Simvastatin Promotes Osteogenic Differentiation of Mouse Embryonic Stem Cells *Via* Canonical Wnt/ β -Catenin Signaling

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Simvastatin, an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, has been known to reduce cholesterol biosynthesis. However, recent studies demonstrate that simvastatin shows diverse cholesterol-independent functions including cellular differentiation. In this study, we investigated the stimulatory effect of simvastatin on the osteogenic differentiation of mouse embryonic stem cells (ESCs). The osteogenic effect of simvastatin was observed at relatively low doses (ranging from 1 nM to 200 nM). Incubation of ESCs in simvastatin-supplemented osteogenic medium significantly increased alkaline phosphatase (ALP) activity at day 7. The matrix mineralization was also augmented and demonstrated pivotal levels after 14 days incubation of simvastatin. Osteogenic differentiation of ESCs by simvastatin was determined by upregulation of the mRNA expression of runt-related gene 2 (Runx2), osterix (OSX), and osteocalcin (OCN) as osteogenic transcription factors. Moreover, the increased protein expression of OCN, osteopontin (OPN), and collagen type I (Coll I) was assessed using Western blot analysis and immunocytochemistry. However, the blockage of canonical Wnt signaling by DKK-1 downregulated simvastatin-induced ALP activity and the mRNA expression of each osteogenic transcription factor. Furthermore, the β -catenin specific siRNA transfection decreased the protein levels of OCN, OPN, and Coll I. Collectively, these findings suggest that simvastatin enhances the differentiation of ESCs toward osteogenic lineage through activation of canonical Wnt/ β -catenin signaling.

INTRODUCTION

The ability of embryonic stem cells (ESCs) to depict self-renewal capacity and differentiation potential toward specific lineages (Bratt-Leal et al., 2009) provides an unlimited source for cell-based regenerative therapy. ESCs are one of many different cell types which are being tested for their feasibility to establish cell-based strategies in bone tissue engineering. Particularly, the transplantation of ESCs and ESC-derived osteo-

genic cells could be a promising treatment to repair skeletal defects with low numbers of osteoprogenitor cells. However, such a transplantation process needs appropriate cells with a well defined differentiation pattern and efficient acquirement by an easy management of cell culture conditions.

Osteogenic differentiation of ESCs has been performed by introducing cells in an osteoinductive medium (Bielby et al., 2004; Sottile et al., 2003). However, the potential use of ESCs for skeletal regeneration requires a more practical manipulation to differentiate cells into osteogenic cell lineages. Recently, a number of cell culture conditions including physiologically active molecules, signaling inducers, and chemical modifications of cell microenvironments have been found to control osteogenic differentiation of ESCs (Buttery et al., 2001; Phillips et al., 2001). However, it is critical to determine whether the dynamic action of each factor directs or restricts the differentiation of ESCs to desired cell lineages.

Statins, 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) - reductase inhibitors, are widely used for lowering serum cholesterol. It has been reported that simvastatin has anabolic effects on bone metabolism (Montagnani et al., 2003; Wang et al., 2007) and stimulates osteoblastic bone formation *in vitro* and *in vivo* (Maeda et al., 2001). There is evidence that simvastatin promotes osteoblast differentiation in human or mouse bone marrow stem cells by increasing the activity of alkaline phosphatase activity and the expression level of bone morphogenetic protein (BMP) 2 or osteocalcin (Baek et al., 2005; Song et al., 2003). Although a number of studies have demonstrated the potential use of simvastatins to stimulate bone regeneration, few studies have reported the osteogenic effect of simvastatin on ESCs (Pagkalos et al., 2010).

The critical function of the canonical Wnt pathway is to activate β -catenin dependent transcription (Kim et al., 2011; Nusse, 2005). It is well known that Wnt signaling is the most extensively studied pathway with direct relevance to bone formation (Kato et al., 2002; Zhang et al., 2004). However, the precise mechanisms between simvastatin and the canonical Wnt/ β -catenin signaling pathway have been unclear at least in the case of osteogenic process.

A cell culture method with simvastatin for osteogenic differen-

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tiation could suggest the progressive strategy that provides large numbers of osteoprogenitor cells for skeletal regeneration. Thus, the present study demonstrates that the application of simvastatin enhances the differentiation of ESCs into the osteogenic lineage by activating canonical Wnt/ β -catenin signaling.

MATERIALS AND METHODS

Materials

The mouse embryonic stem cell (ESC) line D3 was obtained from the American Type Culture Collection (USA). Fetal bovine serum (FBS) was purchased from Gibco-BRL (USA). Unless otherwise specified, chemicals and laboratory wares were purchased from Sigma Chemical Co. (USA) and Falcon Labware (Becton-Dickinson, USA).

ESC culture and embryoid body formation

The ESCs were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL) supplemented with 1.7 mM L-glutamine, 0.1 mM β -mercaptoethanol, 5 ng/ml mouse leukemia inhibitory factor (LIF), 15% FBS, and 1% penicillin and streptomycin, without a feeder layer at 37°C in an atmosphere containing 5% CO₂. To form embryoid bodies (EBs), the cells were dissociated by 0.05% trypsin/EDTA. Then 2,000 cells were hung from the lids of 100-mm culture dishes for two days in 20 μ l DMEM without LIF. EBs were then suspended in an additional medium for three days. Thus, EBs of the same culture age (5 days old) were used in this experiment.

Osteogenic differentiation of ESCs

EBs were plated onto gelatin-coated six-well plates (10 EBs per well) and maintained in an osteogenic medium consisting of a α minimal essential medium (α -MEM) containing 5% FBS, 50 μ g/ml ascorbic acid, 1 μ M dexamethasone, and 3 mM β -glycerophosphate for 1 day before the application of simvastatin. To promote osteogenic differentiation, simvastatin at different concentrations (1 nM to 200 nM) was added to the osteogenic medium, which was changed every other day. Simvastatin was dissolved in dimethyl sulfoxide (DMSO) immediately before use, and the final concentration of DMSO did not exceed 0.1% (v/v) in any of the experiments. DMSO was used as a control; its concentration was always 0.1%.

Alkaline phosphatase activity

Cells were washed twice with PBS and lysed in a 50 mM Tris-HCl buffer (pH 7.0) containing 1% (v/v) Triton X-100 and 1 mM PMSF. The total protein was then quantified using the Bradford procedure (1976). The entire cell lysate was assayed by adding 200 μ l of *p*-nitrophenylphosphate (pNPP) as a substrate (Sigma, USA) for 30 min at 37°C. The reaction was stopped by adding 3 N NaOH and the absorbance was read spectrophotometrically at 405 nm. The enzyme activity was expressed as mM/100 μ g of protein.

Alizarin red staining

The culture media was discarded and the cells were fixed for 30 min in 4% paraformaldehyde fluid, washed three times with ice-cold phosphate-buffered saline (PBS), stained for 5 min with alizarin red (Sigma, USA), and observed under a light microscope. To quantify mineralization, bound dye was solubilized in 10 mM sodium phosphate containing 10% cetylpyridinium chloride and quantitated spectrophotometrically at 562 nm.

RNA isolation and real time RT-PCR

The total RNA was extracted from the cells using TRIzol re-

agent (Invitrogen, USA), following the manufacturer's instructions. The real-time quantification of RNA targets was then performed in the Rotor-Gene 2000 real-time thermal cycling system (Corbett Research, Australia) using a QuantiTect SYBR Green RT-PCR kit (QIAGEN, USA). The reaction mixture (20 μ l) contained 200 ng of the total RNA, 0.5 μ M of each primer, the appropriate amounts of enzymes and fluorescent dyes, as recommended by the supplier. The Rotor-Gene 2000 cyclor was programmed as follows: 30 min at 50°C for reverse transcription; 15 min at 95°C for DNA polymerase activation; 15 s at 95°C for denaturing; and 45 cycles of 15 s at 94°C, 30 s at 55°C and 30 s at 72°C. Data collection was carried out during the extension step (30 s at 72°C). The PCR reaction was followed by melting curve analysis to verify the specificity and identity of the RT-PCR products, which can distinguish the specific PCR products from the non-specific PCR product resulting from primer-dimer formation. The primers used were 5'-CCA ACT TCC TGT GCT CCG TG-3' (sense), 5'-TCT TGC CTC GTC CGC TCC-3' (antisense) for Runx2, 5'-ACC AGG TCC AGG CAA CAC-3' (sense), 5'-GGG CAG TCG CAG GTA GAA-3' (antisense) for OSX, and 5'-CAG GAG GGC AAT AAG GTA GT-3' (sense) and 5'-GAG GAC AGG GAG GAT CAA G-3' (antisense) for OCN. The temperature of the PCR products was increased from 65 to 99°C at a rate of 1°C/5 s, and the resulting data was analyzed using the software provided by the manufacturer.

siRNA transfection

Cells were transfected for 24 h with either a Stealth small interference RNA (siRNA) specific to β -catenin (5'-CCC UCA GAU GGU GUC UGC CAU UGU A-3', 200 pmol/l; Invitrogen, USA) or a non-related control siRNA targeting the green fluorescent protein (GFP) (5'-CCA CTA CCT GAG CAC CCA GTT-3'), using LipofectAMINE 2000 according to the manufacturer's instructions before being subjected to simvastatin treatment.

Western blot analysis

Protein extract samples (20 μ g) were separated by 8-10% SDS-PAGE and blotted onto polyvinylidene difluoride (PVDF) membranes. The blots were washed with TBST [10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.05% Tween-20], blocked with 5% skim milk for one hour, and incubated with the appropriate primary antibody at the dilutions recommended by the supplier. The membrane was then washed, and the primary antibodies were detected with goat anti-rabbit IgG or goat anti-mouse IgG conjugated with horseradish peroxidase. The blots were developed with enhanced chemiluminescence (ECL) (Santa Cruz Biotechnology, USA) and exposed to X-ray film (Eastman-Kodak, USA).

Immunofluorescence staining

The cells were fixed and treated with mouse anti-collagen type I or osteopontin antibody (1:100, Santa Cruz Biotechnology, USA) for 1 h at room temperature. Subsequently, the fluorescein isothiocyanate-conjugated (FITC-conjugated) anti-mouse IgG (1:100) was treated for 1 h at room temperature. Fluorescence images were obtained using a fluorescence microscope (fluoview 300, Olympus).

Statistical analysis

All data are expressed as mean $\pm$ standard deviation (S.D.). One-way ANOVA was used for multiple comparisons (Duncan's multiple range test), using SPSS software ver. 10.0. A *P* values < 0.05 were considered significant.

Fig. 1. Effect of simvastatin on osteogenic differentiation of ESCs. (A) Morphology of the cells under a light microscope. Undifferentiated ES cells (a), suspended EBs (b), EBs incubated in osteogenic medium for 1 day (c), and EBs cultured in osteogenic medium with simvastatin for 7 days (d) (magnification 20 $\times$). Cells were incubated in osteogenic medium with simvastatin (1, 10, 100, 200 nM) for 4, 7, and 14 days each, then (B) ALP activity or (C) Alizarin red staining was assessed as described in "Materials and Methods". Each microscopic image shown is a representative of five separate experiments. The size bars on panel A represent 50 μm . (D) ARS quantification was assessed on days 7 and 14 as described in "Materials and Methods". The values reported are the mean $\pm$ S.D. of five independent experiments. * $P < 0.05$ or # $P < 0.001$ vs. control value.

RESULTS

Stimulatory effects of simvastatin on osteogenic differentiation

To investigate the osteogenic effect of simvastatin on ESCs, simvastatin was added in the osteogenic medium then ESCs were differentiated toward osteoblastogenic lineage up to day 7. Figure 1A shows the stage of osteogenic differentiation from undifferentiated ES-D3 cells (a). The 5 day-old EBs were spherical and structurally intact (b). The EBs were sprouted in osteogenic medium after one day of plating (c) and osteogenic differentiation was continued in the presence of simvastatin (d).

We next examined the effects of simvastatin on ALP activity and mineralization of the cultures, which are markers of osteogenic differentiation. As shown in Fig. 1B, ALP activity was measured at days 4 and 7 following osteogenic induction. The

alteration of ALP activity was not observed in cells with simvastatin compared to the control group at day 4. However, significant increase of ALP activity was established in a dose-dependent manner at day 7. Cultures achieved at day 14, a late stage of osteogenic differentiation, presented positive Alizarin red staining, of which the simvastatin had increased calcium nodule formation and matrix mineralization (Fig. 1C). The quantification of mineralization at days 7 and 14 confirmed that the addition of simvastatin in osteogenic medium increased mineralization of ESCs (Fig. 1D).

Effects of simvastatin on osteogenic associated gene expression

To further support the osteogenic effect of simvastatin, we determined Runx2, OSX, and OCN mRNA expression, which are known as osteogenic target genes, using real time RT-PCR.

Fig. 2. Effects of different concentrations of simvastatin on the mRNA expression of Runx2, OSX, and OCN. The mRNA levels of (A) Runx2, (B) OSX, and (C) OCN were analyzed using the real time RT-PCR technique after a 4 day-osteogenic induction. The values reported are the mean $\pm$ S.D. of three independent experiments. * $P < 0.05$ vs. control value.

Fig. 3. Effect of simvastatin on OCN, OPN, and collagen type I protein levels. (A) Cells were treated with simvastatin (1, 10, 100, 200 nM) for 7 days and then the protein levels of OCN, OPN, and collagen type I were determined by Western blot analysis using total protein lysates. (B) The panels (bars) denote the mean $\pm$ S.D. of three experiments for each condition determined from densitometry relative to β -actin. * $P < 0.05$ vs. control value.

Although there was no dose-dependent increase of each the osteogenic gene, the cultures treated with simvastatin demonstrated higher gene expression compared to the control cultures (Fig. 2). Particularly, a maximal increase in each mRNA level was observed with a stimulation of 100 nM simvastatin and a slightly decreased level was observed with a 200 nM treatment.

Effects of simvastatin on osteocalcin, osteopontin, and collagen type I protein levels

We also analyzed the simvastatin effects on the osteogenic differentiation of ESCs by following the protein level data of osteogenic markers, OCN, OPN, and Coll I at a 7 day osteogenic induction. Western blot analysis showed that the level of each protein was dose-dependently increased in cells incu-

Fig. 4. Immunofluorescence staining with antibodies of OPN and collagen type I. Cells were incubated in the presence of 100 or 200 nM simvastatin for 7 days then (A) OPN and (B) collagen type I were detected by the immunostaining method. The nuclei were stained with DAPI as shown in blue. A representative result from the four independent experiments is shown.

bated with simvastatin at a range from 1 to 100 nM and slightly decreased in the cultures of 200 nM simvastatin (Fig. 3). Moreover, immunofluorescence staining with OPN and Coll I confirmed that the treatment of simvastatin enhances the differentiation of ESCs into osteogenic lineage (Fig. 4).

Involvement of canonical Wnt/ β -catenin signaling in simvastatin-stimulated osteogenic differentiation

In order to confirm whether canonical Wnt/ β -catenin signaling is a stimulating regulator for the osteogenesis of ESCs, cells were incubated with DKK-1, which binds to LRP5/6 co-receptors and inhibits canonical Wnt/ β -catenin signaling, before treatment with 100 nM of simvastatin. Pretreatment of cells with DKK-1 decreased the simvastatin-stimulated ALP activity (Fig. 5A) and the mRNA levels of osteogenic transcription factors to the control levels (Fig. 5B). Moreover, knockdown of β -catenin by its

specific siRNA transfection shows the reduced protein levels of β -catenin, where control siRNA transfection did not affect the β -catenin protein levels (Fig. 6A). Subsequently, the blockage of β -catenin expression by siRNA transfection decreased the simvastatin-induced increases in OCN, OPN, and Coll I protein levels (Fig. 6B).

DISCUSSION

This study presents experimental evidence demonstrating that simvastatin is an efficient candidate to enhance the differentiation of ESCs into osteogenic cell lineage. A beneficial application of ESCs for bone regeneration is that ESCs have the potential to supply unlimitedly the differentiated osteoblast and osteoprogenitor cells for transplantation. However, technically it is significantly more difficult to direct ESCs into the osteogenic

Fig. 5. Inhibitory effect of DKK-1 on simvastatin-induced osteogenic differentiation of ESCs. Cells were pretreated with 0.5 µg/ml DKK-1 1 h before the addition of simvastatin (100 nM) and (A) processed for the analysis of ALP activity after 7 days of incubation. (B) mRNA levels of Runx2, OSX, and OCN were also measured by real time RT-PCR. The results shown are the mean $\pm$ S.D. from five separate experiments. $^{\#}P < 0.001$ vs. the untreated control values, $^{\#}P < 0.05$ and $^{\#\#}P < 0.001$ vs. simvastatin treatment alone.

Fig. 6. Effect of knockdown of β -catenin on simvastatin-stimulated osteogenesis of ESCs. Cells were transfected with either β -catenin- or GFP-specific siRNA using LipofectAMINE 2000 and after 24 h of transfection, and the cells were then treated with simvastatin (100 nM). (A) The protein levels of β -catenin according to the siRNA transfection were determined by Western blotting after 2 days of transfection. (B) The pro-

tein levels of OCN, OPN, and collagen type I were also detected after 7 days of incubation. A representative result from the four independent experiments is shown.

lineage compared with tissue-derived progenitor cells. Osteogenic differentiation was mostly stimulated by culturing the cells with osteoinductive factors such as dexamethasone, ascorbic acid, β -glycerophosphate, and BMP-2 (Bielby et al., 2004; Canalis et al., 2003; Chaudhry et al., 2004). Recently, it has been shown that ESCs can differentiate into osteogenic cells under selective culture conditions (Chaudhry et al., 2004; zur Nieden

et al., 2005). On the other hand, BMP stimulated the differentiation of ESCs into other cell lineages rather than osteogenic cells, unlike mesenchymal stem cells (Chadwick et al., 2003; Xu et al., 2002). Thus, in order to satisfy a cell therapy approach for bone regeneration, cell culture conditions need to be improved to promise appropriate and consistent differentiation of ESCs into osteoprogenitor cells with mass production.

The present study employed the supplement of simvastatin, a potent stimulator of osteoblast differentiation and bone formation (Mundy et al., 1999; Ruiz-Gaspa et al., 2007), to intensify the differentiation of ESCs into osteogenic lineage. The current studies demonstrated the pharmacological doses of simvastatin for bone formation in alternative cell culture conditions (Baek et al., 2005; Maeda et al., 2001; Ruiz-Gaspa et al., 2007). In the present study, the applied doses of simvastatin in the ESCs culture ranged from 1 to 200 nM. The osteogenic effect peaked at 100 nM simvastatin and slightly decreased at 200 nM treatment. Corresponding to previous reports, we found that relatively low doses of simvastatin markedly enhanced the osteogenic differentiation of ESCs. Although the ideal culture condition to enhance the osteogenic differentiation of ESCs *in vitro* should be chemically defined and serum-free, previous protocols for osteogenic differentiation of ESCs used serum in an *in vitro* culture environment because the absence of serum could damage the survival of differentiated cells (Buttery et al., 2001; Pagkalos et al., 2010; Sottile et al., 2003; zur Nieden et al., 2005). Unlike previous studies, the present study used osteogenic medium containing a low concentration (5%) of serum. At a much lower serum concentration, however, the cells became apoptotic (data not shown). This culture condition would at least reduce any undefined growth factors or differentiation-promoting factors and be useful for achieving controlled differentiation of ESCs into the osteogenic lineage *in vitro*.

In the present study, the ALP activity was significantly promoted in ESCs exposed to simvastatin. Moreover, the microscopical analysis and spectrophotometrical quantification revealed that ESCs cultured in the presence of simvastatin show a higher extent of matrix mineralization than control groups. Previous studies have shown that simvastatin has a bone-forming potential proved by enhancing ALP activity and matrix mineralization, as well as increasing the expression of BMP-2, osteocalcin, and collagen type I in various cell types (Chen et al., 2010; Mundy et al., 1999; Ruiz-Gaspa et al., 2007; Sakoda et al., 2006). The present study demonstrated that the expression of osteogenic genes including Runx2, OSX, and OCN was increased in the simvastatin-treated ESCs. The molecular mechanism of Runx2 has been demonstrated to directly stimulate the transcription of osteoblast-related genes (Harada et al., 1999; Kern et al., 2001). In accordance with the Runx2 up-regulation, the protein levels of OCN, OPN, and collagen type I were increased following the supplement of simvastatin in a culture medium. Although the precise mechanism of simvastatin has not been elucidated in ESCs, these results suggest that the modulating effect of simvastatin on osteogenic differentiation of ESCs may be mediated by at least the up-regulation of Runx2 expression.

The canonical Wnt/ β -catenin signaling is induced by extracellular Wnt proteins but also occurs in response to other chemical and mechanical stimuli (Grebénová et al., 2003; Guo et al., 2011; Heo and Lee, 2011). The present study also provides evidence that simvastatin induces the canonical Wnt signaling in processing the osteogenesis of ESCs. Although it is well known that Wnt signaling is considered as a key regulator of bone biology (Guo et al., 2011; Rawadi et al., 2003), the relationship between simvastatin and Wnt/ β -catenin activation has not been broadly identified. One previous study demonstrated that Wnt signaling-related genes were significantly increased by simvastatin treatment, which involves the osteogenic differentiation of BMSCs (Zhang et al., 2009). In another study concept, simvastatin recovers Wnt/ β -catenin signaling from the negative effect of high glucose, where it blocks mesangial cell apoptosis (Lin et al., 2008). Thus, the previous and present results sug-

gest that simvastatin can trigger the Wnt/ β -catenin signaling cascade in modulating various cell functions including promoting osteogenic differentiation.

In conclusion, the present study demonstrated that simvastatin stimulates the osteogenic differentiation of ESCs and canonical Wnt signaling contributes to this processing. Although the stimulatory effects of statins on bone turnover and bone formation remain controversial, the employment of simvastatin in ESCs culture conditions can be considered to achieve the efficient protocol for directing the osteogenic differentiation of ESCs *in vitro*. Moreover, the potential use of simvastatin can be extensively applied in the bone regeneration field. Finally, we suggest that the present study can be a guideline for the development of such well-defined ESCs culture methods and be useful in an *in vitro* model for studying osteogenesis and bone regeneration.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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