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Kruppel-like factor 4 (KLF-4) plays a crucial role in simvastatin (SVT)-induced differentiation of rabbit articular chondrocytes

Seon-Mi Yu, Song Ja Kim*

Department of Biological Sciences, College of Natural Sciences, Kongju National University, Gongju, Republic of Korea

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ABSTRACT

Simvastatin is a cholesterol-lowing reagent that is derived synthetically from the fermentation of Aspergillus terreus. Recently, SVT has been shown to possess a protective effect of chondrocytes.

Kruppel-like factor 4 (KLF-4) is a zinc finger transcription factor that plays crucial roles during the development and maintenance of multiple organs. However, the roles of KLF-4 in chondrocytes have not been well unknown. Here, we investigated whether KLF-4 regulates SVT-caused differentiated phenotype of chondrocytes. A KLF-4 cDNA or KLF-4 siRNA was transfected into SVT-treated chondrocytes. Western blot analysis, RT-PCR and immunofluorescence staining analyzed expression of type II collagen and SOX-9, marker proteins of differentiation. The results showed overexpression of KLF-4 accelerates SVT-induced type II collagen expression, as determined by western blot analysis and causes sulfated-proteoglycan synthesis, as detected by Alcian blue staining. RT-PCR revealed that ectopic expression of KLF-4 induces SVT-caused SOX-9, a transcription factor of type II collagen, expression. Transfection of KLF-4 siRNA reversed SVT-caused type II collagen and SOX-9 expression and inhibited SVT-induced sulfated proteoglycan production. This study indicates that KLF-4 plays critical role in SVT-caused chondrocytes differentiation.

1. Introduction

Osteoarthritis (OA) is the most common disease of joint, affecting almost half of the aged population and is characterized by the progressive degeneration of articular cartilage. They significantly affect the psychosocial status of affected people as well as their families and carers. The major clinical manifestation includes symptoms of chronic knee pain, ankylosis, knee swelling, and limited physical activity [1]. OA leads to severe pain and movement problems during the intermediate or advanced stages and represents a leading socioeconomic burden in the developed world.

Statins is a family of inhibitors of 3-hydroxy-3-methylglutaryl-conenzyme A (HMG-CoA) reductase and it are principal therapeutic agents for the treatment of hypercholesterolemia due to results in a dramatic reduction in circulating low-density lipoprotein (LDL)-cholesterol [2]. Statin are commonly used for treatment of hyperlipidemic cardiovascular disease to reduce morbidity and mortality. Recently, it has been known that the value of statin therapy is not based on lipid decrease, but also involves direct vascular effects. Another study demonstrated that statins have protective, anti-inflammatory effects where they may function in immunoregulation of inflammatory cytokines or more directly as inhibitors on MMP expression in chondrocytes [3]. Recent reports have shown that simvastatin may have the potential to induce differentiation of chondrocytes [4].

Kruppel-like factor 4 (KLF-4) is a zinc finger type transcription factor that is expressed in the gastrointestinal tract, skin, testis, bone and other tissue [5]. KLF-4 has three tandem C_2H_2 zinc finger motifs at its C-terminal end that bind to DNA sequences of 5′-gagaggctgct-3′ or 5′-cacc-3' [6]. KLF-4 is considered as a pleiotropic transcription factor because it can either activate or repress transcription of gene [7].

During skeletal development, KLF-4 is overexpressed in immature osteoblasts and the expression slowly diminishes as osteoblasts mature [5]. KLF-4 has also has been shown to counteract the oncogenic effect of Notch signaling which is intimately involved in skeletal development [8]. Moreover, expression of KLF-4 in fibroblast is highest in growth-arrested cells and lowest in cells in the exponential phase of proliferation [9]. This suggests that the physiological regulation of KLF-4 expression in various cell types is

* Corresponding author. Department of Biological Sciences, Kongju National University, Gongju, 32588, Republic of Korea.
E-mail address: ksj85@kongju.ac.kr (S.J. Kim).

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crucial role for development.

In this study, we describe the role of KLF-4 in SVT-caused differentiation. Here we show that, in rabbit articular chondrocytes, KLF-4 plays a critical role in simvastatin (SVT)-caused differentiation. When KLF-4 was overexpressed under SVT-treated chondrocytes, the differentiation of chondrocytes was induced. Further, KLF-4 knock down with siRNA into the chondrocytes were severely delayed SVT-caused differentiation. These finding indicates that KLF-4 may act as a positive regulator of differentiation by SVT-treated chondrocytes.

2. Materials and methods

2.1. Reagents and treatment

Simvastatin (SVT) was purchased from Sigma-Aldrich (Saint Louis, MO, USA). Cells were treated with 50 μM SVT or vehicle (DMSO, dimethyl sulfoxide) for 24 h. The dosage was chosen based on our previous observation data that SVT induced differentiation [10]. Sodium nitroprussade (SNP; Sigma Chemicals St. Louis, MO, USA) was used as a dedifferentiation of chondrocytes. KLF-4 cDNA (GFP-tagged) and pCMV-AC6 vector were purchased from Origene technologies (Rockville, MD, USA). KLF-4 siRNA was obtained from Bioneer (Daejeon, Korea). Transfection reagent of cDNA and siRNA were performed using the TurboFect transfection reagent (Fisher Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

2.2. Isolation and monolayer culture of rabbit articular chondrocytes

New Zealand White rabbits (2-week-old) were purchased from the Koatech (Pyeoungtaek, Republic of Korea). After the animals were sacrificed by ether anesthesia, they were disinfected with 75% ethanol and rinsed with PBS containing penicillin (100 unit/mL), and streptomycin (100 μg/mL). Cartilage tissues were cut into tiny pieces of 1–3 mm^2, which were digested with DMEM containing 0.2% collagenase II (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for 7 h, and then centrifuged at 1000 rpm for 10 min. After centrifugation, the cells (2×10^4 cells/dish) were resuspended in DMEM supplemented with 10% (v/v) fetal bovine serum, penicillin (50 unit/mL), and streptomycin (50 μg/mL), and maintained as monolayers in a 5% CO_2 incubator at 37 °C. After having been plated overnight, chondrocytes attached to the wall of the culture dish. The medium was changed every two days after seeding. The stretched cells exhibited irregular spindle or polygonal shapes. The subsequent experiments were performed at least three independent times. This study protocol was approved by the Ethics Committee of the Kongju National University.

2.3. Western blot analysis and immunoprecipitation (IP)

Total protein was harvested from chondrocytes and western blot analysis were performed as described previously [11]. An equal amount (20–40 μg) of proteins were separated on 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane. Membrane were blocked with 5% (w/v) nonfat dry milk in Tris-buffered saline-0.01% Tween 20 (TBST, pH 8.0) buffer at room temperature for 1 h and then incubated for overnight with primary antibodies. After washing three times for 10 min with TBST, the membrane was incubated for 2 h with appropriate secondary horseradish peroxidase-conjugated antibodies. An enhanced chemiluminescence reagent (Dogen, Seoul, Republic of Korea) was used to visualize the reactive bands, which were quantified using the LAS4000 camera system (Fuji Film, Tokyo, Japan). For IP, cell extracts were prepared with IP buffer (20 mM Tri- HCl pH 8.0, 137 mM NaCl, 10% glycerol, 1% NP-40, 2 mM EDTA) and then lysed. Supernatants were incubated for overnight with anti-SOX-9 antibody and protein G beads (GenDEPOT, Barker, TX, USA). IP fraction was assessed by WB using an anti-KLF-4 antibody.

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

Total RNA from the cells was extracted using TRIzol reagent (Life Technologies, Gaithersburg, MD, USA). Reverse transcription and cDNA synthesis were performed with the Maxime™ RT premix kit (iNtRON Biotechnology, Seongnam, Korea). RNA (0.2 μg) was reverse transcribed into cDNA according to the manufacturer's instructions. The following primers abundance of mRNA from the perspective target genes was calculated after normalization to GAPDH.

The following primers and conditions were employed for RT-PCR: type II collagen (370 bp product, annealing temperature 52 °C, 27 cycles), 5′-GAC CCC ATG CAG TAC ATG CG-3′ (sense) and 5′-AGC CGC CAT TGA TGG TCT CC-3′ (antisense); SOX-9 (386 bp product, annealing temperature 62 °C, 27 cycles); KLF-4 (253 bp product, annealing temperature 52 °C, 27 cycles), 5′- GTG TGT TTG CGG TAG TGC CTG-3′ (antisense) and 5′- AGC TCA TGC CAC CGG GTT C′ (sense); GAPDH (299 bp product, annealing temperature 56 °C, 25 cycles), 5′-TCA CCA TCT TCC AGG AGC GA-3′ (sense) and 5′-CAC AAT GCC GAA GTG GTC GT-3′ (antisense). The DNA were separated on a 1% agarose gel and stained with Ecodye™ nucleic acid staining solution (BioFact, Daejeon, Republic of Korea).

2.5. Alcian blue staining

For Alcian blue staining, cells (P0) were fixed with 3.5% paraformaldehyde in PBS for 15 min and stained with 1% Alcian blue in 3% acetic acid for overnight, and rinsed three times with 0.1 N HCl. The stained cells were washed three times with distilled water and 6 M guanidine HCl was added for 6 h. The production of sulfated-proteoglycans was measured at 595 nm. Data represent the results from at least four independent experiments.

2.6. Immunofluorescence staining

Chondrocytes was seeded at concentration of 2×10^4 cells in a 35 mm dish with preset sterilized coverslips. After the cells adhered, they were washed with PBS and fixed with 3.5% paraformaldehyde for 30 min. After having been washed with PBS three times, the cells were blocked with 5% BSA at 37 °C for 1 h. Primary antibodies (Mouse Anti-COL2A1/type II collagen, MAB8887; 0.2 μg/mL) at 4 °C overnight. The cells were incubated with fluorescent dye-conjugated secondary antibodies at 37 °C for 1 h, before the cells were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI; Invitrogen, Burlington, ON, Canada) for 5 min at room temperature. Immunofluorescence was observed using the fluorescence microscope. As the cartilage matrix is enriched with type II collagen, the synthesis and secretion of the type II collagen can be used as a specific maker for identifying the differentiation phenotype of chondrocytes [12].

2.7. Data analysis and statistics

Data were presented as the means ± standard deviation (SD). All experiments were performed for at least three times, and representative experiments are shown. Statistical analysis was performed by a one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test and all values are expressed as the