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A Negative Correlation Between *Per1* and *Sox6* Expression During Chondrogenic Differentiation in Pre-chondrocytic ATDC5 Cells

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Abstract. Pre-chondrocytes undergo cellular differentiation stages during chondrogenesis under the influence by different transcription factors such as sry-type high mobility group box-9 (Sox9) and runt-related transcription factor-2 (Runx2). We have shown upregulation by parathyroid hormone (PTH) of the clock gene *Period-1* (*Per1*) through the cAMP/protein kinase A signaling pathway in pre-chondrocytic ATDC5 cells. Here, we investigated the role of Per1 in the suppression of chondrogenic differentiation by PTH. In ATDC5 cells exposed to 10 nM PTH, a drastic but transient increase in *Per1* expression was seen only 1 h after addition together with a prolonged decrease in *Sox6* levels. However, no significant changes were induced in *Sox5* and *Runx2* levels in cells exposed to PTH. In stable Per1 transfectants, a significant decrease in *Sox6* levels was seen, with no significant changes in *Sox5* and *Sox9* levels, in addition to the inhibition of gene transactivation by Sox9 allies. Knockdown of *Per1* by siRNA significantly increased the *Sox6* and *type II collagen* levels in cells cultured for 24 – 60 h. These results suggest that Per1 plays a role in the suppressed chondrocytic differentiation by PTH through a mechanism relevant to negative regulation of transactivation of the *Sox6* gene during chondrogenesis.

Keywords: parathyroid hormone (PTH), Per1, sry-type high mobility group box-6 (Sox6), chondrocyte, siRNA

Introduction

The prevailing view is that mesenchymal precursor cells differentiate into skeletal elements by forming a cartilaginous model during embryogenesis for bone formation known as endochondral ossification in the vertebral column and long bone (1). The cartilaginous rudiment, which is a tightly regulated area of both differentiation and maturation of chondrocytes, undergoes developmental growth for skeletogenesis. Within the cartilaginous rudiment, chondrocytes progressively differentiate through developmental stages from the resting, proliferating, hypertrophic, and calcifying cells, which lead to mineralization of the cartilage matrix around the central region of the rudiment in the area of hypertrophic chondrocytes. Shortly after the mineralization process takes place, most hypertrophic chondrocytes undergo sustained apoptosis. Upon apoptotic death after mineralization, osteoblasts, osteoclasts, and capillaries begin to invade the cartilage matrix to produce new bone, leading to longitudinal bone growth (2). These sequential differentiation steps are not only under the positive control by essential transcription factors including sry-type high mobility group box-9 (Sox9) and runt-related transcription factor-2 (Runx2) (2), but also differentially regulated by a wide range of local autocrine/paracrine factors including parathyroid hormone (PTH)-related peptide (3), indian hedgehog (3), wingless-type mouse mammary tumor virus integration site family (4), and glutamate (5).

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Amongst these endogenous effectors, PTH is believed to negatively regulate chondrogenesis along with positive regulation of osteoblastogenesis to promote osteoclastogenesis required for the elevated levels of circulating Ca^{2+} (2), whereas exposure to PTH led to transient upregulation of the clock gene *Per1* expression through the cAMP/protein kinase A signaling pathway in mouse pre-chondrocytic cell line ATDC5 cells and in organotypic cultured mouse metatarsals isolated before vascularization (6). These previous findings prompted us to elucidate the possible involvement of essential transcription factors such as Sox9 allies and Runx2 in mechanisms underlying the suppression of chondrogenesis by Per1 up-regulated in response to PTH in ATDC5 cells.

Materials and Methods

Materials

Chondrogenic ATDC5 cells were purchased from RIKEN Cell Bank (Saitama). Dulbecco's modified Eagle's medium: Nutrient Mixture F-12 (1:1) Mixture (DMEM/F-12), Lipofectamine, Plus reagent, G418, and Lipofectamine 2000 were purchased from Invitrogen (San Diego, CA, USA). Insulin was obtained from Sigma (St. Louis, MO, USA). Anti-Per1 antibody was supplied by Alpha Diagnostic (San Antonio, TX, USA). Negative control siRNA was purchased from Ambion (Austin, TX, USA). Rat PTH (1 – 34) was purchased from Calbiochem (San Diego, CA, USA).

Cell culture

ATDC5 cells were plated at a density of 1×10^4 cells/cm^2 in DMEM/F12 medium containing 5% fetal bovine serum (FBS). For induction of differentiation, culture media were replaced with medium containing 10 μg/ml transferrin, 3×10^{-8} M sodium selenite, and 10 μg/ml bovine insulin (7).

Determination of mRNA and protein

Reverse transcription polymerase chain reaction (RT-PCR) was conducted as described previously (8). Quantification of PCR products were conducted by real time-based RT-PCR using a MiniOpticon^{TM} (Bio-Rad, Hercules, CA, USA) with an iQ SYBR Green Supermix (Bio-Rad). The relative amount of transcript was normalized by glyceraldehydes-3-phosphate dehydrogenase (GAPDH) expression. The primer sequences of oligonucleotides used for real time-based RT-PCR are as follows: *GAPDH*, 5′-AGGTCGGTGTGAACGGATTTG-3′ (sense) and 5′-TGTAGACCATGTAGTTGAGGTCA-3′ (antisense); *Per1*, 5′-CAGGCTAACCAGGAATATTACCAGC-3′ (sense) and 5′-CACAGCCACAGAGAAGGTGTCC TGG-3′ (antisense); *Runx2*, 5′-CCGCACGACAAC CGCACCAT-3′ (sense) and 5′-CGCTCCGGCCCA CAAATCTC-3′ (antisense); *Sox5*, 5′-GATGGGGAT CTGTGCTTGTT-3′ (sense) and 5′-CTCGCTGGA AAGCTATGACC-3′ (antisense); *Sox6*, 5′-GGATTG GGGAGTACAAGCAA-3′ (sense) and 5′-CATCTG AGGTGATGGTGTGG-3′ (antisense); *Sox9*, 5′-CGA CTACGCTGACCATCAGA-3′ (sense) and 5′-AGACTG GTTGTTCCCAGTGC-3′ (antisense); *type II collagen* (*Col II*), 5′-TGAAGACCCAGACTGCCTCAA-3′ (sense) and 5′-AGCCGCGAAGTTCTTTTCTCC-3′ (antisense). Northern blotting was conducted as described previously (9) by using cRNA probes labeled with digoxigenin (DIG). Western blotting was done by using the anti-Per1 antibody as described previously (8).

Establishment of stable Per1 transfectants

ATDC5 cells were plated at a density of 2×10^4 cells/cm^2 in DMEM/F12 containing 5% FBS on culture dishes (ϕ 35 mm). After 24 h, cells were transfected with pcDNA3.1 containing the full-length coding region of Per1 or with the empty vector (EV) using 2 μg of DNA and Lipofectamine and Plus reagent. After 24 h, and every 48 h thereafter for 2 weeks, culture media were replaced with DMEM/F12 containing 5% FBS and 500 μg/ml G418. Pools of 28 clones of ATDC5 cells resistant to G418 (ATDC5-Per1) were isolated for further studies. Pools of clones between passages 2 and 5 were used for these experiments (10).

Transfection with siRNA

ATDC5 cells were plated at 2×10^4 cells/cm^2 in DMEM/F12 containing 5% FBS, followed by the transfection of siRNA for Per1 (B-Bridge) or negative control siRNA at 30 nM by Lipofectamine 2000 for 24 h and subsequent replacement of the medium to DMEM/F12 containing 5% FBS. BLOCK-iT^{TM} Alexa Fluor Red Fluorescent Oligo and Silencer GAPDH siRNA (Human, Mouse, Rat) was used for the evaluation of siRNA transfection efficiency in ATDC5 cells.

Cloning and constructs

Mouse *Clock* and hamster *brain and muscle aryl hydrocarbon receptor nuclear translocator-like (BMAL1)* expression plasmids were kindly donated by Dr. Reppert (University of Massachusetts Medical School, Worcester, MA, USA), while a 4x48-p89-Luc, which is 4 tandem copies of Sox9 binding site linked to the minimal Col II gene promoter in the luciferase reporter plasmid, is a generous gift from Dr. B. de Crombrugghe (Anderson Cancer Center, Houston, TX, USA). Reporter plasmids for mouse *Sox6* promoter were prepared as follows. Mouse *Sox6* promoter was at first obtained by cloning

with the forward primer 5′-GGTACC (KpnI site)-ATG GGCTGGCTTTGAAAACT-3′ (−2,190 to −2,171) and the reverse primer 5′-CTCGAG (XhoI site)-CAGAGA ATTAATCTAAACA-3′ (+181 to +200) with mouse tail genomic DNA. The deletion mutant of mouse *sox6* promoter plasmid was made from the forward primer 5′-GGTACC (KpnI site)-AATTGTTGTTACTTTCTA-3′ (−1,080 to −1,061) and the reverse primer 5′-CTC GAG (XhoI site)-CAGAGAATTAATCTAAACA-3′ (+181 to +200). The mouse *sox6* promoter fragment (−2,190 to +200) and the deletion mutant of mouse *sox6* promoter fragment (−1,080 to +200) were cloned into the promoterless pGL-3 basic vector, to create the recombinant plasmid −2,190/+200 Sox6-Luc (−1,080 to +200 Sox6-Luc).

Luciferase assay

Reporter vectors were co-transfected with a SV40-Renilla luciferase construct into ATDC5 cells using Lipofectamine and Plus reagent. Two days after transfection, cells were lysed, and luciferase activity was determined using specific substrates in a luminometer according to the manufacturer's protocol. Transfection efficiency was normalized by determining the activity of Renilla luciferase (10).

Chromatin immunoprecipitation (ChIP) assay

ChIP experiments were performed essentially according to the protocol provided by the ChIP assay kit. Cells were treated with formaldehyde for cross-linking, followed by sonication in lysis buffer containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml pepstatin). Immunoprecipitation was performed with the antibody against brain and muscle aryl hydrocarbon receptor nuclear translocator-like (Bmal1), followed by extraction of DNA with phenol/chloroform. PCR was performed using sequences found in the 5′ flanking region (−1,388 to −1,184 upstream 5′-CAGCATGTGAACCCTGAAGA-3′, downstream, 5′-AAGCCAACACCGAGAGAGAA-3′) of the mouse *Sox6* gene as primers (11).

Data analyses

Results are all expressed as the mean ± S.E.M. and statistical significance was determined by two-tailed and unpaired Student's *t*-test or one-way analysis of variance "ANOVA" with the Bonferroni/Dunnett post hoc test.

Results

Effects of PTH on gene expression

We first examined whether PTH affects the mRNA expression of endogenous factors essential for chondrogenic differentiation, including Runx2, Sox5, Sox6, and Sox9, at the concentration effective for inducing *Per1* upregulation (6). Cells were exposed to 10 nM PTH for different periods up to 48 h, followed by determination of the mRNA levels by real time-based RT-PCR. Exposure to PTH led to rapid but transient upregulation of *Per1* expression 1 h after addition, with concomitant downregulation of *Sox6* expression after the exposure for 12 to 48 h in ATDC5 cells (Fig. 1). However, PTH induced a biphasic increase in *Sox9* levels during the exposure for 1 to 48 h, without significantly affecting the mRNA levels of *Runx2* and *Sox5*.

Effects of stable overexpression of Per1 on chondrocytic differentiation

In order to elucidate the significance of rapid *Per1* upregulation in chondrocytes exposed to PTH, we next attempted to establish several stable *Per1* transfectants in ATDC5 cells. We have previously confirmed drastically high endogenous levels of mRNA and corresponding protein for Per1 in the #28 clone cultured for 2 days (11). Real time based RT-PCR analysis again revealed high expression of *Per1* in the #28 clone cultured for 3 days, with unaltered *Runx2* levels (Fig. 2A). Both ATDC5-EV and ATDC5-Per1#28 cells were then cultured in the presence of transferrin, sodium selenate, and bovine insulin for 7 days, followed by determination of the mRNA levels of *Sox9* allies with real time-based RT-PCR. Stable overexpression of Per1 was found to almost completely diminish *Sox6* expression without significantly affecting either *Sox5* or *Sox9* expression (Fig. 2B). In ATDC5-Per1#28 cells cultured for 14 to 21 days, a drastic decrease in different maturation markers including Alcian blue staining and alkaline phosphatase activity was seen in addition to downregulation of several differentiation marker genes such as *type II collagen* (*Col II*) for proliferating chondrocytes and *type X collagen* (*Col X*) for hypertrophic chondrocytes, respectively (11). We next examined the transcriptional activation mediated by Sox9 allies endogenously expressed in stable Per1 transfectant cells using a 4x48-p89-Luc reporter plasmid with four tandem copies of the Sox9 binding site linked to the minimal promoter of *Col II*. The reporter plasmid was transiently transfected into ATDC5-EV and ATDC5-Per1#28 cells, followed by determination of luciferase activity 48 h after transfection. Luciferase activity was significantly attenuated in ATDC5-Per1#28 cells stably overexpressing Per1 compared with that in ATDC5-EV cells (Fig. 2C). Northern blotting analysis confirmed a significant decrease in mRNA expression of the proliferating chondrocytic marker protein Col II in ATDC5-Per1#28 cells cultured for 7 days (Fig. 2D).

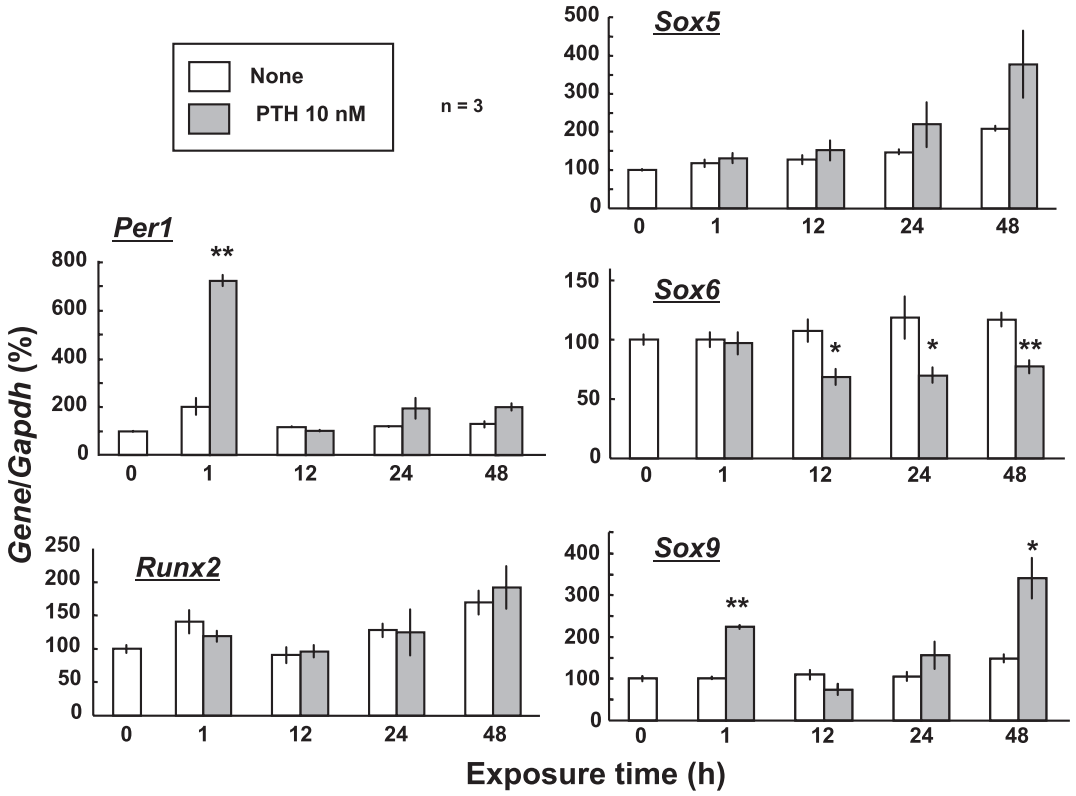

Fig. 1. Expression profiles of different chondrogenic genes in ATDC5 cells exposed to PTH. ATDC5 cells cultured for 1 day were exposed to 10 nM PTH for 1 – 48 h, followed by isolation of total RNA and subsequent real time-based RT-PCR analysis. *$P < 0.05$, **$P < 0.01$, significantly different from each control value obtained in cells cultured in the absence of PTH.

Effects of knockdown of Per1 on chondrocytic differentiation

An attempt was next made to elucidate chondrocytic maturation in ATDC5 cells transfected with small interfering RNA (siRNA) for the knockdown of *Per1* expression. Approximately 90% of cells were transfected with siRNA conjugated with Alexa594 in ATDC5 cells transfected under the present experimental conditions (Fig. 3A). Cells were thus transfected with siRNA for *Per1*, followed by further culture in DMEM/F12 containing 5% FBS for 24 to 60 h and subsequent determination of the *Per1* mRNA levels by real time-based RT-PCR. The *Per1* mRNA levels were significantly decreased in ATDC5 cells transfected with *Per1* siRNA when determined at 24, 36, and 48 h, but not at 60 h, after the transfection (Fig. 3B). In cells transfected with *Per1* siRNA, a marked reduction of Per1 protein was found 60 h after transfection (Fig. 3C). Under these experimental conditions, a significant and sustained increase in *Sox6* expression (Fig. 3D) was seen, along with the increased mRNA levels of *Col II* (Fig. 3E), in ATDC5 cells cultured for 36 to 60 h after transfection.

Effects of overexpression of Per1 on transactivation of Sox6 gene

The *Sox6* promoter regions were subjected to a DBTSS (http://dbtss.hgc.jp) and TRANSFAC (http://www.gene-regulation.com/index.html) database search. A database analysis revealed that 2.2 kb of the 5′ flanking region of the mouse *Sox6* gene contains one putative E-box (CACGTG), which is a target element recognized by the master clock molecules Bmal1/Clock heterodimer. For identification of the promoter element responsible for the gene transactivation, a deletion mutant was made from the 5′ flanking region of the mouse *Sox6* gene for preparation of particular luciferase reporter plasmid constructs. In HEK293 cells with *Sox6*-Luc (−2190/+200), the introduction of Bmal1/Clock led to a significant increase in luciferase activity in a manner sensitive to the inhibition by *Per1* transfection (Fig. 4A). However, luciferase activity was not significantly affected by the introduction of Bmal1/Clock in cells with *Sox6*-Luc (−1080/+200). Stable Per1 transfectants were thus transfected with *Sox6*-Luc (−2190/+200), followed by determination of luciferase activity 48 h after transfection. Luciferase activity was significantly decreased

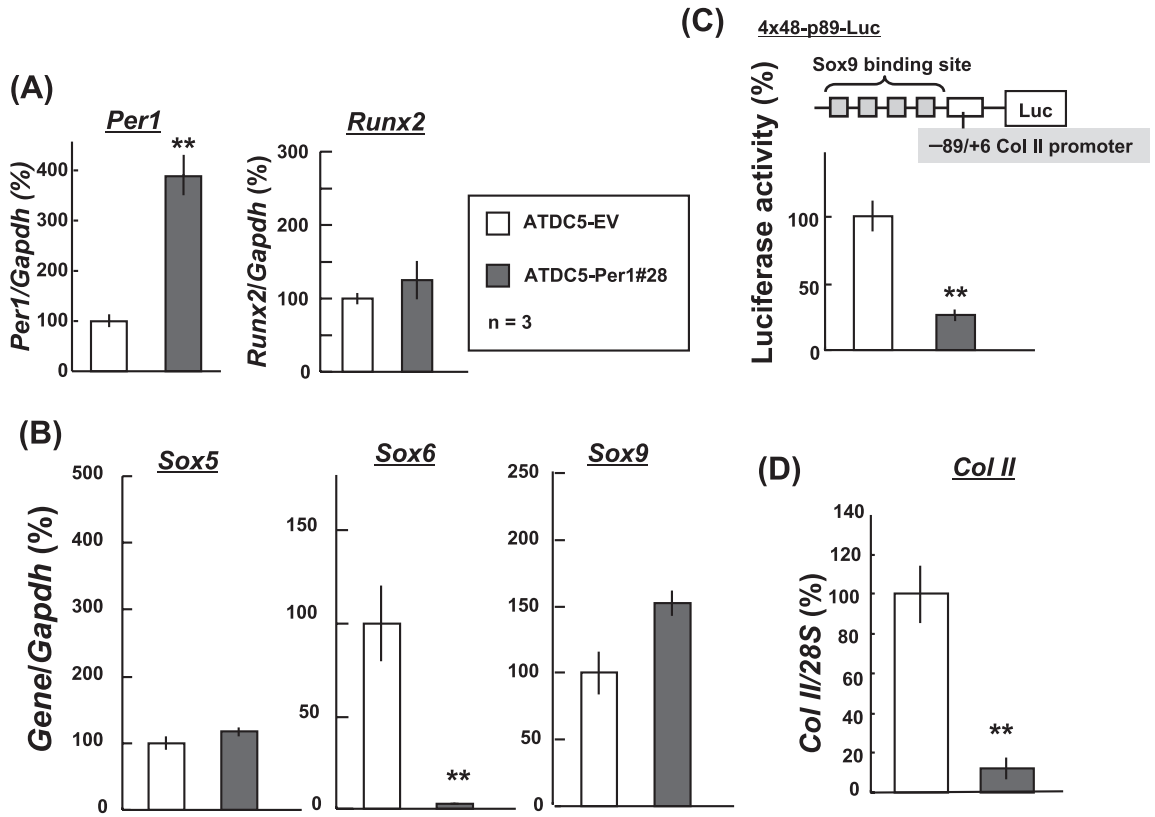

Fig. 2. Stable overexpression of Per1 in ATDC5 cells. A) ATDC5 cells were stably transfected with the *Per1* expression vector or *EV*, followed by culture for 3 days and subsequent determination of the mRNA levels of *Per1* and *Runx2*. B) Stable transfectant cells were cultured for 3 days, followed by isolation of total RNA and subsequent real time–based RT-PCR. C) Stable transfectant cells were transfected with 4x48-p89-Luc, followed by determination of luciferase activity 48 h after transfection. D) Stable Per1 transfectants were cultured for 3 days, followed by isolation of total RNA and subsequent northern blotting analysis for *Col II*. $^{**}P < 0.01$, significantly different from each control value obtained in cells with EV.

in ATDC5-Per1#28 cells transfected with *Sox6*-Luc (−2190/+200) compared to ATDC5-EV cells (Fig. 4B). Transient overexpression of Per1 led to a similarly significant reduction of luciferase activity in ATDC5 cells with *Sox6*-Luc (−2190/+200) (Fig. 4C). We next performed ChIP assays using sequences found in the 5′ flanking region (−1388/−1184) or (+242/+540) of the mouse *Sox6* gene. In immunoprecipitates with the anti-Bmal1 antibody of lysates from ATDC5 cells transfected with *Bmal1*/*Clock*, a PCR product was clearly found with the 5′ flanking region containing an E-box element (−1388/−184), but not with the region devoid of the E-box element (+242/+540), of the *Sox6* gene (Fig. 4D).

Discussion

The essential importance of the present findings is that overexpression of the clock gene *Per1* negatively and preferentially regulated the mRNA expression of *Sox6* amongst different endogenous factors highly responsible for chondrogenic differentiation and maturation in pre-chondrocytic ATDC5 cells. By contrast, knockdown by siRNA of *Per1* led to positive regulation of *Sox6* and *Col II* expression in ATDC5 cells. These findings thus give rise to an idea that Per1 negatively regulates chondrocytic differentiation for maturation through a mechanism relevant to downregulation of *Sox6* expression during chondrogenesis. Both Sox5 and Sox6 are defective of a DNA binding domain, but essential for promotion of the transactivation of particular target genes encoding cartilage-specific extracellular matrix components including Col II. By contrast, the transcription factor Sox9 is a nuclear protein with a DNA binding domain highly required for converting mesenchymal stem cells locally condensed in bone marrows into chondrocytes, in conjunction with Sox5 and Sox6 to orchestrate a trimetric protein complex (2). To our knowledge, this is the first direct demonstration of a negative correlation between *Per1* and *Sox6* expression at the level of gene transactivation in pre-chondrocytic

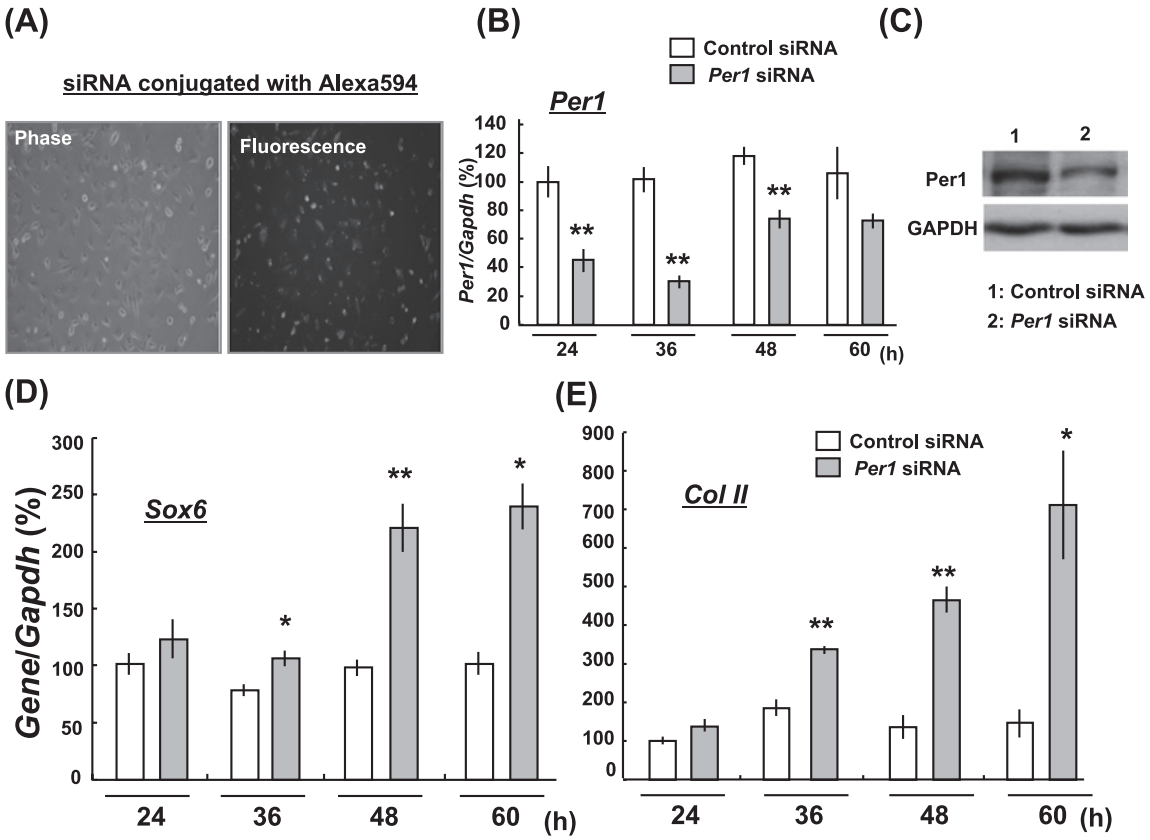

Fig. 3. Knockdown of Per1 in ATDC5 cells. A) ATDC5 cells were transfected with siRNA conjugated with Alexa594, followed by observation under a fluorescence microscope. ATDC5 cells were transfected with either *siControl* or *Per1* siRNA, followed by further culture for different periods from 24 to 60 h and subsequent determination of the *Per1* levels at 24 to 60 h after transfection (B) and the Per1 levels on western blotting analysis 60 h after transfection (C), respectively. Cells were also subjected to determination of *Sox6* (D) and *Col II* levels (E). $*P < 0.05$, $**P < 0.01$, significantly different from each control value obtained in cells with Control siRNA.

ATDC5 cells. Mutations in a single allele of Sox9 result in a severe skeletal malformation syndrome such as campomelic dysplasia in humans (12), while Sox9 heterozygous mutant mice exhibit similar skeletal anomalies seen in campomelic dysplasia patients (13). By contrast, transgenic mice overexpressing Sox9 show delayed endochondral bone formation (14). Accordingly, Per1 could interfere with *Sox6* expression essential for transactivation by the Sox9 family member complex of target genes in chondrocytes. The final conclusion should await the demonstration of failure of Per1 to suppress chondrogenic differentiation in chondrocytes isolated from mice defective in Per1.

The inconsistent results of *Sox9* expression in stable Per1 transfectants argue in favor of an idea that PTH negatively regulates chondrogenic differentiation for maturation through upregulation of *Per1* expression in a manner associated with downregulation of Sox6 rather than upregulation of Sox9 during chondrogenesis in prechondrocytic ATDC5 cells. Expression of mouse *Per* genes is positively regulated by other clock proteins belonging to the basic helix-loop-helix period/aryl hydrocarbon receptor nuclear translocator/single minded class, which are Clock and Bmal1, whereas Per proteins constitute multimeric complexes with products of the cryptochrome (Cry) genes such as *Cry1* and *Cry2*, which in turn negatively regulate the gene transactivation mediated by the Bmal1/Clock complex (15 – 18). The data from ChIP assays give support to the proposal that the Bmal1/Clock complex promote transcription of the *Sox6* gene through the E-box element located between −1389 and −1185 bp upstream in a manner sensitive to the inhibition by Per1. Taken together, downregulation of Sox6 would lead to suppression of the gene transactivation mediated by the Sox9 trimeric complex upstream of the *Col II* gene whose translation is essential for chondrogenesis in ATDC5 cells. The possibility that Per1 may play a role in mechanisms underlying the suppressed chondrocytic differentiation mediated by PTH in conjunction with downregulation of *Sox6*

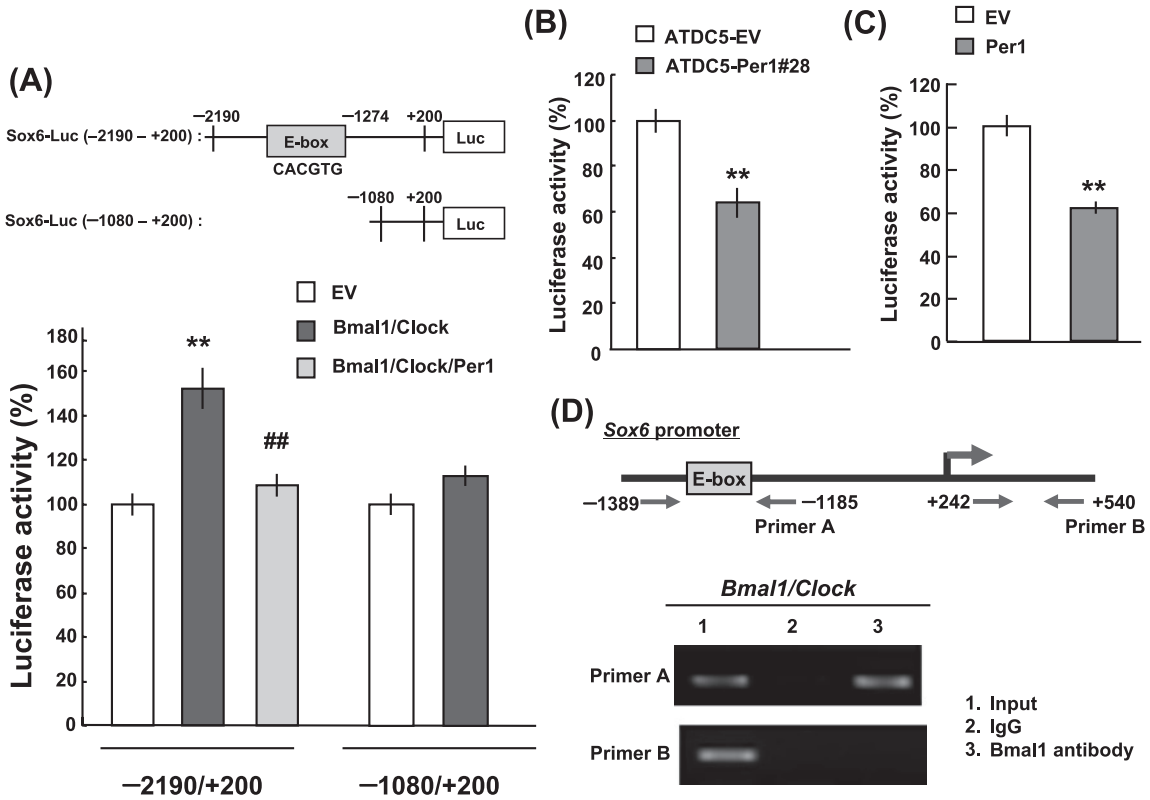

Fig. 4. Effect of Per1 on Sox6 promoter. A) A putative E-box (CACGTG) element exists within the promoter region from −2190 to −1274 bp upstream of the mouse *Sox6* gene. HEK293 cells were transiently transfected with deletion mutants of the *Sox6* promoter reporter plasmid in either the presence or absence of expression vectors of Bmal1, Clock, and Per1, followed by further culture for 48 h and subsequent determination of luciferase activity. B) Stable transfectants were introduced with Sox6-Luc (−2190/+200), followed by determination of luciferase activity 48 h after transfection. C) ATDC5 cells were transiently transfected with Sox6-Luc (−2190/+200) in either the presence or absence of Per1 expression vectors, followed by determination of luciferase activity 48 after transfection. D) ATDC5 cells were transfected with *Bmal1*, *Clock*, and *Per1* expression vectors, followed by immunoprecipitation with the anti-Bmal1 antibody and subsequent RT-PCR using either primer A or primer B. $*P < 0.05$, $**P < 0.01$, significantly different from each control value obtained in cells with EV. $^{\#\#}P < 0.01$, significantly different from the value obtained in cells with Bmal1/Clock.

expression during chondrogenesis is not ruled out. The physiological and pathological significance of peripheral clock genes expressed outside the hypothalamic suprachiasmatic nucleus, however, still remains to be elucidated. Pancreatic islets are shown to have a self-sustained molecular clock system apart from control by the central clock to coordinate insulin secretion with a sleep-wake cycle (19).

It thus appears that there is a negative correlation between expression profiles of the circadian rhythmicity oscillator *Per1* and the chondrogenesis regulator *Sox6* in pre-chondrocytic ATDC5 cells. Evaluation of the underlying mechanism could give a clue for the future discovery and development of innovative drugs useful for the treatment and therapy of a variety of cartilage diseases relevant to abnormal development and maturation of chondrocytes in human beings.

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Conflicts of Interest

The authors declare no conflicts of interest.

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