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Twist Negatively Regulates Osteoblastic Differentiation in Human Periodontal Ligament Cells

Motohiro Komaki,[1]* Takeo Karakida,[3] Mayumi Abe,[1] Shinichiro Oida,[3] Kaori Mimori,[2] Kengo Iwasaki,[2] Kazuyuki Noguchi,[2] Shigeru Oda,[2] and Isao Ishikawa[2]

[1]Department of Nanomedicine (DNP), Tokyo Medical and Dental University Graduate School, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8549, Japan
[2]Periodontology, Department of Hard Tissue Engineering, Tokyo Medical and Dental University Graduate School, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8549, Japan
[3]Department of Chemistry, School of Dentistry, Tsurumi University, 2-1-3 Tsurumi, Tsurumi-ku, Yokohama city, Kanagawa 230-8501, Japan

Abstract Periodontal ligament (PDL) is a thin fibrous connective tissue located between two mineralized tissues, alveolar bone and cementum, which maintains a constant width physiologically. The mechanisms by which PDL resists mineralization are not well understood. Twist is a basic helix loop helix protein that plays a central role in regulation of early osteogenesis. We investigated the localization of Twist in PDL and compared the expression of Twist and osteoblast-related genes in PDL cells with those in osteoblast-like cells in the presence or absence of recombinant human bone morphogenetic protein (BMP)-2. Histochemical analysis showed that Twist was expressed along alveolar bone surface in PDL. PDL cells constitutively expressed *Twist* gene and the expression level was higher than that in osteoblast-like cells. In osteoblast-like cell culture, BMP-2 enhanced osteoblast-related gene expression, while Twist expression was slightly decreased. In contrast, BMP-2 increased runt-related transcription factor (Runx)-2, but failed to enhance alkaline phosphatase (ALP) and osteocalcin (OCN) gene expression in PDL cells. Interestingly, unlike in osteoblast-like cells, Twist expression was upregulated by BMP-2 in PDL cells. We transiently knocked down *Twist* gene in PDL cells using a short interference RNA expression vector (siTwist) and found that ALP, osteopontin (OPN), bone sialoprotein (BSP) genes expression and basal level of ALP activity were slightly increased, whereas *Runx2* and *OCN* genes were not affected. Collectively, these results suggest that Twist may act as a negative regulator of osteoblastic differentiation in PDL cells. J. Cell. Biochem. 100: 303–314, 2007.

Key words: periodontal ligament cells; osteoblasts; Twist; BMP; siRNA

Periodontal ligament (PDL) is a highly vascular and cellular thin fibrous connective tissue interposed between two mineralized tissues, that is, alveolar bone and cementum of tooth root. The principal function of PDL is to anchor the tooth to alveolar bone and to absorb mechanical stress, such as mastication or orthodontic forces during orthodontic tooth movement [Shore and Berkovitz, 1978, 1979; Shore et al., 1985, 1991]. Despite the mechanical stress, the PDL preserves a thin space and remains unmineralized under physiological conditions. PDL cells are fibroblastic cells which share some markers such as runt-related transcription factor (Runx)-2, alkaline phosphatase (ALP), and type I collagen with osteoblasts [Saito et al., 2002]. Previous reports including one from our group have shown that PDL cells are heterogeneous and that PDL cells have cementoblasts and/or osteoblast subpopulations which are capable of forming mineralized nodules in vitro under certain conditions [Cho et al., 1992; Lekic and McCulloch, 1996; Lekic et al., 2001; Murakami et al., 2003]. Recently, Seo et al. [2004] reported that cells released from PDL expressed stem cell markers such as STRO-1 and CD146/MUC18

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*Correspondence to: Motohiro Komaki, Department of Nanomedicine (DNP), Tokyo Medical and Dental University Graduate School, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8549, Japan. E-mail: komaki.peri@tmd.ac.jp

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and formed cementum/PDL-like structure when transplanted into subcutaneous pocket of immunocompromised rodents, suggesting PDL contains stem-cell like population. Therefore, PDL cells are thought to play a crucial role in maintenance and regeneration of periodontium. Runx2 plays an essential role in the early differentiation of osteoblasts and bone formation. It has been reported that Runx2 is expressed during tooth and craniofacial tissues [Yamashiro et al., 2002; Aberg et al., 2004]. Recently, Saito et al. [2002] reported that a cell line mimicking the gene expression of PDL, PDL-L2 showed high-Runx2 expression in vivo, but never formed mineralized nodules in differentiation medium containing 1 μM dexamethasone, 10 mM β-glycerophosphate, and 50 μg/ml ascorbic acid, suggesting that the function of Runx2 was suppressed in PDL cells. Recently, Yoshizawa et al. [2004] reported that PDL-L2 expressed homeobox protein Msx2 and that Msx2 expression was higher in PDL-L2 than in osteoblasts. Msx2 was co-localized with Runx2 and suppressed Runx2 transcriptional activity in PDL-L2 [Yoshizawa et al., 2004]. Taken together, these results suggest that PDL has regulatory mechanisms to prevent PDL cells from differentiating into osteoblasts in vitro and mineralizing in vivo. However, the regulatory mechanisms that control osteoblastic differentiation in PDL are not fully understood.

Bone morphogenetic protein (BMP) is a member of the transforming growth factor (TGF)-β family which stimulates osteoblastic differentiation in both osteoblast progenitor cells and cells from non-osteogenic lineage such as fibroblasts and myoblasts [Katagiri et al., 1990, 1994; Komaki et al., 1996]. Since BMPs are powerful inducers of osteogenesis and are involved in development of the tooth root [Thomadakis et al., 1999; Yamashiro et al., 2003], they may have therapeutic potential for periodontal regeneration [Ripamonti et al., 1994, 1996]. In spite of the ability of BMP to induce ectopic bone formation in skeletal muscle, BMP did not induce bone formation in PDL leading to cementum-alveolar bone fusion, namely ankylosis [Rajshankar et al., 1998]. Maintenance of Msx2 at a higher level prevented a PDL cell line, PDL-L2 from forming mineralized nodules, however PDL-L2 formed mineralized nodules when Msx2 expression was downregulated by BMP-2 [Yoshizawa et al., 2004]. It is still unknown how PDL cells resist BMP-induced osteoblastic differentiation in vitro and bone formation in vivo.

Twist is a basic helix loop helix (bHLH) protein that plays a central role in cell type determination and differentiation and has been shown to regulate early osteogenesis [Lee et al., 1999]. It has been also reported that Twist is expressed in cranial sutures and that a mutation of *Twist* gene results in premature fusion of the cranial sutures, craniosynostosis [El Ghouzzi et al., 1997, 1999, 2000; Carver et al., 2002]. Twist and Msx2 have been reported to control skeletogenic mesenchyme co-operatively in mice [Ishii et al., 2003] and a mutation of *Msx2* gene also causes craniosynostosis. Lee et al. [1999] reported that the level of *Twist* gene could influence osteogenic gene expression. It has been also reported that a transfection of muscle-specific bHLH protein, MyoD, which binds to Twist through basic region, enhanced BMP-induced osteoblastic differentiation in fibroblasts [Hamamori et al., 1997; Komaki et al., 2004]. Bialek et al. [2004] reported that Twist interacted with the DNA-binding domain of Runx2 to inhibit its function, without altering its expression. Afanador et al. [2005] reported that Twist was expressed in mice PDL and its expression was transiently decreased by occlusal hypofunction, suggesting the functional role of Twist in PDL. However, the function of Twist in human PDL is entirely unknown.

Based on these observations, we hypothesized that Twist might be involved in regulation of osteoblastic differentiation of human PDL cells. To test this hypothesis, we first checked Twist localization in rat PDL by immunohistochemistry. We then compared Twist and osteoblast-related genes in human PDL cells with those in human osteoblast-like cells, Saos-2 in the absence or presence of BMP-2. Finally we examined the effect of *Twist* gene suppression on osteoblastic differentiation in human PDL cells by transient transfection of short interference RNA (siTwist) expression vector. Histochemical analysis showed that Twist was localized along alveolar bone surface in rat PDL. Real-time polymerase chain reaction (PCR) analyses demonstrated that Twist mRNA expression was differentially regulated in human PDL cells compared to osteoblast-like cells. We also found that the transient transfection of siTwist slightly increased ALP, osteopontin (OPN), and bone sialoprotein (BSP) gene expression and significantly stimulated

basal ALP activity in PDL cells. These results suggest that Twist may act as a negative regulator of osteoblastic differentiation in human PDL cells.

MATERIALS AND METHODS

Growth Factor

Recombinant human BMP-2 was kindly provided by Astellas Pharma Inc. (Tokyo, Japan).

Cell Cultures

Human PDL cells, obtained from patients undergoing therapeutic third molar extraction or extraction of premolars for orthodontic reasons, were retrieved as described previously [Ishikawa et al., 2004]. All procedures were performed with informed consent from the subjects, and the Ethics Committee of Tokyo Medical and Dental University approved the protocol. Briefly, extracted teeth were rinsed twice with phosphate-buffered saline (PBS) supplemented with 3% antibiotic-antimycotic. PDL tissue attached to the middle-third of the root was removed carefully with a surgical scalpel. The PDL tissue was minced and placed in a 6-well culture plate. The explants were then covered with sterilized glass cover slips and kept in Dulbecco's modified Eagle medium (D-MEM) high glucose (Wako Pure Chemical Industries, Ltd., Osaka, Japan) with 10% fetal bovine serum (FBS) at 37°C in 5% CO_2 in humidified air, until cells grew out of the explants and reached confluent. The cells were then trypsinized and cells from passages three to eight were used in subsequent experiments. Human osteogenic sarcoma cell line, Saos-2, a gift from Dr. Suzuki, N., Nihon University, Tokyo, Japan was cultured in D-MEM supplemented with 10% FBS.

For osteoblastic differentiation, cells were inoculated at the cell density of 2×10^4 cells/cm^2 in D-MEM supplemented with 10% FBS (growth medium) and incubated for 24 h. The medium was replaced by D-MEM containing 5% house serum (differentiation medium) with 250 ng/ml of BMP-2. The medium was changed every 3 days.

Construction and Transfection of siTwist Expression Vector

Before construction of siTwist expression vectors, two siRNA for human Twist, Silencer™ Pre-designed siRNA were purchased from Ambion, Inc. (Austin, TX). Their inhibitory effects on Twist in PDL cells were tested by real-time PCR (Roche Diagnostic, Tokyo, Japan) according to manufacturer's instructions. Briefly, $2.5 \times 10^4/cm^2$ of PDL cells were inoculated and incubated for 24 h in growth medium. 20 μM of each pre-annealed siRNA oligonucleotide was mixed with 15 μl of lipofectamine 2000 reagent (Invitrogen Co., Tokyo, Japan) in total 1 ml of OPTI-MEM® I Reduced-Serum Medium and pre-incubated for 5 min at room temperature to make a complex, and added to the PDL cell culture. Cells were incubated at 37°C in 5% CO_2 in humidified air for 6 h. And then, the transfection reagents were withdrawn from the culture, and cells were incubated over night in growth medium. For BMP treatment, the growth medium was replaced with differentiation medium containing BMP and cultured for another 3 days. According to sequence data provided, we purchased siRNA oligonucleotides with restriction enzyme sites (Apa I and EcoR I), annealed and extended at 45 and 72°C, respectively for 5 min. The annealed siRNA with Apa I and EcoR I sites were cloned into pSilencer 1.0-U6 (Ambion, Inc.). To knock down Twist mRNA in PDL cells, $2.5 \times 10^4/cm^2$ of PDL cells were inoculated and transfected with either an siTwist expression vector or an empty vector. Twist mRNA expression was checked by real-time PCR.

ALP Activity

For quantitative analysis of ALP activity, the culture medium was removed and cell layers were rinsed twice in ice-cold PBS and lysed in Tris buffered saline containing 1% Triton- X-100 and 1% phenymethylsulfonyl fluoride (PMSF). Then cell lysate was assessed at 37°C for an hour in the buffer containing 0.1 M 2-amino-2-methyl-1, 3-propanediol-HCl, and 5 mM $MgCl_2$ (pH 10.0) using 10 mM ρ-nitrophenylphosphate as a substrate. The reaction was stopped by adding 0.5 N NaOH. The absorbance was read at 405 nm. The enzyme activity was expressed as micromoles of ρ-nitrophenol produced per minute per milligram of protein. Protein concentration was determined using BCA Protein Assay kit (TaKaRa, Tokyo, Japan).

Histochemical Analyses for Twist and ALP Activity

To analyze expression of Twist and ALP activity in vitro, the cells were fixed for 10 min

at room temperature with 4% formaldehyde in PBS and treated with ice-cold acetone/ethanol (v/v = 50/50) for a minute to permeate the cell and nuclear membrane. Endogenous peroxidase was inhibited with 3% H_2O_2 in methanol for 5 min at room temperature. Cells were incubated with the primary antibody, rabbit anti-Twist polyclonal IgG (Santa Cruz Biotechnology, Inc.) for 2 h at room temperature. Cells were then rinsed with PBS, and incubated with the secondary antibody, simple stain MAX-PO (MULTI) (Nichirei, Tokyo, Japan) for 30 min at room temperature. After rinsing with PBS, cells were incubated with naphthol AS-MX phosphate (Sigma-Aldrich, Tokyo, Japan), 0.5% N, N-dimethylformamide, 2 mM $MgCl_2$, and 0.6 mg/ml fast blue BB salt (Sigma-Aldrich) in 0.1 M Tris-HCl (pH 8.7) at room temperature for 20 min. Twist was visualized using simple stain AEC solution (Nichirei) for 5 min at room temperature. Twist and ALP staining were evaluated under light microscope.

Tissue Preparation for Histochemistry

C57BL/6J mouse, 35 days old, was generous gift from Dr. Sata, M (The University of Tokyo, Tokyo, Japan) and was perfused transcardially with 4% paraformaldehyde in PBS under ether anesthesia. The mandibles were dissected out, fixed by agitating in 4% paraformaldehyde in PBS for 48 h, trimmed and then decalcified in 10% EDTA (pH 7.2) at room temperature for a week. Specimens were dehydrated and embedded in Technobit 8100® (Heraeus Kulzer, Wehrheim, Germany). Serial sections of 4 μm thickness were prepared in the bucco-lingual plane. Sections were blocked with 0.5% goat serum in PBS at room temperature for 15 min and incubated with the primary antibody, rabbit anti-Twist polyclonal IgG at 4°C overnight. The sections were washed three times with PBS for 5 min each, and treated with the secondary antibody, biotin conjugated-goat anti-rabbit IgG antibody at room temperature for an hour. The specimens at intervals of 60 μm were visualized with Vecstain® ABC-AP kit (Vecter Laboratories, Inc., Burlingame, CA) at room temperature for an hour, counter–stained with Harris's hematoxyline and evaluated under a microscope.

Real-Time PCR

Total RNA was prepared using ISOGEN (Nippon Gene, Tokyo, Japan) according to manufacturer's instructions. Two micrograms of total RNA was used for cDNA synthesis using Ready-To-Go You-Prime First-Strand Beads, according to manufacturer's instructions. Quantitative real-time PCR was carried out using Light Cycler™ FastStart DNA Master SYBR Green I, Light Cycler™ Primer set for Human GAPDH, Runx2, ALP, OPN, BSP, osteocalcin (OCN), and Twist (Roche Diagnostic) according to the manufacturer's protocol. Amplification conditions were 95°C for 10 min for denaturing, 35 cycles at 95°C for 10 min, 68°C for 10 min, 72°C for 16 min followed by melting curve from 58 to 95°C. Reaction product was quantified with the Light Cycler Software Ver. 3.5 (Roche Diagnostic). The value was normalized to internal control, GAPDH.

Statistical Analysis

The effect of siTwist on basal ALP activity in human PDL were analyzed for statistical differences by Fisher's protected least significance (PLSD) test in the post hoc comparison of specific groups. Differences with $P < 0.01$ were considered significant. The values represent the mean with the error bar (standard deviation) derived from triplicate results.

RESULTS

Twist Expression in Periodontal Ligament In Vivo

As the first step, we performed histochemical analysis to determine whether Twist was expressed in murine PDL. Twist (stained in red) was detected in PDL sparsely and the staining was intense along the surface of alveolar bone (Fig. 1A). Twist was also detected in dental pulp. However, Twist was very weak or undetectable in cementum, dentin, gingival, and alveolar bone.

Twist Gene Expression in PDL Cells

PDL cells are fibroblast-like cells and predominant in PDL. Since Twist was detected in PDL, we compared Twist expression in human PDL cells to that in an osteoblast-like cell line, Saos-2, using real-time-PCR. Cells were inoculated and cultured in growth media for 24 h (day 0) and the medium was replaced with differentiation medium and cultured for 3 and

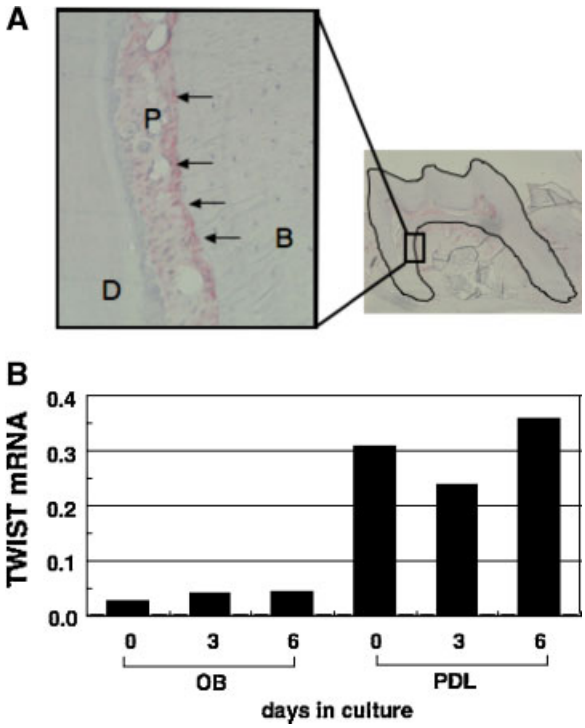

Fig. 1. Twist was preferably expressed in periodontal ligament. **A**: Immunohistochemical analysis of murine Twist in mandibular first molar. The image was digitally captured. The first molar was outlined with a solid line. Square area was magnified in left. (Note the intense staining of murine Twist was distributed along the alveolar bone surface (arrows)). D, dentin of tooth root; B, alveolar bone; P, periodontal ligament. **B**: Expression of Twist mRNA in human periodontal ligament (PDL) cells and human osteoblast-like cells (OB) analyzed by real-time PCR. Cells were inoculated in growth medium (day 0) and cultured in differentiation medium for 3 and 6 days. Data present one of three independent experiments with similar results. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

6 days. Real-time PCR analysis showed that the expression of Twist in PDL cells was higher than that in osteoblast-like cells over the culture period (Fig. 1B). These results were consistent with the localization of Twist in periodontal tissues, predominantly along alveolar bone surface in PDL.

Twist is Differentially Regulated in PDL Cells

Twist has been previously shown to interact with osteoblast-specific transcription factor, Runx2 to inhibit its function in osteoblasts, resulting in preclusion of osteoblastic differentiation [Bialek et al., 2004]. We first examined the effects of a strong osteogenic inducer, BMP-2, on osteoblast-like cells, Saos-2. When the cells reached confluent (day 0), the medium was replaced by differentiation medium supplemented with 250 ng/ml of BMP-2 and cultured for 3 and 6 days. Figure 2A shows a double staining for Twist (brown) and ALP activity (blue) in osteoblast-like cells (3 days). In control culture, osteoblast-like cells expressed both early osteoblastic differentiation marker, ALP and Twist. When osteoblast-like cells were treated with BMP-2, ALP activity was markedly increased, while Twist expression was decreased (Fig. 2A,B). Interestingly, ALP activity and Twist expression appeared mutually exclusive in control culture. Quantitative analyses for Twist and osteoblast-related genes by real-time PCR showed that osteoblast-specific transcription factor, Runx2 was increased by BMP-2 on day 3, and the expression level was maintained until day 6. ALP and OCN, early and late markers for osteoblastic differentiation, were upregulated in a time-dependent manner. In contrast, Twist expression was decreased in the presence of BMP-2 on day 3 in osteoblast-like cells (Fig. 2A,B). The decrease of Twist expression by BMP-2 was clear on day 3, but became slight on day 6 (Fig. 2B). Taken together, these results suggest that Twist may inhibit early osteoblastic differentiation in osteoblast-like cell culture. PDL cells are thought to play important roles such as self-renewal and regeneration in PDL. In fact, PDL cells formed bone-like nodules when cells were cultured in defined differentiation medium [Arceo et al., 1991]. In spite of osteogenic potential of PDL cells in vitro, it has been reported that a powerful osteogenic inducer, BMP-2 does not induce osteoblastic differentiation, and does not alter the width of PDL in vivo [Rajshankar et al., 1998]. Therefore, we also examined the effects of BMP-2 on Twist and osteoblast-related gene expression as well as ALP activity in PDL cells. In control culture, there were some PDL cells exhibiting a positive staining for ALP on day 3 (Fig. 3A, arrowhead). BMP-2 slightly increased number of ALP-positive cells but the intensity of ALP staining remained weak as compared to that seen in osteoblast-like cell culture (Figs. 2A and 3A). As for Twist expression, most of the PDL cells showed a positive staining for Twist in control culture (day 3), and BMP-2 enhanced the intensity of Twist staining on day 3 (Fig. 3A). Real-time PCR analysis showed that Runx2 expression was enhanced by BMP-2 in PDL cells (Fig. 3B). On the other hand, BMP-2 inhibited ALP gene expression and did not increased

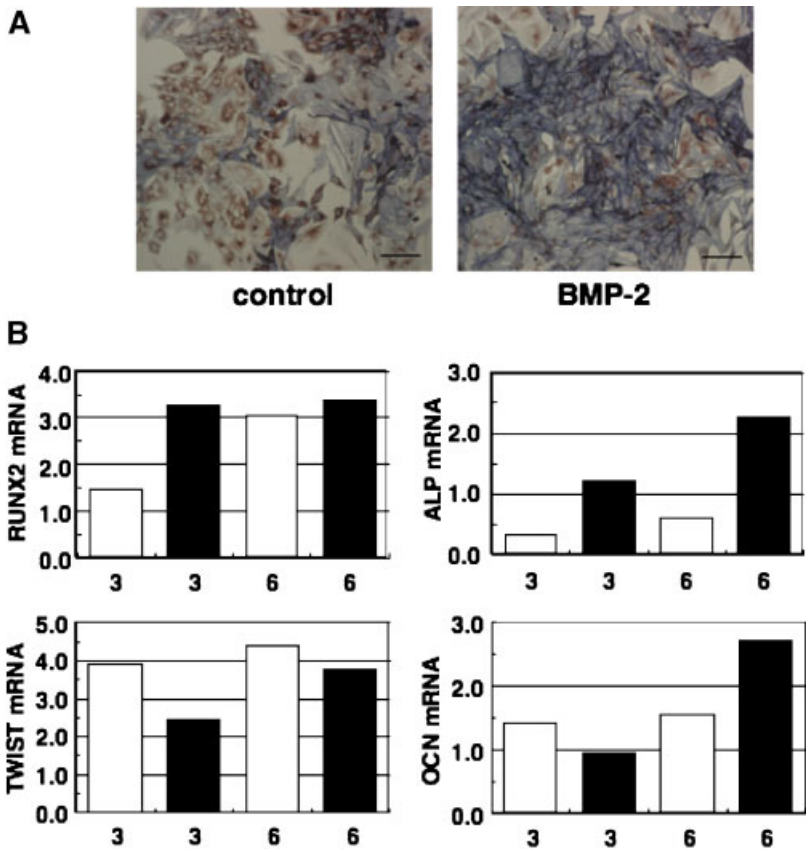

Fig. 2. Effects of BMP-2 on Twist and osteoblast-related gene expression in osteoblast-like cell. **A**: Histochemical analysis of Twist and ALP activity. Cells were cultured in differentiation medium with or without BMP-2 (250 ng/ml) for 3 days. Note BMP increased ALP and decreased Twist in osteoblast-like cells. **B**: Quantitative real-time PCR analyses for Twist and osteoblast-related genes. Cells were treated with (closed bar), or without BMP-2 (open bar) for 3 and 6 days. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

OCN gene expression, regardless of the upregulation of Runx2 gene expression, an important factor for commitment of osteoblastic differentiation (Fig. 3B). Since both protein and RNA levels of Twist were decreased when osteoblast-like cells were cultured in the presence of BMP-2 (Fig. 2A,B), we examined if Twist expression was also decreased in PDL cells in the presence of BMP-2. Surprisingly, both protein and RNA levels of Twist were dramatically increased in PDL cells. These results imply that Twist may function as a negative regulator of BMP-induced osteogenesis in PDL cells.

Suppression of Endogenous Twist Gene Enhances Early Osteoblastic Differentiation in PDL Cells

BMP-2 increased Twist expression in both protein and RNA levels in PDL cells (Fig. 3), suggesting that Twist may suppress BMP-induced osteoblastic differentiation in PDL cells. To see if endogenous Twist also affects osteoblastic differentiation in PDL cells, we constructed an siTwist expression vector and transiently transfected it into PDL cells to suppress endogenous Twist gene expression. Real-time PCR analysis demonstrated that Twist expression was decreased by 80% and early to middle osteoblastic differentiation markers, ALP, OPN, and BSP were slightly increased (Fig. 4) and basal ALP activity was also increased in PDL cells (Fig. 5). In siTwist-expressing PDL cells, BMP-2 did not increase the number of ALP positive cells but elevated the intensity of ALP staining (Fig. 5A). In contrast to the results from osteoblast-like cells, BMP-2 increased Twist expression in both control and siTwist-expressing PDL cells, suggesting that Twist expression is differentially regulated in PDL cells.

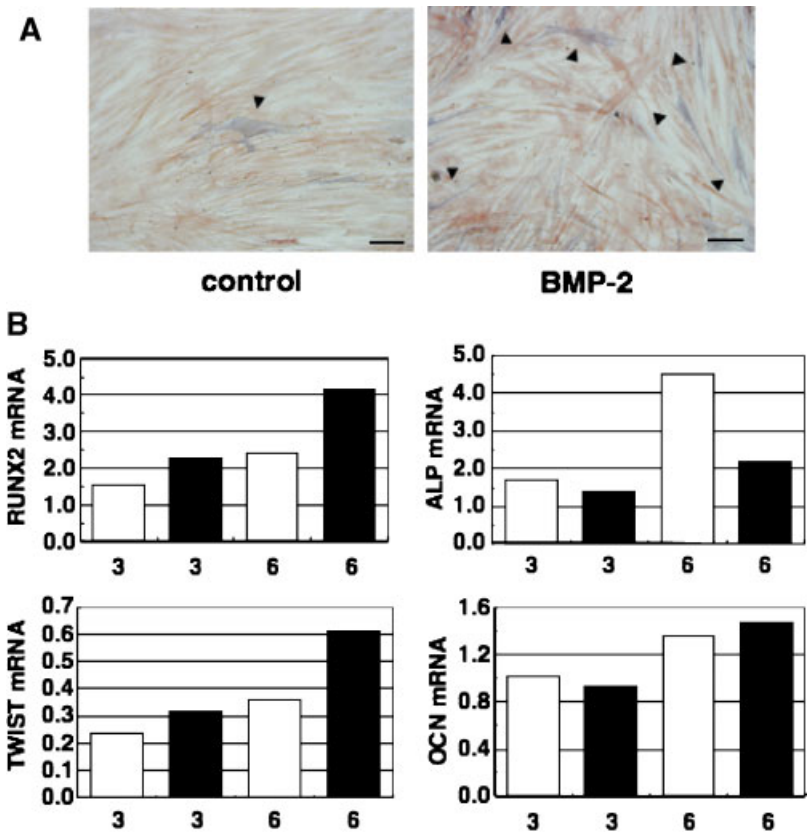

Fig. 3. Effect of BMP-2 on Twist and osteoblast-related gene expression in PDL cells. **A**: Histochemical analysis of Twist and ALP activity. Cells were cultured in differentiation medium with or without BMP-2 (250 ng/ml) for 3 days. Note Twist expression was enhanced by BMP-2 in PDL cells. Arrowheads indicate ALP-positive cells. **B**: Quantitative real-time PCR analyses for Twist and osteoblast-related genes. Cells were treated with (closed bar), or without BMP-2 (open bar) for 3 and 6 days. (Note Twist mRNA expression was increased by BMP-2 in PDL cells.) [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DISCUSSION

PDL is a thin fibrous connective tissue located between two mineralized tissues, that is, alveolar bone and tooth root, which maintains a thin space under physiological conditions as an unmineralized tissue, despite the mechanical stress due to mastication or orthodontic forces during tooth movement. Rajshankar et al. [1998] reported that BMP-7 promoted the proliferation but did not significantly induce osteogenic differentiation of cells in mouse periodontal wound model. Occlusal hypofunction decreased Twist gene expression [Afanador et al., 2005] and increased BMP-2-induced transient ankylosis [King and Hughes, 1999] in rodent PDL, suggesting Twist plays a role in maintenance of PDL. It is, therefore, important to understand the mechanisms of PDL to resist mineralization. We demonstrated that Twist was detected sparsely within mouse periodontium and relatively intense staining was specifically distributed along the alveolar bone surface in PDL immunohistochemically (Fig. 1A), which is consistent with previous report showing that Twist and its target gene periostin are expressed in mice PDL [Afanador et al., 2005]. It is also known that a calvarial suture is formed where two ossification centers expand and confront each other under the regulation of growth factors [Kim et al., 1998; Rice et al., 2005]. Twist has been reported to be expressed adjacent to the suture where osteogenic activity was high [Rice et al., 2000] and a mutation of Twist causes premature fusion of calvarial sutures, known as craniosynostosis [El Ghouzzi et al., 1999, 2000, 2001; Rice et al., 2000]. Although PDL and cranial suture are different tissues histologically and developmentally, both tissues interpose between two mineralized connective tissues. These reports suggest that Twist may function in regulation of

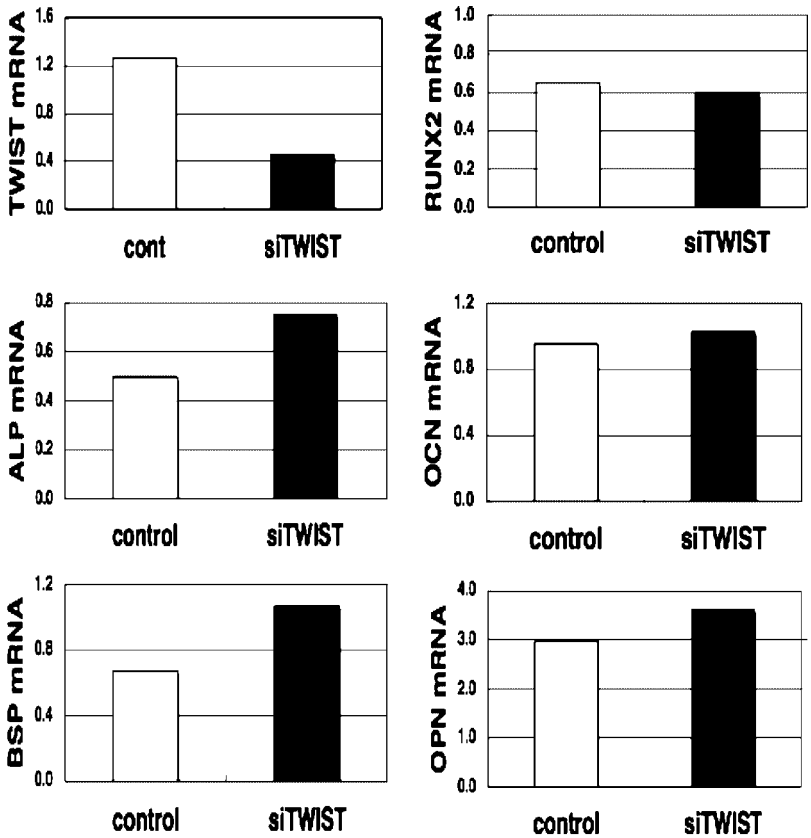

Fig. 4. Real-time PCR analyses of osteoblast-associated genes. Cells were transiently transfected with an siTwist vector or an empty vector (control). RNA was isolated 3 days after the transfection. Data present one of five independent experiments with similar results. (Note repression of Twist by 80% and slightly increased relatively early osteoblast differentiation markers such as ALP, OPN, and BSP.)

mineralization in PDL, as does Twist in developing skull [Bialek et al., 2004].

Since Twist was detected in PDL, we then analyzed Twist expression in human PDL cells and showed that Twist was constitutively expressed in human PDL cells and the expression was higher than that in osteoblast-like cells over the culture period (Fig. 1B). We assumed the reason why the localization of Twist in PDL is higher along alveolar bone surface than cementum surface because alveolar bone is more actively remodeled compared with tooth root cementum. We also postulated that PDL cells expressed Twist to resist osteogenic differentiation when the cells were challenged by osteogenic stimuli. Therefore, we compared Twist expression in PDL cells with osteoblast-like cells during BMP-induced osteoblastic differentiation in vitro. We found that BMP-2 enhanced maturation of osteoblast-like cells but not of PDL cells (Figs. 2 and 3). Although expression of osteoblast-specific transcription factor, Runx2 was slightly increased by BMP-2 in both PDL cells and osteoblast-like cells, ALP and OCN, early and late markers for osteoblastic differentiation were not upregulated in PDL cells, suggesting that the function of Runx is somehow suppressed in PDL cells. Yoshizawa et al. [2004] reported that Msx2, a homeobox protein was expressed higher in clonal PDL cells than that in osteoblasts, and co-localized with Runx2 and that Msx2 suppressed Runx2 transcriptional activity. Twist and Msx2 have been reported to control skeletogenic mesenchyme co-operatively in mice [Ishii et al., 2003]. Most recently, Bialek et al. [2004] reported that Twist inhibited differentiaton of osteoblastic cells by interacting with the DNA-binding domain of Runx2 to inhibit its function, but not its expression. Collectively, these reports suggest that Twist regulates osteoblastic differentiation in PDL cells. It has to be clarified whether Twist interacts with Runx2 in PDL cells, as previously demonstrated in osteoblasts.

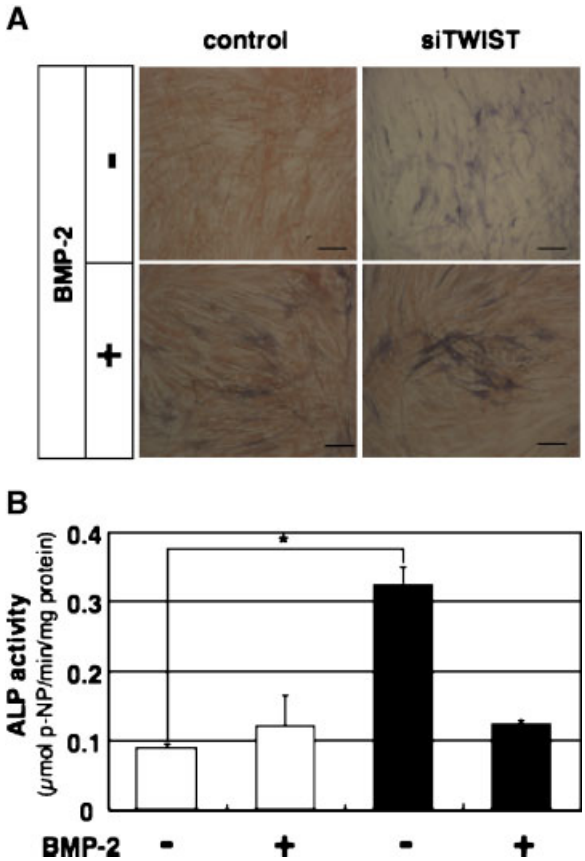

Fig. 5. Transfection of siTwist vector increased basal ALP activity in PDL cells. **A**: Histochemical analysis of Twist and ALP activity. Cells were transiently transfected with an siTwist or an empty vector (control) and treated with 250 ng/ml of BMP-2 for 3 days. (Note basal level of ALP activity was enhanced by siTwist.) **B**: Effects of siTwist transfection in combination with BMP-2 on ALP activity. Cells were transfected with an empty vector as control (open bar) or an siTwist vector (closed bar) and treated with or without BMP-2 for 3 days. Each value represents the mean with the error bar (standard deviation) derived from triplicate results. * $P < 0.01$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

To elucidate this, further experiment such as pull-down assay will be required. We then tested Msx2 expression in PDL cells in the presence of BMP-2 by real-time PCR and found that Msx2 was decreased by BMP-2 treatment in human PDL cells (data not shown), in agreement with the previous report [Yoshizawa et al., 2004]. Interestingly, we showed that BMP-2 increased Twist gene expression in human PDL. On the other hand, Twist expression was decreased in the presence of BMP-2 in osteoblast-like cells. Taken together, these data imply that Twist plays a role in resisting BMP-induced osteoblastic differentiation in PDL cells. Donor age for PDL collection may affect Twist expression and response to rhBMP-2, since previous cellular aging studies showed dramatic differences in human Twist expression in young versus senescent fibroblasts [Doggett et al., 1992; Wang et al., 1996]. It is unknown what signaling molecules control Twist expression and its interaction with Runx2 in PDL cells. Recently, Twist1 was reported to be induced by canonical Wnt signaling and inhibits chondrogenesis in mice mesenchymal cells [Reinhold et al., 2006]. It is also known that Msx2 is regulated by canonical Wnt signaling. We are currently investigating Wnt expression and its regulatory effect on Twist gene expression in human PDL cells.

PDL cells are predominant in PDL and are thought to play a crucial role in maintenance and regeneration of periodontium. There are many reports showing the osteogenic potential of PDL cells. In fact, under defined culture conditions (supplement of 1 μM dexamethasone, 10 mM β-glycerophosphate, and 50 μg/ml ascorbic acid), PDL cells exhibit properties of osteoblasts and are capable of forming mineralized nodules in vitro [Cho et al., 1992; Lekic and McCulloch, 1996; D'Errico et al., 1999; Lekic et al., 2001]. By contrast, although BMP has been known as a strong osteogenic factor to cause ectopic and orthotopic bone formation in vivo [Takaoka et al., 1988; Si et al., 1998; Stoeger et al., 2002], BMP promoted proliferation of PDL cells, but never induced osteoblastic differentiation in rat PDL cells [Rajshankar et al., 1998]. Saito et al. [2002] also reported that BMP-2 induced ALP and mineralized nodule formation, although the extent of mineralization was much less compared to that in osteoblasts. Recently, Seo et al. [2004] showed that PDL expressed stem cell markers such as STRO-1 and CD146/MUC18 and had the potential to generate cementum-, bone-, and PDL-like tissue in vivo. Therefore, PDL cells are assumed to be a source of osteoblasts and cementoblasts. It has been reported that Twist is overexpressed in young quiescent cells and underexpressed in senescent fibroblast [Wang et al., 1997]. As cells are passaged, they gradually lose their stem cell population that expresses Twist, suggesting that Twist may be involved not only in regulation of osteogenic differentiation, but in maintenance of stemness in PDL cells.

Finally, we constructed an siTwist expression vector and transiently transfected it into PDL cells to see if endogenous Twist affects

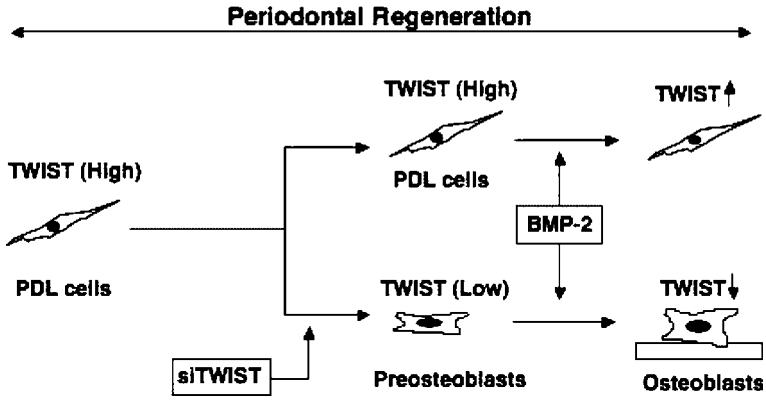

Fig. 6. Hypothetical model of Twist function in PDL cells. During periodontal regeneration, PDL cells differentiate into PDL cells and osteoblasts (cementoblasts). Decreased level of Twist shifts PDL cells to preosteoblasts. BMP-2 decreases Twist expression and stimulates osteoblastic differentiation in preosteoblasts but not in PDL cells.

osteogenic property of PDL cells. Real-time PCR analysis showed 80% reduction of Twist expression and slight increase in ALP, OPN, and BSP after the transient transfection of an siTwist expression vector in PDL cells (Fig. 4A). Basal level of ALP was apparently increased in siTwist transfected-PDL cells in the absence of BMP-2 (Fig. 5). In contrast, transient transfection of a Twist expression vector slightly decreased basal level of ALP activity (data not shown). It is an interesting question to ask if Twist affects mineralization of human PDL cells in vitro. To elucidate this, PDL cells stably expressing siTwist are required, since more than 3 weeks of culture is necessary to cause mineralization in PDL cells. We would like to assess whether Twist affects mineralized nodule formation in future.

In summary, the present study demonstrats that Twist is constitutively expressed in PDL cells. Twist expression is increased when PDL cells are treated with BMP-2. Repression of endogenous Twist stimulates osteoblastic differentiation. Therefore, Twist negatively regulates osteoblastic differentiation of PDL cells in both presence and absence of BMP-2 (Fig. 6). We believe this is the first evidence that Twist is involved in the mechanism to suppress osteoblastic differentiation in PDL cells.

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