Calcyclin, a Ca^{2+}ion Binding Protein, Plays a Role for Anabolic Effects of Simvastatin on Bone

Thesis by

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Calcyclin, a Ca^{2+}ion Binding Protein, Plays a Role for Anabolic Effects of Simvastatin on Bone

Directed by Professor Sung-Kil Lim

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This certifies that the Master's Thesis of Ranjoo Hwang is approved.

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ABSTRACT

Calcyclin, a Ca^{2+}ion Binding Protein, Plays a Role for Anabolic Effects of Simvastatin on Bone

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(Directed by Professor Sung-Kil Lim)

Simvastatin is a pro-drug of a potent 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor and inhibits cholesterol synthesis in humans and animals. In the previous studies, in vitro treatment of pharmacological dose of simvastatin stimulated bone

formation. To identify the mediators of the anabolic effects of simvastatin on osteoblasts, we tried to identify and characterize simvastatin-induced proteins by using proteomic analysis. Simvastatin stimulated the proliferation of primary cultured osteoblast cells significantly even at the lowest concentration(10^{-9} M) after 6 hour exposure, and the expression of osteoblast differentiation markers such as alkaline phosphatase (ALP), type I collagen and osteocalcin in the presence of simvastatin was remarkable in dose-dependant manner. Calcyclin was up-regulated more than 10 times, and annexin I, III, vimentin and tropomyosin, were also up or down regulated by simvastatin significantly. Up-regulated calcyclin mRNA by simvastatin was validated by reverse transcription in mouse calvarial cells. In confocal microscope analysis, GFP-cacy fusion protein was visualized in cytoplasm of MC3T3-E1 cells transfected with GFP-calcyclin cDNA containing plasmid and quickly shifted to the nucleus 20 min after simvastatin treatment. The rate of ALP mRNA expression and proliferation were significantly increased without exposure to simvastatin in MC3T3-E1 cells overexpressing calcyclin cDNA. In conclusion, simvastatin stimulates the

proliferation and early differentiation of osteoblast. Calcyclin is one of the candidate proteins playing a role in osteoblastogenesis in response to simvastatin, although the precise functions of calcyclin in osteoblast remain unknown.

Key words: simvastatin, anabolic effect, osteoblast differentiation markers, 2DE, calcyclin

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Ⅰ. Introduction

Osteoporosis is a skeletal disorder characterized by compromised bone strength predisposing to an increased fracture[1,2]. The mainstay of therapy for osteoporosis in these days are still anti-resorptive agents[3].

Anti-resorptive agents stabilize the bone remodeling by reducing the number and/or the activity of osteoclasts and thereby reducing the prevalence of fracture without increments of true bone mass[4]. In contrast, anabolic agents such as parathyroid hormone, statin and fluoride directly stimulate bone formation and increase bone mass[5,6]. Recently, FDA approved PTH(1-34) as a therapeutic agent for severe osteoporosis. However, there are several unanswered questions regarding cortical porosity and tumorigenesis induced by PTH(1-34). Certainly, development of new anabolic agents is necessary.

Statin is a pro-drug of a potent 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor and, thus, inhibits the conversion of HMG-CoA to mevalonic acid, needed for hepatic cholesterol biosynthesis [6, 7]. Mevalonic acid is a precursor not only of cholesterol but also of proteins such as geranylgeranyl pyrophosphate which is important in the control of osteoclast-mediate bone resorption [8,9]. Many previous studies have indicated that some statins, lipophilic statins in particular, have a potent stimulatory effect on bone formation by inducing the expression of BMP-2[6,7,10]. Therefore statins are expected to be a future anabolic agent for treatment of

osteoporosis[11]. However, the detailed mechanism by statins promote bone formation has yet to be elucidated. Little is known about the intracellular statin-induced protein profiles as well as mediator proteins specific for the anabolic function of statins so far.

Calcyclin (S100A6), a small acidic protein weighs about 10 kDa, is a member of the S100 calcium-binding protein family [12]. These family members share a common S100 calcium-binding motif and are involved in several regulations of protein phosphorylation, some enzyme activity, the dynamics of cytoskeletal components, transcription factors, Ca^{2+} homeostasis, and cell proliferation and differentiation [13, 14]. An interesting feature of the S100 proteins is that they are expressed by epithelial cells and fibroblast in a cell specific way[15]. Calcyclin is also expressed by osteoblast and up-regulated markedly during osteoblast differentiation, however, its roles in bone physiology are unknown [16].

2-D PAGE analysis has been the technique of choice for analyzing the protein composition of a given cell type and for monitoring change in gene activity through the quantitative and qualitative analysis of the thousands of proteins that organize various cellular events [17, 18].

The introduction of silver staining has greatly enhanced the sensitivity of 2D-PAGE analysis, allows detection of protein to the nanogram range [19]. Proteomic analysis is a useful technique to compare changes in protein expression, modification and degradation between treated and untreated samples [20].

In the present study, we tried to reassess whether simvastatin regulates the proliferation and differentiation of osteoblast delivered from primary cultured calvarial cells. We also explored new protein profiles by proteomic techniques in response to simvastatin. Here, we found that calcyclin, calcium-binding protein, is one of the simvastatin-induced proteins up-regulated more than 10 times and over expression of calcyclin induced proliferation and differentiation of osteoblast. Calcyclin might be one of mediator molecules inducing osteogenic function of simvastatin.

Ⅱ. Materials and Methods

1. Cell cultures

Mouse calvariae were dissected aseptically from postnatal 1-day ICR mouse. Frontal and parietal bones were cleaned of loose soft connective tissue and submerged in alpha modification of Eagle's medium (α-MEM) (Invitrogen Corporation, New York, USA). Calvariae were digested at 37°C for 10 minutes with shaking of an enzymatic solution containing 0.1% collagenase (Invitrogen Corporation, New York, USA) and 0.05% trypsin containing 0.53 mM EDTA (Invitrogen Corporation, New York, USA) in α-MEM. This procedure was repeated to yield a total of five digests. The cells were collected by centrifugation at 1200 rpm for 5 minutes. The cells were then resuspended in α-MEM containing 10% FBS and antibiotics (penicillin, 100 unit/ml and streptomycin, 100 mg/ml, all from Invitrogen Co.). Cells were grown to 70-80% confluence over the next 3-4 days at 37°C in 5% CO_2 with humidification.

MC3T3-cells were grown in α-MEM with 10% fetal bovine serum. After cells reached confluence, they were cultured in differentiation

medium (α-MEM containing 50μg/ml of phosphate ester of ascorbic acid (Sigma, St Louis, MO, USA) 10 mM β-glycerophosphate (Sigma, St Louis, MO, USA) and 10^{-8} M Dexamethasone (Sigma, St Louis, MO, USA) for 5 days and subjected to transfection

2. Cell proliferation assay

Primary-cultured calvarial cells were cultured in 96-well plate and stimulated only for 6 hours with 10^{-7} M simvastatin in serum free α-MEM. For long-term effect of simvastatin, cells were cultured for 8 days being treated with simvastatin every other day for 6 hours intermittently. Cell proliferation assay was performed using Cell Proliferation Reagent WST-1 (Roche, Mannheim, Germany) under manufacturer's instruction.

3. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the cultured cells with a commercial RNeasy kit (Quiagen, Maryland, USA) following the manufacturer's instructions. The purity and amount of isolated RNA were assessed by spectrophotometric measurement at 260 and 280 nm.

Total RNA (5μg) was reverse transcribed to cDNA at 42°C for 50 minutes in a volume of 20 μl containing the following reagents: 0.5 mM dNTP mixture; 10 mM dithiothreitol (DTT); 0.5 mg Oligo(dT), buffer (250 mM Tris, 375 mM KCl and 15 mM $MgCl_2$, pH 8.3) and 5 U of AMV (RNase H-free reverse transcriptase) (all from Promega, WI, USA). Then, the reaction is terminated at 70°C for 15 minutes. Aliquots of the cDNA were diluted from 1 : 1 to 1 : 100. RT-PCR analysis were done essentially as described with the following conditions: 94°C, 5 minutes; 94°C, 45 seconds; 60°C, 45 seconds ; 72°C, 35 seconds (repeated for 30 cycles); and 72°C, 15 minutes. The following oligonucleotides were used for RT-PCR amplification: calcyclin (sequences of EcoRI restriction enzyme site are under-lined), forward 5' GAGAATTCCAGTGATCAGTCATGG 3'; reverse 5' CAGAATTCAACGGTCCCATTTTAT 3', Alkaline phosphatase (ALP), forward 5' GGGACTGGTACTCGGATAACG 3'; reverse 5' CTGATATGCGATGTCCTTGCA 3', Osteocalcin, forward 5' CGGCCCTGAGTCTGACAAA 3'; reverse 5' GCCGGAGTCTGTT CCTCCTT 3', Type I Collagen, forward 5' GAGGCATAAAGGGTCA TCGTGG 3'; reverse 5' CATTAGGCGCAGGAAGGTCAGC 3', β-

actin, forward 5' TTCAACACCCCAGCCATGT 3'; reverse 5' TGTGGTACGACCAGAGGCATAC 3'.

4. pcDNA-*cacy* and pEGFP-*cacy* construct

Calcyclin full cDNA amplified by RT-PCR described above was digested with EcoRI restriction enzyme and purified by agarose gel electrophoresis. The resulting restriction fragments containing entire calcyclin coding region were ligated into the vector pcDNA 3.0 and pEGFP-N1.

5. Transient transfection

MC3T3-E1 cells were maintained in α-MEM medium supplemented with 10% FBS, 100 mg/ml streptomycin sulfate, 100 units/ml penicillin G and 250 mg/ml amphotericin B.

MC3T3-E1 cells were transfected with pcDNA-*cacy* and pEGFP-*cacy* construct using LipofectAMINE reagent (Invitrogen Co. New York, USA). Briefly, MC3T3-E1 cells are 60 ~ 70% confluence were washed once with α-MEM (serum-free, Invitrogen Co.) and a mixture of 1μg plasmid in a total volume of 1 ml per well in 6-well plate. Transfection

was allowed to proceed for 6 hours and removal of the transfection medium and addition of 2 ml α-MEM containing 10% FBS. Assays were performed the day after transfection.

6. Two-dimensional gel electrophoresis and gel staining

Protein samples of mouse calvarial cells used for 2-DE analysis were obtained 6 hours after simvastatin stimulation and non-stimulated samples were used as a control. The samples were homogenized in double the volume of lysis buffer (8 M Urea, 4% CHAPS, 40 mM Tris and 20 mM DTT). Then cell lysates were centrifuged at 12,000 rpm for 15 minutes at 4°C. The protein concentration was determined by the Bradford protein assay and 100 μg proteins were used for each. Commercial strips with a non-linear immobilized pH4-7 gradient (Amersham Pharmacia Biotech, Uppsala, Sweden) were used for isolelectric focusing. These strips were wetted by rehydration buffer containing 8 M Urea, 2% CHAPS, 10 mM DTT and IPG buffer pH4-7(Amersham Pharmacia Biotech, Uppsala, Sweden) for 12h at 50 voltage. Isoelectric focusing was performed in Multiphore II apparatus (Amersham Pharmacia Biotech, Uppsala,

Sweden) for total 40,000 vhrs. In the second dimension, proteins were separated by size in 7.5 - 17.5% T polyacrylamide gradient gel. Two slab gels were run using Protean II xi Cell (Biorad, Richmond, CA, USA) at a constant current of 20 mA for 14 hours. After electrophoresis, the separated proteins were either silver-stained or Coomassie brilliant blue stained. For silver staining, gels were first fixed for 1h in 40% ethanol/ 5% acetic acid, then washed with 50 ml of double-distilled water followed by a second fixing in 5% ethanol/5% acetic acid. Gels were washed with 50 ml of double-distilled water and incubate with a 0.02% sodium thiosulfate (Sigma, St Louis, MO, USA) for 30 minutes at room temperature. Gels were washed with 2 x 10 ml of double-distilled water and incubated for 90 minutes at 4ºC with 0.1% silver nitrate solution that was pre-cooled to 4ºC. The gels were rinsed with 50 ml of double-distilled water and proteins were visualized using a developing solution containing 0.5% formaldehyde v/v 2% KCO_4 w/v until proteins were visible. Acetic acid solution (1%) was used to stop the reaction. For MALDI-TOF-MS, gels were stained with Coomassie blue G-250 (0.25%) in 50% methanol/12% acetic acid overnight at room temperature. Coomassie blue stained

gels were destained with 40% methanol/12% acetic acid until the background was clear.

7. Image analysis using PDQuest software and MALDI-TOF-mass spectrometry

Qualitative analysis of digitized images was carried out using PDQuest 2D analysis software (Biorad, Richmond, CA, USA).

Samples were loaded onto the target plate using the three layers method [19]: 0.6 µl of matrix solution (10 mg/ml sinapinic acid in 60% acetonitrile, 40% water, 0.1% TFA, prepared fresh every day) were loaded onto the sample plated and left to dry at room temperature. One µl of sample solution was deposited on the matrix layer and left to dry. The spot was then covered with 0.6 µl of the matrix solution. Mass spectra were recorded using a Tof-Spec 2DE MALDI-TOF instrument (Micromass, Manchester, UK), equipped with a pulsed nitrogen laser (337 nm, pulse width 4ns) and operated in delayed extraction linear mode, with an acceleration voltage of 22.5 kV.

8. Confocal microscopic analysis

For calcyclin translocation assay, MC3T3-E1 cells were transfected with GFP-*cacy* plasmid. After various treatments, 10^{-7} M simvastatin, 10^{-7} M PTH(1-34) and co-incubation for 20 minutes. Confocal microscopic experiment was performed on a Zeiss LSM-510 laser scanning microscopy by using a Zeiss 100 x oil-immersion lens. Fluorescent signals were collected by using a Zeiss LSM software in the line switching mode with dual excitation (488, 568 nm) and emission (515-540 nm, 590-610 nm) filter sets.

9. Statistical analysis

Results are expressed as mean value±SE. Statistical analysis was performed by student's t-test. Relationships were considered statistically significant when p value was less than 0.05.

Results

1. The effect of simvastatin on osteoblast proliferation

Proliferation of mouse calvarial cells was increased after 6 hours of simvastatin incubation. Cells were sensitive to the lower concentration of simvastatin and largely increased in the lowest concentration, $10^{-9.}$ M (Fig. 1-A). For the study of long-term effect of simvastatin, Mouse calvarial cells were cultured for 8 days with intermittent simvastatin treatment (6 hours/ 2days) in various concentrations (10^{-9} ~ 10^{-6} M). We treated simvastatin intermittently because it has been reported that statins exert a number of effects such as stabilizing cells and inhibition of cell proliferation. The proliferation was increased during simvastatin exposure periods dose-dependently and, reached a maximum at 10^{-7} M (Fig. 1-B).

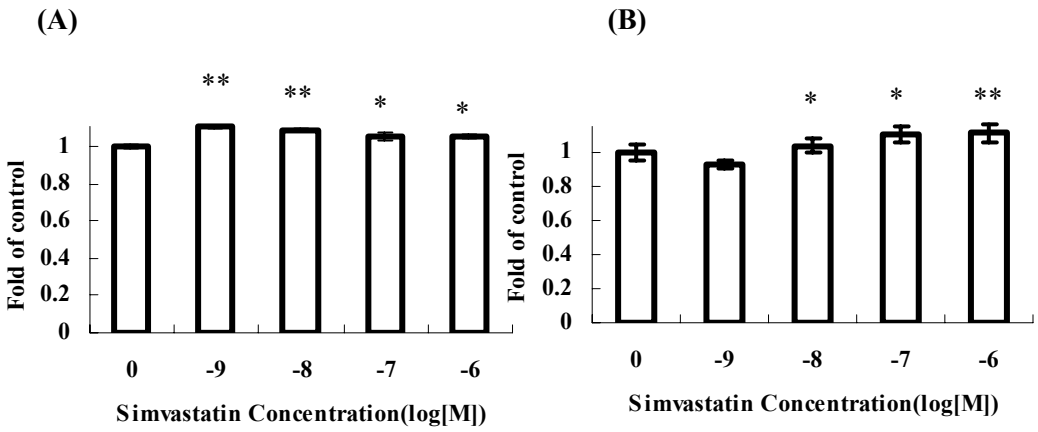

Fig 1. The effect of short- and long-term exposure of simvastatin on proliferation of mouse calvarial cells. The short-term effect of simvastatin on mouse calvarial cells was determined after 6 hours of treatment (A). For the study of long-term effect of simvastatin, Mouse calvarial cells were cultured for 8 days with intermittent simvastatin treatment (6 hours/ 2days) in various concentrations (10^{-9} ~ 10^{-6} M) (B). After incubation periods, the absorbance was determined by an ELISA reader. *: P<0.05, **: P<0.01 simvastatin vs. control.

2. The effects of simvastatin on mRNA expression of the osteoblast differentiation makers: ALP, Type I collagen and osteocalcin

We examined the effect of simvastatin on the mRNA expression of osteoblast differentiation markers in primary cultured mouse osteoblasts. We determined the mRNA level by quantitative RT-PCR. As shown in Fig. 2, the incubation with 10^{-7} M simvastatin for 6 hours

enhanced the expression of ALP, type I collagen and osteocalcin with statistical significance compared to that in un-stimulated condition in cultured mouse calvarial cells. As this increase was mostly dose-dependent (10^{-9} – 10^{-6} M), the following experiments were performed with the stimulation by final 10^{-7} M simvastatin for 6 hours, otherwise indicated. Quiescent mouse osteoblast expressed a low level of ALP mRNA, and 10^{-7} M simvastatin significantly enhanced the expression of ALP mRNA (more than 100-fold) (Fig. 2-B). Similar to the case of ALP, the increase in type I collagen expression was significant at a concentration of 10^{-7} M and the expression was down to the basal level at the highest concentration of final 10^{-6} M (about 10-fold, Fig. 2-C). In case of osteocalcin, the mRNA expression increased dose dependently and reached a maximum level at a concentration of 10^{-6} M (about 30-fold, Fig. 2-D).

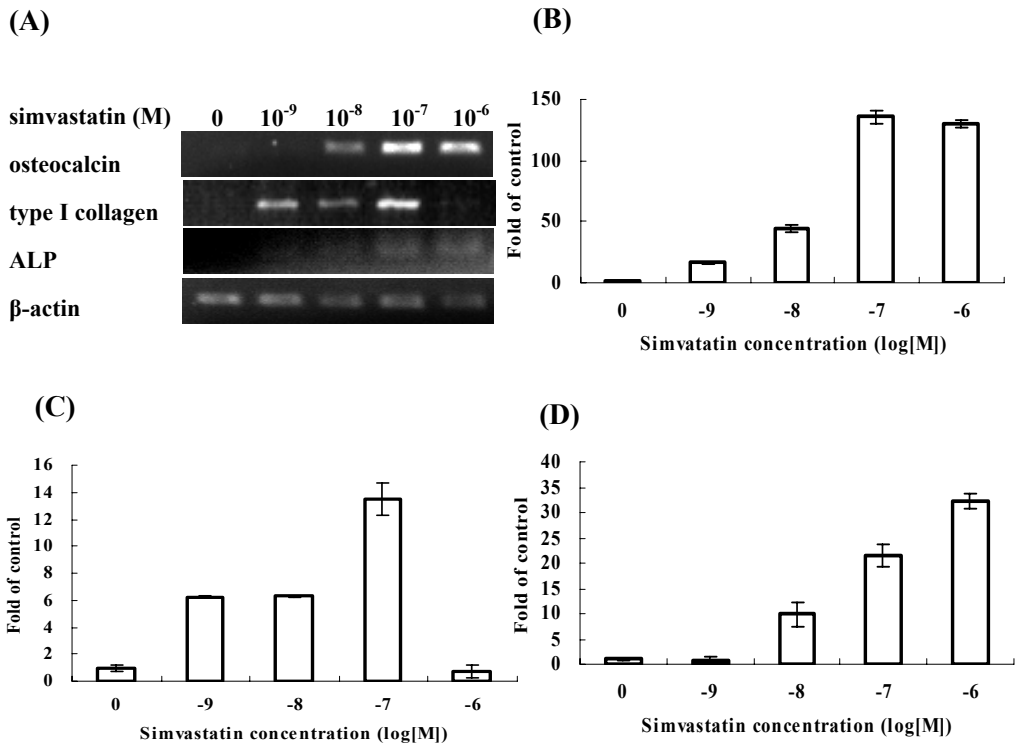

Fig. 2. The effect of simvastatin treatment on mRNA expression of osteoblast differentiation markers. ALP, type I collagen and osteocalcin mRNA expressions were increased progressively with the simvastatin exposure for 6 hours dose-dependently, whereas β-actin mRNA expression was not modified. The expression of each differentiation markers was investigated by gel electrophoresis and visualized by EtBr staining (Fig.2.A). (B) ALP, (C) type I collagen and (D) osteocalcin illustrate the mean values of 3 different experiments.

3. Induction of calcyclin protein after simvastatin treatment in mouse calvarial cells

Figure 3-A show an example of a silver-stained 2DE images aimed at determining the any change after 6 hour treatment with simvastatin and these images were analyzed by PDQuest software (described above). 6 spots that showed changes in expression were abtained selectively from this analysis and characterized by MALDI-TOF (Fig. 3-B and Table 1). Calcyclin protein was largely increased by 6 hours of simvastatin treatment about 10-fold. Some of S100 protein family inhibit protein phosphorylation and in this case, annexin I, II and vimentin are some of revealed protein targets so far. Furthermore, S100 proteins regulate all three major constituents of cytoplasmic cytoskeleton, i.e. MTs, Ifs, and microfilaments, and tropomyosin and myosin. S100A6, specially interact with tropomyosin, however suggested functions are unknown. Thus, we did focused experiment on calcyclin although expression levels of other proteins we characterized was increased or decreased about 4-fold.

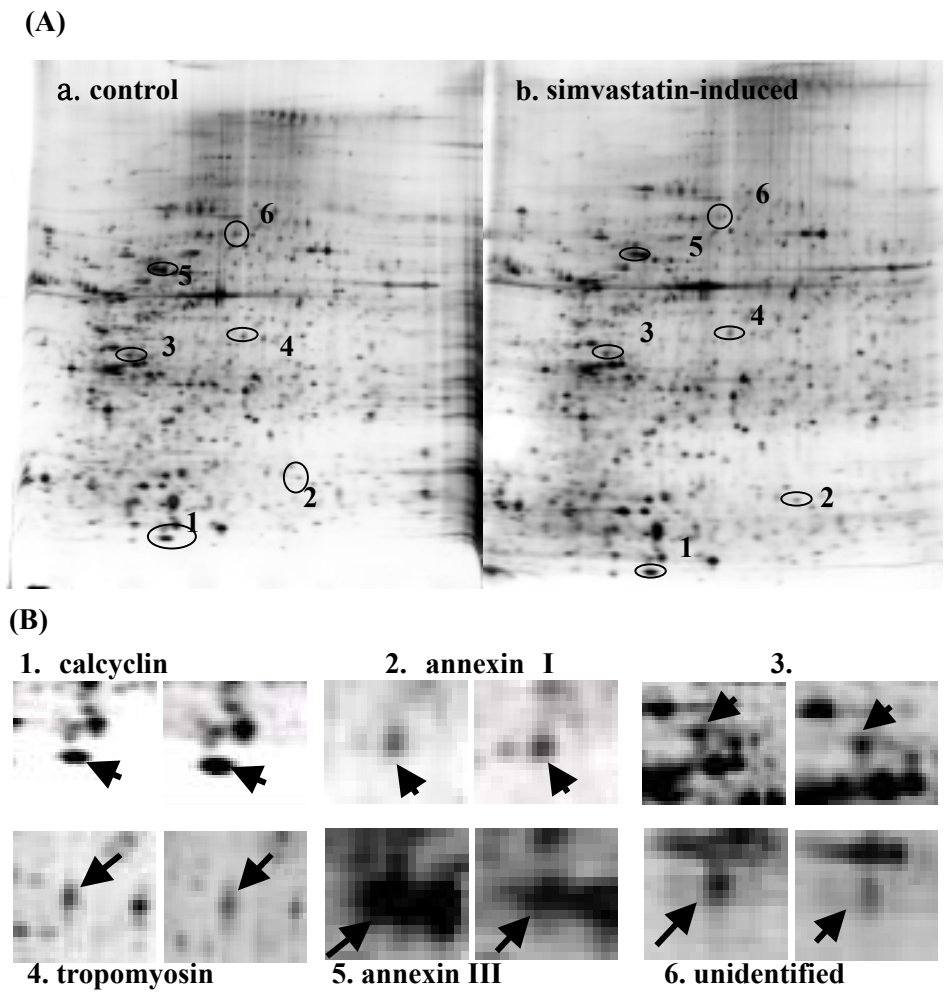

Fig. 3. 2D gel-protein profiles 6 hours after simvastatin treatment in mouse calvarial cells. (A) Black circles indicate proteins whose expression was found to be lower or higher in simvastatin-induced culture relative to control. Numbers refer to protein identities in Table 1. The horizontal axis of the gels is the isoelectric focusing dimension, which stretches from pH 4 (left) and to pH 7 (right). The vertical axis is the polyacrylamide gel dimension, which stretches from about 8 kDa (bottom) to about 80 kDa (top). (B) Insets from Fig.3-A showing altered expression upon stimulating with simvastatin for 6 hours.

Table 1. Identities of the proteins characterized by MALDI-TOF-MS

	protein I.D.	PI/M.W. (kD)	increase/decrease
1.	calcyclin	5.4/10.5	increase
2.	annexin I	6.97/39	increase
3.	vimentin	5.06/53.7	decrease
4.	tropomyosin alpha chain	4.71/32.7	decrease
5.	annexin III	5.96/36.5	decrease
6.	unidentified	-	decrease

4. The effects of simvastatin on mRNA expression of calcyclin in mouse calvarial cells

Figure 4 illustrating the mean values of 3 different experiments shows calcyclin mRNA increased progressively with the time of simvastatin exposure. Calcyclin mRNA induction was significantly increased as soon as 30 minutes after simvastatin addition with a maximal effect after 1 hours (about 60% increase) thereafter, calcyclin mRNA expression returned to the control level at 24 hours. Figure 5 shows an example of RT-PCR aimed at determining the effective simvastatin concentration of calcyclin mRNA induction in

mouse calvarial cells. The expression was increased dose-dependently and reached a maximum level at a concentration of 10^{-6} M.

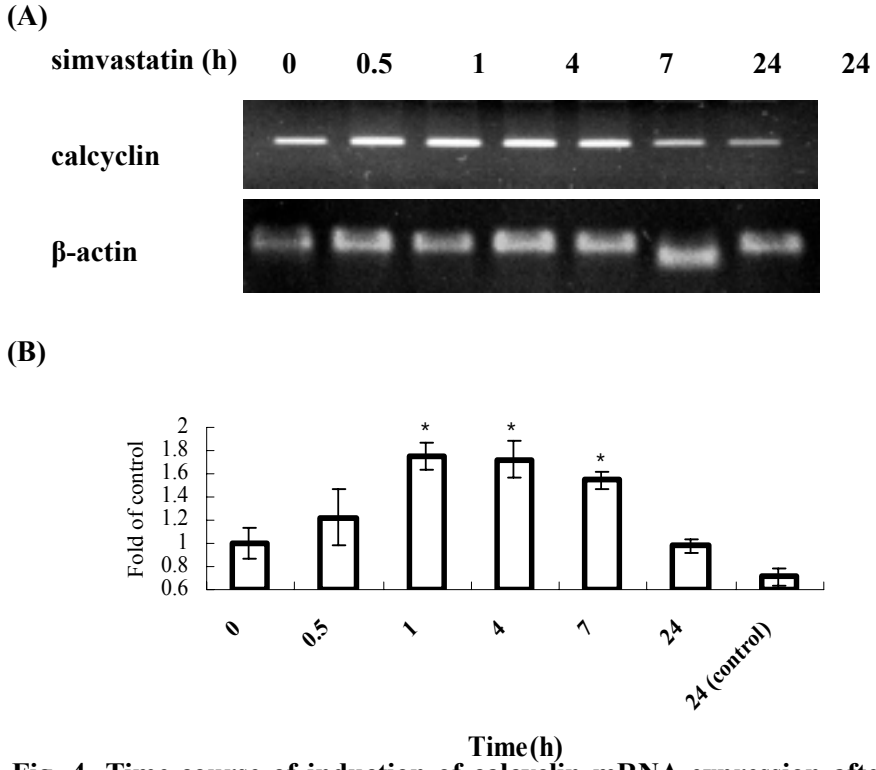

Fig. 4. Time-course of induction of calcyclin mRNA expression after simvastatin treatment in mouse calvaria cells. The effect of treatment of mouse calvaria cells with simvastatin 10^{-7} M for different elapses of time was studies by RT-PCR. Calcyclin appears as a single band at about 310 bp and β-actin was used as an internal standard. Calcyclin mRNA expression is significantly increased by simvastatin after 1 hours and then increases up to 7 hours. Thereafter, it returns to control value at 24 hours. * : $P<0.05$, simvastatin vs. control.

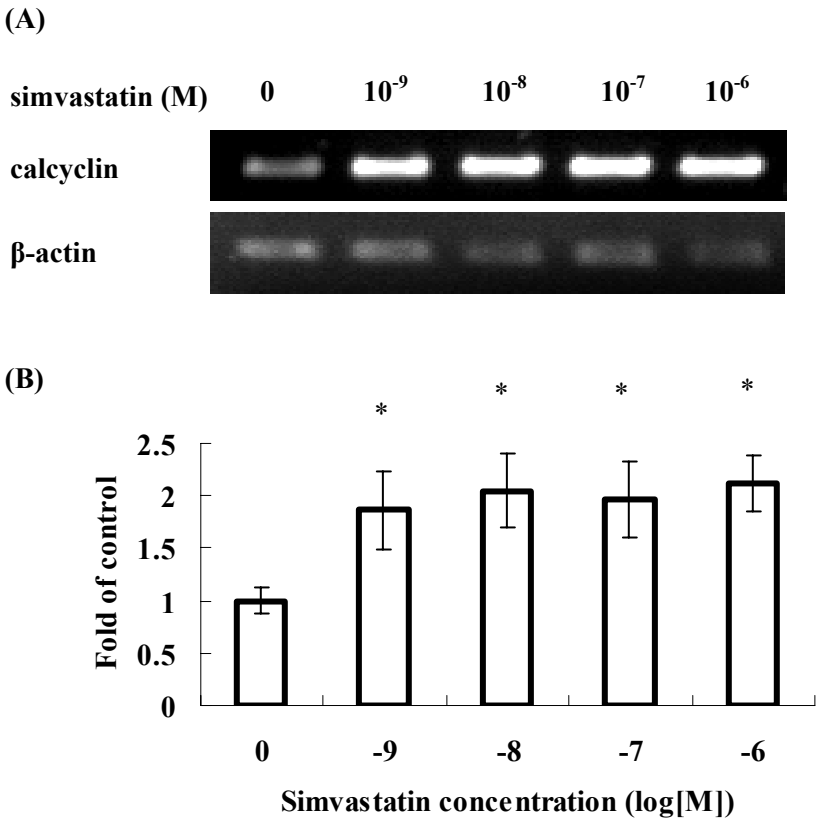

Fig. 5. Induction of calcyclin mRNA by various concentrations of simvastatin treatment. The effect of treatment of mouse calvaria cells with simvastatin in various doses was studied by RT-PCR. Calcyclin mRNA expression was saturated even at the lowest 10^{-9} M concentration 6 hours after simvastatin treatment and showed similar values at 10^{-8}, 10^{-7} and 10^{-6} M. * : $P<0.05$, simvastatin vs. control

5. The distribution of calcyclin after simvastatin treatment in MC3T3-E1 cells

To determine the effect of simvastatin stimulation on the cellular distribution of calcyclin, pEGFP-*cacy* encoding calcyclin coding region was transiently expressed in MC3T3-E1 cells. As shown in Fig 6, the fluorescence distributions of the calcyclin were localized in the cytoplasm ubiquitously in un-stimulated cells. 20 minutes after stimulation with simvastatin, the cellular GFP-*cacy* was quickly shifted to nucleus and a clear image was observed. The effects of PTH(1-34) treatment and co-treatment PTH(1-34) with simvastain on GFP-*cacy* localization were added to results because PTH(1-34) is known to exert osteoblastogenic effects (no further experiment was performed in related to PTH).

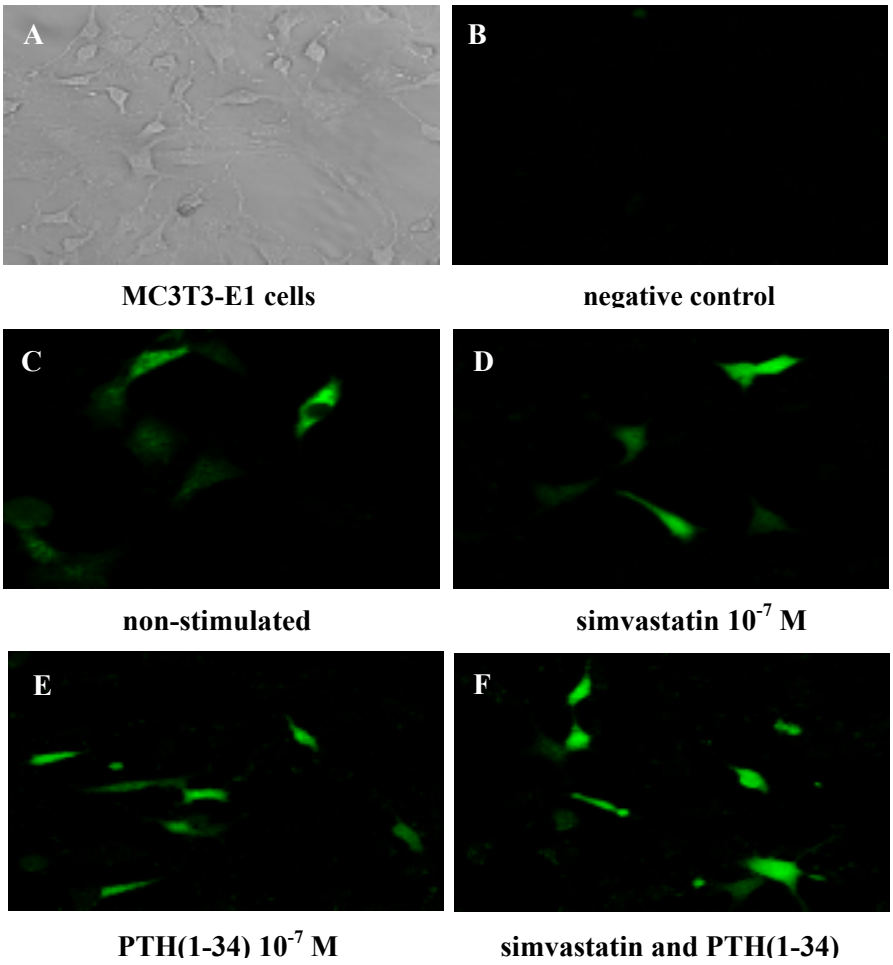

Fig. 6. Localization of calcyclin by confocal microscope in MC3T3-E1 cells. In MC3T3-E1 cells (A), no signal was observed in the absence of pEGFP-*cacy* plasmid (B). In the presence of GFP-*cacy* fusion protein, a clear signal was evidenced in the cytoplasm of the cells (C). GFP-*cacy* fusion protein was quickly moved to nucleus as soon as 20 minutes after simvastatin treatment (D). A potent anabolic hormone, PTH, gave a similar effect on calcyclin localization as well as co-treatment with simvastatin (E, F).

6. The effects of transient transfection of pcDNA-*cacy* plasmid on proliferation of MC3T3-E1 cells

In order to examine the in vitro effect of calcyclin in terms of proliferation, we transiently transfected pcDNA-*cacy* in MC3T3-E1 cells. In compare to the control, vehicle only transfected MC3T3-E1 cells, calcyclin over-expressed cells showed higher proliferation with statistical significance (Fig. 7).

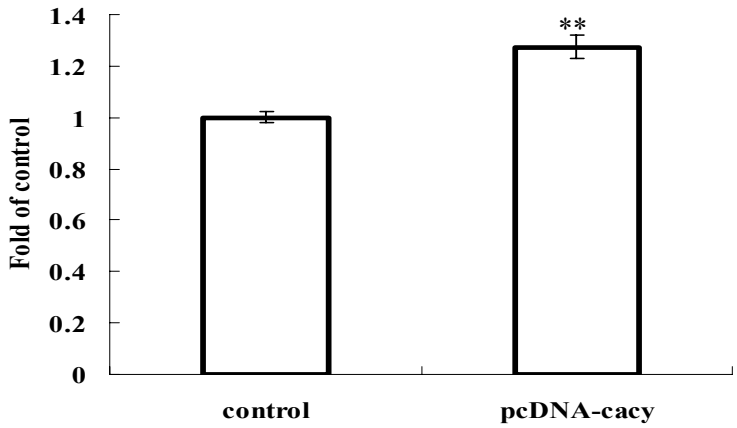

Fig. 7. The effect of pcDNA-*cacy* transfection on proliferation of MC3T3-E1 cells. pcDNA-*cacy* plamid was introduced into MC3T3-E1 using LipofectAmine and proliferation level was determined using WST-1 reagent the day after transfection. ** : P<0.01 pcDNA-*cacy* vs. vehicle.

7. The effects of transient transfection of pcDNA-*cacy* plasmid on ALP mRNA expression of MC3T3-E1 cells

In order to examine the in vitro osteogenic effect of calcyclin, we transiently transfected pcDNA-*cacy* in MC3T3-E1 cells. The expression of ALP mRNA, early osteoblast differentiation marker, was significantly increased the day after the transfection and the effects were normalized to β-actin mRNA expression. It might show osteogenic potential of calcyclin in osteoblast in vitro.

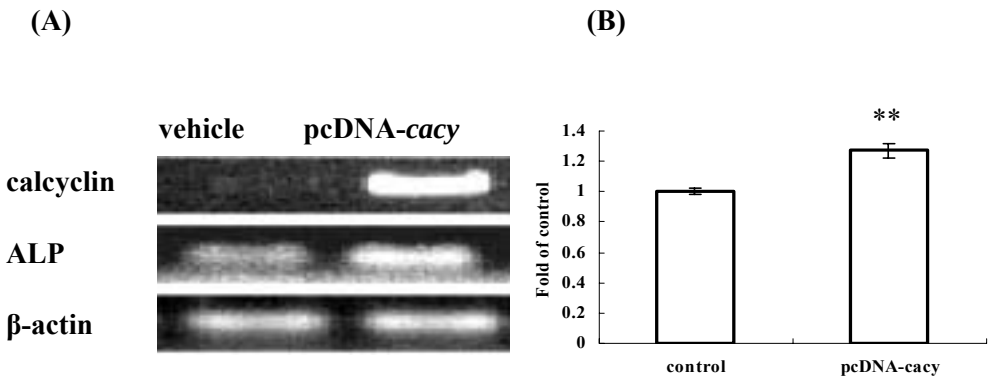

Fig. 8. The effect of pcDNA-*cacy* transfection on ALP mRNA expression of MC3T3-E1 cells. RNA was harvested from MC3T3-E1 cells the day after transfection with vehicle only (pcDNA 3.0) or pcDNA-*cacy* and screened by RT-PCR for ALP expression. ** : P<0.01 pcDNA-*cacy* vs vehicle.

Ⅳ. Discussion

Recently, many experimental observations revealed that statins, pro-drugs of a potent 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor, stimulated bone formation. Subsequent epidemiological studies have suggested that treatment of statin for hyperlipidemia may be associated with increased bone mineral density (BMD) and reduced fracture risk in humans, even though there are some controversies. The effects of statin have been suggested to be mediated through induction of potent bone-forming growth factors, the bone morphogenetic proteins (BMPs). However, action mechanisms of statin for inducing pleiotropic effects on bone have not been well documented yet.

In this study, simvastatin was proved to be a rapid and strong transcriptional inducer of osteoblast differentiation. Simvastatin exerted stimulatory effects on both proliferation and differentiation of mouse osteoblasts dose-dependently, however, more stimulatory effects on differentiation than on proliferation were observed. Only 6 hours induction of simvastatin enhanced the mRNA expression of osteoblast markers, alkaline phosphatase, Type I collagen and

osteocalcin remarkably.

Our aim was to identifying some key molecules playing a role in anabolic effects of simvastatin through proteomics. In cultured mouse calvarial cells, calcyclin was induced more than 10 times by simvastatin. Other 4 proteins, annexin I, III, vimentin and tropomyosin, were also up or down regulated by simvastatin significantly. Calcyclin (S100A6) is a 10.5 kDa protein which belongs to the family of calcium-binding proteins. Calcium-binding proteins are divided into two groups: the first groups is constituted by annexin, the second group by EF-hand proteins as calmodulin[13,14]. Calcyclin has first been identified as a cellular cycle-dependent protein highly induced by growth conditions but its precise role remains unknown. S100 protein family has been known to conduct an inhibitory role on protein phosphorylation targeting intracellular proteins such as vimentin, annexin I and III. Calcyclin, S100A6, interacts with tropomyosin in intracellular level[14]. And tropomyosin, a type of cytoskeleton, was down regulated by simvastatin along with up regulation of calcyclin in this study. Therefore, we focused our experimental efforts on calcyclin rather than the other proteins we

had found as simvastatin-regulated proteins.

The mRNA expression of calcyclin was rapidly induced by 10^{-7} M simvastatin only 30 minutes after treatment. And mRNA expression was degraded rapidly to the control level one day after simvastatin induction. Simvastatin is a strong and effective inducer of calcyclin because mRNA expression of calcyclin was very sensitive to even the low dosage of simvastatin (10^{-9} M). Furthermore, simvastatin also exerted a nuclear localization effect of calcyclin quickly after exposure. Calcyclin localized in the cytoplasmic compartment of the cell in basal conditions, shifted quickly to the nucleus after 20 min treatment of simvastatin. Parathyroid hormone, a potent anabolic hormone on bone, also induced nuclear translocation of calcyclin in MC3T3E-1 cells. This may suggest that nuclear transferred calcyclin may turn on or off some important genes related to the anabolic effects on osteoblast induced by both simvastatin and PTH(1-34). To clarify the role of calcyclin on the anabolic effects of simvastatin on bone, calcyclin was over-expressed in MC3T3-E1 cells without treatment of simvastatin. Interestingly, both the rate of proliferation and the expression of alkaline phosphatase mRNA were elevated significantly

one day after transfection. These results strongly indicate that calcyclin plays some important roles as a mediator of simvastatin induced anabolic effects on bone.

Ⅴ. Conclusion

The effects of simvastatin on bone have suggested an exciting new direction for research in bone formation that may lead to advances in the therapy of osteoporosis. In this present study, we identified calcyclin as a new simvastation-induced protein in mouse osteoblast cells and found calcyclin could play some roles mediating the effects of simvastatin on bone.

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국문요약

심바스타틴의 골 형성능에 있어서의 칼사이클린 단백질의 기능연구

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황 난 주

스타틴은 강력한 HMG-CoA reductase 억제제로서 사람과 동물에서 콜레스테롤의 합성을 억제하는 약제로 알려져 있다. 선행된 연구에 의하면 임상적으로 쓰이고 있는 양만으로도 in vitro 실험에서 골 형성능을 촉진하는 것으로 보고되었다. 이에 심바스타틴에의한 조골세포의 동화작용 유도 매개체를 찾고자 하며 프로테오믹스 기법을 이용하여 이를 동정하고자 본연구를 수행하였다. 생후 하루된 마우스의 두개관(頭蓋冠)에서 조골세포를 얻었으며 이후 10^{-9}~`10^{-6} M 농도의 심바스탄틴을

처리하였다. 증식실험을 위해 스타틴은 이틀에 6시간만을 간헐적으로 처리 하였고, 총 8일 동안 반복하였다. 친지질성 스타틴은 세포내에서 세포를 안정화 하거나 증식을 억제시키는 등의 작용을 하므로, 단기간 처치 방법을 택하였으며, 배지 성분도 Fetal Bovine Serum이 아닌 1% Bovine Serum Albumin 성분이 포함된 α-MEM을 택하였다. 조골세포의 증식은 스타틴 처리 농도에 의존적으로 증가하였으며 이는 대조군 대비하여 통계학적으로 의미 있는 값을 보여주었다. 또한 심바스타틴이 조골세포의 분화에 미치는 영향은 조골세포 분화 마커들의 표현정도를 RT-PCR로서 확인하고자 하였다. ALP, Type I Collagen 그리고 osteocalcin으로 확인하였고, 조골세포 분화 또한 심바스타틴 처리 농도에 의존적으로 현저한 증가를 보였다. 심바스타틴을 처리하지 않은 대조군과 10^{-7} M 로 6시간 처리한 실험군에서 단백질을 얻어서 프로테오믹스를 시행하였다. 2차원적으로 분리된 단백질 양상은 PDQuest 소프트웨어로 분석되었으며 이후에 선별적으로 6개의 단백질이 MALDI-TOF로 동정되었다. 이중 칼사이클린은 심바스타틴에 의해 10배 이상의 높은 증가를 보였으며 mRNA 수준에서도 심바스타틴에 의해 그 발현이 촉진됨을 확인하였다. **GFP-*cacy***

fusion 단백질을 만들어 세포내 칼사이클린의 위치를 확인하였다. 심바스타틴을 20분 처리시 빠르게 그 위치가 cytoplasm에서 핵쪽으로 이동하였음을 confocal 현미경으로 확인하였다. 또한 조골세포에 있어 칼사이클린의 작용을 연구하기 위해 **pcDNA-*cacy*** 재조합 유전체를 만들어 **MC3T3-E1** 세포에 과발현 시켜 보았다. 이때 조골세포의 증식은 물론 첫단계 분화마커인 **ALP**가 현저하게 증가되었다. 결론적으로, 심바스타틴은 조골세포의 증식과 분화를 모두 촉진하였으며 이때 칼사이클린은 심바스타틴의 조골능 유도에 있어 일부 중요한 역할을 할 것으로 사료되었다.

핵심되는 말 : 심바스타틴, 골 형성능, 조골세포 분화 마커, 프로테오믹스, 칼사이클린

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