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The role of sirtuin 1 in osteoblastic differentiation in human periodontal ligament cells

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Background and Objective: Activation of sirtuin 1 (SIRT1) promotes the differentiation of keratinocytes and mesenchymal stem cells, but inhibits the differentiation of muscle and fat cells. However, the involvement of SIRT1 in the differentiation of human periodontal ligament cells into osteoblast-like cells remains unclear. To identify the role of SIRT1 in human periodontal ligament cells, we measured *SIRT1* mRNA and SIRT1 protein levels during the osteoblastic differentiation of human periodontal ligament cells. Additionally, we investigated the effects of overexpressing and underexpressing SIRT1 on the differentiation of human periodontal ligament cells, and the signaling mechanisms involved.

Material and Methods: Expression of SIRT1 and osteoblastic differentiation markers was assessed by RT-PCR, real-time PCR, Alizarin red staining and western blotting.

Results: Marked upregulation of *SIRT1* mRNA and SIRT1 protein was observed in cells grown for 3 d in osteogenic induction medium (OM). Activation of SIRT1 using resveratrol and isonicotinamide stimulated osteoblastic differentiation in a dose-dependent manner, as assessed by the expression of mRNAs encoding alkaline phosphatase, osteopontin, osteocalcin, osterix and Runx2, and induced calcium deposition. In contrast, inhibition of SIRT1 using sirtinol, nicotinamide and gene silencing by RNA interference suppressed mineralization and the expression of osteoblast marker mRNAs. Further mechanistic studies revealed that resveratrol treatment increased the phosphorylation of Akt, adenosine monophosphate kinase (AMPK), Smad 1/5/8 and c-Jun N-terminal kinase, but reduced OM-induced activation of nuclear factor-κB. Conversely, application of sirtinol suppressed the phosphorylation of Akt, AMPK, Smad 1/5/8, p38, ERK and c-Jun N-terminal kinase, and enhanced nuclear factor-κB activity, in OM-stimulated cells.

Conclusion: These data suggest that SIRT1 is a potent regulator of differentiation of human periodontal ligament cells and may have clinical implications for periodontal bone regeneration.

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Periodontal disease leads to destruction of components of the periodontium, such as alveolar bone, cementum, the periodontal ligament and gingiva. The aim of periodontal therapy is to regenerate and restore the various periodontal components affected by disease to their original form, function and consistency (1). New therapeutic approaches available to achieve periodontal regeneration include the use of barrier membranes

for guided tissue regeneration and application of signaling molecules, such as growth factors and enamel matrix proteins, to root surfaces (2–5); however, the effectiveness of these approaches is not predictable. Furthermore, the defined molecular factors specifically inducing the osteoblastic phenotype have not been well explored in periodontal ligament cells.

The periodontal ligament is a non-mineralized connective tissue, which surrounds the tooth and exhibits osteoblast-like features, such as high alkaline phosphatase (ALP) activity and expression of osteopontin, bone sialoprotein and osteocalcin (6). MacNeil *et al.* (7) demonstrated that bone sialoprotein was expressed in cells lining the root surface during molar root development and in the developed root in mice *in situ*, but expression in cultured periodontal ligament cells was minimal. In addition, RNA expression of bone sialoprotein and osteopontin was detectable in cells obtained from freshly isolated periodontal ligament tissue, with much lower levels in cultured periodontal ligament cells (8). Periodontal ligament cells have been shown to possess stem cell-like properties, including self-renewal, clonogenicity and multi-tissue differentiation potential (9). Thus, the regulation of osteogenic differentiation in human periodontal ligament cells has important implications for the development of new therapeutic strategies for treating periodontal defects (10). However, comprehensive understanding of the molecular mechanisms controlling the osteogenic differentiation of periodontal ligament cell progenitors remains poor.

Sirtuin 1 (SIRT1; a member of the sirtuin family) is a nicotinamide adenosine dinucleotide (NAD)-dependent deacetylase and a class III histone deacetylase (11). It has been shown to play a central role in regulating cellular differentiation and senescence and to control metabolic pathways in response to changes in nutrient availability in a wide variety of tissues (11,12). However, the effects of SIRT1 overexpression or activation on differentiation remain unclear. Overexpression of SIRT1 in muscle and fat cells inhibits differentiation (13,14). The potent SIRT1 activator resveratrol was recently shown to inhibit the differentiation of pig preadipocytes, while nicotinamide, a SIRT1 inhibitor, greatly stimulated the proliferation and differentiation of these cells (15). Sirtinol, another SIRT1 inhibitor, impaired the differentiation into neurons and oligodendrocytes (16). In contrast, overexpression of SIRT1 and SIRT1 knockdown have, respectively, been shown to promote and block keratinocyte differentiation (17). Additionally, resveratrol, as well as isonicotinamide (another SIRT1 activator), increased the expression of osteoblast markers in mouse mesenchymal stem cells and primary rat bone marrow stromal cells (18). However, no information is available regarding the role of SIRT1 in the osteogenic potential of human periodontal ligament cells. The purpose of this study was to investigate the role of SIRT1 in the osteoblastic differentiation of periodontal ligament cells and to identify the underlying signal transduction pathways involved.

Material and methods

Reagents

Dulbecco's modified Eagle's medium (DMEM), α-minimal essential medium (α-MEM), fetal bovine serum (FBS) and other tissue culture reagents were purchased from Gibco BRL Co. (Grand Island, NY, USA). Anti-SIRT1 antibody (Ab) was purchased from Abcam (Cambridge, UK). Antibodies against ERK, phospho-ERK (p-ERK), p38, phospho-p38 (p-p38), c-Jun N-terminal kinase (JNK), phospho-JNK (p-JNK), adenosine monophosphate kinase (AMPK), phospho-AMPK, nuclear factor-κB (NF-κB) p65, and inhibitor of NF-κB (IκBα) were purchased from Cell Signaling (Beverly, MA, USA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated.

Cell culture

We used an immortalized human periodontal ligament cell line produced by transfection with a telomerase catalytic subunit (*hTERT*) gene (19). Cells were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified atmosphere of air containing 5% CO_2 at 37°C. To induce differentiation, human periodontal ligament cells were cultured with osteogenic medium (OM; DMEM supplemented with 10% FBS, 50 μg/mL ascorbic acid, 10 mM β-glycerophosphate and 10^{-7} M dexamethasone) as described previously (6,20,21). The culture medium was replaced every 2 d during the incubation period.

Human cementoblast-like cells, immortalized by transfection of the *hTERT* gene, were established by Kitagawa *et al.* (22). Human cementoblast-like cells express bone/cementum-related proteins, such as type I collagen, runt-related transcription factor 2, osteocalcin, bone sialoprotein and CP-23, show strong ALP activity and form calcified nodules (22). This study was approved by the institutional review board and ethical committee at Wonkwang University.

Primary culture of human periodontal ligament cells

Pieces of periodontal ligament were obtained exclusively from the middle of tooth roots extracted for orthodontic reasons, to exclude intermixture of gingivae and dental pulp. The ligament samples were cultured in α-MEM containing 10% FBS and supplemented with antibiotics in a 35 mm primary culture dish (Falcon Becton Dickinson, Franklin Lakes, NJ, USA). Cells that proliferated from the extracts were passaged. The periodontal ligament cells used in these experiments underwent four to eight passages.

Primary culture of human alveolar bone marrow stem cell-derived osteoblasts

Human bone marrow was obtained from the alveolar bone according to a protocol approved by ethical authorities at Wonkwang University, as previously described (23,24). Briefly, bone marrow was added to a container filled

with 30 mL of α-MEM containing 20% FBS and 350 units of heparin. The mixture was centrifuged at 4°C and 472*g* for 10 min, after which the supernatant was discarded, and 20 mL of culture medium was added to the remaining pellets. The mixture was filtered (Falcon, Franklin Lakes, NJ, USA), and 10 mL of the medium was then added per T-75 culture flask (Corning Science Products, Corning, NY, USA), and the culture was initiated. The next day, 50 μg/mL L-ascorbic acid (Sigma), 10 mM glycerophosphate and 10^{-7}M dexamethasone were added to facilitate cell differentiation into osteoblasts. On day 14 of culture, Alizarin red staining was performed to detect newly produced calcium. Cells were trypsinized and replated, and passage three to five was used for the experiment.

Western blot

Cells (1×10^6) were grown in 100 mm dishes and preincubated for 16 h in DMEM containing 10% FBS. Cells from each set of experiments were harvested and washed twice in cold Tris-buffered saline (TBS). Cells were solubilized in ice-cold 1% Triton X-100 lysis buffer. After 30 min on ice, the lysates were clarified by centrifugation. Protein contents were measured by using a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Proteins (20 μg per lane) were then separated by 6–10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blotted onto Hybond-N nitrocellulose. The membrane was then rinsed once with TBS and blocked for 60 min in blocking buffer (TBS with 5% nonfat dry milk and 0.1% Tween-20), and then incubated with primary antibody diluted in 2.5% blocking buffer. Unbound primary antibody was subsequently removed by washing three times in TBS containing 0.05% Tween-20, before bound primary antibody was labeled through incubation at room temperature for 60 min with horseradish peroxidase-conjugated secondary antibody. Following three washes in TBS containing 0.05% Tween-20, protein bands were visualized using electrochemiluminescence and X-ray film.

Isolation of RNA and RT-PCR

Total RNA was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. It was then reverse transcribed using AccuPower RT Premix (Bioneer, Daejeon, Korea). The PCR amplification of the resulting cDNA samples was performed using a GeneAmp PCR System 2400 thermal cycler (PerkinElmer, Wellesley, MA, USA). The primers used were as follows: *ALP*, 5′-ACGTGGCTAAGAA TGTCATC-3′ and 5′-CTGGTAGGC-GATGTCCTTA-3′ (475 bp); osteopontin, 5′-CCAAGTAAGTCCAACG AAAG-3′ and 5′-GGTGATGTCCT CGTCTGTA-3′ (347 bp); osteocalcin, 5′-CATGAGAGCCCTCACA-3′ and 5′-AGAGCGACACCCTAGAG-3′ (310 bp); osterix, 5′-GTGAATTCAC CTTTCAGCCCCCAAAACC-3′ and 5′-TGGGATCCCAGCTGTGAATG GGCTTCTT-3′ (746 bp); and Runx2, 5′-AACCCACGAATGCACTATC-CA-3′ and 5′-CGGACATACCGAGG GACCTG-3′ (75 bp). The following PCR conditions were used: 30 cycles of denaturation at 94°C for 30 s, primer annealing at 56°C for 30 s, and extension at 72°C for 30 s. The PCR products were resolved on 1–2% agarose gels and stained with ethidium bromide.

Quantitative real-time PCR

Total RNA was prepared using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. The RNA (2 μg) was reverse transcribed using AccuPower RT Premix (Bioneer) according to the instructions of the manufacturer. The resulting cDNA (equivalent to 40 ng total RNA) was used in quantitative real-time PCR using FastStart DNA MasterPLUS SYBR Green I (Roche Applied Science, Indianapolis, IN, USA) and LightCycler according to the instructions of the manufacturer. The following primers were used: SIRT1, 5′-TCAGTGTCATGGTTCCTTTGC-3′ and 5′-AATCTGCTCCTTTGCCA CTCT-3′; and β-actin, 5′-GCGCGG CTACAGCTTCA-3′ and 5′-TCTCCT TAATGTCACGCACGAT-3′. Each assay was run in triplicate. A linear regression was done for each primer set to calculate the amount of starting material in each sample. All samples were normalized to GAPDH. A comparison was done for each sample using a relative cycle threshold (C_t) comparison. An average C_t was calculated for the triplicate reactions and normalized to GAPDH ($\Delta C_t = C_{t\text{Sample}} - C_{t\text{GAPDH}}$). The C_t values were then compared between different treatments and time points ($\Delta\Delta C_t$) with normalization to the untreated samples. Finally, a fold change was calculated from the $\Delta\Delta C_t$ (fold change $= 2^{\Delta\Delta c_t}$).

Alizarin red staining

After incubation in OM for 3 and 14 d, cell mineralization was determined using Alizarin red staining. Cells were washed twice in distilled water and fixed by incubation in ice-cold 70% (v/v) ethanol for 1 h. They were then rinsed twice in deionized water and stained with Alizarin red (dissolved in deionized water, pH 4.2) for 10 min at room temperature. Following staining, excess dye was removed through gentle washing in running water. To determine the optical density of the Alizarin red staining, each well was destained for 30 min with 500 mL 10% cetylpyridinium chloride monohydrate (Acros Organics, Morris Plains, NJ, USA), and the absorbance measured at 540 nm on an ELISA plate reader (Bio-Rad Laboratories). Quantitative analysis was performed through three repetitions.

Electrophoretic mobility-shift assay (EMSA)

Nuclear extracts were prepared from cells as described previously (25). Oligonucleotides containing NF-κB and activating protein-1 (AP-1) sequences (Promega, Madison, WI, USA) were 5′-end labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Subsequent binding reactions were performed on ice for 15 min in a buffer consisting of

10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 0.5 mM dithiothreitol, 10 mM $MgCl_2$, 10% glycerol, 0.05% NP-40, and 2 μg/mL poly(dI-dC). Assay mixtures were incubated with the radiolabeled oligonucleotides for 30 min at room temperature. Following the addition of 6× dye solution (0.1% bromophenol blue, 30% glycerol), mixtures were immediately electrophoresed on nondenaturing 6% polyacrylamide gels using 0.25 × Tris-borate-EDTA. The gels were then dried in a vacuum drier at 80°C for 1 h and autoradiographed on Fuji RX X-ray films.

SIRT1 siRNA transfection

Small interfering RNAs (siRNAs) were synthesized as purified duplexes using Bioneer technology (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The sequences of the sense and antisense strands of the human *SIRT1* siRNA were as follows: 5′-GAUGAAGUUGACCUCCUCA-3′ (sense) and 5′-UGAGGAGGUCAACUUCAUC-3′ (antisense). Cells were plated in six-well plates at a density of 5×10^5 cells per well, grown for 16 h, and transfected with siRNA duplexes (250 pM) using Lipofectamine RNAi-Mix (Invitrogen), according to the manufacturer's instructions. Silencer Negative Control siRNA (Invitrogen) was introduced into cells by the same method. Following transfection, cells were cultured in six-well plates at 37°C until required.

Statistical analysis

Data are expressed as the means ± SD. Statistical comparisons were performed using analysis of variance (ANOVA). A p-value < 0.05 was deemed to indicate statistical significance.

Results

Change in time course of expression of *SIRT1* mRNA and SIRT1 protein during osteoblastic differentiation of human periodontal ligament cells

To investigate the expression of *SIRT1* mRNA and SIRT1 protein during osteoblastic differentiation of human periodontal ligament cells, they were cultured in OM for 14 d, and the samples collected were analyzed by western blotting (Fig. 1A), conventional RT-PCR (Fig. 1A) and real-time PCR (Fig. 1B). Expression of *SIRT1* mRNA and SIRT1 protein was detected in unstimulated human periodontal ligament cells; it increased in a time-dependent manner following exposure to OM until 3 d after the initiation of treatment, after which it decreased. The increase in SIRT1 protein expression in human periodontal ligament cells correlated with a parallel increase in *SIRT1* mRNA levels (Fig. 1A,B).

Alkaline phosphatase is considered to be an early marker of osteoblastic differentiation, while osteopontin and osteocalcin are intermediate and late bone differentiation markers, respectively (25). To verify that OM had stimulated osteoblastic differentiation in human periodontal ligament cells, we examined calcium nodule formation by Alizarin red staining and the expression of early, intermediate and late differentiation markers by RT-PCR. Expression of the early differentiation marker alkaline phosphatase and late differentiation marker osteocalcin increased in a time-dependent manner, while osteopontin mRNA expression peaked on day 7, before subsequently decreasing (Fig. 1C). As shown in Fig. 1D, mineralization, and hence osteoblastic differentiation, was confirmed by positive Alizarin red staining.

Effects on osteoblastic differentiation of a SIRT1 activator and a SIRT1 inhibitor in human periodontal ligament cells

To investigate the potential of human periodontal ligament cells to undergo osteoblastic differentiation following SIRT1 activation and inhibition, we performed Alizarin red staining and assessed human periodontal ligament cell expression of mRNA species encoding differentiation makers and the essential transcriptional factors Runx2 and osterix (26,27). As maximal induction of SIRT1 expression was observed at 3 d of growth in OM, we examined mRNA expression at this time point. Treatment of human periodontal ligament cells with the SIRT1 activator resveratrol increased the expression of mRNAs encoding the OM-induced differentiation markers ALP, osteopontin and osteocalcin and the transcriptional factors osterix and Runx2 (Fig. 2A). In contrast, treatment with the SIRT1 inhibitor

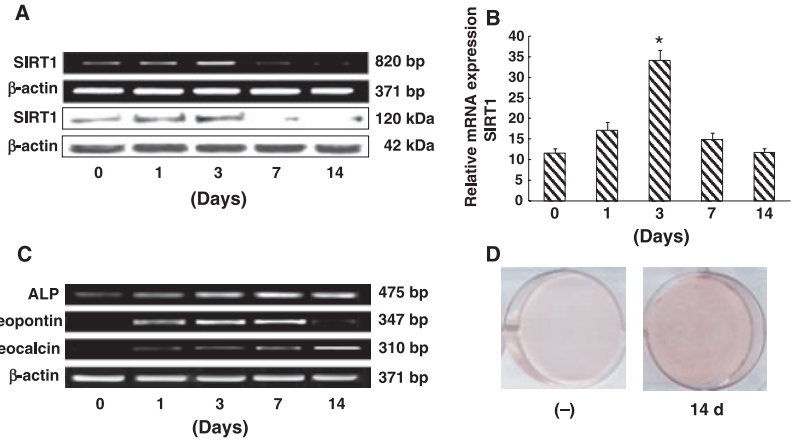

Fig. 1. Time course of *SIRT1* mRNA and SIRT1 protein expression (A,B), mRNA expression of osteoblastic markers (C) and mineralized nodule formation (D) in human periodontal ligament cells. Cells were cultured in osteogenic medium (OM) containing 10 mM β-glycerophosphate, 50 μg/mL ascorbic acid and 10^{-7} M dexamethasone for 0, 1, 3, 7 or 14 d. Total RNA was isolated and analyzed by RT-PCR (A,C) and real-time PCR (B). The SIRT-1 protein levels were analyzed by western blotting (A). Mineralized nodule formation was assessed by Alizarin red staining (D). *$p < 0.05$ vs. control. The data presented are representative of three independent experiments.

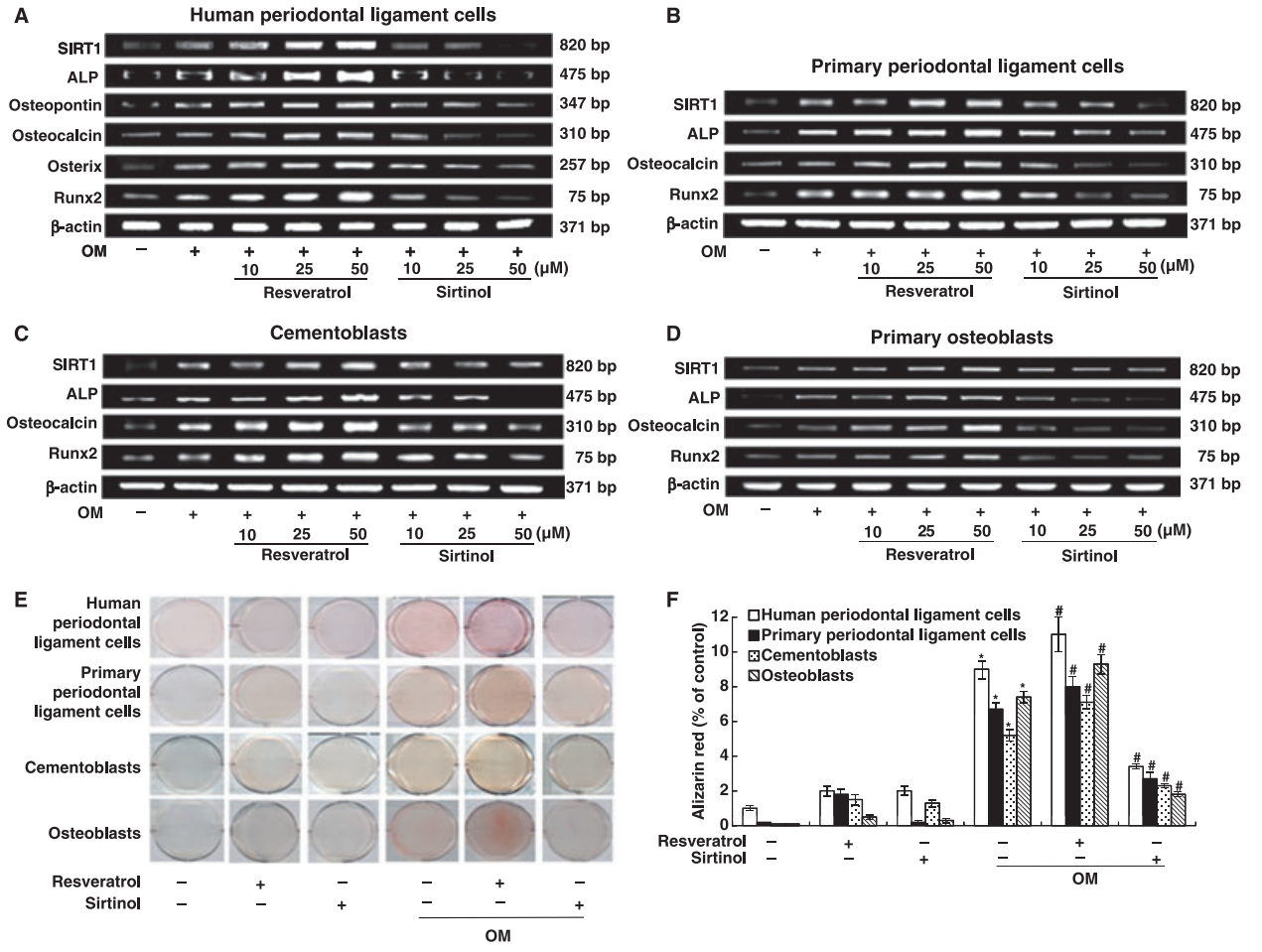

Fig. 2. Effects of resveratrol (SIRT1 activator) and sirtinol (SIRT1 inhibitor) on OM-induced differentiation in human periodontal ligament cells (A), primary periodontal ligament cells (B), cementoblasts (C) and primary osteoblasts (D) as assessed by RT-PCR (A–D) and Alizarin red staining (E,F). Cells were treated with resveratrol or sirtinol for 3 (A–D) or 14 d (E,F). The intensity of Alizarin red staining was analyzed by optical density measurement (F) and is presented as the means ± SD relative to control values. Control values are set at onefold. The data presented are representative of three independent experiments. $*p < 0.05$ with respect to untreated control. $p < 0.05$ with respect to the group treated with OM.

sirtinol reduced the expression of these same five mRNAs (Fig. 2A). However, while human periodontal ligament cells showed no changes in calcified nodule formation following exposure to resveratrol and sirtinol for 3 d, exposure to resveratrol and sirtinol for 14 d, respectively, increased and reduced mineral deposition (Fig. 2E,F).

Effects of a SIRT1 activator and a SIRT1 inhibitor on osteoblastic differentiation in cementoblasts, osteoblasts and primary cultured periodontal ligament cells

To investigate whether osteoblastic differentiation due to SIRT1 activation or inhibition is of broader significance for cells involved in periodontal regeneration, we examined primary cultured human periodontal ligament cells, a cementoblast cell line and alveolar bone marrow stem cell-derived osteoblasts. Induction SIRT1 by resveratrol in primary cultured human periodontal ligament cells, cementoblasts and osteoblasts increased mineralization and upregulated the mRNA expression of osteoblastic markers, such as *ALP*, osteocalcin and Runx2 (Fig. 2B–F). In contrast, sirtinol treatment significantly decreased calcified nodule formation and the mRNA expression of *ALP*, osteocalcin and Runx2 in primary periodontal ligament cells, cementoblasts and osteoblasts. Together, these results suggest that SIRT1 activation and inhibition by resveratrol and sirtinol may have broad relevance for periodontal regeneration-related cells.

Effects of isonicotinamide and nicotinamide on osteoblastic differentiation in human periodontal ligament cells

In order to extend the investigation of efficacy to another SIRT1 activator and SIRT1 inhibitor, human periodontal ligament cells were treated with isonicotinamide or nicotinamide.

The SIRT1 inducer isonicotinamide also increased the nodule formation and expression of *SIRT1* mRNA and several osteoblastic differentiation markers in a dose-dependent manner (Fig. 3). The treatment of cells with nicotinamide inhibited Alizarin red staining intensity and the mRNA expression of *SIRT1* and differentiation markers, including *ALP*, osteopontin, osteocalcin, osterix and *Runx2*, compared with the OM control (Fig. 3A–C).

Effects of *SIRT1* siRNA on OM-induced differentiation in human periodontal ligament cells

We next assessed the effects of using RNA interference to knock down SIRT1 expression on differentiation following OM treatment. Treatment of human periodontal ligament cells with *SIRT1* siRNA blocked OM-induced upregulation of *ALP*, osteopontin, osteocalcin, osterix and *Runx2* mRNA expression, whereas transfection of cells with an equivalent amount of nonspecific siRNA produced no effect (Fig. 4).

Involvement of Akt, AMPK and MAP kinases in osteoblastic differentiation and effects of SIRT1 activation and inhibition on OM-induced kinase expression in human periodontal ligament cells

To investigate the molecular basis of the osteoblastic differentiation of human periodontal ligament cells, we examined the effects of OM on the phosphorylation of three MAPKs (p38, JNK and ERK), Akt, Smad 1/5/8, and adenosine monophosphate kinase (AMPK). The OM treatment increased the phosphorylation, but not overall levels, of p38, JNK, ERK, Akt, Smad 1/5/8 and AMPK (Fig. 5A). Maximal expression of p-ERK and p-JNK was detected following incubation of human periodontal ligament cells in OM for 30 min, while p-p38, p-Akt and p-AMPK levels peaked at 240 min. The phosphorylation of Smad 1/5/8 was increased after treatment with OM for 60 min (Fig. 5A). As p38 MAPK, JNK, ERK, Smad 1/5/8, Akt and AMPK were activated by OM in human periodontal ligament cells, we next tested whether SIRT1 activation or inhibition might influence the phosphorylation of these same signaling molecules. Treatment with the SIRT1 activator resveratrol enhanced OM-induced phosphorylation of Akt, Smad 1/5/8, AMPK and JNK, but had no effect on the activation of p38 or ERK. In contrast, pretreatment with the SIRT1 inhibitor sirtinol decreased OM-induced phosphorylation of Akt, Smad 1/5/8, AMPK, p38, ERK and JNK.

Involvement of NF-κB in osteoblastic differentiation, and effects of SIRT1 activation and inhibition on OM-induced activation of NF-κB and AP-1 in human periodontal ligament cells

We examined the effects of OM on NF-κB activation by measuring IκBα

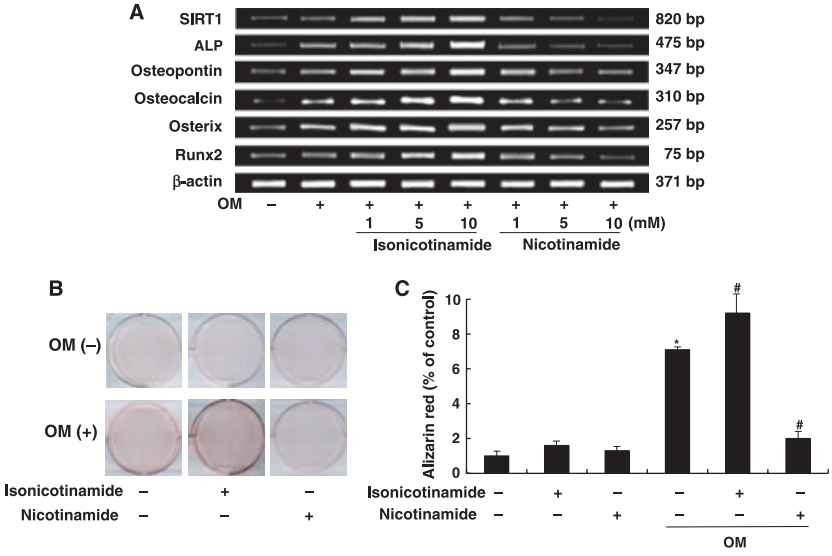

Fig. 3. Effects of isonicotinamide (SIRT1 activator) and nicotinamide (SIRT inhibitor) on the OM-induced mRNA expression of *SIRT1* (A), the mRNA expression of osteoblastic markers (A), the formation of mineralized nodules (B) and the intensity of Alizarin red staining in human periodontal ligament cells. Cells were treated with isonicotinamide and nicotinamide for 3 (A) or 14 d (B,C). $*p < 0.05$ with respect to untreated control. $p < 0.05$ with respect to the group treated with OM. Data presented are representative of three independent experiments.

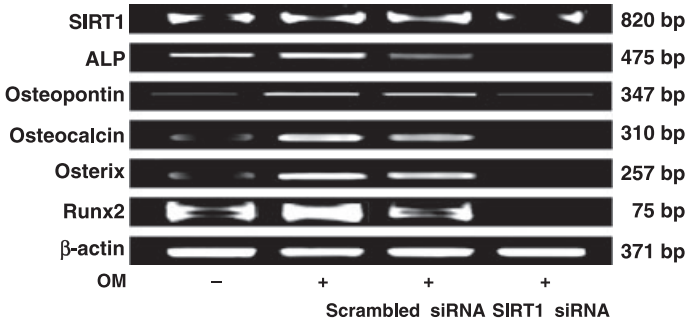

Fig. 4. Effects of *SIRT1* siRNA on OM-induced differentiation of human periodontal ligament cells. Cells were transiently transfected with *SIRT1* siRNA and then cultured in OM for 3 d. Total RNA was isolated and analyzed by RT-PCR. The data presented are representative of three independent experiments.

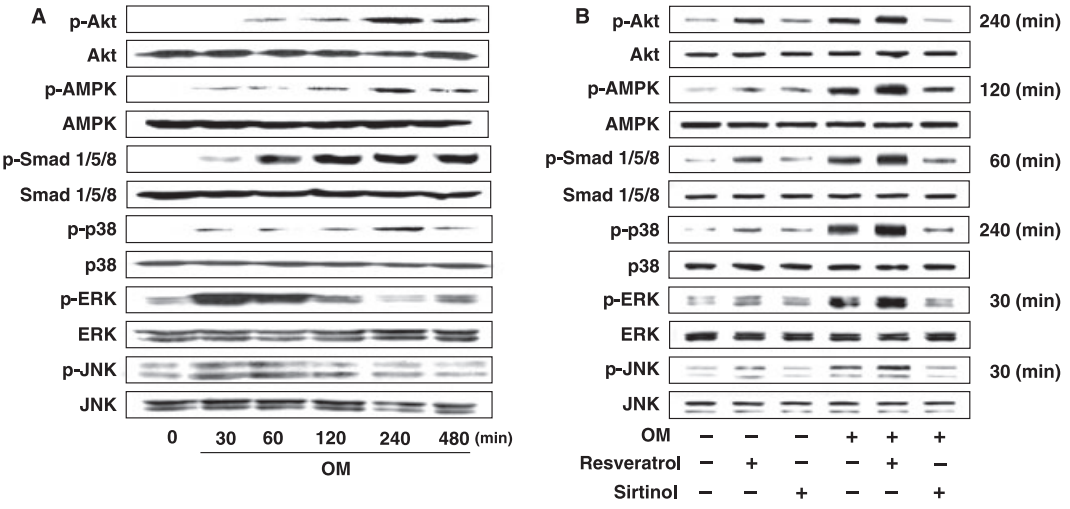

Fig. 5. (A) Expression of signal transduction molecules during osteoblastic differentiation. Cells were cultured in OM for the indicated periods of time. (B) Effects of resveratrol and sirtinol on OM-induced expression of kinases. Cells were treated with resveratrol (50 μM) or sirtinol (50 μM) for the indicated times. Similar results were obtained in three independent experiments.

degradation and nuclear translocation of p65. Maximal degradation of IκBα and expression of p65 protein were detected in human periodontal ligament cells that had been incubated in OM for 90 min (Fig. 6A). In an attempt to determine whether SIRT1 activation and/or inhibition influenced OM-induced activation of NF-κB, we tested the effects of treatment with resveratrol and sirtinol on OM-induced degradation of IκBα and nuclear translocation of p65. As shown in Figure 6B, resveratrol and sirtinol, respectively, inhibited and enhanced both these processes.

To ascertain whether p65 nuclear translocation following exposure to resveratrol or sirtinol was accompanied by the binding of NF-κB to DNA, NF-κB activity was assessed by EMSA. Sirtinol significantly increased NF-κB binding activity in nuclear fractions from cells incubated with OM for 90 min (Fig. 6C); however, pretreatment with resveratrol abolished this DNA-binding activity. Binding specificity was confirmed by competition analysis using an excess of unlabeled or cold probe. To determine whether sirtinol specifically activated NF-κB, the DNA binding of a second transcriptional factor, AP-1, was assessed by EMSA. It was found that neither resveratrol nor sirtinol affected the DNA binding of AP-1 in nuclear fractions from OM-exposed cells (Fig. 6D).

Discussion

Effective regulation of human periodontal ligament cells contributes to successful periodontal tissue regeneration (2–5). We previously showed that substance P and nitric oxide stimulate the osteoblastic differentiation of human periodontal ligament cells (20,21) To our knowledge, this is the first reported study to examine the expression of SIRT1 during osteoblastic differentiation, and the effects of activating SIRT1 with resveratrol and isonicotinamide and inhibiting it using sirtinol, nicotinamide and *SIRT1* siRNA, on the differentiation of human periodontal ligament cells.

The periodontal ligament is a source of pluripotential cells and molecular factors controlling cellular events in the surrounding tissues (10). Once the periodontal ligament is severely damaged by inflammation such as periodontitis and lost, its regeneration is well known to be difficult. Therefore, to clarify the regenerative mechanism of periodontal ligament is an important subject in conserving teeth (28). As primary periodontal ligament fibroblasts are heterogeneous cell populations with varied proliferative potential, long-term culture of periodontal ligament fibroblasts is apt to reduce the number of cells with low proliferative potential, so that their primary phenotype is lost (6–9). Consequently, reproducible results are difficult to obtain, and the biology and regenerative mechanism of periodontal ligament is difficult to clarify by using primary cells (28). Therefore, to address this issue, we used an immortalized human periodontal ligament cell line, which retains the features of their original primary cells, viz., human periodontal ligament cells (19). In addition, Kitagawa *et al.* (19) demonstrated that F-spondin increased the expressions of *ALP*, osteocalcin and bone sialoprotein mRNA and ALP activity in an immortalized human periodontal ligament cell line. Thus, an immortalized human periodontal ligament cell line is a helpful tool for studying the biology and regenerative mechanisms of human periodontal ligament.

Osteoblastic differentiation and mineralization typically involve an initial period of cell proliferation and extracellular matrix biosynthesis, followed by cell differentiation (29). We demonstrated in this study that human periodontal ligament cells differentiate into osteoblasts that produce mineralized nodules and express early (ALP), intermediate (osteopontin) and late markers (osteocalcin) of osteoblastic differentiation when cultured in osteoinductive medium for 14 d. The induction of osteoblastic differentiation by this medium was consistent with the results of previous *in vitro*

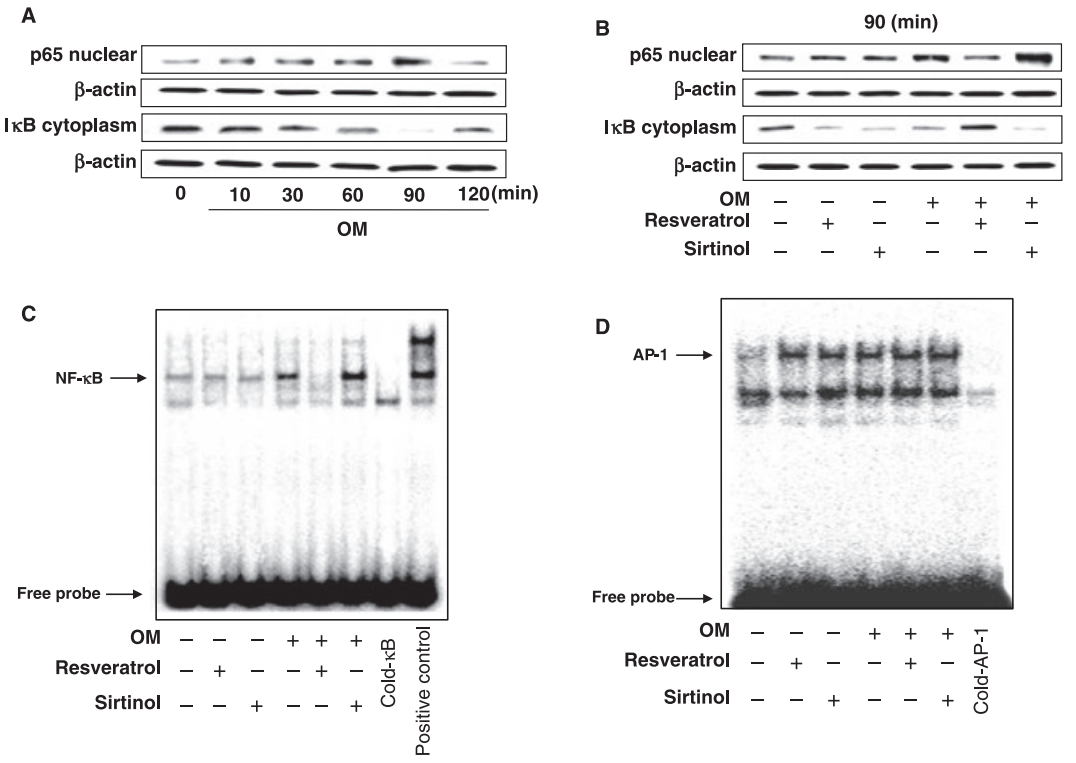

Fig. 6. Effects of resveratrol and sirtinol on OM-induced activation of nuclear factor-κB (NF-κB; A–C) and activating protein-1 (AP-1; D). (A) Effects of OM on the activation of NF-κB during osteoblastic differentiation of human periodontal ligament cells. (B) Cells were treated with resveratrol (50 μM) or sirtinol (50 μM) for the indicated times. Levels of IκB in the cytosolic fraction and p65 NF-κB in the nuclear fraction were measured by western blotting (A,B). (C) EMSA was performed using ^{32}P-labeled oligonucleotide probes containing NF-κB sequences and equal amounts of nuclear lysates prepared from cells treated with resveratrol or sirtinol. (D) Autoradiogram showing AP-1 binding activity by EMSA in human periodontal ligament cells. Cold-κB and Cold-AP-1 represent unlabeled κB and AP-1, respectivelly (C,D). The NF-κB positive control is the p65 subunit of NF-κB. The data presented are representative of three independent experiments.

studies performed using human bone marrow stromal cells (30), MC3T3E1 osteoblasts (31) and human periodontal ligament cells (32).

To assess the role of SIRT1 in the osteoblastic differentiation of human periodontal ligament cells, we tested the effects of SIRT1 activation, inhibition and gene silencing on the expression of key differentiation markers. Our results indicate that activation of SIRT1 by resveratrol in human periodontal ligament cells, cementoblasts, oseoblasts and primary cultured periodontal ligament cells increased mineralized nodule formation and upregulated the expression of mRNAs encoding the osteoblastic markers ALP, osteopontin, osteocalcin, osterix and Runx2 in a dose-dependent manner. These results are consistent with previous data obtained using mouse mesenchymal stem cells and rat primary bone marrow stromal cells (18), osteoblastic MC3T3-E1 cells (33) and human bone marrow-derived mesenchymal stem cells (34). Furthermore, downregulation of *SIRT1* expression through transfection with *SIRT1* siRNA and inhibition of SIRT1 activity, using sirtinol and nicotinamide, blocked OM-induced osteoblastic differentiation of human periodontal ligament cells. Our results suggest that osteoblastic differentiation is stimulated by SIRT1 activation and repressed by inhibition of SIRT1. Thus, the differentiation of human periodontal ligament cells into osteoblasts appears to be controlled, at least in part, by a SIRT1-dependent mechanism.

In the present study, *SIRT1* mRNA and SIRT1 protein were found to be widely expressed throughout osteoblast differentiation in human periodontal ligament cells, in accordance with results obtained for adipocyte differentiation (15). However, the mRNA and protein levels of SIRT1 in differentiating human periodontal ligament cells increased significantly after 3 d of osteogenic induction, and then declined steadily. This result differs from that in a study of pig preadipocytes, which showed that *SIRT1* mRNA expression peaked on day 4 during adipogenic differentiation (15). The difference in maximal expression of SIRT1 during differentiation in human periodontal ligament cells and preadipocytes might be caused by differences between the cell types or their state of differentiation.

We next investigated whether SIRT1 influenced the phosphorylation of kinases, because activation of Akt (35) and MAP kinases, including p38, ERK and JNK (36), has been reported to contribute to osteoblastic differentiation in bone cells and osteoblasts. In the present study, OM-induced phosphoactivation of JNK and Akt, but not p38 or ERK, was significantly enhanced by

resveratrol. Resveratrol has previously been shown to inhibit 4β-phorbol 12-myristate 13-acetate-induced activation of ERK1/2 and p38 signaling in human monocyte cell lines (37), but to activate ERK in PC12 cells (38). However, it was shown that resvaratrol rapidly activates ERK and p38 signaling in human bone marrow-derived mesenchymal stem cells (34). These discrepancies may stem from differences in treatment protocols or cell types tested. The results of the present study show that sirtinol attenuated OM-induced phosphorylation of p38, ERK, JNK and Akt. Consistent with these findings, sirtinol was shown to attenuate epidermal growth factor- and insulin-like growth factor 1-stimulated phosphorylation of ERK, JNK and p38 in MCF-7 and H1299 cells (39).

Adenosine monophosphate-activated protein kinase is a eukaryotic heterotrimeric protein kinase that senses nutritional and environmental stresses and functions as a metabolic master switch (40). Its activation mediates the effects of glucose restriction on skeletal muscle differentiation (41). As signal-specific Smad proteins physically interact with Runx2 (42), we surmised that bone morphogenetic protein (BMP)-specific Smad proteins might be additionally required for the induction of osteoblast-specific gene expression. In the present study, exposure of resveratrol-treated cells to OM was found to increase the phosphorylation of AMPK and Smad 1/5/8 protein above the levels detected in cells not treated with resveratrol, while exposure to sirtinol decreased the expression of p-AMPK and p-Smad 1/5/8. This suggests that AMPK signaling and BMP pathways activated by Smad 1/5/8 may contribute to the ability of SIRT1 to stimulate the osteoblastic differentiation of human periodontal ligament cells.

Activation of NF-κB is dependent on the degradation of IκB, an endogenous inhibitor of NF-κB signaling that binds to NF-κB in the cytosol (43). Recent studies demonstrated that resveratrol inhibits NF-κB activation (44,45). As this transcription factor is strongly linked to differentiation responses (34,42), we hypothesized that resveratrol and/or sirtinol influence differentiation, at least in part, by suppressing or enhancing NF-κB activation. Our results indicate that resveratrol decreased the degradation of IκB and the nuclear translocation and DNA binding activity of p65 NF-κB in OM-stimulated human periodontal ligament cells. In contrast, treatment with sirtinol enhanced OM-induced NF-κB activation. These findings suggest that resveratrol and sirtinol influence the osteoblastic differentiation of human periodontal ligament cells by modulating the activation of NF-κB and degradation of IκB.

In summary, the present study is the first report of the expression of *SIRT1* mRNA and SIRT1 protein being induced in human periodontal ligament cells during the early phase of osteoblastic differentiation. Furthermore, while SIRT1 overexpression promotes the differentiation of human periodontal ligament cells into osteoblast-like cells and stimulates the latter's subsequent mineralization, *SIRT1* knockdown blocks human periodontal ligament cell differentiation. These findings support the hypothesis that SIRT1 may play an important regulatory role in osteoblastic differentiation for periodontal regeneration.

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