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Research article

Simvastatin enhances proliferation and pluripotent gene expression by canine bone marrow-derived mesenchymal stem cells (cBM-MSCs) *in vitro*

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ABSTRACT

Establishing the intervention to enhance proliferation and differentiation potential is crucial for the clinical translation of stem cell-based therapy. In this study, the effects of simvastatin on these regards were explored. Canine bone marrow-derived mesenchymal stem cells (cBM-MSCs) were treated with 4 doses of simvastatin, 0.1, 1, 10, and 100 nM. Simvastatin in low-dose range, 0.1 and 1 nM, enhanced dose-dependent cell proliferation at day 5 and 7. Exploration of the mechanisms revealed that simvastatin in low-dose range dose-dependently upregulated sets of cell cycle regulators, *Cyclin D1* and *Cyclin D2*; proliferation marker, *Ki-67*; and anti-apoptotic gene; *Bcl-2*. Interestingly, pluripotent markers, *Rex1* and *Oct4*, were dramatically increased upon the low-dose treatment. Contrastingly, treatment with high-dose simvastatin suppressed the expression of those genes. Thus, the results suggested beneficial effects of simvastatin on cBM-MSCs proliferation and expansion. Further study regarding differentiation potential and underlying mechanisms will accelerate the clinical application of the molecule on veterinary stem cell-based therapy.

1. Introduction

Mesenchymal stem cells (MSCs) have been isolated from various types of tissues. Among them, MSCs isolated from bone marrow (BM), so-called bone marrow-derived MSCs (BM-MSCs), contained a distinguished potential for regenerative treatment due to their availability, multi-potentiality, immunomodulating ability, and homing capability (Schäfer et al., 2016). Isolation of canine BM-MSCs (cBM-MSCs) has been reported previously by using different isolation and expansion techniques (Sawangmake et al., 2019; Ippokratis et al., 2007; Kadiyala et al., 1997). The most sites for BM collection were ilium and femur (Catana et al., 2008; Eca et al., 2009). The collected BM was then processed for MSCs isolation using various cell isolation techniques i.e. physiological-buffered solution washing, red blood cell (RBC) lysis, and gradient centrifugation (Juopperi et al., 2007). The normalized isolated MSCs number per tissue mass of cBM-MSCs was higher than other MSCs resources e.g. adipose tissue and synovial fluid (Giai Via, Frizziero and Oliva, 2012; Liao and Chen, 2014). However, the expansion rate of the cells was lower than canine adipose-derived MSCs (cAD-MSCs). The lowered cell proliferation and altered cell morphology were found in higher cell passage (Lennon et al., 2012). These suggested the burdens in cBM-MSCs expansion during the process of cell amplification.

Besides, the multipotentiality of cBM-MSCs has been illustrated by the *in vitro* induction toward osteogenic, chondrogenic, and adipogenic lineages (Ciuffreda et al., 2016; Csaki et al., 2007). These evidences suggested the plasticity of cells and the potential application for stem-cell based regenerative treatment. However, comparing with embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), the differentiation potential of MSCs was much lower (Zomer et al., 2015). This burden led to the gap for introducing MSCs toward clinical application. Many

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strategies have been studied and proposed for improving MSCs plasticity and proliferative capability.

Simvastatin, a statin cholesterol-lowering drug, has been proposed as a potential enhancing molecule for MSCs culture. In addition to the effect as a 3-hydroxy-3methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, it has been illustrated that the drug contained beneficial pleiotropic effects on cell proliferation and differentiation *in vitro* (Pagkalos et al., 2010; Qiao et al., 2011). Previous publications reported the promoting effect of simvastatin on the osteogenic differentiation potential of BM-MSCs as illustrated by an increased osteogenic marker expression (Pagkalos et al., 2010; Tai et al., 2015). Positive effect of simvastatin on proliferation has been reported as a dose-dependent enhanced periodontal ligament (PDL) cells proliferation assessed by BrdU assay (Yazawa et al., 2005). Toxicity testing of the molecule in mouse ESCs, concentrations ranging from 0.1 nM to 10 mM, showed approximately 60% of viability reduction in micromolar but not nanomolar dose ranges (Pagkalos et al., 2010). The evidences suggested the potential application of simvastatin along with the importance of dose adjustment on cBM-MSCs culture aiming for enhancing the proliferation and differentiation capabilities.

Thus, this study was aimed for exploring the effect and optimizing the simvastatin dose on cBM-MSCs proliferation and pluripotent marker expression along with the exploration of related mechanisms.

2. Materials and methods

2.1. Bone marrow collection

The protocol was approved by the Institutional Animal Care and Use Committee (IACUC), the Faculty of Veterinary Science, Chulalongkorn University. Bone marrow (BM) was collected from the healthy canine donors with the owners' consents. BM was collected from ilium or femur (Kraus and Kirker-Head, 2006). Aseptic technique was employed throughout the procedures. BM aspiration was performed by using 18-gauge Jamshidi® bone marrow biopsy needles (BD, USA) assembled with 10 mL syringe containing heparin solution (2,500 IU heparin/1 mL bone marrow aspirate). The volume of the aspirated bone marrow was not exceeded 1 mL/kg body weight. The collected BM was kept in sterile bag, placed on ice, and immediately transported to the laboratory.

2.2. cBM-MSCs isolation, culture, and expansion

For cBM-MSCs isolation, the aspirated bone marrow was mixed with 10 mL Hank's Balanced Salt Solution (HBSS) (Thermo Fisher Scientific Corporation, USA) as a washing solution. After centrifugation at 300 rpm for 15 min, the supernatant, solution above the white ring layer lied over red blood cell, was gently discarded. The second wash with 20 mL HBSS was performed at 1,000 rpm for 15 min, and the supernatant was gently discarded. The pellet was gently resuspended with 10 mL Gibco™ Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) (Thermo Fisher Scientific Corporation, USA) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific Corporation, USA), 2 mM L-glutamine, and 1% Antibiotic-Antimycotic solution (Thermo Fisher Scientific Corporation, USA). Cells were seeded in T-75 Corning® treated tissue culture flasks (Corning, USA) containing pre-warmed culture medium and subsequently incubated under 5% CO_2 and 95% air at 37°c condition. The culture medium, 10% FBS-DMEM/F12, was replaced every 48 h. Cells were subcultured once the confluence reached 80% using 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) (Thermo Fisher Scientific Corporation, USA). Cells in passage 2–5 were used for the experiments.

2.3. Proliferation assay

3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) assay was used to assess the cell proliferation at day 1, 5, and 7. To explore the proliferative effects of simvastatin, cells were starved with serum free (SF)-DMEM/F12 for 3 h and then maintained in 2% FBS-DMEM/F12 supplemented with 4 simvastatin doses (0.1, 1, 10, and 100 nM). The medium was routinely changed every 48 h. At day 1, 5, and 7 post-treatment, MTT assay was performed according to the following protocol. Briefly, cells were gently washed with 500 μL pre-warmed phosphate buffer saline (PBS) solution per well, and then incubated with 300 μL 0.5 mg/mL MTT solution under 5% CO_2 and 95% air at 37 °C condition for 15 min. After discarding the MTT solution, 1 mL of glycine buffered-dimethylsulfoxide (DMSO) solution was added for elution. The optical density was measured at 570 nm wavelength (Riss et al., 2013). The data were presented as a relative proliferation normalized with untreated control group.

2.4. Gene expression analysis

For the analysis of gene expression profiles, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used. At 48 h post-treatment, the mRNA expressions of simvastatin-treated cells were analyzed according the following protocols. Briefly, total RNA was harvested by using TRIzol-RNA isolation reagent (Thermo Fisher Scientific Corporation, USA) and DirectZol-RNA isolation kit (ZymoResearch, USA) according to manufacturer's protocols. The obtained RNA products were converted to complementary DNA (cDNA) by using reverse transcriptase enzyme kit (Promega, USA). The relative mRNA expressions were normalized with reference gene, Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*), and with untreated control according to the following Formula: $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = [Ct^{\text{target gene}} - Ct^{GAPDH}]_{\text{treated}} - [Ct^{\text{target gene}} - Ct^{GAPDH}]_{\text{control}}$. Ct referred to cycle threshold. The amplification primers were summarized in Table 1.

2.5. Fluorescence staining

To illustrate the occurrence of apoptotic and necrotic cells, live/dead (NUCLAER-ID® Blue/Red cell viability) (Enzo Life Sciences, USA) and Annexin V-FITC/propidium iodide (PI) (BD Biosciences, USA) staining was performed according to previous report and manufacturer's protocol (Gelzo et al., 2014). The results were observed under a fluorescent microscope incorporated with Carl ZeissTM Apotome.2 apparatus (Carl Zeiss, Germany).

2.6. Statistical analysis

Data was expressed as mean ± standard deviation (SD). For comparison of data among groups, analysis of variance (ANOVA) was used, and Dunnett's test was performed as *post hoc* analysis. Statistical significance was recognized when p-value < 0.05.

3. Results

3.1. cBM-MSCs culture and expansion

cBM-MSCs could be isolated, cultured, and expanded *in vitro*. Morphology of the isolated cells were fibroblast-like structure (Fig. 1). Expression of pluripotent markers, *Rex1* and *Oct4*, was routinely analyzed in every isolation batch using RT-qPCR (data not shown).

3.2. cBM-MSCs proliferation property

Effect of simvastatin (0.1, 1, 10, and 100 nM) on cBM-MSCs

Table 1
Primer sequences.

Gene	Accession number	Sequences	5′ 3′	Length (bp)	Tm (°C)
Zinc finger protein 42 (ZFP42 or Rex1)	XM_022425491.1	Forward	GCTGACCACCAGCACACTAGGC	199	59.24
		Reverse	TTTCTGGTGTCTTGTCTTTGCCCG		60.73
Octamer-binding transcription factor 4 (Oct4)	XM_538830.3	Forward	GACCATCTGCCGCTTTGAGGCTCTG	100	60.55
		Reverse	GCGCCGGTTACAGAACCACACTCGG		59.50
G1/S-specific cyclin-D1 (Cyclin-D1)	NM_001005757.1	Forward	TGAACTACCTGGACCGCT	137	60.74
		Reverse	CAGGTTCCACTTGAGYTTGT		59.50
G1/S-specific cyclin-D2 (Cyclin-D2)	XM_849493.5	Forward	TACTTCAAGTGCGTGCAGAAGGAC	147	74.80
		Reverse	TCCCACACTTCCAGTTGCGATCAT		74.40
Proliferation marker protein Ki-67 (Ki67)	XM_022411692.1	Forward	CGTAGCAGCACAGAAAT	124	58.49
		Reverse	TGATGGTTGAGGTCGTTCCTTGATG		58.49
Tumor protein p53 (TP53)	NM_001003210.1	Forward	ATGGGAGGCATGAACCGGCG	109	75.30
		Reverse	CGGGACAGGCACAAACGCGT		74.60
B-cell lymphoma2 (Bcl-2)	NM_001002949.1	Forward	CCTGTGGATGACTGAGTACC	100	65.00
		Reverse	GAGACAGCCAGGAGAAATCA		65.80
BCL2 like 1 (BCL-2-L1)	NM_001003072.1	Forward	ACTGTGCGTGGAGAGCGTAG	77	65.60
		Reverse	TCAGGTAAGTGGCCATCCAA		65.40
Caspase 3 (CASP3)	NM_001003042.1	Forward	GCGGAAACCCACGGGGTTCG	79	76.40
		Reverse	CGGATGCGAGCCCGGGAAAG		76.60
Caspase 8 (CASP8)	NM_001048029.1	Forward	ACAAGGGCATCATCTATGGCTCTGA	70	76.40
		Reverse	CCAGTGAAGTAAGAGGTCAGCTCA		76.60
Caspase 9 (CASP9)	NM_001031633.1	Forward	TCAGTGACGTCTGTGTTCAGGAGA	97	76.40
		Reverse	TTGTTGATGATGAGGCAGTAGCCG		76.60
Glyceraldehyde 3-phosphate dehydrogenase (Gapdh)	NM_001003142.1	Forward	CCAACTGCTTGGCTCCTCTA	100	59.38
		Reverse	GTCTTCTGGGTGGCAGTGAT		59.67

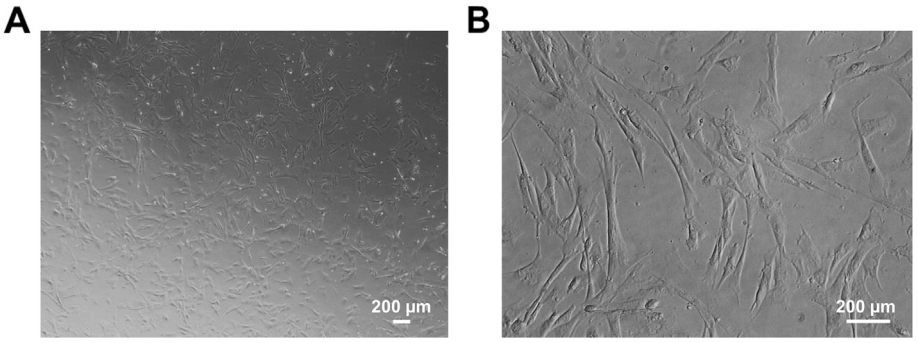

Fig. 1. Morphology of canine bone marrow-derived mesenchymal stem cells (cBM-MSCs). Morphology of the isolated cBM-MSCs was observed under phase-contrast microscope. Magnifications were 40X (A) and 100X (B), respectively.

proliferation was explored. The relative proliferation at day 1, 5, and 7 was plotted (Fig. 2). The significant beneficial effect of simvastatin on proliferation was found at day 5 and 7. At day 7, 1 nM simvastatin showed the highest proliferative effect compared to other doses. Cell morphology of the treated cells were explored in order to investigate the cytotoxicity of the treatment. Cell morphology upon treatment at day 1 and 5 was normal (Fig. 3).

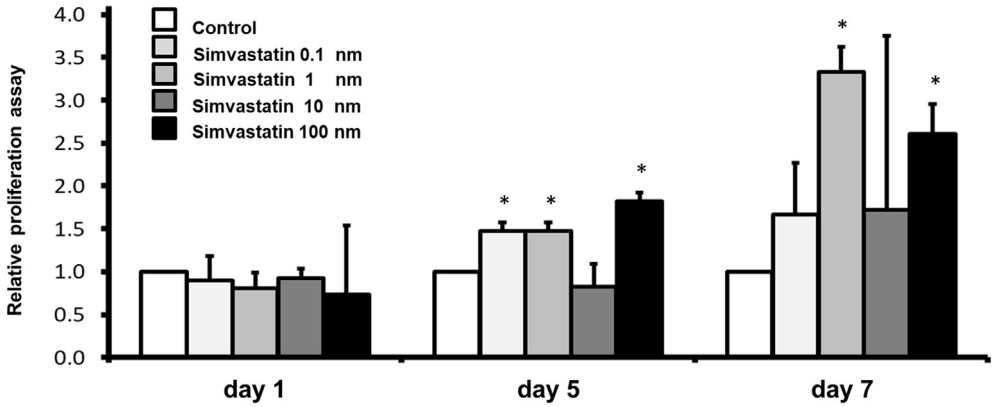

Fig. 2. Relative proliferation of cBM-MSCs upon simvastatin treatment. Proliferative effect of simvastatin was tested. Four simvastatin doses (0.1, 1, 10, and 100 nM) were used, and the MTT assay was explored at day 1, 5, and 7 after treatment. The results were normalized with untreated control group in each day and illustrated as relative cell proliferation. The asterisks indicate statistical difference compared with the control (p-value < 0.05).

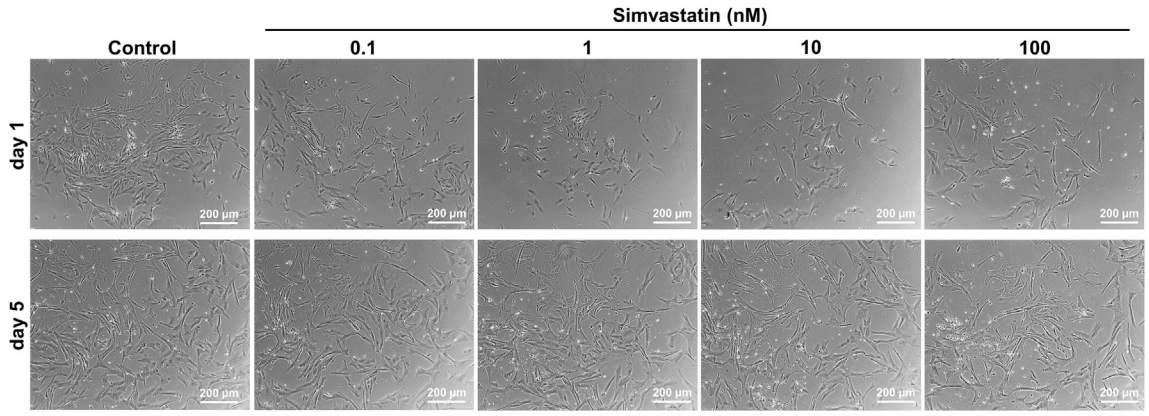

Fig. 3. cBM-MSCs morphology upon simvastatin treatment. Morphology of cBM-MSCs was explored at day 1 and 5 upon simvastatin (0.1, 1, 10, and 100 nM) treatment. Magnification was 40X.

3.3. Simvastatin enhances cBM-MSCs pluripotent gene expression

To assess the effect of simvastatin on stemness property of cBM-MSCs. Expression of pivotal pluripotent gene markers, *Rex1* and *Oct4*, were evaluated using RT-qPCR (Fig. 4). After 48 h post-treatment, simvastatin (0.1, 1, and 100 nM) could enhance the expression of *Rex1* significantly. For *Oct4* expression, only 1 nM simvastatin could enhance the significant upregulation. Interestingly, 1 nM simvastatin exerted dramatic effects on the upregulation of both markers.

3.4. Simvastatin promotes cBM-MSCs cell cycle and exerts anti-apoptotic gene expression

To explore the related mechanisms of beneficial effects of simvastatin on cBM-MSCs proliferation, sets of gene that represent cell cycle regulation were analyzed at 48 h post-treatment. Simvastatin at 1 nM increased the level of *Cyclin D1*, while Simvastatin at 0.1 and 1 nM increased *Cyclin D2* expression in cBM-MSCs. On the contrary, mRNA expressions of *Cyclin D1* and *Cyclin D2* were decreased in simvastatin-treated cBM-MSCs in high-dose, ranging from 10 nM to 100 nM. There was no significant effect of simvastatin on *Ki-67* expression, while significant downregulation of *TP53* was found in 10 nM treatment (Fig. 5).

For representative anti-apoptotic marker, low-dose simvastatin (0.1 and 1 nM) dose-dependently exerted *Bcl-2* expression, while trend of suppression was found in simvastatin high-dose range (10 and 100 nM). There was no significant effect of simvastatin on *Bcl-2-L1* and *Caspase 3*. All dose range of simvastatin significantly suppressed *Caspase 8* expression, while *Caspase 9* was significantly suppressed in 100 nM treatment (Fig. 6).

Live/dead and Annexin V-FITC/PI staining was performed in order to distinguish the occurrence of cell early apoptosis or necrosis. The results illustrated that 0.1, 10, and 100 nM simvastatin treatment triggered an early apoptosis of some cell population, but there was no necrotic cell found in any group of treatment (Fig. 7A and B).

4. Discussion

Various strategies for enhancing the success of MSCs application have been proposed. Cell expansion and differentiation capacities have been included in the top priority since most of MSCs contain limited ability in these regards. For cell expansion issue, each MSCs require different *in vitro* culture condition due to their source of origin and cellular characteristics (Elahi et al., 2016). Most MSCs contain limited potential of differentiation comparing with ESCs and iPSCs (Diederichs and Tuan, 2014). Variety of culture media and supplements have been utilized in MSCs culture in order to enhance expansion and differentiation potential. However, the responses were varied in particular cell types suggesting the necessary for study in individual circumstance. Simvastatin also illustrated particular pleiotropic effects on various cell types e.g. osteogenic enhancement in pluripotent stem cells (Kavalipati et al., 2015). In this study, we illustrated that simvastatin could enhance proliferation capacity of cBM-MSCs. However, only 1 nM simvastatin could show an

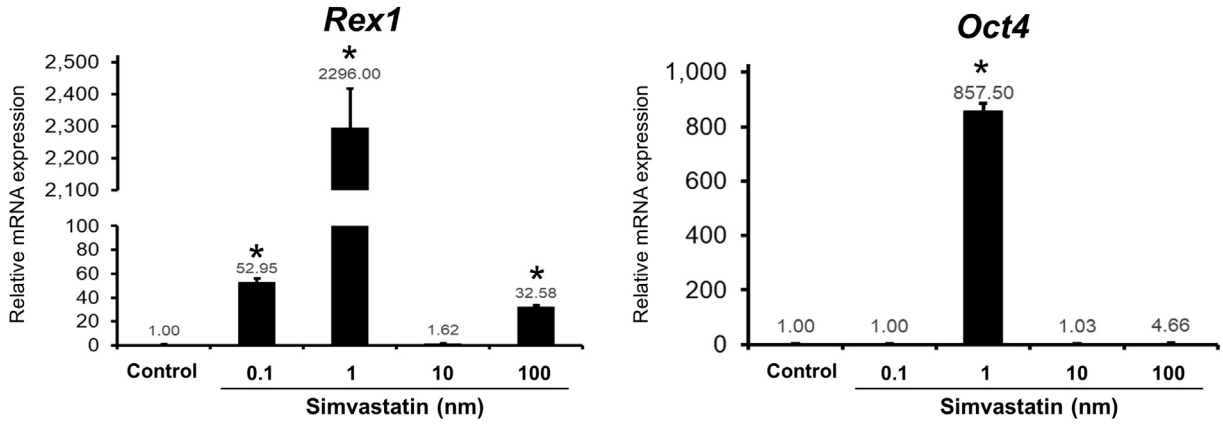

Fig. 4. Effect of simvastatin on cBM-MSCs pluripotent genes expression *in vitro*. The effect of simvastatin (0.1, 1, 10, and 100 nM) on cBM-MSCs pluripotent gene expression was analyzed by using RT-qPCR. mRNA expressions of *Rex1* and *Oct4* were explored at 48 h post-treatment. mRNA expressions of the genes were presented as relative expression by normalizing with reference gene, *Gapdh*, and the control. The asterisks indicate statistical difference compared with the control (p-value < 0.05).

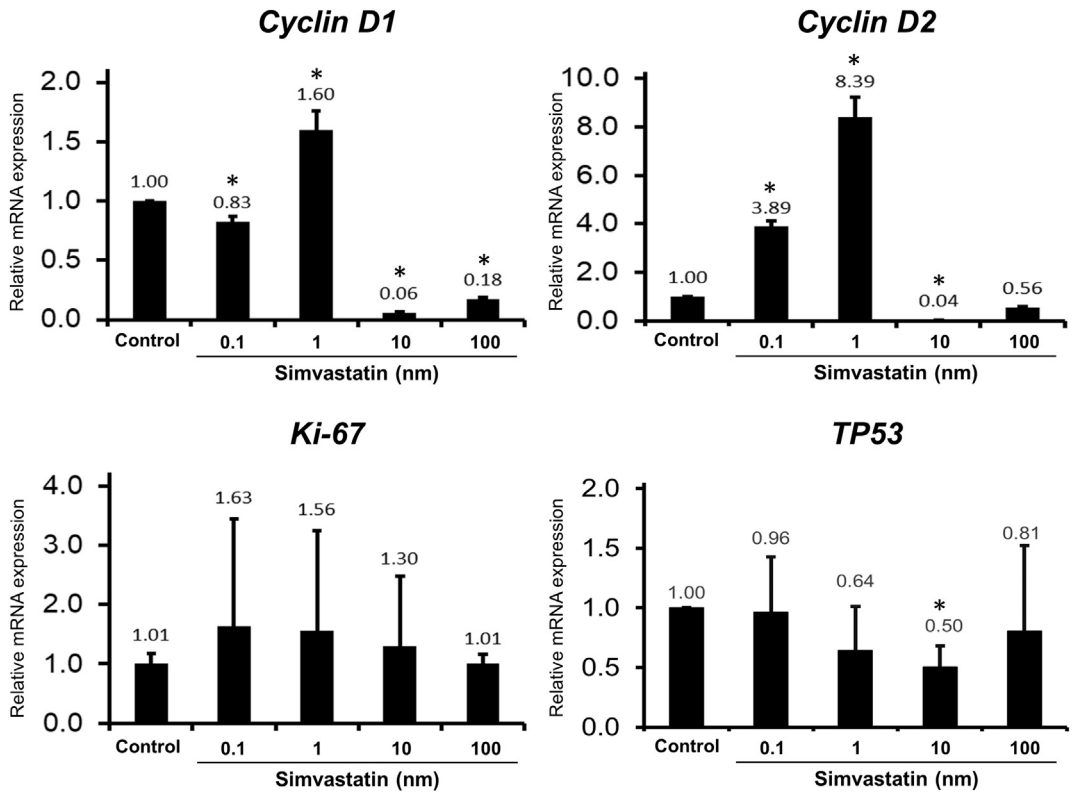

Fig. 5. Effects of simvastatin on cBM-MSCs cell cycle-regulated gene expression *in vitro*. The effect of simvastatin (0.1, 1, 10, and 100 nM) on cBM-MSCs cell cycle-regulated gene expression was analyzed by using RT-qPCR. The mRNA expressions were analyzed at 48 h post-treatment. *Cyclin D1, Cyclin D2, Ki-67,* and *TP53* were explored. mRNA expressions of the genes were presented as relative expression by normalizing with reference gene, *Gapdh*, and the control. The asterisks indicate statistical difference compared with the control (p-value < 0.05).

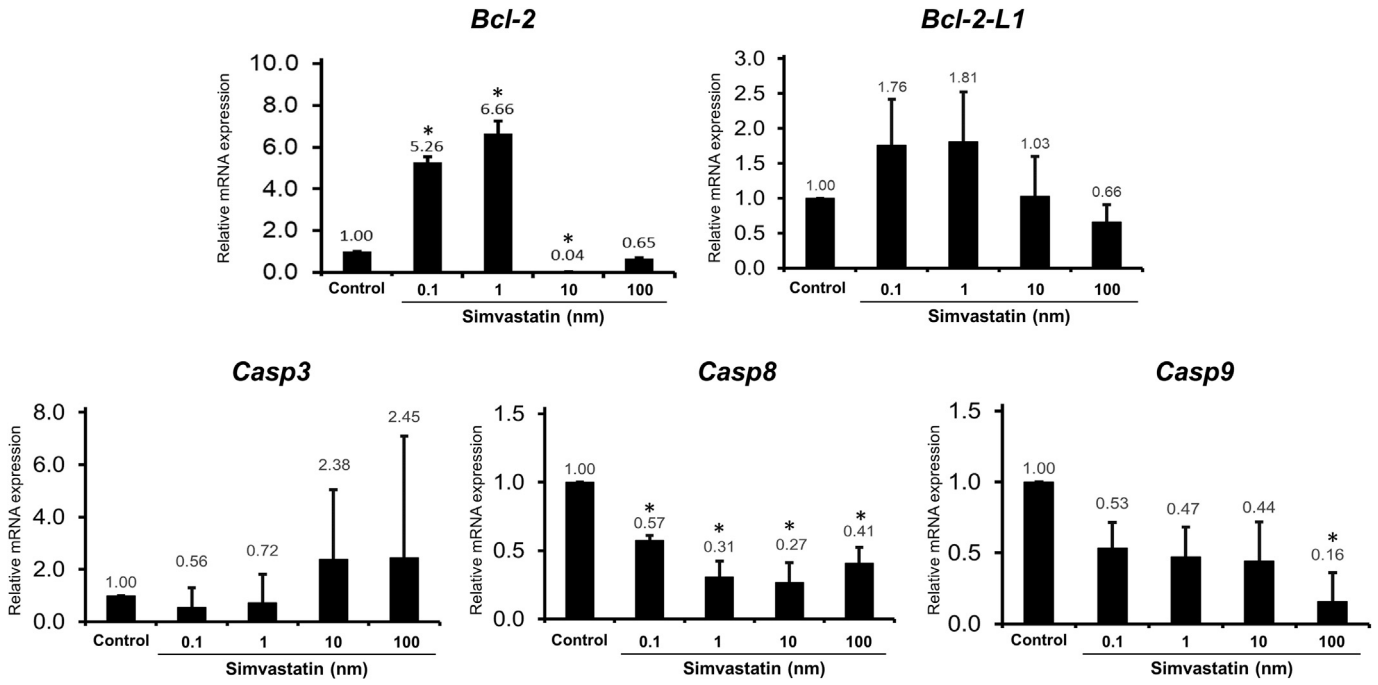

Fig. 6. Effects of simvastatin on cBM-MSCs apoptotic gene expression *in vitro*. The effect of simvastatin (0.1, 1, 10, and 100 nM) on cBM-MSCs apoptotic gene expression was analyzed by using RT-qPCR. The mRNA expressions were analyzed at 48 h post-treatment. *Bcl-2, Bcl-2-L1, Caspase 3, Caspase 8,* and *Caspase 9* were explored. mRNA expressions of the genes were presented as relative expression by normalizing with reference gene, *Gapdh*, and the control. The asterisks indicate statistical difference compared with the control (p-value < 0.05).

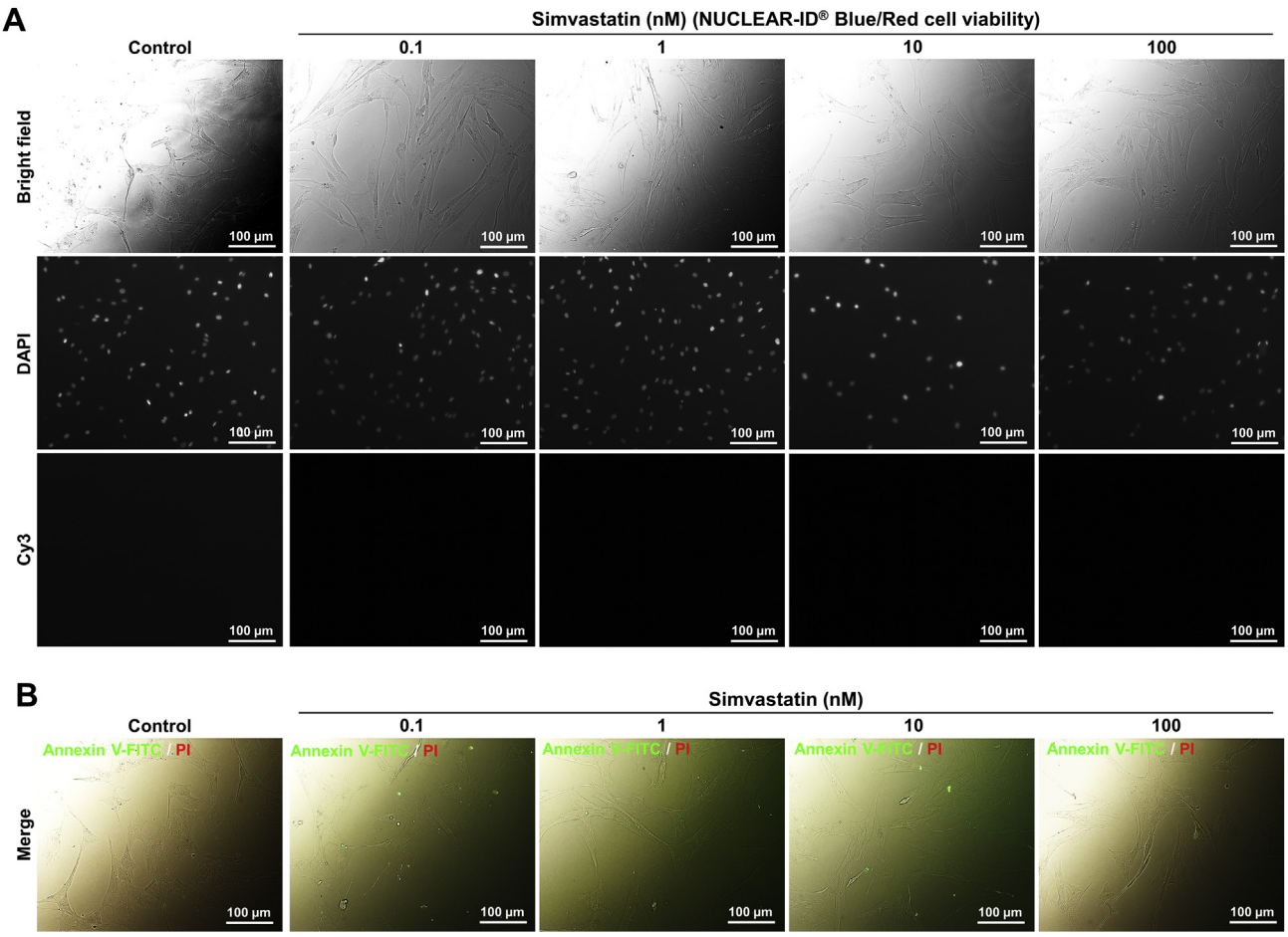

Fig. 7. Live/dead and Annexin V-FITC/PI staining of simvastatin-treated cBM-MSCs. The occurrence of apoptotic and necrotic cBM-MSCs upon simvastatin treatment (0.1, 1, 10, and 100 nM) was analyzed using live/dead (NUCLAER-ID® Blue/Red cell viability) (A) and Annexin V-FITC/PI (B) staining at day 7. For live/dead staining, all nuclei and necrotic cells were showed in DAPI and Cy3 panels, respectively. For Annexin V-FITC/PI staining, early apoptosis was showed as FITC-stained cell, while late apoptosis/necrosis was PI-stained cells.

obvious proliferative effect *in vitro*. These findings correlate with the previous reported by Zhao et al. that demonstrated the positive effects of simvastatin at 0.01 and 0.1 μM on the proliferation of periodontal ligament stem cells (PDLSCs) after 5-day treatment. In that report, simvastatin at 1 and 10 μM significantly suppressed the proliferation at day 3 and day 5 in comparison with the control ($p < 0.01$) (Zhao and Liu, 2014).

In addition, 1 nM simvastatin also dramatically enhanced the expression of pluripotent markers, *Rex1* and *Oct4*, suggesting the beneficial effects of the optimal dose simvastatin on cBM-MSCs proliferation and pluripotent marker expressions. In various molecular study, self-renewal marker like *Rex1* (*ZFN42*) and *Oct4* were of interest (Guercio et al., 2012; Kolf et al., 2007; Shi et al., 2006). These genes were recognized as the markers for undifferentiated ESCs and could indicate the pluripotent stage of cells (Kolf et al., 2007; Shi et al., 2006). MSCs from various mammalian species could express ESCs-related gene markers e.g. *Rex1* and *Oct4* (Kolf et al., 2007). The increase of these factors correlated with differentiation potential toward osteogenic and adipogenic lineages (Roche et al., 2007).

The exploration of related mechanisms governing proliferative effects illustrated that simvastatin in low-dose range (0.1 and 1 nM) upregulated cell cycle regulators, *Cyclin D1* and *Cyclin D2*, while 10 nM simvastatin suppressed tumor suppressor *TP53* expression. Cell cycle was normally controlled by regulating molecules at specific internal checkpoints (Alberts et al., 2015). Cyclins and cyclin-dependent kinases (CDKs) were two groups of protein working coordinately in order to drive the cell cycle forward to further stages throughout the various checkpoints. Therefore, cyclin proteins were able to promote progress of the cell division by partnering with specific CDKs allowing it to be functional enzymes and to activate target proteins along the process. From the previous study revealed that human endothelial progenitor cells (EPCs) treated with atorvastatin, one of the HMG-CoA reductase inhibitors, modulated expression of cell cycle genes including up-regulation of cyclins (cyclin A and cyclin F) and downregulation of the cell cycle inhibitor (p27) corresponded with the result in this study (Assmus et al., 2003). Recent report suggested that high dose simvastatin ranging from (6.25×10^3 nM to 50×10^3 nM) could induce G1 arrest by down-regulating *CyclinD1* and *Cyclin E1* in human primary colorectal cancer cells, regardless of *P53* status (Chen et al., 2018).

The additional exploration revealed an effect on anti-apoptotic gene, *Bcl-2*, upregulation. Bcl-2 family is apoptotic related genes controlling program cell death (Czabotar et al., 2014; Thomadaki and Scorilas, 2006) which consists of both anti-apoptotic and pro-apoptotic genes (Danial, 2007). The expressions of Bcl-2 family such as B cell lymphoma 2 (*Bcl-2*) which is anti-apoptotic gene marker was found to block the program rather than promote cell proliferation rate (Hardwick and Soane, 2013; Korsmeyer, 1999). *Bcl-2* gene increased in low-dose of simvastatin, 0.1 and 1 nM, and corresponded with the result of Dong et al. report which

demonstrated that atorvastatin, one of the statin group, reduced apoptosis of porcine BM-MSCs and increased *Bcl-2* gene (Dong et al., 2011).

All dose of simvastatin suppressed *Caspase 8* expression, while *Caspase 9* downregulation was found in 100 nM treatment. The initiator Caspases play a crucial role in triggering the executioner Caspases (Kim et al., 2015; Tummers and Green, 2017). It seemed that simvastatin downregulated the initiator of extrinsic pathway *(Caspase 8)*, but only high dose simvastatin could suppress the intrinsic pathway initiator (*Caspase 9*) which might result in a proliferative effect of 100 nM treatment. In addition, according to the live/dead and Annexin V-FITC/PI staining, simvastatin (0.1, 10, and 100 nM) triggered early apoptosis of cells, but there was no necrotic cell found upon treatment. This might suggest the anti-apoptotic effect of a low-dose simvastatin treatment.

In conclusion, Simvastatin in low-dose (0.1 and 1 nM) illustrated *in vitro* proliferative effects on cBM-MSCs expansion by upregulating cell cycle regulators (*Cyclin D1* and *Cyclin D2*), and anti-apoptotic gene (*Bcl-2*). Apoptotic initiators, especially *Caspase 8*, was also downregulated. Besides, the pluripotent markers, *Rex1* and *Oct4*, were upregulated. Further study regarding molecular mechanisms and differentiation potential should be conducted in order to facilitate the clinical application of the molecule.

Declarations

Author contribution statement

S. Nantavisai, P. Wikran, P. Kitcharoenthaworn, S. Smithiwong and S. Archasappawat: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

W. Rodprasert: Contributed reagents, materials, analysis tools or data; Wrote the paper.

K. Pathanachai: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

C. Sawangmake: Conceived and designed the experiments; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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