namiki, J., et al., *Nestin protein is phosphorylated in adult neural stem/progenitor cells and not endothelial progenitor cells*. Stem cells international, 2012. **2012**.
44. Li, D., et al., *Human dental pulp stem cells and gingival mesenchymal stem cells display action potential capacity in vitro after neuronogenic differentiation*. 2019. **15**(1): p. 67-81.

## Table 1

Table 1 Forward and reverse primers for qRT-PCR

Genes	Primers	Sequences (5'-3')	References
<i>NES</i>	Forward	CTGCTACCCTTGAGACACCTG	NM_006617.1
	Reverse	GGGCTCTGATCTCTGCATCTAC	
<i>MAP-2</i>	Forward	CGAAGCGCCAATGGATTCC	NM_001039538.1
	Reverse	TGAACTATCCTTGACAGACACCT	
<i>TUBB3</i>	Forward	GGCCAAGGGTCACTACACG	NM_006086.3
	Reverse	GCAGTCGCAGTTTTTCACACTC	
<i>GAPDH</i>	Forward	CTGGGCTACTGAGCACC	NM_001256799
	Reverse	AAGTGGTCGTTGAGGGCAATG	

## Figures

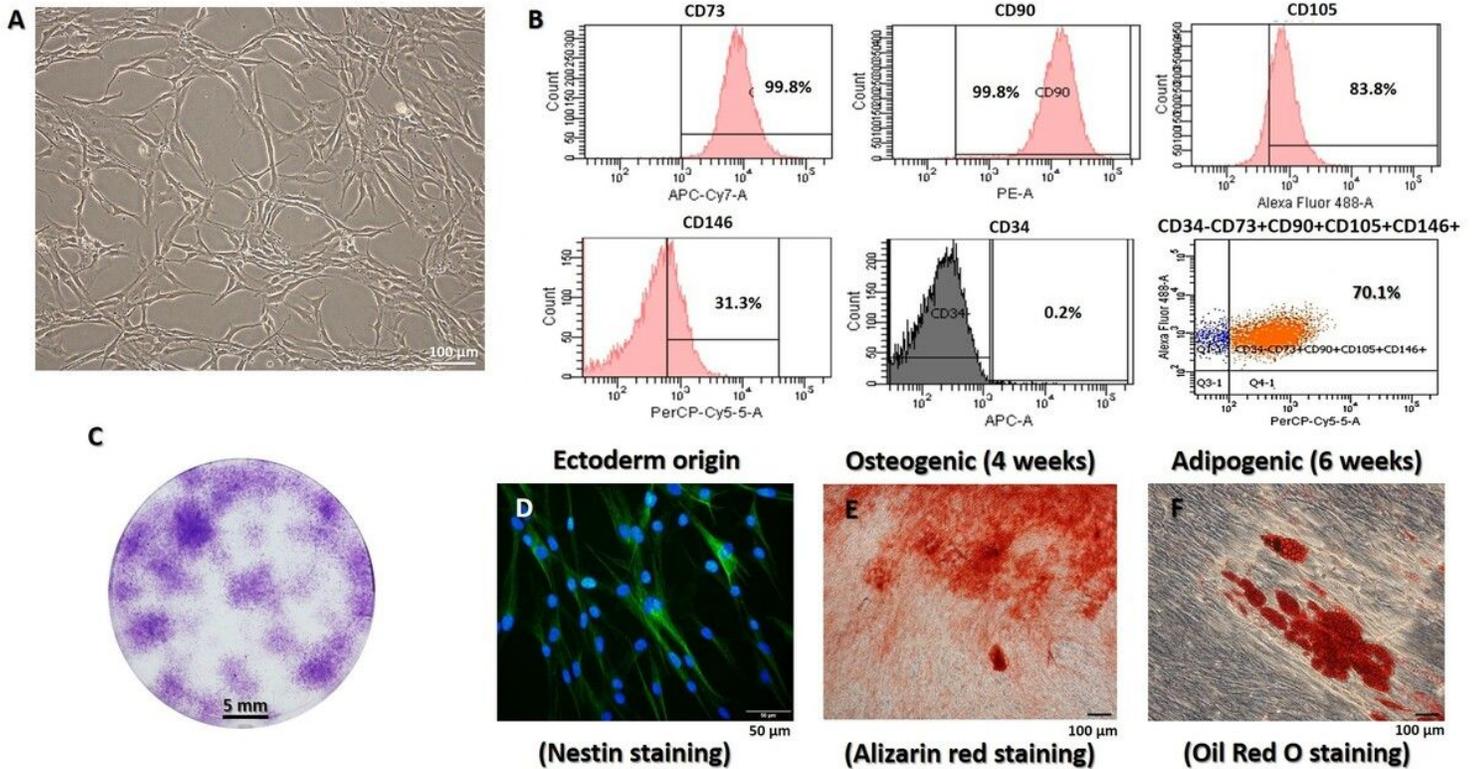
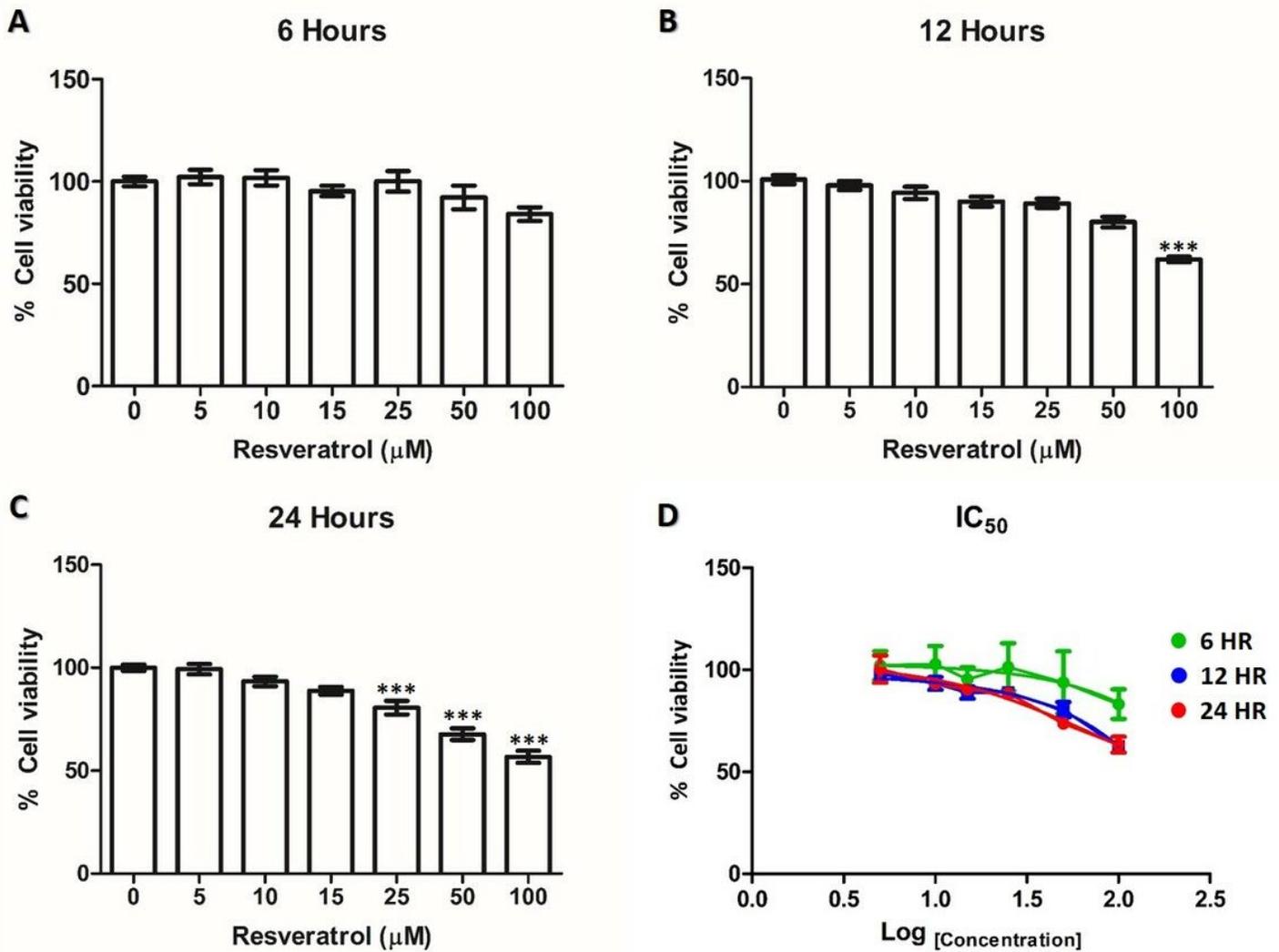


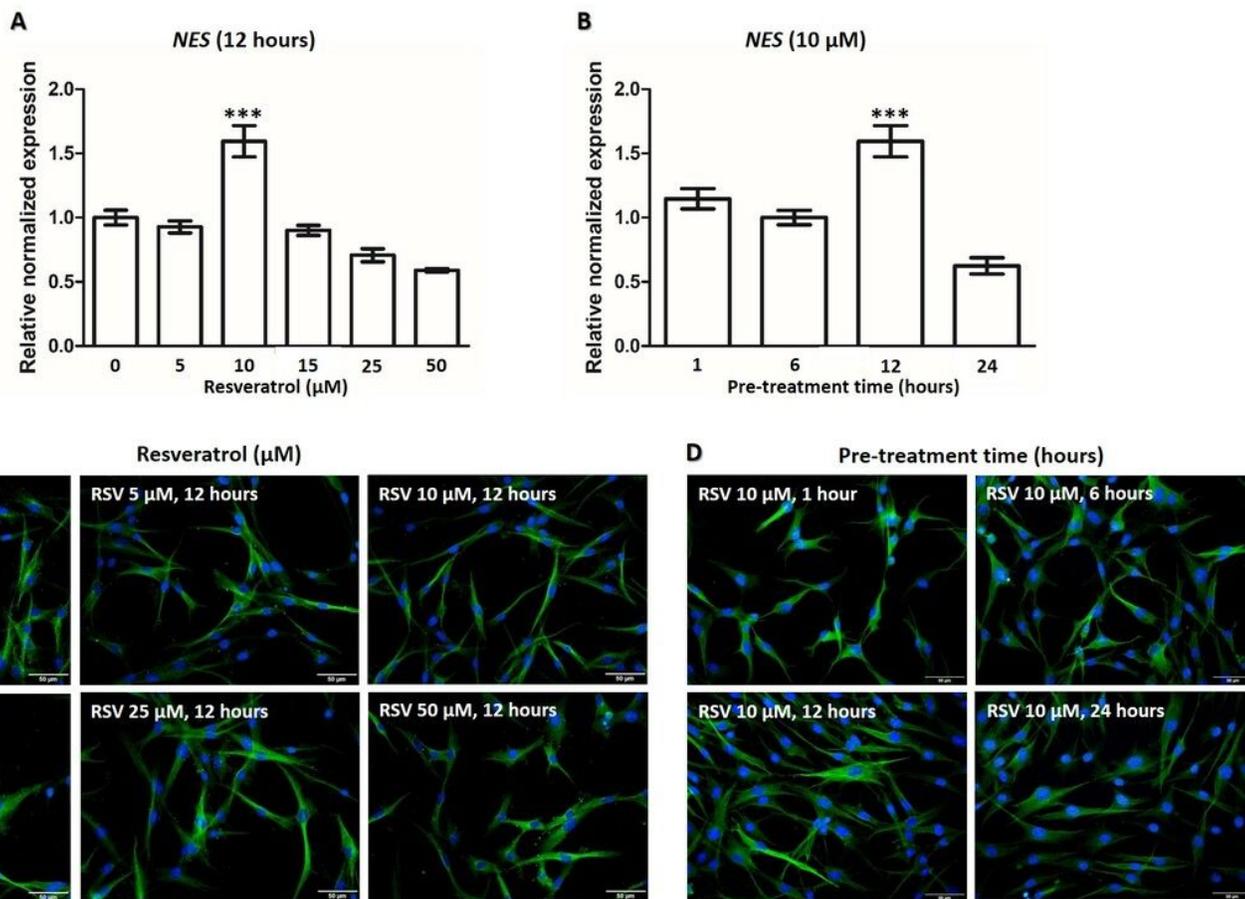
Figure 1

Characterization of hSCAPs. (A) Isolated cells present the typical fibroblast and spindle-like shaped morphology. (B) The cells are positive for CD73, CD90, CD105, and CD146 but negative for CD34. The amount of isolated cells that expressed these markers (CD73+, CD90+, CD105+, CD146+, and CD34-) are highly expressed. (C) The cells efficiently form colonies. (D) The cells reveal the neural crest derivative origin with nesting staining. (E) Osteogenic differentiation was demonstrated with Alizarin red staining of calcified nodule. (F) Adipogenic differentiation was revealed with lipid droplets stained by Oil Red O. Scale bars: A, E, and F = 100  $\mu$ m, C = 5 mm, and D = 50  $\mu$ m.



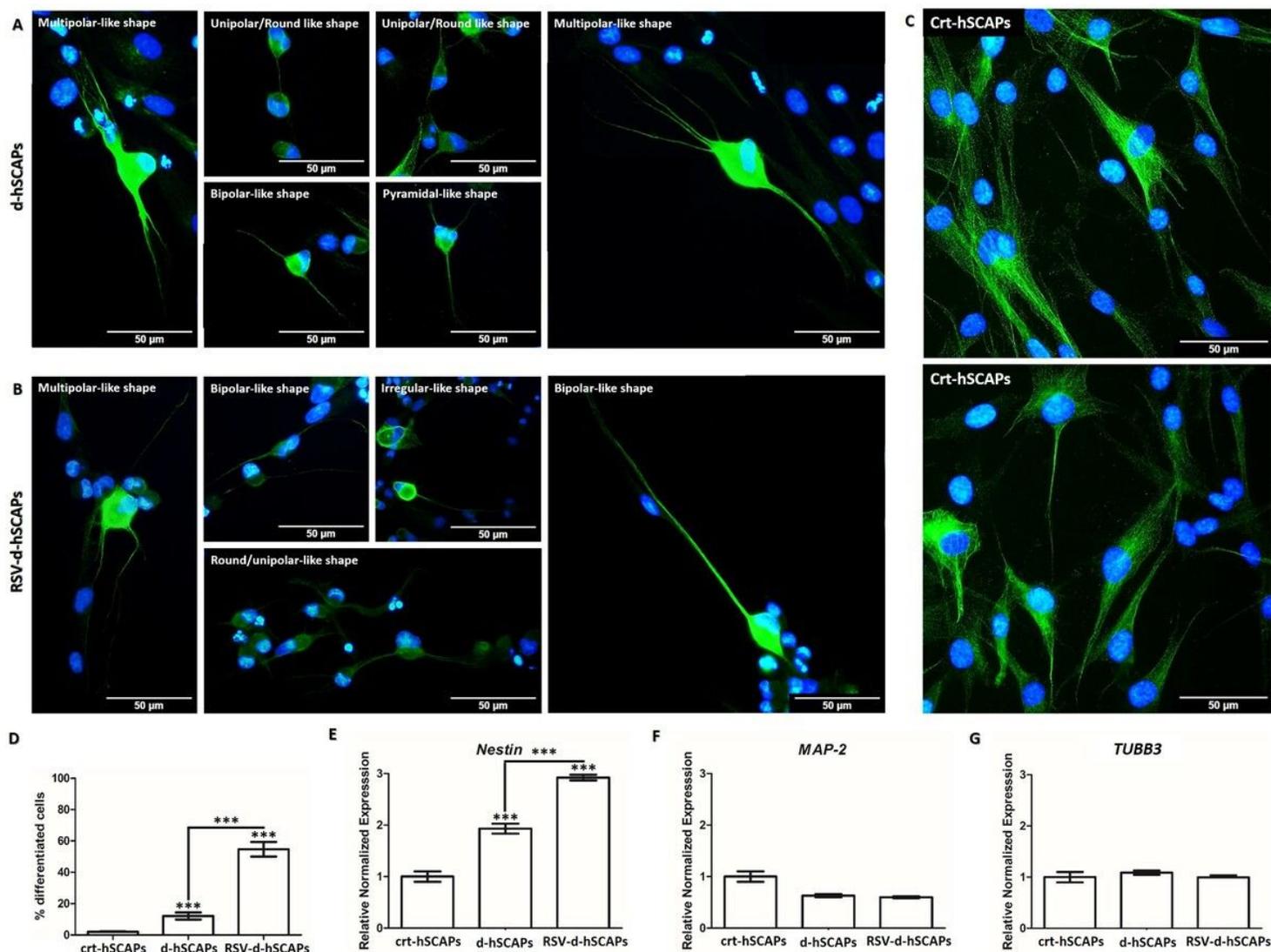
**Figure 2**

The cellular toxicity of RSV-hSCAPs. (A-C) The percentage of cell viability of RSV-hSCAPs during 0-100  $\mu$ M of resveratrol for 6, 12, and 24 hours, respectively. Cellular toxicity was not observed for up to 100  $\mu$ M for 6 hours, 50  $\mu$ M for 12 hours, and 15  $\mu$ M for 24 hours. (D) The IC<sub>50</sub> of resveratrol treatment on hSCAPs for 6, 12, and 24 hours. Data were expressed as the mean  $\pm$  SEM; n = 3, \*\*\*p < 0.001.



**Figure 3**

Optimal condition of resveratrol pre-treatment. (A) The expression of NES of resveratrol pre-treatment for 12 hours ranging from 0-50  $\mu\text{M}$ . The NES expression was significantly increased at 10  $\mu\text{M}$ , and dropped at 50  $\mu\text{M}$ . (B) The NES expression of resveratrol pre-treatment at 10  $\mu\text{M}$  for 1, 6, 12, and 24 hours. The highest expression of NES was observed at 12 hours, and determined as an optimal condition. Data were expressed as the mean  $\pm$  SEM;  $n = 3$ , \*\*\* $p < 0.001$ . (C) The cell morphology visualized by  $\beta$ -III tubulin staining of RSV-hSCAPs during 0-50  $\mu\text{M}$  of resveratrol for 12 hours. (D) The  $\beta$ -III tubulin profiling of RSV-hSCAPs at 10  $\mu\text{M}$  for 1, 6, 12, and 24 hours. These outcomes have strongly demonstrated that pre-treatment of resveratrol effectively induces neural progenitor gene marker expression but insufficiently triggers morphological change of the hSCAPs. Scale bars: C, and D = 50  $\mu\text{m}$ .



**Figure 4**

Neuronal induction. (A-C) The immunofluorescences profiling ( $\beta$ -III tubulin) of crt-hSCAPs, d-hSCAPs, and RSV-d-hSCAPs. These visualizations have revealed that a neuronal induction medium successfully promotes morphological change of d-hSCAPs and RSV-d-hSCAPs into neuronal-like cells, while the crt-hSCAPs still clearly exhibit as fibroblast-like cells. (D) The percentage of neuronal differentiation between d-hSCAPs and RSV-d-hSCAPs. Resveratrol pre-treatment efficiently enhances the percentage of differentiated cells from  $12.11 \pm 5.08$  up to  $54.71 \pm 10.39$ . Data were expressed as the mean  $\pm$  SEM;  $n = 5$ ,  $***p < 0.001$ . (E-G) The genes expression profiling (NES, MAP-2, and TUBB3) of crt-hSCAPs, d-hSCAPs, and RSV-d-hSCAPs. Under neuronal induction, the increasing expression was highly expressed in NES but not for MAP-2 or TUBB3. Resveratrol pre-treatment synergistically promotes the neural progenitor marker gene. Data were expressed as the mean  $\pm$  SEM;  $n = 3$ ,  $***p < 0.001$ . Scale bars: A, B, and C = 100  $\mu$ m.