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A Novel Mechanism for the Regulation of Osteoblast Differentiation: Transcription of Periostin, a Member of the Fasciclin I Family, is Regulated by the bHLH Transcription Factor, Twist

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Abstract Periostin is a secreted protein that is highly expressed in early osteoblastic cells in vitro and in periosteum and periodontal ligament tissues in vivo. It is known that periostin supports cellular adhesion and spreading in vitro. Although, the mechanisms of transcriptional regulation of periostin are poorly understood, gene-profiling data have revealed that overexpression of Twist, a basic helix-loop-helix (bHLH) transcription factor, resulted in increased periostin expression as validated by Northern blot and reverse transcription-polymerase chain reaction (RT-PCR) analyses. Twist is an important transcription factor for cell type determination and differentiation and has been shown to play an important regulatory role in early osteogenesis. In situ hybridization of mouse calvarial bones indicated that periostin and Twist mRNA are co-localized at the osteogenic fronts of calvarial bones. To characterize the 5′ flanking region of the periostin gene, primer extension was carried out to identify the transcription start site, and DNA sequence analysis confirmed the presence of a 'Twist-box' response element. The results of electrophoretic mobility shift assay (EMSA) using nuclear extracts of MC3T3-E1 cells revealed that Twist bound to the Twist-box sequence on the periostin promoter. In vivo footprinting experiments using ligation-mediated PCR (LM-PCR) indicated that the Twist-box sequence was protected in undifferentiated MC3T3-E1 preosteoblasts but not in differentiated MC3T3-E1 osteoblasts. To determine whether Twist actually regulates the periostin expression, 293T cells were transiently co-transfected with the periostin promoter construct and the human Twist expression vector. Reporter analysis indicated that the periostin promoter activities were enhanced by overexpression of Twist. These data suggest that Twist can bind to the periostin promoter in undifferentiated preosteoblasts and up-regulate periostin expression, consistent with the up-regulation of periostin expression by Twist as observed in the gene-profiling data. J. Cell. Biochem. 86: 792–804, 2002.

Key words: periostin; Twist; osteoblast differentiation; transcriptional regulation

Periostin is a secreted protein that was isolated by using the techniques of subtraction hybridization and differential screening between cDNA libraries of MC3T3-E1 and NIH3T3 cells [Takeshita et al., 1993]. Mouse periostin comprises 811 amino acids, and has four repeats of a characteristic domain. A similar structure has been reported for fasciclin I, a homophilic cell–cell adhesion molecule expressed in the central nervous system of insects [Zinn et al., 1988], and for βig-h3, a molecule induced by transforming growth factor-β (TGF-β), which promotes fibroblast attachment and spreading [LeBaron et al., 1995]. Periostin was found to support the attachment and spreading of MC3T3-E1 cells, and this effect was impaired by anti-periostin antiserum, suggesting that periostin is involved in cell adhesion and recruitment [Horiuchi et al., 1999]. Immunohistochemistry in adult mice revealed that periostin is preferentially expressed in periosteum and periodontal ligament tissues. Periostin is also expressed in the mouse embryonic

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and fetal heart, where it is localized to the endocardial cushions [Kruzynska-Frejtag et al., 2001]. Moreover, the expression of periostin was increased by mechanical stress in periodontal ligament tissues [Wilde et al., 2000]. These findings indicate that periostin is temporally and spatially expressed in bone cells and may play a potential role in maintenance of structure. However, the mechanisms of transcriptional regulation of periostin are still poorly understood.

Basic helix-loop-helix (bHLH) transcription factors are important for cell type determination and differentiation during myogenesis [Olson, 1990], neurogenesis [Anderson et al., 1997], cardiogenesis [Thomas et al., 1998], hematopoiesis [Lister and Baron, 1998], and osteogenesis [Murray et al., 1992; Cserjesi et al., 1995]. These transcription factors form either homo- or heterodimers with other bHLH family proteins and bind to a core sequence E-box (CANNTG), on the promoter region of target genes through the basic region [Murre et al., 1989]. Twist, a member of the bHLH family protein, was originally identified in *Drosophila* as one of the zygotic genes required for dorsoventral patterning during embryogenesis [Simpson, 1983]. Twist-null mice die at embryonic day 11.5 due to defects in the head mesenchyme, somites, limb buds, and failure of neural tube closure in the cranial region [Chen and Behringer, 1995]. Twist-heterozygous mice display skull defects resulting in poorly developed squamosal bones and of over-developed intraparietal bones and limb abnormalities [Bourgeois et al., 1998]. It has been demonstrated that Twist expression is down-regulated during osteogenic cell differentiation. Results also suggest that Twist expression maintains cells in an osteoprogenitor or a preosteoblast-like state, thus preventing premature or ectopic osteoblast differentiation [Lee et al., 1999]. It has been reported that Twist protein is expressed early in the undifferentiated mesenchymal layer beneath the epidermis that will develop into a distinct dermal layer at a later stage [Fuchtbauer, 1995]. Twist protein expression was detected in secreting glandular tissues and tubules. These include chief cells in gastric glands that secrete enzymes. Twist expression was also detected in glandular epithelium, testicular seminiferous tubules, granulosa cells of ovarian follicles, renal collecting and secretory tubules, uterus glands, adrenal cortex, and bronchioles and alveoli of the lung [Lee et al., 2000]. In addition, immunohistochemistry demonstrated that Twist protein is expressed predominantly in the cytoplasm in many differentiated adult tissues [Lee et al., 2000]. In contrast, in undifferentiated embryonic tissues, Twist protein expression was mainly localized to the nucleus [Gitelman, 1997]. Although, the significance of this finding is not known at the present time, a number of studies have shown that nucleo-cytoplasmic trafficking constitutes an important regulatory mechanism for transcription factor activities. These results indicate that the function of Twist may regulate cell fate differently depending on the level of expression and cellular localization during embryo and adult tissue development [Lee et al., 2000]. Although, it is known that Twist negatively regulates osteoblast differentiation [Murray et al., 1992; Lee et al., 1999], the mechanisms by which Twist regulates osteoblast differentiation by activation of target genes is not clear. Therefore, the search for the target genes that are regulated by Twist is critical for understanding of the molecular mechanisms involved in transcriptional regulation during osteoblast differentiation.

According to gene expression profiling, Northern blot and reverse transcription-polymerase chain reaction (RT-PCR) analyses, periostin expression was up-regulated by Twist during osteogenesis. Because periostin and Twist are expressed in the undifferentiated mesenchymal cell lines, we performed RNA in situ hybridization of neonatal mouse calvarial bones. The results demonstrate that both Twist and periostin are co-expressed in the sutures of calvarial bones. To demonstrate the relationship between periostin and Twist, we investigated the 5′ flanking region of the periostin gene and the effects of Twist on periostin transcription. Results of these studies demonstrate that Twist binds to the periostin 5′ flanking region, and up-regulates transcription of periostin in undifferentiated and not in differentiated MC3T3-E1 cells.

MATERIALS AND METHODS

Antibodies

Rabbit polyclonal antibodies against human Twist were established as described previously [Lee et al., 1999], and they cross-react to mouse Twist.

Cell Cultures

MC3T3-E1 (the mouse calvaria-derived osteoblast-like cell line) was grown in alpha modified Eagle's medium (ICN Biomedicals, Aurora, OH). 293T (the human embryonal kidney cell line) was grown in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical Co, Tokyo, Japan). SaOS-2 (the human osteoblast-like sarcoma cell line) was grown in low glucose DMEM (Invitrogen, Carlsbad, CA). Each medium was supplemented with 10% fetal calf serum (FCS; INTERGEN, Purchase, NY), 100 U/ml penicillin G and 100 μg/ml streptomycin. In the differentiated condition, MC3T3-E1 cells were cultured with the addition of 10 mM disodium β-glycerol phosphate, 10 μg/ml L-ascorbic acid, and 4 μg/ml bovine insulin (Sigma, St. Louis, MO) as described previously [Glackin et al., 1992]. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

Preparation of Hybridization Probes for Microarray Analysis

Cy-3 and Cy-5 labeled cDNA probes were prepared from 2.5 μg of poly(A)$^+$ RNA purified from Twist sense and antisense transfected SaOS-2 cell lines using the Fastrack^{TM} 2.0 (Invitrogen) RNA purification kit. The poly(A)$^+$ RNA was resuspended in dH_2O and sent immediately to Incyte (Incyte Genomics, Palo Alto, CA). Incyte performed RT-PCR and annealed with 2 μl of oligo(dT) for 3–5 min at 65°C. RT-PCR was performed to produce cDNAs incorporating Cy-3 or Cy-5-dUTP labels. Both Cy-3 and Cy-5 labeled cDNAs were mixed and hybridized to the glass slides containing the cDNA arrays containing 10,000 human cDNAs and processed according to the protocol outlined by the TSA labeling kit (Perkin Elmer, Shelton, CT). Arrays were scanned immediately on the Axon 4000B scanner according to the manufacturer's instructions (Axon, Union City, CA). Data analysis was performed using the GenePix software package that is provided with the Axon 4000B scanner. All microarray data was downloaded to a browser. The investigator using the Incyte web-based analysis programs performed further statistical analysis of the microarray data according to methods described previously [Kerr et al., 2000; Kerr and Churchill, 2001a,b].

RT-PCR Analysis of the Periostin Expression in Twist Transfectants

Total RNA was isolated using Trizol reagent (Invitrogen). One microgram of total RNA from SaOS-2 transfected with TWIST sense or antisense construct was used for cDNA synthesis. The reaction was carried out at 42°C for 2 h in a 20 μl mix of 1 × avian myeloblastosis virus reverse transcriptase (AMV-RT) incubation buffer, 2.5 μM oligo(dT) primer, 1 mM each of dATP, dCTP, dGTP, and dTTP, 20 U of RNase inhibitor and 20 U of AMV-RT. After stopping the reaction by heating to 95°C for 5 min, the volume was diluted to 200 μl. Five microliters of reverse transcribed cDNA was amplified in the PCR reaction using a Taq polymerase amplification kit (Qiagen, Inc., Valencia, CA).

The primer sequences for human periostin gene were as follows: the forward primer, 5′-ATGATTCCCTTTTTACCCATGTTTTCTCTA-3′ and the reverse primer, 5′-GAAGGAATA-ATCATGCCATTTTTTAAGTCC-3′. The cycling conditions were as follows: initial denaturation at 94°C for 5 min, followed by 32 cycles at 95°C for 30 s, 55°C for 30 s, 72°C for 1 min, and finally, 72°C for 5 min. Amplified cDNA were separated on 1% agarose gels and the bands were visualized with ethidium bromide and photographed under translumination. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The primer sequences for human GAPDH gene were as follows: the forward primer, 5′-GGCAAATTCCATGGCACCGTCAA-3′ and the reverse primer, 5′-CAGCAGAGGGGG-CAGAGATGAT-3′. The result represents three independent experiments.

RNA In Situ Hybridization of Mouse Skull

Skulls from neonatal mice were isolated and fixed in 4% paraformaldehyde in Mg^{2+}/Ca^{2+}-free phosphate-buffered saline (PBS) at 4°C overnight. Skull tissues were dehydrated in a graded series of 25, 50, 75, and 100% methanol in PBST (PBS with 0.1% Tween-20). Tissue samples were rehydrated and bleached at 4°C for 2 h in a 4:1 mixture of PBST/30% hydrogen peroxide. They were then treated in PBST containing 14 μg/ml proteinase K (Roche, Indianapolis, IN) at 37°C for 20–30 min and then washed three times in PBST at 4°C for 10 min each. Samples were re-fixed in 0.2% glutaraldehyde/4% paraformaldehyde in PBST

at room temperature for 20 min. Riboprobes labeled with digoxigenin (DIG)-conjugated UTP (Roche) in the sense and antisense orientations were generated, respectively. Prehybridization and hybridization solution contained 50% formamide, 5 × SSC (pH 7.0), 50 μg/ml yeast total RNA, 1% SDS, and 50 μg/ml heparin. Samples were prehybridized for 3 h at 65°C followed by hybridization overnight at 65°C in the fresh hybridization solution containing 1 μg riboprobe/ml. Wash solutions were as follows: solution 1, 50% formamide, 5 × SSC (pH 7.0), 1% SDS; solution 2, 50% formamide, 2 × SSC (pH 7.0). Washes were carried out as follows: solution 1 for 60 min at 65°C twice; solution 2 for 60 min at 65°C twice. Samples were then washed with TBST (Tris-buffered saline, TBS, with 0.1% Tween-20) for 20 min five times and then incubated for 3 h in TBST containing 10% heat-inactivated lamb serum and 2 mM levamisole. Then, samples were incubated at 4°C overnight in 500 μl of 1:2,000 diluted preabsorbed anti-digoxigenin-AP Fab fragments (Roche). Samples were washed extensively in TBST containing 2 mM levamisole at room temperature, and then in NTMT [100 mM NaCl, 100 mM Tris (pH 9.5), 50 mM $MgCl_2$, 0.1% Tween-20] twice at room temperature for 20 min. Samples were incubated in BM purple AP substrate (Roche). Reactions were carried out at room temperature in the dark.

Primer Extension Analysis

Antisense oligonucleotides, complementary to nucleotides 36–59 of the mouse periostin cDNA sequence, were IRD-labeled at the 5′-end. This primer (1.5 pmol) was added to 5 μg of total RNA isolated from MC3T3-E1 cells in 20 μl hybridization buffer [50% formamide, 0.4 M NaCl, 40 mM PIPES (pH 6.4), 1 mM EDTA] and incubated at 80°C for 15 min. The labeled oligonucleotide and mRNA were hybridized at 25°C for 1 h. Reverse transcription was performed using the first-strand cDNA synthesis kit according to the manufacturer's instructions (Life Sciences, St. Petersburg, FL). The hybridized sample was reverse-transcribed using 25 U of AMV-RT at 42°C for 1 h. After completion of the reaction, the sample was extracted with phenol/chloroform and precipitated with ethanol. The primer extension product was dissolved in a denaturing dye solution and analyzed on a 4% polyacrylamide-urea gel.

DNA Sequencing

The nucleotide sequence of the mouse periostin primer extension product and its corresponding promoter sequence was determined using the dideoxynucleotide chain termination method [Sanger et al., 1977] and the Thermo Sequenase Cycle Sequencing Kit (USB, Cleveland, OH) on an automated fluorescence DNA sequencer LI-COR model 4000L (Aloka, Tokyo, Japan).

Transfection Assay

Cells were transfected by lipofection using the LIPOFECTAMINE 2000 reagent according to the manufacturer's instructions (Invitrogen). Cells were plated in 60-mm diameter tissue culture dishes to achieve 90–95% confluence in 24 h. Lipid and DNA were diluted separately in 500 μl each of DMEM containing 10% FCS without antibiotics and incubated for 5 min at room temperature. The two solutions were mixed and allowed to form complexes by incubating for 30 min at room temperature. Cells were washed with culture medium prior to the addition of lipid–DNA complexes. Cells were incubated with the complexes for 48 h under normal culture conditions.

Luciferase Assay

Cells were washed twice with PBS and 400 μl of Reporter Lysis Buffer (Promega, Madison, WI) were added. After incubation for 15 min at room temperature, cells were scraped and transferred into a microcentrifuge tube. Cell lysates were vortexed for 15 s and centrifuged at 12,000*g* for 2 min at 4°C to pellet the cell debris. Cell extracts (supernatant) were transferred into a new tube, and 20 μl of cell extracts were used to measure luciferase activities with 100 μl of Luciferase Assay Reagent (Promega). Luciferase activities were measured with a Lumitester K-100 (Kikkoman, Noda, Japan). To normalize luciferase activities for differences in transfection efficiency, pKM18, a β-galactosidase expression vector, was co-transfected with reporter vectors. One hundred fifty microliters of cell extracts were mixed well with equal volume of Assay Buffer [200 mM sodium phosphate buffer (pH 7.3), 2 mM $MgCl_2$, 100 mM β-mercaptoethanol, 1.33 mg/ml *o*-nitrophenyl-β-D-galactopyranoside] and incubated for 30 min at 37°C. The reactions were stopped by adding 500 μl of 1 M sodium carbonate. The absorbance was read at 420 nm, and β-galactosidase

activities were estimated from the standard curve prepared simultaneously.

Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared as described by Dignam et al. [1983]. The sequences of the Twist binding oligonucleotides used as a radioactive DNA probe were Twist-box (WT); 5′-AGGGGCATGTGTCTCT-3′ and Twist-box (MT); 5′-AGGGGCTTGGGTCTCT-3′, respectively. These oligonucleotides were radiolabeled with dATP by MEGALABEL (Takara, Otsu, Japan). The DNA binding reactions were performed at room temperature in a volume of 20 μl. Competition assays were performed in a total volume of 15 μl, with 1 × binding buffer [10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 4% glycerol, 100 mM NaCl, 5 mM ditiothreitol (DTT), 100 mg/ml bovine serum albumin (BSA)], 1 μg of poly (dI-dC), 0–200 fold excess of corresponding unlabeled probes and 4 μg of nuclear proteins. After incubation of the mixture for 20 min, 5 μl (2×10^5 cpm) of ^{32}P-labeled probes were added to the sample, and the mixture was incubated for 20 min. Supershift assays were performed in a total volume of 15 μl, with 1 × binding buffer, 1 μg of poly (dI-dC), 2×10^5 cpm of ^{32}P-labeled probes and 4 μg of nuclear proteins. After incubation for 20 min, human Twist polyclonal antibodies were added to the sample up to 20 μl, and the mixture was incubated for 20 min. All samples were electrophoresed on native 5% acrylamide gels prepared in Tris acetate–EDTA. The gels were then dried and exposed to X-ray films by autoradiography.

Alkaline Phosphatase Assay

MC3T3-E1 cells were cultured in differentiation media (10% FCS, αMEM, β-glycerol-phosphate, and ascorbic acid) in 12-well plates at a density of 7×10^4 cells/well. Cells cultured in the undifferentiated condition (10% FCS, αMEM) were cultured 1 day prior to the alkaline phosphatase (ALP) assays. Two wells from each condition were washed twice with PBS and lysed with 1ml of 0.1% Triton X-100 (per well). Fifty microliters of lysate was pipetted into a 96-well plate. This was done in triplicate for each sample. Two wells with only 50 μl of 0.1% Triton X-100 were used as blanks. Substrate (*p*-nitrophenyl phosphate; pNPP) was prepared as follows: 26.6 mM pNPP, 0.133 M carbonate buffer (pH 10.3), 1.33 mM $MgCl_2$. One hundred fifty microliters of this substrate was added to each sample (including blanks), and the absorbance was taken at 410 nm immediately and at 30 min intervals for 3 h. Total protein content was measured using the BCA Protein Assay Kit (Pierce, Rockford, IL). The ALP activity was calculated as the ratio of A410/total protein in μg/ml. The time point at which the A410 readings stabilized was chosen for this calculation (150 min in this case). The total protein content in each cell sample was extrapolated from the BSA standard curve.

LM-PCR Footprinting

Cell lines and DNA purification. MC3T3-E1 cells undifferentiated 0 days and differentiated for 8 days were cultured according to the conditions reported [Glackin et al., 1994] and assayed for ALP activity as previously described. Undifferentiated and differentiated MC3T3-E1 cells were treated with dimethyl suberimidate (DMS) in vivo for ligation mediated-PCR (LM-PCR) footprinting as described previously [Dai et al., 2000], then lysed and the DNA was purified and treated with piperidine according to the methods described previously [Tornaletti and Pfeifer, 1995]. Nested periostin oligonucleotide primers were designed and synthesized for LM-PCR in vivo footprinting according to the primer design program, ospX [Hillier and Green, 1991]. OspX was utilized to construct two primer sets with progressively increasing T_m and overlapping sequences. Primers consisted of (PerTwiR1, $T_m = 59.8°C$), 5′-ATCTCTCTGCCTTCTGTCTCTGCCAC-3′; (PerTwiR2, $T_m = 63.1°C$), 5′-ATCTCTCTGCCTTCTGTCTCTGCCACCAAC-3′; (PerTwiR3:IRD800, $T_m = 65.0°C$), 5′-TGCCTTCTGTCTCTGCCACCAACACTCTGTG-3′. The primers labeled PerTwiR1, PerTwiR2, and PerTwiR3, respectively, were used to map the non-transcribed strand of the periostin promoter region surrounding the Twist-box.

Hot start primer extension. The bottom premix (6.5 μl) contained 1 μl of DNA, 0.92 μl of 10 × buffer (supplied with cloned Pfu by Stratagene), 0.2 μl each of dATP, dCTP, dGTP, and dTTP (2.5 mM), 0.6 μl of 2 μM primer 1, and 3.18 μl of dH_2O. First, 1 μl of DNA was added manually after the rest of 5.5 μl of bottom premix was transferred to the tube by the Biomek® 2000 robotic workstation (Beckman Coulter, Fullerton, CA). Next, 7.5 μl of mineral oil and about 20 μl of warm ampligrease were added to top of the bottom premix. The warm ampligrease was set up by incubating petroleum jelly

in VWR heat block and transferred with a hair blower-preheated Biomek P200 pipette tip. Finally, 142 μl of mineral oil was pipetted on top of the petroleum jelly, followed by 3.5 μl of top premixes (0.2 μl of 2.5 U/μl Pfu Turbo, 0.08 μl of 10 × cloned Pfu buffer, 1.7 μl of 70% sucrose, and 1.52 μl of dH_2O). The hot start primer extension was then performed in the thermocycler by a denaturing step at 95°C for 5 min, an annealing step at the oligonucleotide T_m (calculated by ospX) of primer 1 (59.8°C) for 30 min, and a primer extension step at 76°C for 10 min.

Ligation step. After the primer extension step, about 95% of the suspension of ampligrease in mineral oil was removed. About 6.4 μl of ligation premix was transferred to the tube with the primer extension products and mixed well by the Biomek 2000 robotic workstation. The ligation premix contains the following: 0.4624 μl of 1 M Tris-HCl (pH 7.5), 0.1024 μl of 1 M $MgCl_2$, 0.32 μl of 1 M DTT, 0.1568 μl of 100 mM ATP, 0.0784 μl of 10 mg/ml BSA, 1.6 μl of 20 μM linker, 0.32 μl of 20 U/μl T4 DNA ligase (Promega), and 3.36 μl of dH_2O. The ligation was carried out at 17°C for 2 h or overnight.

Hot start PCR. Warm ampligrease was added directly to top of the ligation product and the pre-existing mixture of mineral oil and ampligrease. After adding an additional 142 μl of mineral oil, 3.5 μl of PCR premix was added to the top. The PCR premix consisted of 1.7 μl of 70% sucrose, 0.67 μl of 10 × Pfu cloned buffer, 0.11 μl of 25 mM dNTP, 0.11 μl of 20 μM primer 2, 0.11 μl of 20 μM LP25, 0.4 μl of 2.5 U/μl Pfu turbo, and 0.4 μl of dH_2O. The PCR conditions were: one cycle of 95°C for 2 min, T_m of primer 2 (63.1°C) for 2 min and 76°C for 3 min; 18 cycles of 95°C for 45 s, T_m of primer 2 for 2 min and 76°C for 3 min; and one cycle of 95°C for 45 s, T_m of primer 2 for 2 min and 76°C for 10 min. About 95% of the mixture of ampligrease and mineral oil was removed. One microliter of 1 U/μl *E. coli* exonuclease I (10 U/μl from USB diluted to 1 U/μl with 1 × PCR buffer) was added to the PCR products. The reaction tube(s) were put in the thermocycler first at 37°C for 30 min and then at 72°C for 15 min to inactivate the exonuclease. After exonuclease treatment, about 20 μl of warm ampligrease, 142 μl of mineral oil, and 3.5 μl direct labeling premix were added, in order, to the top of the exonuclease treated samples. The direct labeling premix contained 0.45 μl of dH_2O, 1.70 μl of 70% sucrose, 0.35 μl of 10 × Pfu cloned buffer, and 1 μl of 1 μM IRD-labeled primer. This step was performed under yellow light to protect the IRD-labeled primer, which is kept as dark and cold as possible between steps. The direct labeling was carried out in the thermocycler with conditions of 95°C for 2 min, five cycles of 95°C for 45 s, T_m of primer 3 (65.0°C) for 3 min, 76°C for 2 min, and 76°C for 10 min. Electrophoresis and scanning were performed in a LI-COR DNA sequencer according to the manufacturer's instructions (LI-COR, Lincoln, NE). The collected images were analyzed by Adobe® PhotoShop® and RFLP software programs.

RESULTS

Gene Profiling Studies

Microarray analysis is a powerful tool for the discovery of new Twist targets expressed in osteoblast differentiation. We performed gene expression profiling technology to gain insight into the role of Twist and its possible targets in the regulation of osteogenesis (Fig. 1A). The experimental system consisted of cultures of SaOS-2 cells, a human osteoblastic cell line, which overexpressed Twist in the sense (TS) or antisense (TAS) orientations. To identify Twist target genes that may have key functional roles in osteoblast differentiation, RNA was isolated from SaOS-2 cells transfected with TS and TAS and analyzed for representational differences by means of hybridization to a microarray from Incyte containing 10,000 human genes. Six different genes were identified as the positive clone whose expression was upregulated between 20- and 35-fold by Twist, and two different genes were identified as the negative clone whose expression was downregulated between two and sevenfold by Twist (data not shown). One of the targets that was up-regulated by Twist about 35-fold has been identified as a new early osteoblast adhesion molecule, periostin. For the validation of periostin targets detected in microarray, Northern blot (data not shown) and RT-PCR (Fig. 1B) analyses were performed using GAPDH as a control, comparing mRNA from SaOS-2 cells transfected with TS versus those transfected with TAS (Fig. 1B). The result demonstrated that the level of expression of periostin, an early marker of osteogenesis, was increased in TS cells compared with TAS cells.

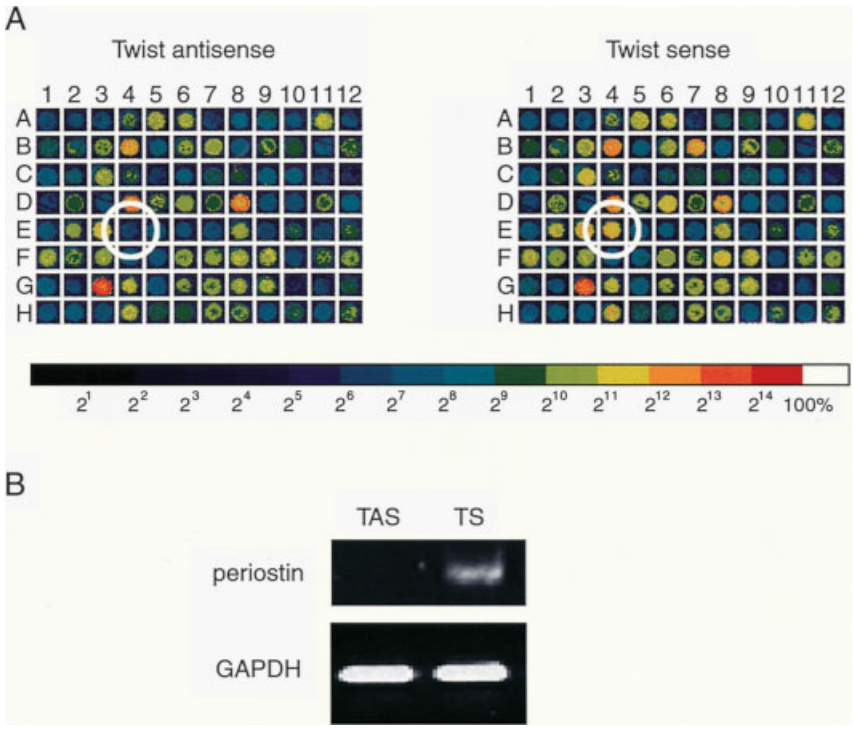

Fig. 1. Microarray analysis of the twist target genes. (**A**) Microarray analysis. Experimental procedures were detailed in Materials and Methods. The circle shows the periostin expression. (**B**) RT-PCR analysis of human periostin mRNA was performed using mRNAs from Twist sense overexpressing SaOS-2 (TS) and Twist antisense overexpressing SaOS-2 (TAS) cells. GAPDH was a control. The result represents three independent experiments.

Expression Patterns of Mouse Periostin and Twist mRNA in Calvarial Bone

Because it was demonstrated that Twist regulates expression of the periostin gene in the human, we further examined Twist and periostin expressions in the mouse in vivo. To compare the mRNA expression patterns of periostin with those of Twist in calvarial bone of neonatal mice, we performed RNA in situ hybridization. This result indicated that both periostin (Fig. 2A) and Twist (Fig. 2B) were co-expressed at the osteogenic fronts, sagittal suture, and coronal suture of calvarial bones.

Determination of the Mouse Periostin Transcription Start Site

To analyze the mouse periostin promoter region, we used a positive clone of 10 kb that we had previously isolated from a mouse genomic library using the plaque hybridization method (data not shown), that had been subcloned into pHSG396 [Takeshita et al., 1987]. To identify the transcriptional start site of the mouse periostin gene, we performed primer extension analysis. An antisense primer was extended on MC3T3-E1 total RNA. Then, the extension product was compared with a DNA sequence of a mouse genomic clone using the same primer. The result showed that only one potential site of transcription initiation was seen at −34 relative to the ATG translation start codon (Fig. 3A). To investigate regulatory *cis*-elements of mouse periostin transcription, we determined the mouse periostin promoter sequence and transcription factor binding sites by computer analysis. The results showed the presence of an E-box, a putative binding site for bHLH transcription factors, on the mouse periostin promoter (Fig. 3B). Moreover, this type of E-box (CATGTG) is thought to be a putative binding site for Twist. We have designated this putative binding site as 'Twist-box.' We also identified a putative TATA-box at positions −62 to −57 from the translation start site (Fig. 3B).

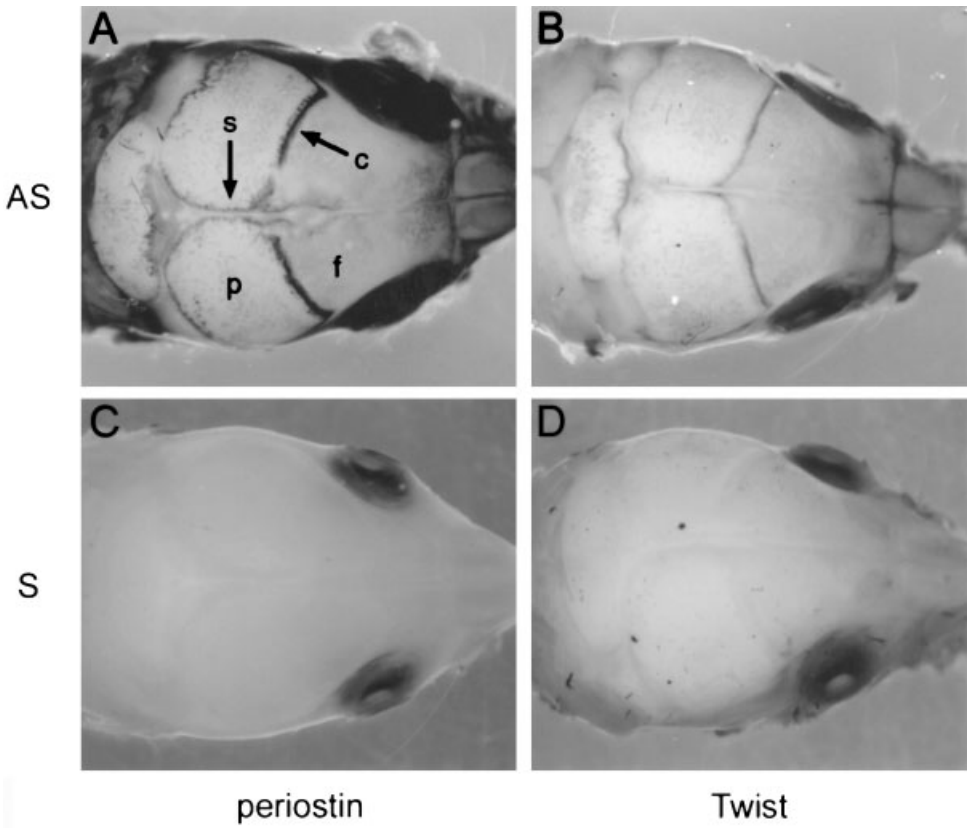

Fig. 2. RNA in situ hybridization of periostin and Twist in calvarial bone. Heads were isolated from C57BL/6 newborn mice, and their skins were removed. RNA in situ hybridization was performed by DIG-labeled antisense (**A**, **B**) or sense (**C**, **D**) RNA probes of periostin (A, C) and Twist (B, D). This result indicates that the periostin and Twist co-express at the osteogenic fronts of calvarial bones. c, Coronal suture; f, frontal bone; p, parietal bone; s, sagittal suture.

Promoter Activity of the 5′-Flanking Region of the Mouse Periostin Gene

To determine if the promoter region of the mouse periostin gene is responsible for transcriptional regulation by Twist, we transiently transfected MC3T3-E1 cells with the periostin promoter sequence (−563 to +1) or with promoter constructs with sequential deletions lacking the Twist-box, fused with pGL3-Basic luciferase reporter vector (Promega). The promoter activities were examined by analyses of luciferase expression in the transfected cell extracts, and the luciferase activity in the each extract was normalized by the activity of co-transfected β-galactosidase construct. Luciferase activity was significantly increased in all the periostin promoter constructs in comparison with control (Fig. 4). Moreover, the promoter construct containing the region −334 to −563 of the mouse periostin gene gave greater luciferase activity compared to the constructs lacking the Twist-box. Therefore binding of Twist to Twist-box on the mouse periostin promoter may enhance the activity of −563 construct. Because even the construct containing the shortest fragment of the periostin promoter possesses the transcriptional activity, the nucleotide sequence between −130 and +1 appears essential for basal transcription of the mouse periostin gene.

Binding of Twist to the Twist-Box on the Mouse Periostin Promoter

To determine if Twist binds to this putative binding site on the mouse periostin promoter, we performed electrophoretic mobility shift assay (EMSA). Radiolabeled oligonucleotides spanning the Twist-box sequence were incubated with MC3T3-E1 nuclear extracts. The results showed a band representing the DNA–protein complex (Fig. 5A, lane 2). To determine

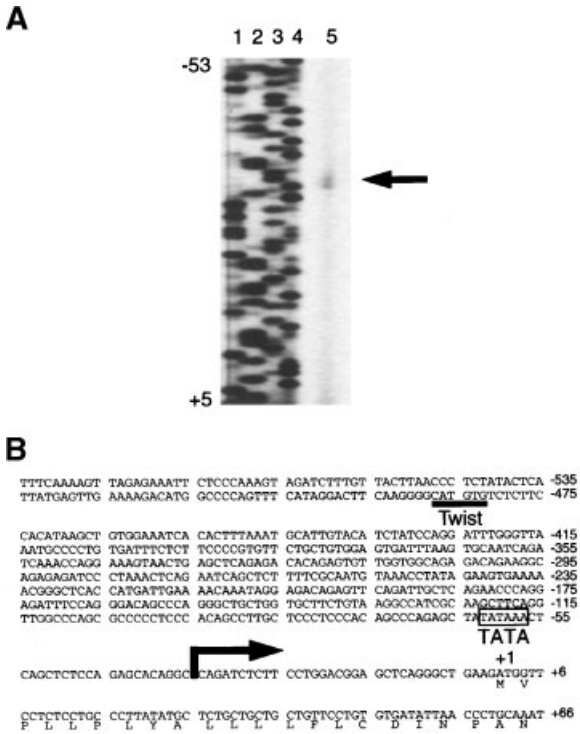

Fig. 3. Identification of the putative binding site of Twist on the mouse periostin promoter. (**A**) Identification of the mouse periostin transcription start site by primer extension. Antisense oligonucleotides complementary to nucleotides 5′-GGGTTAA-TATCACACAGGAACAGC-3′ of the mouse periostin cDNA sequence were used as a primer in a reverse transcription reaction using total RNA isolated from MC3T3-E1 cells. Primer extension analysis revealed the transcription of the mouse periostin is initiated at −34 (**lane 5**; indicated with arrow), positioned due to the translation start site, +1. **Lanes 1–4**, corresponding to T, G, C, and A respectively, represent the nucleotide sequencing ladders of pKT61, containing 5′ flanking region and first exon of the mouse periostin gene. (**B**) The promoter sequence of the mouse periostin gene. An arrow indicates the transcription start site. Twist-box, a putative binding site of Twist, is indicated by underline. The putative TATA-box sequence was boxed. The translation start site was indicated by ''+1''.

the specificity of binding, we added unlabeled oligonucleotides to the reaction mixture. The results showed that unlabeled competitors inhibited binding of protein to radiolabeled oligonucleotides dose-dependently (Fig. 5A, lanes 3–5). To determine if the shifted band was due to binding of Twist protein to radiolabeled oligonucleotides, we added polyclonal antibodies against Twist to the reaction mixture. The result showed that supershifted bands (SS) appeared and that the signals increased dose-dependently (Fig. 5B, lanes 3–5). Further analyses were performed by using mutated oligonucleotides. When mutated oligonucleotides

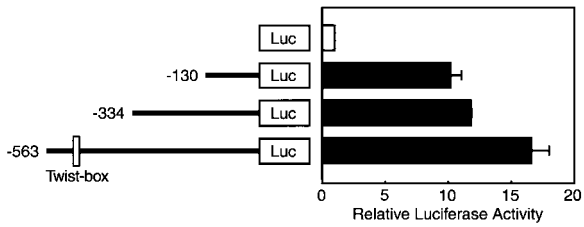

Fig. 4. Transient expression analyses of the promoter-regulatory region of the mouse periostin gene. Luciferase expression vectors were generated by inserting the periostin transcription start site and various portions of the 5′ flanking region to the promoterless luciferase vector, pGL3-Basic. These expression vectors were transfected into MC3T3-E1 cells. This result indicates that all these portions of periostin promoter have basal transcriptional activity. The pKM18, an expression vector of β-galactosidase, was co-transfected with luciferase expression vectors for measuring transfection efficiency utilized for normalization of luciferase activities. The results are the means ± SD and represent more than three independent experiments in triplicate for each fragment.

were used as an unlabeled competitor, the shifted band did not disappear (Fig. 6A, lane 4). Alternatively, when mutated oligonucleotides were used as a labeled probe, the shifted band did not appear (Fig. 6B, lane 4). Taken together, Twist protein can specifically bind the putative binding site, the Twist-box, on the mouse periostin promoter in vitro.

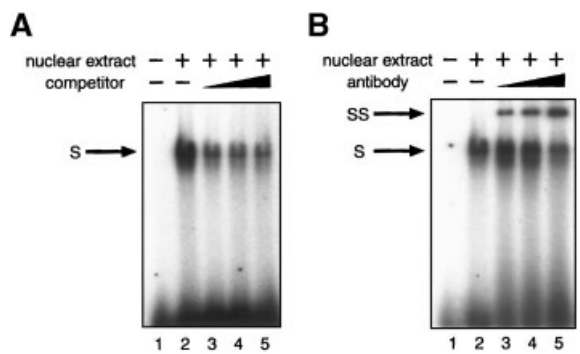

Fig. 5. Binding of Twist to Twist-box sequence in the mouse periostin promoter analyzed by EMSA. Radiolabeled oligonucleotides spanning the Twist-box sequence (**lanes 1–5**) were incubated in the absence (lane 1) or the presence (lanes 2–5) of MC3T3-E1 nuclear protein. (**A**) Competition assays were performed by adding 0- (lane 2), 50- (lane 3), 100- (lane 4), and 200- (lane 5) fold excess of corresponding unlabeled oligonucleotides. DNA-protein complex (S; indicated with arrow) decreased by competition. (**B**) Supershift experiments were performed by adding 0 (lane 2), 2 (lane 3), 4 (lane 4), and 8 (lane 5) μl of anti-Twist polyclonal antibodies. Bands of DNA–protein complex (S) and supershifted complex (SS) are indicated with arrows. This result indicates that Twist can bind to the Twist-box sequence in the mouse periostin promoter.

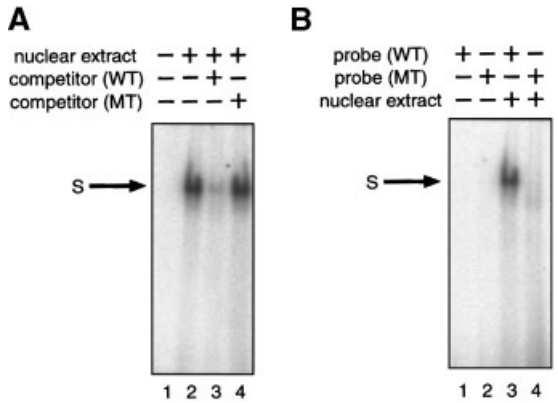

Fig. 6. Inhibitory effects of Twist binding to mutated oligonucleotides. Mutated oligonucleotides spanning the Twist-box sequence (**lanes 1–4**) were incubated in the absence (lane 1) or the presence (lanes 2–4) of MC3T3-E1 nuclear protein. (**A**) Competition assays using wild-type (WT) or mutated (MT) oligonucleotides as unlabeled competitors were performed. In this assay, 200-fold excess of unlabeled competitors were used. DNA–protein complex (S; indicated with arrow) did not decrease by competition with MT oligonucleotides (lane 4). (**B**) EMSA using wildtype (WT) or mutated (MT) oligonucleotides as labeled probes were performed. MT oligonucleotides did not form DNA–protein complex (lane 4). This result indicates that Twist can bind specifically to the Twist-box sequence in the mouse periostin promoter.

In Vivo Footprinting Experiment on the Mouse Periostin Promoter

To further determine if Twist binds to the Twist-box on the periostin promoter in vivo, we performed LM-PCR in vivo footprinting experiments. To confirm the cellular stage of MC3T3-E1 cells in the undifferentiated or the differentiated growth conditions, ALP activities were measured (Fig. 7A). While ALP activities in MC3T3-E1 cells cultured in the proliferated condition for 6 days showed almost the same ALP activity level, MC3T3-E1 cells cultured in the differentiated condition for 6 days dramatically increased ALP activity. These results confirm that MC3T3-E1 cells used for the LM-PCR in vivo footprinting studies were undifferentiated preosteoblasts and differentiated osteoblasts. We examined the twist expression in undifferentiated preosteoblasts and differentiated osteoblasts by EMSA. The result showed that the amount of the twist protein in differentiated osteoblasts was much less, compared with that in undifferentiated preosteoblasts, because the band of Twist protein–DNA complex was hardly detected (data not shown). MC3T3-E1 cells in both cell states, the undifferentiated preosteoblasts and the differentiated osteoblasts, were treated with DMS. Automated LM-PCR footprinting analyses of the mouse periostin promoter sequence from −360 to −535 were performed. Control DNA was also treated with DMS without proteins bound as "in vitro" controls. The result in vivo indicated that protection of the Twist-box sequence was observed in undifferentiated MC3T3-E1 preosteoblasts but not in differentiated MC3T3-E1 osteoblasts (Fig. 7B). Especially, the signals of two guanines in the Twist-box, CATGTG, were enhanced in differentiated cells in vivo (Fig. 7B, lane 3) compared to the in vitro control (Fig. 7B, lane 4), however, those signals in undifferentiated cells in vivo (Fig. 7B, lane 1) were significantly reduced. No significant changes at the Twist-box site were observed in the in vitro controls. These results suggest that Twist regulates periostin expression in undifferentiated MC3T3-E1 cells by binding to the DNA element at the "Twist-box" on the periostin promoter on the in vivo protein bound DNA.

Up-Regulation of Mouse Periostin Transcription by Twist Overexpression

Having established that Twist binds to the mouse periostin promoter, we determined the functional role of Twist in regulating the activity of mouse periostin transcription in MC3T3-E1 osteoblasts. The 563-bp periostin promoter-luciferase reporter construct containing the Twist-box sequence (Fig. 4) was transiently co-transfected into 293T cells together with pcDNA3 TWI-S vector containing human Twist [Lee et al., 1999]. The results in Figure 8 demonstrate that Twist up-regulates mouse periostin transcription by the enhanced transcriptional activity of the luciferase reporter construct containing the "Twist-box" compared to the control vector, pcDNA3, in Twist overexpressing 293T cells.

DISCUSSION

The search for target genes that are regulated by Twist is important for understanding the mechanism of bone development at the molecular level. Our results from the microarray and the binding protein analyses clearly demonstrate that periostin is a target of Twist. The periostin gene is selectively expressed in periosteum and periodontal ligament, and periostin protein is secreted and localized in the

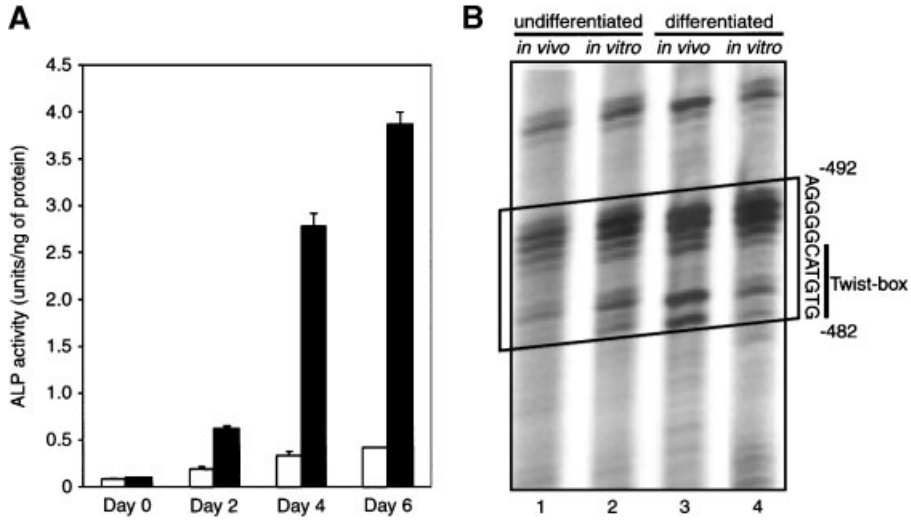

Fig. 7. LM-PCR in vivo footprinting experiments in undifferentiated and differentiated MC3T3-E1 cells. (**A**) ALP activities in MC3T3-E1 cells were measured to examine the cell state, the undifferentiated or the differentiated. The clear bars represent ALP activities with MC3T3-E1 cells cultured in the proliferated condition. The black bars represent ALP activities with MC3T3-E1 cells cultured in the differentiated condition. These results validate the cell states of undifferentiated and differentiated MC3T3-E1 cells prepared for LM-PCR in vivo footprinting. The results are the means ± SD and represent six independent experiments. (**B**) In vivo and in vitro footprinting analyses of the mouse periostin promoter sequence from −360 to −535. This result indicates that protection of the Twist-box sequence was observed in undifferentiated MC3T3-E1 preosteoblasts and was not observed in differentiated MC3T3-E1 osteoblasts.

extracellular matrix [Horiuchi et al., 1999]. Although, the function of periostin in these tissues is still unknown, recent results demonstrated that periostin expression was upregulated by the mechanical stress in the periodontal ligament [Wilde et al., 2000]. Periodontal ligament shows constant adaptive remodeling of the fetal-like soft tissue, and is poorly mineralized. If the function of periostin in periodontal ligament tissue is the negative regulation of osteoblast differentiation, so called anti-mineralization, this is consistent with periostin being a target gene of Twist. Twist behaves as a negative regulator in osteoblast differentiation in vitro; therefore, cells overexpressing Twist remain in an undifferentiated, osteoprogenitor-like state, and cells expressing Twist antisense progress to more differentiated, mature osteoblasts [Lee et al., 1999].

The Twist-box is located between −334 and −563 on the mouse periostin promoter. Luciferase activity was enhanced by the promoter fragment containing the Twist-box (−563 compared with the −334 promoter fragment), although, the level of enhancement is not high, only 1.4-fold. This is consistent with the reporter assay using the 293T cells. Twist may not be able to bind efficiently to the promoter region of periostin due to competition with other transcription factors, or the indirect regulation of Twist, controlling positive or negative transcriptional activity, may exist. However, in the calvarial bones of neonatal mice, Twist and periostin expression is co-localized to undifferentiated cells of mesenchymal origin at osteogenic

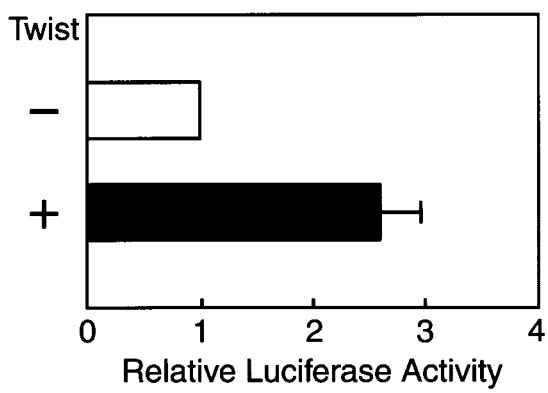

Fig. 8. Overexpression of Twist transactivates the reporter genes consisting of the periostin promoter. About 293T cells were transiently co-transfected with the 563-bp periostin promoter-luciferase reporter construct as shown in Figure 4, the β-galactosidase expression vector and either the human Twist expression vector (black bar) or a control vector (pcDNA3; white bar). This result indicated that the luciferase activity was increased by overexpression of Twist. The luciferase activity was measured, and was normalized by β-galactosidase activity. The results are the means ± SD and represent more than three independent experiments in triplicate for each fragment.

fronts and sutures. Therefore, it is possible that Twist expression maintains cells in an osteoprogenitor-like state by enhancing periostin expression.

Further investigations into the functions of periostin and Twist in the embryo and the adult, as well as the comparison of the phenotypes of periostin-null mice with that of Twist-deficient mice should broaden our understanding of the interactions of Twist and periostin during bone development.

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