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Regulatory Role of Stanniocalcin 1 in Osteoblastic and Osteoclastic Differentiation

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이 논문의 치의과학박사 학위논문으로 제출함

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ABSTRACT

Regulatory Role of Stanniocalcin1 in Osteoblastic and Osteoclastic Differentiation

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According to the pressure-tension theory in orthodontics, the first physiologic response in the pressure side during tooth movement is hypoxia in periodontal ligament (PDL). We recently performed a preliminary microarray analysis to examine differentially expressed genes in human PDL exposed to hypoxic condition for 24 hours and identified Stanniocalcin 1 (STC1) as one of the most significantly upregulated genes. The purpose of this thesis was to study the functional role of STC1 in the regulation of cell viability, osteoblast differentiation and osteoclast differentiation.

To address the purposes, the primary cultured PDL cells were purchased from ATCC. Gaspak pouch was used to induce hypoxia. To evaluate

apoptosis, quantification of the cells positively stained with Annexin V/Propidium Iodide and western blot analysis against Caspase 3 were performed. To investigate the effect of STC1, overexpression or knockdown of STC1 was induced. Treatment of cells with recombinant STC1 protein or neutralizing antibody to STC1 was also used. Osteogenic differentiation was evaluated by examining the expression levels of osteogenic marker genes, the activity of alkaline phosphatase, and matrix calcification. Osteoclastogenesis was induced by treating RAW264.7 or primary cultured mouse bone marrow-derived macrophages with RANKL. Osteoclast differentiation was evaluated by examining the expression levels of osteoclastogenesis related genes and the number of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells.

Under the hypoxic condition, PDL cells exhibited increased the expression levels of STC1. In addition, hypoxia increased the number of apoptotic cells and activation of caspase 3, which were attenuated by addition of rhSTC1. Under the normoxic conditions, knockdown of STC1 promoted apoptosis. When PDL cells were induced to undergo osteogenic differentiation, the expression of STC1 increased in a time-dependent manner. Overexpression of STC1 or recombinant STC treatment significantly increased osteogenic differentiation of PLD cells, whereas knockdown of STC1 or addition of STC1-neutralizing antibody attenuated osteogenic

differentiation. STC1 induced the expression of VEGF, and knockdown of VEGF significantly suppressed STC1-induced osteogenic differentiation. STC1 inhibited the formation of TRAP-positive multinucleated cells as well as the expression of osteoclast marker genes.

Taken together, these results suggest that hypoxia-induced STC1 in compression side contribute to the prevention of excessive alveolar bone loss or root resorption by protecting the cells from apoptotic cell death, enhancing osteogenic differentiation and inhibiting osteoclast differentiation.

Key words: human periodontal ligament cells, Stanniocalcin 1, hypoxia, osteogenic differentiation, apoptosis, osteoclast differentiation

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I. LITERATURE REVIEW

I.1. Stanniocalcin 1

Stanniocalcin (STC) is a 50-kDa secreted disulfide-linked homodimeric glycoprotein hormone which was first found in the corpuscles of Stannius in fish. Regulation of calcium and phosphate homeostasis in order to prevent hypercalcemia was suggested as a function of STC based on the finding showing that decreased STC secretion was accompanied with an elevation of plasma calcium concentration (Lafeber et al., 1988). The proposed functions through which STC regulates the level of Ca/P were as follows; reduction of calcium uptake in the gills (Lafeber et al., 1988), inhibition of calcium absorption in the gut (Sundell et al., 1992) and enhancement of phosphate reabsorption in the kidney (Lu et al., 1994). In addition to Ca/P regulation, STC was known to respond to extracellular Na/Cl concentration, especially under the hypotensive conditions (Butler et al., 2003). Although it has been reported that expression levels of Ca^{2+} channel mRNA were suppressed by zSTC1 in zebrafish (Tseng et al., 2009), the definite mechanism how STC regulates ion balance is needed to be further elucidated.

Although there were no corpuscles of Stannius gland or similar organs in mammals, the searching for mammalian cDNA counterpart to fish STC

identified STC1 (Chang et al., 1996; Chang et al., 1995; Olsen et al., 1996) and STC2 (Chang and Reddel, 1998; DiMattia et al., 1998; Ishibashi et al., 1998), which presented the similarity of amino acid sequence about 50 ~ 80 % and 35%, respectively. In spite of relatively low similarity between STC1 and STC2, the exon-intron boundaries, the cysteine residues and glycosylation sites are known to be conserved (Wagner and Dimattia, 2006). Subsequent studies have revealed that STC1 gene was expressed in multiple organs in mammals, including kidney, heart, lung, liver, adrenal glands, prostate and ovary (Chang et al., 1995; Varghese et al., 1998). These results lead to the search for the organs other than gills which express STC in fish; heart, gill, intestine, muscle, brain, gonads, and kidneys of salmon (McCudden et al., 2001) and turbot (Shin et al., 2006) were further incorporated, although the function of STC remained to be clarified. Based on the finding that mammalian STC1 and/or STC2 were not normally detected in blood in healthy status, it was suggested that STC acts in an autocrine and/or paracrine manner (Yeung et al., 2012).

I.2. STC1 and bone

The fundamental studies using transgenic overexpression (Varghese et al., 2002; Yeung et al., 2012) and conventional knockout (Chang et al., 2005)

of STC1 gene have revealed that gain-of-function induced growth retardation in multiple organs (Filvaroff et al., 2002; Varghese et al., 2002) while no significant change was noted in null mice model (Chang et al., 2005). In skeletal tissues, STC1 is highly expressed in osteoblasts (Yoshiko et al., 2002; Yoshiko et al., 1999). Chondrocytes also express STC1 especially in growth plate (Filvaroff et al., 2002; Yoshiko et al., 2002) and it was demonstrated that STC1 negatively regulates chondrogenesis (Johnston et al., 2010). Time-dependent expression pattern of STC1 has been demonstrated in the process of differentiation and maturation of rat calvarial osteoblasts (Yoshiko et al., 2003), which suggests the modulatory role of STC1 in the bone development. However, the regulatory role of STC1 in the progression of osteogenesis and its mechanism of action remains to be further clarified.

I.3. Markers used to examine the osteogenic differentiation of cells

To examine the regulatory role of STC1 in osteogenic differentiation of human periodontal ligament fibroblasts, the expression levels of several marker genes were examined in the present study. Runt-related transcription factor 2 (RUNX2) is an early stage transcription factor involved in osteogenic differentiation. RUNX2 is regarded as a key transcription factor for osteogenesis because loss-of-function of RUNX2 leads to significant defect

in skeletal development (Stein et al., 2004). Interestingly, this gene was hypothesized to relate Neanderthals closer to modern Europeans rather than Africans (Green et al., 2010). Osterix (OSX) is a zinc finger-containing transcription factor, which is expressed in all of the osteoblasts during endochondral and intramembranous bone formation. RUNX2 controls the expression of OSX in the upstream (Nakashima et al., 2002).

Osteocalcin (OCN) is the most abundant non-collagenous protein in bone matrix and present in both bone and dentin. It is produced by mature osteoblasts, and often to be used as a specific marker for osteoblastogenesis (Lian et al., 1989). STC1 transgenic mice demonstrated the reduction of osteocalcin expression level in the calvaria accompanied with delayed suture closure (Johnston et al., 2010). Ostepontin (OPN) is a non-collagenous phosphorylated sialoprotein located in extracellular matrix, linking cells to minerals. It is expressed in both osteoblasts and osteoclasts, contributing to bone remodeling (Sase et al., 2012). Increase in intracellular phosphate level induces OPN expression (Beck and Knecht, 2003; Beck et al., 2000). With respect to STC1, the expression of Pit1, a sodium-dependent phosphate transporter, was reported to be increased with recombinant human STC1 protein (rhSTC1) treatment at the later stage of osteoblast differentiation (Yoshiko et al., 2003). Alkaline phosphatase (ALP), a tissue non-specific alkaline phosphatase, plays an important role in bone matrix calcification. It

binds to the osteoblast membrane and degrades pyrophosphates, which lead to increase in inorganic phosphate concentration that is necessary for matrix mineralization. Therefore, ALP is also a representative marker of osteogenesis (Siffert, 1951).

Alizarin red S dye stains free calcium and calcium depositions a red color. Free calcium is precipitated by Alizarin red. Of interest, Alizarin red was detected in 1957 in animals whose teeth and bone became red after they ate it. Alizarin red staining is used to examine matrix mineralization (Puchtler et al., 1969).

I.4. STC1 and tumorigenesis

It has been demonstrated that STC1 promotes cancer cell proliferation in multiple organs (Liu et al., 2010). The expression levels of STC1 mRNA increased with the stimulation of hypoxia in human nasopharyngeal, ovarian and colorectal adenocarcinoma cell lines. Exposure of cells to hypoxic conditions induced STC mRNA expression in the human nasopharyngeal carcinoma cell lines in a dose-dependent manner, and hypoxia-inducible factor 1α (HIF1α) was pointed to be a mediator of hypoxic stress to STC1 induction (Yeung et al., 2005). STC1 overexpression resulted in tumorigenesis in human ovarian cell lines and nude mice. Promotion of cell

cycle progression and inhibition of apoptosis were suggested as plausible mechanisms (Liu et al., 2010). STC1 was also reported to stimulate angiogenesis, which is crucial for tumorigenesis (Chakraborty et al., 2007; Gerritsen et al., 2002). Overexpression of STC1 in gastric cancer cells increased the volume of tumor mass in nude mice and expression levels of vascular endothelial growth factor (VEGF). VEGF was noted as a primary factor involved in STC1-induced angiogenesis (Chang et al., 1995; Erdem et al., 2007; He et al., 2011; Liu et al., 2003). Activation of protein kinase C (PKC) βII and extracellular signal-regulated kinase (ERK) 1/2 was suggested as a mechanism through which STC1 upregulated VEGF expression (He et al., 2011).

I.5. VEGF

VEGF is a key growth factor for angiogenesis. It has been demonstrated that VEGF is concentrated at the site of intramembranous and endochondral bone formation (Dai and Rabie, 2007; Furumatsu et al., 2003). It has also been shown that the expression levels of VEGF decrease with aging (Pola et al., 2004; Rivard et al., 1999). During bone development, angiogenesis and osteogenesis are coupled both spatially and temporally (Wang et al., 2014). BMP2, OSX and HIF1α are involved in the upregulation of VEGF expression.

OSX directly binds to the VEGF promoter and thereby transactivates VEGF expression (Chen et al., 2012). VEGF stimulates osteogenic differentiation, while inhibiting adipogenic differentiation of mesenchymal stem cells. Previously, it was suggested that VEGF induces osteoblast differentiation via the intracrine mode of action. The addition of VEGF and/or VEGF neutralizing antibody to culture medium did not exert any effects on osteogenic differentiation of mesenchymal stem cells, whereas knockout of VEGF significantly suppressed osteogenic differentiation, which was rescued by VEGF retroviral infection (Liu et al., 2012). VEGF is secreted by mesenchymal stem cells (Quarto and Longaker, 2006). The cooperation of VEGF and fibroblast growth factor 2 was suggested during osteogenesis and angiogenesis (Yanagita et al., 2014). Collectively, these studies indicate the crucial role of VEGF in bone development and bone formation.

I.6. Periodontal ligament stems cells

Adult stem cells are present in dental organs such as periodontal ligament, dental pulp, apical papilla and alveolar bone marrow (Lee et al., 2007). Periodontal ligament is a fibrous connective tissue which interlinks cementum to alveolar bone. It contains undifferentiated mesenchymal stem cells, fibroblasts, epithelial cells and others (Seo et al., 2004). Depending on

the culture conditions, human periodontal ligament (PDL) stem cells demonstrated osteogenic, chondrogenic and adipogenic characteristics (Gay et al., 2007). A quantitative study about stem cell properties of PDL indicated that depending on donor, 15-30% of prepared PDL cells showed replicative potential. Mesenchymal stem cell markers such as CD105, CD166 and STRO-1 were shown positive in approximately 90% of PDL stem cells. STRO-1 was expressed with efficiency of 63% after 14 days, which was reduced to 29% after 21 days. The proportions of ALP positive colonies increased at the 21th day and approximately 28% stained positive with Alizarin red staining. Adipogenic colony-forming efficiency was about 20%. Culture of human bone marrow cells demonstrated colony-forming efficiency about 27% (Sakaguchi et al., 2004). These reports indicate that PDL stem cells possess definite potentials of replication and differentiation toward multiple lineages, which may contribute to orthodontic tooth movement and subsequent remodeling of periodontal tissues.

I.7. Osteoclast differentiation

Osteoclasts are differentiated from the precursor cells of monocyte and macrophage lineage. Osteoclastogenesis is a multi-step process: differentiation proceeds from early osteoclast precursors to committed

osteoclasts and the cells are finally fused to form multinucleated osteoclasts with polarization in order to resorb bone matrix (Teitelbaum and Ross, 2003). Receptor activator of nuclear factor κB ligand (RANKL) is expressed on the surface of osteoblasts/stromal cells and interacts with its receptor RANK on the osteoclast precursor cells. The genes involved in osteoclast differentiation and function are well known, including nuclear factor of activated T cells 1 (NFATc1), Fos-related antigen (Fra) 1, Fra2, c-Fos, c-Src, intergrin β3, cathepsin K (CTSK) and tartrate-resistant acid phosphatase (TRAP) (Ishida et al., 2002; Kuroda and Matsuo, 2012). Nfatc1 is a key transcription factor for osteoclast differentiation, and overexpression of Nfatc1 induces osteoclastogenic differentiation of precursor cells in the absence of RANKL (Takayanagi et al., 2002). Fos gene family members including c-Fos, Fra1 and Fra2 dimerize with Jun family member, forming activator protein 1 transcription factor. Intergin β3 knockout mice showed osteosclerotic phenotype due to dysfunctional osteoclasts, suggesting that integrin β3 is essential for osteoclast to recognize and attach to bone matrix (McHugh et al., 2000). Integrin activates c-Src which organizes the acidifying vesicles to align through microtubules, eventually differentiates committed osteoclast to polarized osteoclast (Duong et al., 1998; Yokouchi et al., 2001). At the cell-bone interface, resorbing osteoclast generates protons, which were produced by carbonic anhydrase II. Proton pumps located at the ruffled border export

protons to extracellular matrix, which dissolve bone crystals and expose the organic matrix (Teitelbaum, 2000), which is degraded by a low pH optimized collagenase, CTSK (Gelb et al., 1996). TRAP is detected in mature osteoclast, which contributes to production of reactive oxygen species (ROS) and dephosphorylates OPN and bone sialoprotein (Oddie et al., 2000).

II. INTRODUCTION

PDL is a fibrous connective tissue which is composed of collagen fibers, glycoproteins, glycolipid, glycosaminoglycan and cells including undifferentiated mesenchymal stem cells. PDL connects alveolar bone to the root surface of a tooth (Lekic and McCulloch, 1996). Orthodontic mechanical force is known to induce bone formation in tension side and bone resorption in compression side (Niklas et al., 2013). Osteoblast differentiation in the tension side usually starts within three days without angiogenesis. In the compression side, active osteoclastogenesis is appeared seven days after the force applies and continuously observed to fourteen days with accompanied angiogenesis. Orthodontic pressure causes blood vessel occlusion in the compression side of PDL, while causing enlargement of the blood vessels in the tension side. It is, therefore, expected that the compression side underwent hypoxia due to deficient blood supply (Khouw and Goldhaber, 1970; Kitase et al., 2009).

In general, hypoxia indicates a deficiency of oxygen in a body or a part of the body (Greijer and van der Wall, 2004). Mostly, hypoxic conditions stimulate physiologic responses in multiple pathways, especially in vital organs such as the brain, heart and lung. Prolonged hypoxia induces pathologic responses, including cyanosis, *cor pulmonale*, tachycardia, pallor,

breathlessness, extreme headaches, confusion, hallucination, disorientation and low blood pressure causing death (Illingworth and Simpson, 1998). Because oxygen plays a crucial role in oxidative phosphorylation, the hypoxia triggers compensatory signaling pathways that target gene expression, leading to activation of angiogenesis and wound healing. Dysfunction in such compensatory signaling pathways may be the cause of tumor angiogenesis and chronic inflammation (Nakayama, 2009).

Local hypoxia induced by orthodontic tooth movement is also known to stimulate multicellular responses in human PDL (Niklas et al., 2013). HIF1α plays a critical role in cellular responses to local oxygen concentrations in PDL (Arnett et al., 2003; Niklas et al., 2013). In particular, the modulatory function of HIF1α is noted in terms of cell survival and death under chronic or extreme hypoxic conditions. HIF1α not only induces angiogenesis but also plays dual roles in cell survival and apoptosis (Semenza, 2009). In hypoxic conditions, HIF1α is known to activate enolase, glucose transporter 1, erythropoietin and VEGF, which lead to cell survival. On the other hand, under anoxic situation, HIF1α binds to p53 which activates Bax, a pro-apoptotic protein, and results in apoptosis (Niklas et al., 2013). In the context of cell survival, hypoxia is known to play a critical role in the maintenance of stemness of stem cells. Hematopoietic stem cells showed enhanced survival rate and self-renewal stemness in low oxygen tension (Schmidt et al., 1998;

Xiao et al., 2000). To overcome the technical difficulties of maintaining low numbers of adult stem cells, it was suggested that growing the cells in hypoxic conditions is a method to expand the number of stem cells while maintaining their stemness. Treatment of cells with $CoCl_2$, which mimics hypoxia, inhibited osteogenic differentiation, while inducing stem cell marker OCT4 in mesenchymal stem cells (Osathanon et al., 2015). However, sustained hypoxia resulted in periodontal destruction through ROS production. Hypoxia and lipopolysaccharide (LPS)-induced inflammation conditions induced drastic ROS formation, which increased periodontal destruction (Golz et al., 2014).

The severity of hypoxia was considered as an important modulator for cell maintenance, proliferation and death (Choi et al., 2014). Anoxia or severe hypoxia under 2% of oxygen significantly induced cell death; however, over 8% hypoxia was estimated to be physiological oxygen tension in multiple stem cells (Ezashi et al., 2005; Grayson et al., 2006). Immunocytochemical analysis utilizing pimonidazole adducts to detect hypoxic response demonstrated that PDL cells showed 19% increase in cell proliferation rate with augmented HIF1α expression when they were exposed to 8% oxygen tension (Grayson et al., 2006). Other studies also demonstrated that hypoxic microenvironment (2% oxygen) stimulated cellular proliferation and increased pluripotency marker expression in PDL cells. Furthermore, enhanced

differentiation potentials toward osteoblasts, chondrocytes and even adipocytes were demonstrated (Zhou et al., 2014).

Previously, we conducted a preliminary study in order to identify differentially expressed genes in human PDL cells exposed to hypoxia for 24 h compared to the cells incubated in normoxic conditions using the cDNA microarray analysis. Among the upregulated genes, STC1 showed the highest fold-change (data not shown). STC1 and STC2 are mammalian homologues of STC, which is a glycoprotein hormone first found in the corpuscles of Stannius in fish (Bonga and Pang, 1991). STC1 is expressed in multiple organs, including kidney, heart, lung, liver, adrenal gland, prostate and ovary (Chang et al., 1995; Varghese et al., 2002). STC1 transgenic mice demonstrated growth retardation in multiple organs (Filvaroff et al., 2002), whereas no significant change was noted in STC1 null mice (Chang et al., 2005). STC1 is also expressed in osteoblasts (Yoshiko et al., 2002; Yoshiko et al., 1999). A time-dependent expression pattern of STC1 was demonstrated during osteogenic differentiation and maturation of rat calvarial cells, which suggest a modulatory role of STC1 in the osteoblastic bone formation (Yoshiko et al., 2003). Of interest, STC1 expression was also identified in human PDL cells in response to compression. STC1 showed the highest expression levels after 24 h of mechanical compression (Li et al., 2013).

STC1 is induced by HIF1α and plays a crucial role in hypoxia (Yeung et al., 2012). Effect of hypoxia on STC1 has been well-documented in cancer cell lines. In renal carcinoma cell lines, a positive correlation between STC1 and HIF1α was demonstrated (Yeung et al., 2005). Knockdown of HIF1α using RNA interference technology demonstrated significantly reduced levels of STC1. In clear cell renal carcinoma, hypoxia increased expression of HIF1, followed by VEGF expression. VEGF enhanced STC1 mRNA expression in the cells exposed to desferoxamine, an inducer of HIF1α accumulation. In addition, STC1 has been demonstrated to promote angiogenesis by enhancing VEGF expression (Gerritsen et al., 2002; Wang et al., 2014). Recent findings suggested that VEGF enhances osteoblastogenesis as well as angiogenesis (Dai and Rabie, 2007; Furumatsu et al., 2003). In addition to the regulatory role in osteogenesis, a study noted that STC1 may have a possible effect on osteoclasts. TRAP staining of STC1 transgenic mice calvaria demonstrated broader distribution of osteoclasts compared to wild-type mice, suggesting a decreased osteoclast activity in STC1 transgenic mice (Filvaroff et al., 2002). However, the relationships between STC1 and osteogenesis/osteoclastogenesis have not been clearly elucidated.

In addition to the regulatory role in cell differentiation and angiogenesis, protective effect of STC1 against cell death has been also investigated. STC1 was shown to have a protective effect on hypoxia-induced cell damage

(Westberg et al., 2007). Studies using cells from different origins have also indicated that STC1 reduces ROS production (Huang et al., 2009; Liu et al., 2012; Wang et al., 2009). STC1 upregulated expression levels of uncoupling protein 3 in cardiomyocytes, thereby attenuating mitochondrial membrane potential and suppressing superoxide production (Liu et al., 2012).

III. PURPOSE OF THE STUDY

The preliminary microarray study using PDL cells under hypoxic conditions, which mimic the physiologically relevant environmental setting during orthodontic tooth movement, identified STC1 as the most highly upregulated genes, suggesting that STC1 may have a role in alveolar bone remodeling. Previous studies suggest a controversial role of STC1 in bone formation: STC1 promoted osteoblast differentiation in rat calvalria cell cultures, while STC1 transgenic mice exhibited reduced cranial intramembranous bone growth and STC1 null mice showed no significant bone abnormalities. Similarly, it remains to be elucidated whether STC1 regulates osteoclastogenesis.

Considering that PDL cells in compression side are exposed to hypoxic conditions as well as mechanical compression, which is a stimulatory signal for STC1 induction, it is presumed that STC1 may play some roles in PDL during orthodontic tooth movement. Therefore, the present study was performed to test following working hypotheses.

Working hypothesis:

1. **STC1 may enhance osteogenic differentiation via VEGF induction in hPDL cells.**
2. **STC1 may play a protective role in hPDL cells against hypoxia-induced cell death.**
3. **STC1 may inhibit osteoclast differentiation, thereby exerting negative feedback regulation to suppress excessive resorption of alveolar bone and/or tooth root in compression side.**

IV. MATERIALS AND METHODS

IV.1. Reagents and antibodies

Minimum essential medium Eagle alpha modifications (α-MEM), fetal bovine serum (FBS) and other reagents for cell culture were purchased from Hyclone (Logan, UT, USA). A GasPak™ EZ CO2 Pouch System which was used for induction of hypoxia, was obtained from BD Biosciences (Franklin Lakes, NJ, USA). The easy-BLUE, PRO-PREP and WEST-ZOL (plus) were acquired from iNtRON Biotechnology (Sungnam, Korea). The AccuPower RT-PreMix was purchased from Bioneer (Daejeon, Korea), and the SYBR Premix Ex Taq was from TaKaRa (Otsu, Japan). Alizarin red S was purchased from Sigma (St. Louis, MO, USA). QuantiChrome Alkaline Phosphatase Assay kit was obtained from BioAssay Systems (Hayward, CA, USA). LipofectAMINE 2000 transfection reagent was purchased from Invitrogen (Waltham, MA, USA). Synthesized PCR primers were purchased from CosmoGenetech (Seoul, Korea). Antibodies to beta-actin, STC1, VEGF were purchased from Santa Cruz Biotechnology (Dallas, TX, USA) and HRP-conjugated secondary antibodies were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Antibodies to Caspase 3, Caspase 8, BAX and BCL2 were obtained from Cell Signaling Technology (Danvers, MA, USA). Recombinant human STC1

(rhSTC1) and recombinant mouse $VEGF_{164}$ (rmVEGF-A) proteins were purchased from BioVendor R&D (Asheville, NC, USA) and R&D Systems (Minneapolis, MN, USA), respectively.

IV.2. Culture of PDL cells and hypoxic treatment

Human PDL fibroblasts were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA) and maintained in the medium consisted of α-MEM, 10% heat-inactivated FBS and antibiotics (100 μg/ml of streptomycin and 100 U/ml of penicillin). After reaching over 90% of confluence, subculture of the cells was performed by treating 1x trypsin/EDTA solution, followed by harvesting, centrifuging, and resuspension. PDL cells of 4-8 passages were used for the following experiments.

Osteogenic differentiation of PDL cells was induced by adding 10 mM β-glycerophosphatate, 50 μg/ml *L*-ascorbic acid and 100 nM dexamethasone to growth medium for the indicated periods.

Hypoxic conditions were induced by incubating PDL cells in a GasPak pouch EZ, which is known to provide less than 1% of oxygen concentration (Chae et al., 2011).

IV.3. Evaluation of matrix mineralization

PDL cells were cultured for 28 days in growth medium or osteogenic medium in the presence or absence of the indicated reagents. At the end of culture period, cells were fixed with 70% ethanol, washed with phosphate-buffered saline (PBS) and stained with Alizarin red S solution. To quantify matrix mineralization, the stain was eluted by 0.5 N HCl containing 5% sodium dodecyl sulfate (SDS) and optical density of the eluent was measured at 405 nm.

IV.4. ALP staining and ALP activity assay

PDL cells were cultured for 7 days in growth medium or osteogenic medium in the presence or absence of the indicated reagents. At the end of culture period, ALP staining was conducted employing an ALP staining kit following the instructions of manufacturer. ALP activity was also assessed using a QuantiChrom ALP assay kit according to the manufacturer's instructions. Concentration of reaction product *p*-nitrophenol was then determined by measuring absorbance at 405 nm. ALP activity was normalized to the amount of total protein.

IV.5. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using easy-BLUE™ RNA extraction reagents and complementary DNA was synthesized from 1 μg of total RNA using the AccuPower RT-PreMix. Quantitative PCR was conducted using SYBR premix EX Taq in an AB7500 or AB7300 Fast real-time system (Applied Biosystem; Foster City, CA, USA).

Expression levels of genes were compared after normalization to internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Human and mouse genes and their primer sequences for PCR reactions were shown in Table 1 and Table 2.

IV.6. Western blot analysis

At the end of the culture period, the cells were harvested, lysed using PRO-PREP™ and sonicated briefly. Samples containing equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis and subsequent electrotransfer into a PVDF membrane. The membrane was blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20. The membrane was incubated with the indicated primary antibody, followed by incubation with the HRP-conjugated secondary antibody. Immune complexes were visualized using the Sensi-view™ Pico ECL Reagent (Lugen

Sci Inc; Buncheon, Korea) and detected with a MicroChemi (DNR; Jerusalem, Israel).

Table 1. Human genes and their PCR primer sequences

Genes	Forward (5′–3′)	Reverse (5′–3′)
ALP	AACTTCCAGACCGGCTTGA	TTGCCGCGTGTCTT
OCN	GTGCAGAGTCCAGCAAAGGT	CGATAGGCCTCCTGAAAGC
OSX	ACCTACCCATCTGACTTTGCT	CCACTATTTCCCACTGCCTTG
RUNX2	CAGATGGGACTGTGGCTGT	GTGAAGACGGTTATGAAGG
STC1	CACACCCACGAGCTGACTTC	CTCCCTGGTTATGCACTCTCA
VEGF	ATGATTCTGCCCTCCTCCTTCT	GCTGTCTTCGGTGCATTGGA
GAPDH	CCATCTTCCAGGAGCGAGATC	GCCTTCTCCATGGTGGTGAA

ALP, tissue non-specific alkaline phosphatase; OCN, osteocalcin; OSX, osterix; STC1, stanniocalcin 1; VEGF, vascular endothelial growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Table 2. Mouse osteoclast differentiation marker genes and their PCR primer sequences

Genes	Forward (5′–3′)	Reverse (5′–3′)
c-Fos	CCAGTCAAGAGCATCAGCAA	AAGTAGTGCAGCCCGGAGTA
c-Src	CCAGGCTGAGGAGTGGTACT	CAGCTTGCGGATCTTGTAGT
CTSK	GGCCAACTCAAGAAGAAAAC	GTGCTTGCTTCCCTTCTGG
Fra1	AGAGCTGCAGAAGCAGAAGG	CAAGTACGGGTCCTGGAGAA
Fra2	ATCCACGCTCACATCCCTAC	GTTTCTCTCCCTCCGGATTC
Integrin β3	TGACATCGAGCAGGTGAAAG	GAGTAGCAAGGCCAATGAGC
Nfatc1	AATAACATGCGAGCCATCATC	TCACCCTGGTGTTCTTCCTC
TRAP	ACACAGTGATGCTGTGTGGCAACTC	CCAGAGGCTTCCACATATATGATGG
GAPDH	TCAATGACAACTTTGTCAAGC	CCAGGGTTTCTTACTCCTTGG

CTSK, cathepsin K; TRAP, tartrate-resistant acid phosphatase

IV.7. Overexpression and knockdown of genes using small interfering RNA (siRNA)

Overexpression of human STC1 gene was induced by transient transfection of cells with STC1 plasmid (GeneCopoeia; Rockville, MD, USA) using LipofectAMINE 2000 reagent. Gene knockdown was induced by transient transfection of cells with the corresponding siRNA using DharmaFECT 1 transfection reagent (Dharmacon; Lafayette, CO, USA). TARGETplus SMARTpool siRNAs (a mixture of four siRNAs targeting independent sequences of target mRNA) to human STC1 and VEGF-A and non-targeting control siRNA (ON-TARGETplus non-targeting siRNA #2 D-001810-02-5) were obtained from Dharmacon (Lafayette, CO, USA). The sequences of each siRNA are as follows: STC1 siRNA (5'-AAA CGC ACA UCC AUG AGA-3'; 5'-GGG AAA AGC AUU CGU AAA-3'; 5'-GUA CAG CGC UGC UAA AUU U-3'; 5'-CAA CAG AUA CUA UAA CAG A-3') and VEGF-A siRNA (5'-GCA GAA UCA UCA CGA AGU G'-3'; 5'-CAA CAA AUG UGA AUG CAG A-3'; 5'-GGA GUA CCC UGA UGA GAU C-3'; 5'-GAU CAA CCU CAC CAA GGC-3'). The efficacy of knockdown was assessed by quantitative PCR and/or western blot analysis.

IV.8. Function blocking of STC1 and VEGF-A

Extracellular function of STC1 and VEGF-A were blocked using the neutralizing antibody obtained from R&D Systems. Concentration of the antibody (500 ng/ml) used in this study was determined according to the preliminary study and the previous reports (Gerber et al., 1999; Liang et al., 2006; Liu et al., 2010).

IV.9. Enzyme-linked immunosorbent assay (ELISA) of STC 1 and VEGF-A proteins

To measure the amount of secreted STC1 and VEGF-A, PDL cell-conditioned medium was prepared as described in the figure legends. Levels of STC1 and VEGF-A in conditioned medium were analyzed according to the manufacturer's instructions using the ELISA kit from CUSABIO (CSB-EL022821HU; College Park, MD, USA) and Abcam (ab119566-VEGFA; Cambridge, MA, USA), respectively.

IV.10. Osteoclastogenesis

Effect of STC1 on osteoclast differentiation was examined using the RAW264.7 (a murine monocyte/macrophage cell line) and mouse bone marrow-derived macrophage (BMM) cells. RAW264.7 cells were maintained with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and antibiotics. To induce osteoclastogenesis, RAW264.7 cells were incubated for 4 days in the presence of 100 ng/ml of RANKL (ProSpec; Rehovot, Israel). Primary bone marrow cells from the tibiae and femur of BALB/c mice were kindly provided by Prof. Hong-Hee Kim of Seoul National University School of Dentistry. Adherent BMM cells were prepared by incubating the bone marrow cells for 3 days in αMEM supplemented with 10% FBS, antibiotics and 30 ng/ml of macrophage colony stimulating factor (M-CSF; ProSpec). BMM cells were then further incubated for 6 days in the presence of 100 ng/ml of RANKL and 30 ng/ml of M-CSF. When indicated, 100 ng/ml of STC1 was added to the culture medium.

IV.11. TRAP staining and TRAP activity assay

TRAP staining was conducted using a TRAP staining kit (Takara Bio; Kusatsu, Japan) according to the manufacturer's instructions. When indicated, the number of TRAP-positive multinuclear cells (> 3 nuclei/cell) was counted under the microscope (X100). TRAP activity assays were also performed using the cell lysates, employing an ALP and TRACP assay kit (Takara Bio) following the manufacturer's protocol. TRAP activity was normalized by the amount of total protein in the cell lysates.

IV.12. Cell proliferation assay

PDL cells were plated at a density of $5x10^4$ cells per well in 96-well tissue culture plates and incubated in growth medium for 48 and 72 h. At the end of culture periods, cell viability was assessed using Ez-Cytox Cell Viability Assay Kit (Daeilbiotech; Suwon, Korea) according to the manufacturer's instructions.

IV.13. Flow cytometry analysis of apoptosis

PDL cells were incubated for 24 h under hypoxic or normoxic conditions in the presence or absence of rhSTC1. Cells were then harvested using 0.25% trypsin and subjected to Annexin V and propidium iodide (PI) staining using

Annexin-V-FITC apoptosis detection kit I (BD Biosciences). Briefly, single-cell suspension of 2×10^6 cells in 200 μl of binding buffer was incubated for 15 min after addition of 5 μl each of Annexin-V-FITC and PI. Then, 400 μl of binding buffer was further added and flow cytometric analysis was immediately performed adopting a BD FASC caliber system. The fraction of apoptotic cells was presented as the percentage of cells stained positive to both Annexin V and PI.

IV.14. Statistics

All of the quantitative results were demonstrated as the mean ± SD. Student's *t*-test or multiple-comparison was conducted using Prism 6 software (GraphPad, La Jolla, CA, USA). *P* values less than 0.05 were regarded as statistically significant.

V. RESULTS

V.1. Hypoxic conditions induce the expression of STC1 in human PDL cells

To confirm that hypoxic conditions can induce STC1 expression in PDL cells, the cells were incubated in GasPak pouch for 0, 4, 12 and 24 h. The mRNA and protein levels of STC1 were examined by using quantitative PCR and western blot analyses. The data demonstrated that mRNA levels of STC1 significantly increased at 12 and 24 h after incubation under hypoxic conditions. Consistent with this finding, western blot analysis also confirmed that hypoxia increased the levels of STC1 protein at the same time points (Figure 1).

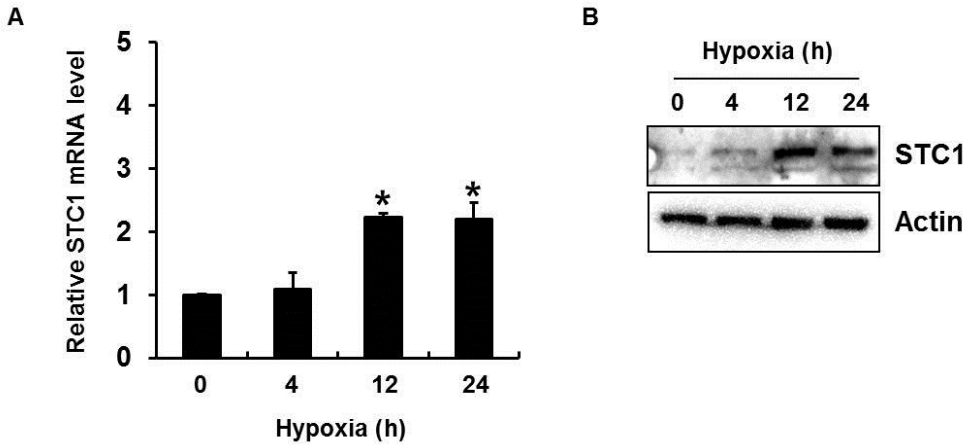

Figure 1. Hypoxia induced STC1 expression in human PDL cells. Human PDL cells were incubated in GasPak pouch for the indicated periods and the expression levels of STC1 mRNA and protein were examined by quantitative PCR (A) and western blot analysis (B). Quantitative data were presented as the mean ± SD (* significantly different from 0 h).

V.2. STC1 expression is highly upregulated during osteogenic differentiation of PDL cells

To examine whether the expression levels of STC1 are regulated during osteogenic differentiation of PDL cells, PDL cells were incubated in osteogenic medium for 0.5, 1, 2, 4 and 7 days and quantitative PCR were performed. Significant increase in the expression levels of osteogenic markers, including RUNX2, DLX5, OSX and ALP, appeared only after the incubation for 4 days (Figure 2). VEGF also showed similar induction pattern to those of osteogenic marker genes. However, STC1 exhibited significant induction after 2 days of incubation, which was earlier than other osteogenic markers (Figure 2). In addition, fold change was highest in STC1, compared to other osteogenic markers.

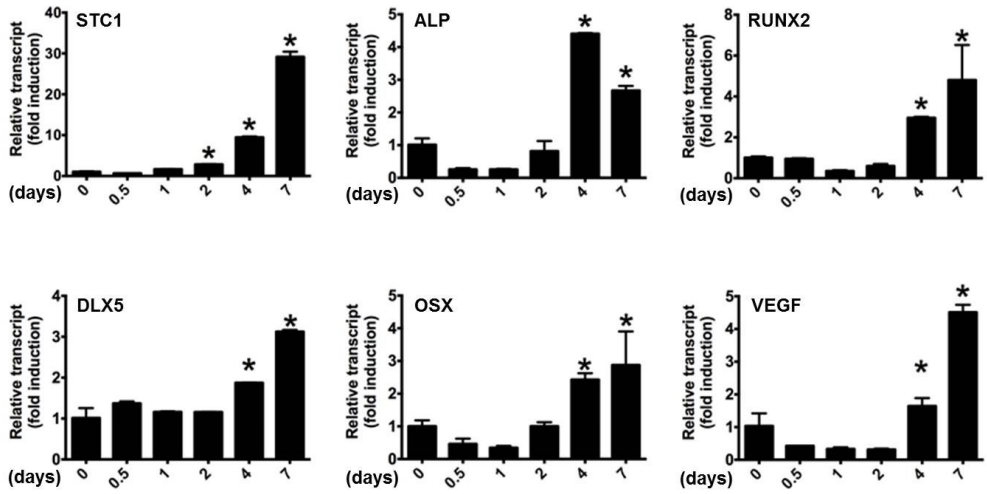

Figure 2. Osteogenic stimuli led to earlier and stronger induction of STC1 compared to other osteogenic marker genes. PDL cells were incubated in osteogenic medium for the indicated periods, and quantitative PCR of osteogenic markers and STC1 were performed. Data represent the mean ± SD of fold changes in mRNA levels compared to the 0 day samples (* significantly different from 0 day).

V.3. Overexpression of STC1 enhances osteogenic differentiation of PDL cells

Because STC1 was strongly induced by osteogenic stimuli, the role of STC1 in osteogenic differentiation was examined by overexpressing STC1 in PDL cells. Two days after transient transfection of STC1 plasmid, the levels of STC1 were examined by quantitative PCR and western blot analysis. The mRNA and protein levels of STC1 were significantly elevated in STC1-transfected cells (Figure 3A). To access the functional consequences of STC1 overexpression, PDL cells were incubated in osteogenic medium for 7 days. At the end of the culture period, ALP staining, ALP activity assay and quantitative PCR of osteogenic marker genes were performed. The data showed that STC1 overexpression significantly enhanced ALP staining and ALP activity (Figure 3B). In addition, the mRNA levels of osteogenic marker genes were much higher in STC1-overexpressing cells compared to PCDNA-transfected control cells (Figure 3C).

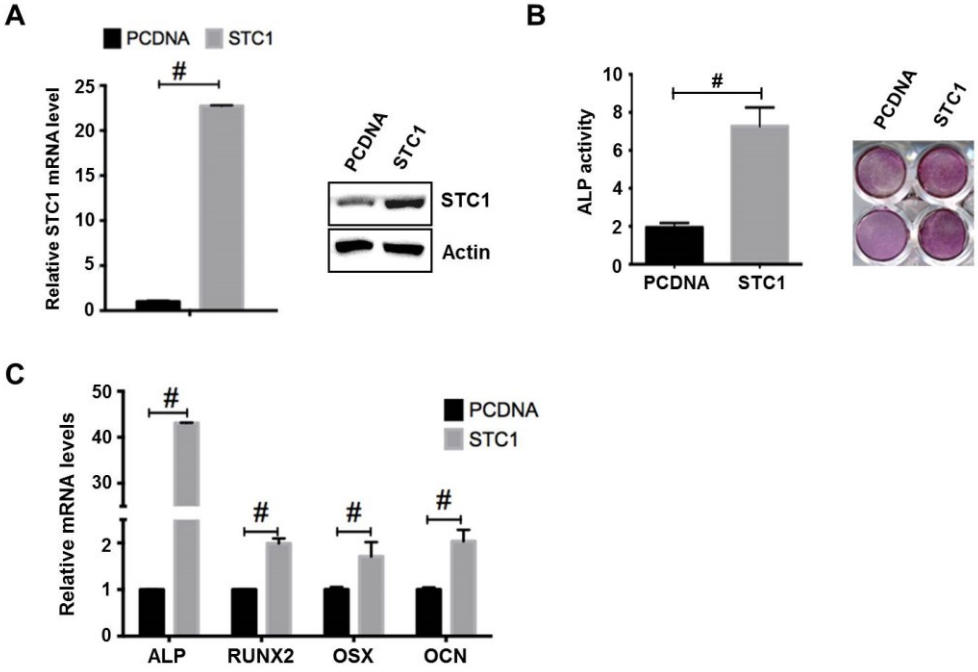
A
PCDNA
STC1
#
Relative STC1 mRNA level
25
20
15
10
5
0
PCDNA
STC1
STC1
Actin
B
#
ALP activity
10
8
6
4
2
0
PCDNA
STC1
PCDNA
STC1
C
#
Relative mRNA levels
50
40
30
2
1
0
PCDNA
STC1
#
#
#
ALP
RUNX2
OSX
OCN

Figure 3. Overexpression of STC1 enhanced osteogenic differentiation of PDL cells. (A) PDL cells were transiently transfected with PCDNA or STC1 expression plasmid and incubated for 2 days. Increase in expression levels of STC1 mRNA (left panel) and protein (right panel) were confirmed by quantitative PCR and western blot analysis. (B, C) After transient transfection of cells with PCDNA or STC1 expression plasmid, PDL cells were incubated in osteogenic medium for 7 days, followed by ALP activity assay (B, left panel), ALP staining (B, right panel) or quantitative PCR (C). Quantitative data were presented as the mean ± SD (#, significantly different from PCDNA-transfected cells). ALP activity represents the normalized amount of *p*-nitrophenol produced in the reaction per unit time (μmol/μg total protein/min).

V.4. rhSTC1 promotes osteoblastic differentiation of PDL cells

To verify the functional effect of exogenous STC1 on osteogenic induction, PDL cells were incubated in the presence or absence of rhSTC1 (100 ng/ml). Of interest, incubation of PDL cells in the presence of rhSTC1 for 2 days increased the expression levels of STC1 protein in the PDL cells, which implies that STC1 has an auto-induction property in PDL cells (Figure 4A). When PDL cells were incubated in osteogenic medium for 7 days, rhSTC1 significantly increased mRNA expression levels of osteogenic marker genes and ALP activity compared to control cells incubated in osteogenic medium alone (Figure 4B&4C). Similarly, rhSTC1 significantly enhanced matrix mineralization when PDL cells were incubated in osteogenic medium for 28 days (Figure 4D). However, rhSTC1 could not induce ALP activity or matrix mineralization when cells were incubated in growth medium (Figure 4C&4D), indicating that rhSTC1 alone is not sufficient to induce osteogenic differentiation of PDL cells.

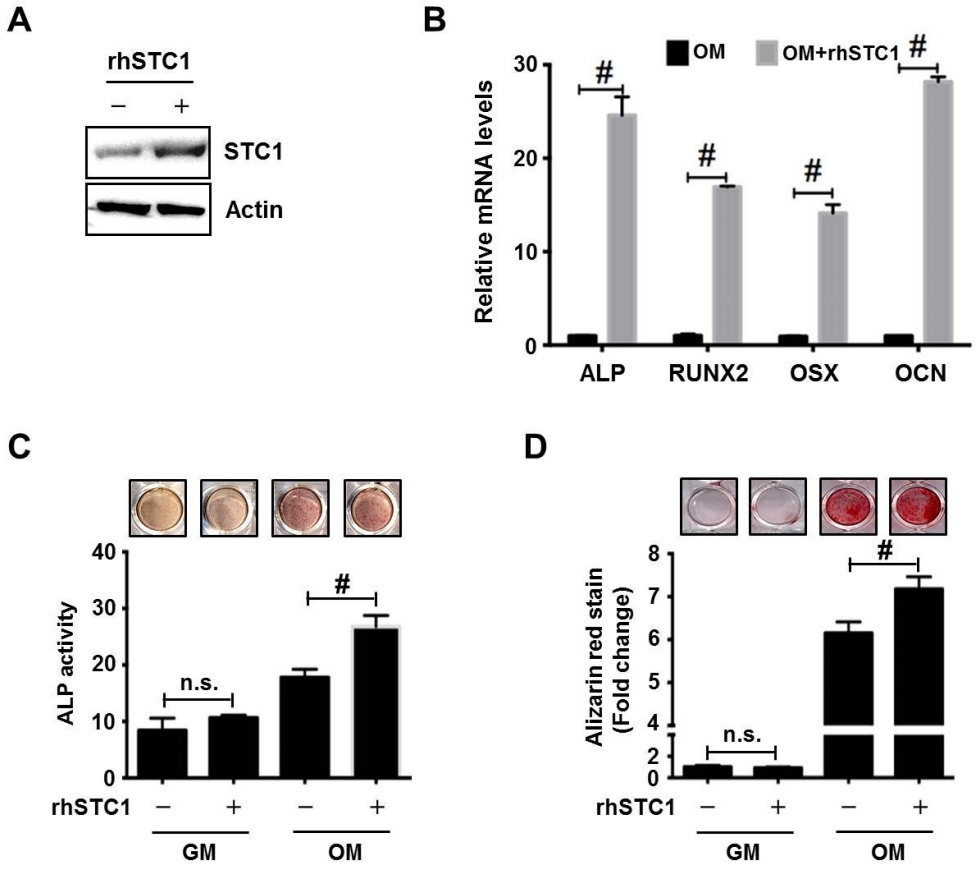
A
rhSTC1
– +
STC1
Actin
B
OM
OM+rhSTC1
Relative mRNA levels
30
20
10
0
#
ALP
RUNX2
OSX
OCN
C
ALP activity
40
30
20
10
0
n.s.
#
rhSTC1 – + – +
GM
OM
D
Alizarin red stain
(Fold change)
8
7
6
5
4
2
0
n.s.
#
rhSTC1 – + – +
GM
OM

Figure 4. rhSTC1 enhanced STC1 expression and osteogenic stimuli-induced osteoblastic differentiation of PDL cells. (A) PDL cells were incubated in the presence or absence of rhSTC1 (100 ng/ml) for 2 days, followed by western blot analysis. (B) PDL cells were incubated in osteogenic medium (OM) for 7 days in the presence or absence of rhSTC1, followed by quantitative PCR. (C, D) PDL cells were incubated in growth medium (GM) or osteogenic medium (OM) for 7 days (C) or for 28 days (D). At the end of the culture periods, the cells were subjected to ALP staining (C, upper panel), ALP assay (C, lower panel) or Alizarin red staining (D, upper panel) and subsequent quantification of Alizarin red stain (D, lower panel). #, significantly different in the indicated pair; n.s., not significantly different

V.5. STC1 knockdown attenuates osteogenic differentiation of PDL cells

To further confirm the role of STC1 in the osteogenic differentiation of PDL cells, the expression of STC1 was blocked using siRNA against STC1. Silencing efficiency was verified by quantitative PCR (Figure 5A). STC1 siRNA did not exert significant effect on the expression levels of osteogenic marker genes when PDL cells were incubated in growth medium. However, STC1 knockdown significantly suppressed osteogenic stimuli-induced gene expression (Figure 5A). Interestingly, VEGF mRNA levels in STC1-silenced cells were much lower than those in control siRNA-transfected cells grown in growth medium (Figure 5A). In a similar manner, STC1 knockdown significantly decreased osteogenic stimuli-induced ALP activity (Figure 5B). To clarify that STC1 siRNA-mediated downregulation of osteogenic markers depends on the knockdown of STC1 in PDL cells, a rescue experiment was performed using rhSTC1. The data in Figure 5B demonstrated that ALP activity of STC1 siRNA-transfected cells was fully recovered to that of control siRNA-transfected cells when exogenous rhSTC1 was added to the culture medium.

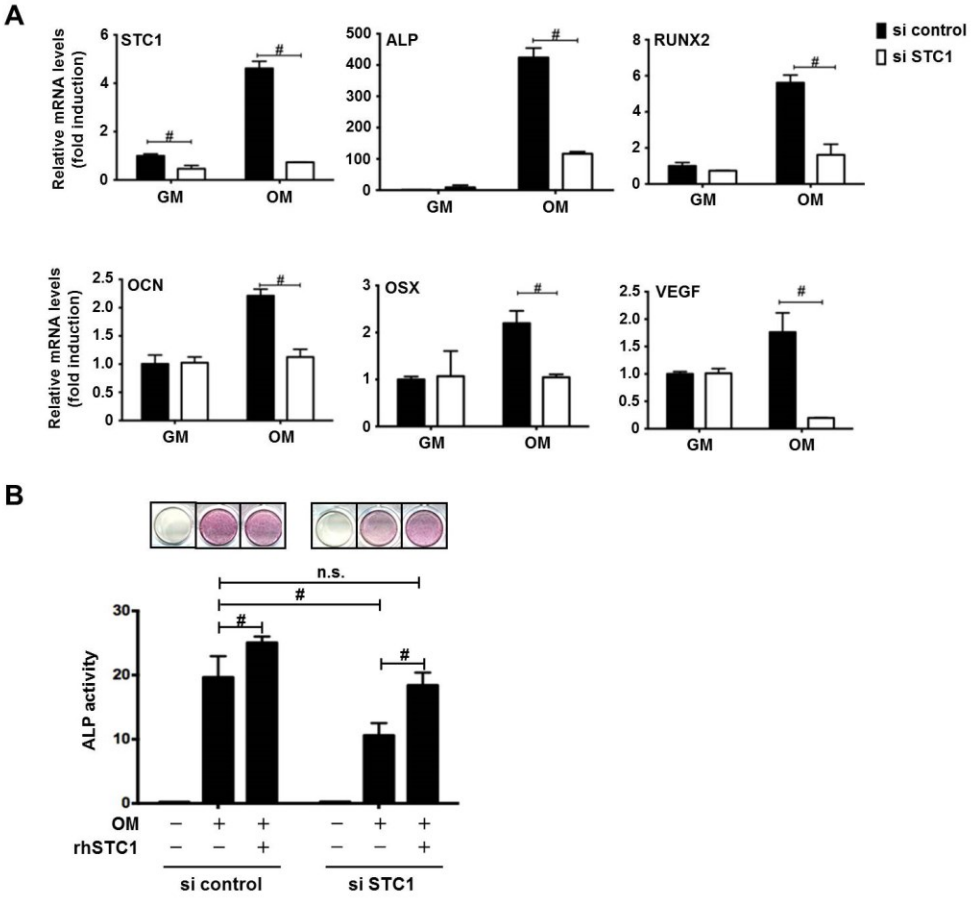
A
si control
si STC1
STC1
ALP
RUNX2
OCN
OSX
VEGF
Relative mRNA levels (fold induction)
GM
OM
#
B
n.s.
ALP activity
OM
rhSTC1
si control
si STC1

Figure 5. Silencing of STC1 attenuated osteogenic stimuli-induced osteogenic differentiation of PDL cells. PDL cells were transiently transfected with control siRNA (si control) or STC1 siRNA (si STC1) and incubated in growth medium (GM) or osteogenic medium (OM) for 7 days. Quantitative PCR (A) or ALP assay (B) was then performed. Data are presented as the mean fold change to those of control siRNA-transfected cells incubated in growth medium. (B) When indicated, rhSTC1 (100 ng/ml) was added to culture medium. #, significantly different in the indicated pair; n.s., not significantly different

V.6. STC1 neutralizing antibody inhibits osteogenic differentiation of PDL cells

Previous studies have suggested that STC1 has a paracrine effect (Ishibashi and Imai, 2002; Luo et al., 2004; Wu et al., 2006). To investigate whether pro-osteogenic role of endogenous STC1 depends on the secreted STC1 protein, the amount of secreted STC1 protein was examined. PDL cells were incubated under the osteogenic conditions and conditioned medium was collected at day 0, 3, 6, 9, 12 and 15. The quantity of STC1 protein in conditioned media was then determined using ELISA kit. The data demonstrated that secreted STC1 protein levels significantly increased in a time-dependent manner (Figure 6A). The concentration of STC1 protein at day 15 was 120±9 ng/ml. In order to block the activity of secreted STC1, PDL cells were incubated in the presence of STC1 neutralizing antibody. Preliminary experiments using C2C12 cells (mouse myoblastic cells) to determine the concentration of STC1 antibody that significantly block rhSTC1-induced osteogenic differentiation demonstrated that 500 ng/ml of STC1 antibody was enough to block the STC1 activity (data not shown). Therefore, osteogenic differentiation of PDL cells was induced in the presence or absence of 500 ng/ml of STC1 antibody, and the levels of osteogenic marker genes and ALP activity were examined at day 7 and matrix mineralization was analyzed at day 28. As shown in Figure 6B, STC1

neutralizing antibody almost completely blocked the induction of osteogenic marker gene expression, including RUNX2, OCN, OSX and VEGF genes. The levels of ALP mRNA and ALP activity were also significantly downregulated by STC1 antibody (Figure 6B&6C). In addition, blocking of extracellular STC1 action significantly decreased matrix mineralization to less than 50% of cells incubated in osteogenic medium only (Figure 6D). Further addition of rhSTC1 to the culture medium significantly rescued the levels of matrix mineralization, although not complete (Figure 6D).

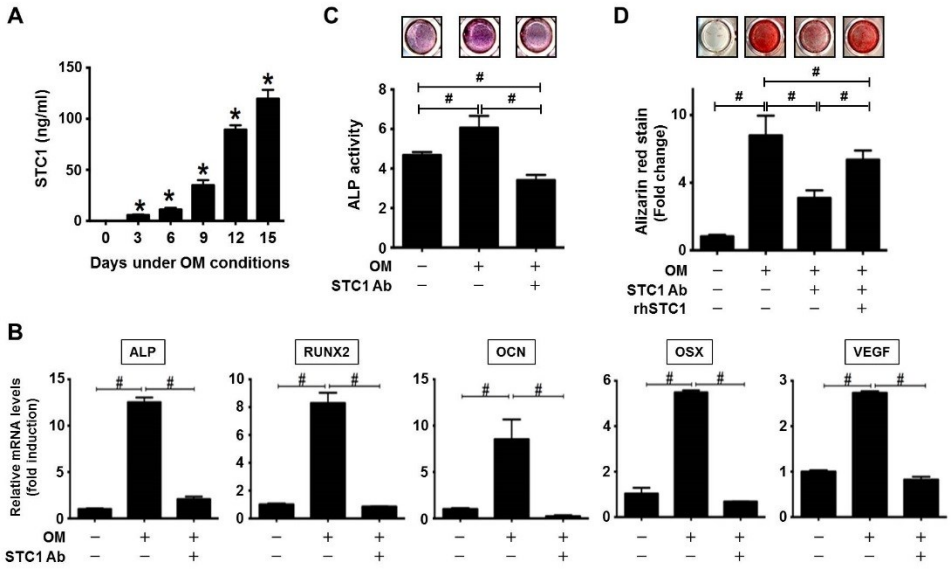

Figure 6. STC1 neutralizing antibody significantly attenuated osteogenic differentiation of PDL cells. (A) PDL cells were incubated in osteogenic medium and conditioned medium was collected at the indicated time points, followed by quantification of STC1 protein levels in conditioned medium using commercial ELISA kit. (B, C) PDL cells were incubated in growth medium or osteogenic medium for 7 days in the presence or absence of STC1 neutralizing antibody (500 ng/ml). At the end of the culture periods, quantitative PCR (B) or ALP staining and activity assays (C) were performed. (D) PDL cells were incubated in growth medium or osteogenic medium for 28 days in the presence or absence of STC1 antibody and rhSTC1 (100 ng/ml), followed by Alizarin red staining. *, significantly different from 0 day; #, significantly different in the indicated pair

V.7. rhSTC1 induces VEGF expression in PDL cells

VEGF is known to promote osteogenic differentiation (Mayer et al., 2005). In this study, expression levels of VEGF mRNA increased in a time-dependent manner during osteogenic differentiation of PDL cells (Figure 2). In addition, knockdown or function blocking of endogenous STC1 inhibited osteogenic stimuli-induced VEGF expression (Figure 5&6). Therefore, it was next examined whether STC1 induces VEGF expression in PDL cells. PDL cells were treated with rhSTC1 (100 ng/ml) for 0, 2, 4 and 7 days in growth medium and expression levels of VEGF mRNA and VEGF-A protein in PDL cells were examined. Data showed that rhSTC1 increased VEGF expression in a time-dependent manner (Figure 7A&7B). To further examine the role of STC1 in VEGF expression in PDL cells, the cells were incubated for 3, 7 and 14 days in the presence or absence of rhSTC1 and STC1 neutralizing antibody. VEGF-A protein levels in conditioned media were then determined using VEGF-A ELISA kit. The data demonstrated that VEGF secretion increased as incubation time went on and that further addition of rhSTC1 significantly increased VEGF production at all the time point examined (Figure 7C). Furthermore, function blocking of STC1 using neutralizing antibody significantly suppressed both basal and rhSTC1-induced VEGF production (Figure 7C).

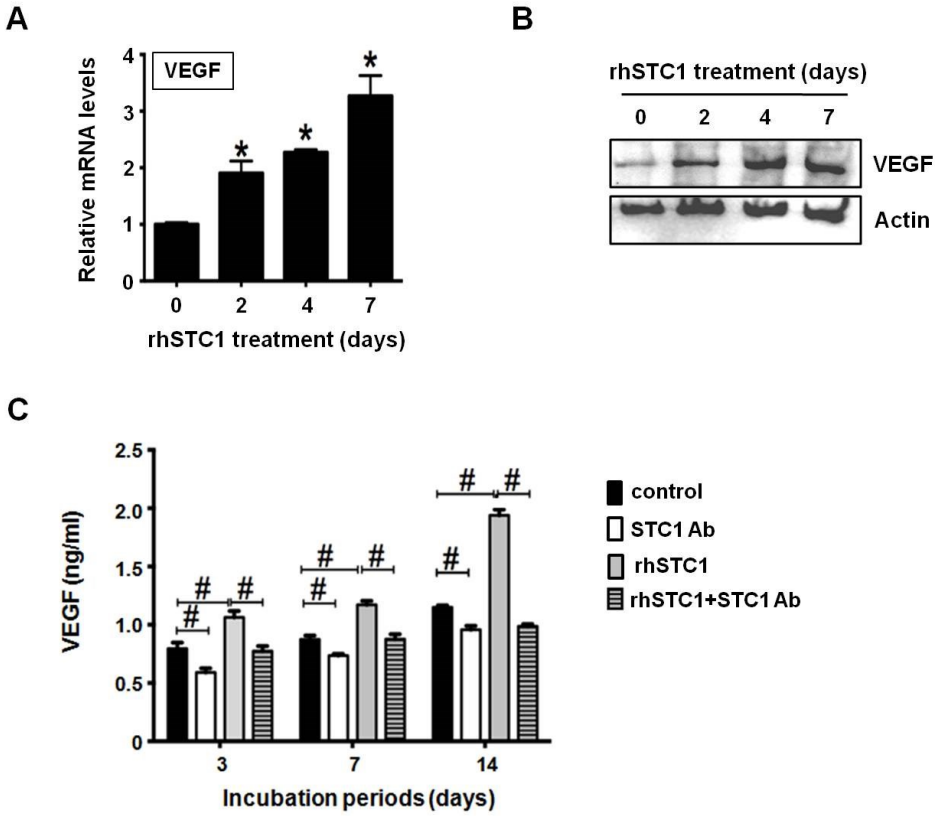

Figure 7. rhSTC1 induced VEGF expression in PDL cells. (A, B) PDL cells were incubated for the indicated periods in the presence of rhSTC1 (100 ng/ml) and quantitative PCR (A) and western blot analysis were performed. (C) PDL cells were incubated in the presence or absence of the indicated reagents, and conditioned medium was prepared at the indicated time points, followed by ELISA of VEGF-A. STC1 Ab, 500 ng/ml of STC1 neutralizing antibody; *, significantly different from 0-day cells

V.8. VEGF enhances osteogenic differentiation of PDL cells

To clarify the hypothesis that VEGF is a downstream mediator of STC1 in osteogenic induction of PDL cells, regulatory effect of VEGF on osteogenic differentiation of PDL cells were first examined. PDL cells were incubated in osteogenic medium for 7 days in the presence or absence of rmVEGF-A (1-20 ng/ml). VEGF significantly increased ALP mRNA expression even at the concentration of 1 ng/ml (Figure 8A, lower panel). ALP staining result also showed similar pattern of induction by VEGF treatment (Figure 8A, upper panel). In addition to the effect on ALP expression, VEGF (20 ng/ml) significantly increased mRNA expression levels of osteogenic marker genes, including RUNX2, OSX and OCN, but not that of VEGF (Figure 8B). Further addition of neutralizing antibody to VEGF-A (500 ng/ml) blocked VEGF-induced expression of osteogenic marker genes (Figure 8B).

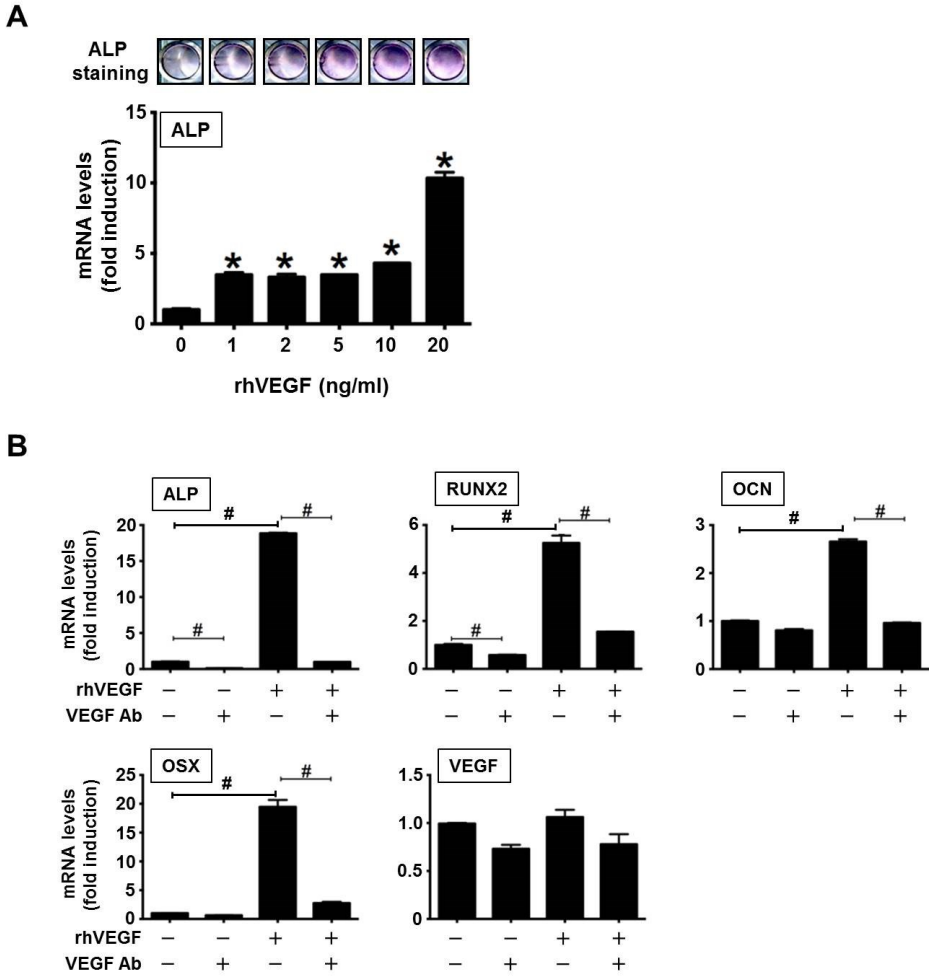

Figure 8. VEGF increased expression levels of osteogenic marker genes, which were blocked by neutralizing antibody to VEGF. (A) PDL cells were incubated for 7 days in the presence of rmVEGF-A at the indicated concentration. At the end of culture period, ALP staining (upper panel) or quantitative RT-PCR was performed (lower panel). * significantly different from the vehicle-treated control cells. (B) PDL cells were incubated for 7 days

in the presence or absence of rmVEGF-A (20 ng/ml) and neutralizing antibody to VEGF-A (500 ng/ml). At the end of culture period, quantitative RT-PCR was performed. #, significantly different in the indicated pair

V.9. VEGF mediates STC1-induced osteogenic differentiation of PDL cells

Because VEGF showed significant stimulatory effect on osteogenic differentiation of PDL cells, it was next examined whether VEGF mediates osteogenic effect of STC1. When PDL cells were incubated in osteogenic medium for 7 days, function blocking of secreted VEGF-A protein with neutralizing antibody significantly suppressed ALP activity (Figure 9A). Furthermore, VEGF neutralizing antibody blocked the induction of ALP activity by rhSTC1 treatment (Figure 9A). To further confirm the role of VEGF in STC1-induced osteogenic differentiation, PDL cells were transiently transfected with non-targeting control siRNA or VEGF siRNA and incubated for 7 days in osteogenic medium in the presence or absence of rhSTC1. Knockdown efficiency of VEGF siRNA was examined by western blotting (Figure 9B) and quantitative PCR (Figure 9D). Of interest, knockdown of VEGF increased both basal and rhSTC1-induced protein levels of endogenous STC1 (Figure 9B). Consistent with the results from VEGF neutralizing antibody experiments, VEGF knockdown significantly attenuated osteogenic stimuli-induced ALP activity and mRNA expression levels of osteogenic marker genes (Figure 9C&9D). Furthermore, VEGF knockdown suppressed rhSTC1-induced ALP activity and osteogenic marker gene expression (Figure 9C&9D). To further confirm the specificity of VEGF

knockdown, rescue experiment with rmVEGF-A was performed. Although the range of recovery was diverse depending on the genes, the addition of rmVEGF-A significantly recovered the expression levels of osteogenic marker genes which were suppressed by VEGF knockdown (Figure 9E).

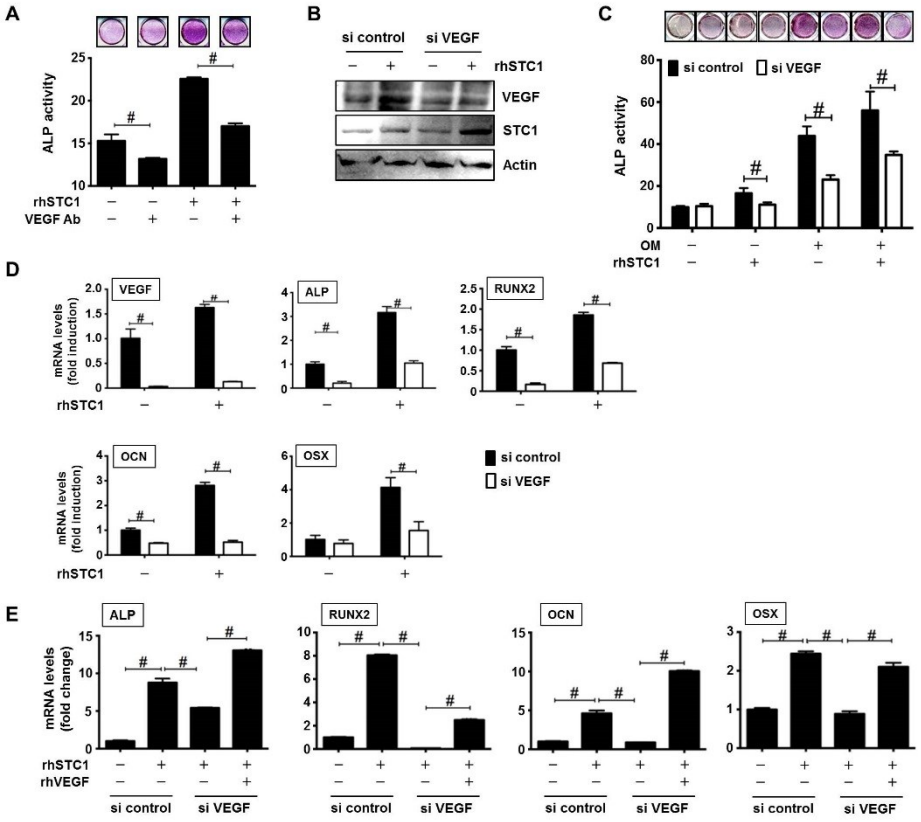

Figure 9. Function blocking or knockdown of VEGF-A attenuated STC1-induced osteogenic differentiation of PDL cells. (A) PDL cells were incubated in osteogenic medium for 7 days in the presence or absence of rhSTC1 (100 ng/ml) and neutralizing antibody to VEGF-A (500 ng/ml). At the end of culture period, ALP staining (upper panel) and ALP activity assays were performed. (B-E) PDL cells were transiently transfected with non-targeting control siRNA (si control) or VEGF siRNA (si VEGF) and incubated in osteogenic medium for 7 days in the presence or absence of the indicated reagents. At the end of culture period, western blotting (B), ALP staining and

activity assay (C) or quantitative PCR (D, E) was performed. rmVEGF, 20 ng/ml rmVEGF-A

V.10. rhSTC1 increases the cell viability of PDL cells

Because rhSTC1 enhances osteogenic differentiation of PDL cells, the effect of STC1 on the cell viability was also examined. PDL cells were incubated in growth medium for 48 and 72 h in the presence or absence of rhSTC1 (10-1000 ng/ml). STC1-induced change in cell viability was not observed at 48 h. However, after incubation for 72 h, STC1 significantly enhanced cell viability at the concentration of 10 and 100 ng/ml (Figure 10).

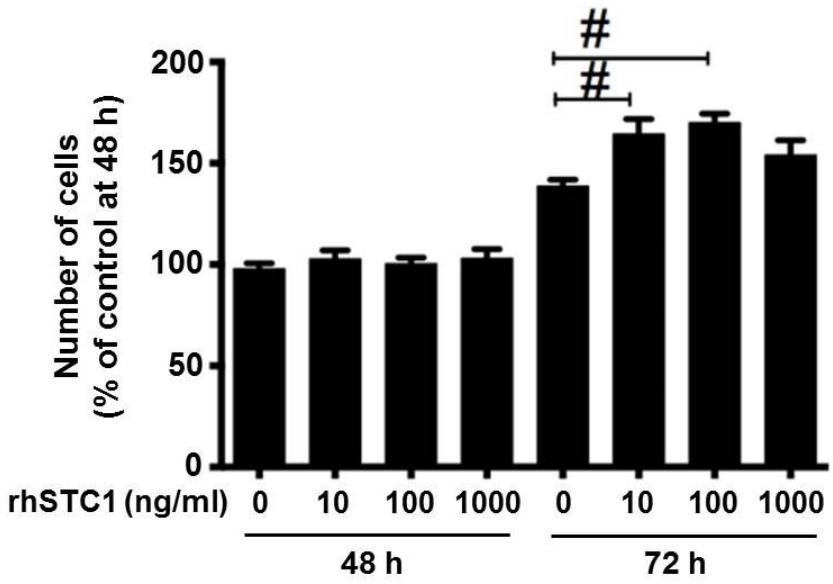

Figure 10. rhSTC1 significantly increased cell viability of PDL cells. PDL cells were incubated in growth medium for 48 and 72 h in the presence or absence of rhSTC1 at the indicated concentration. At the end of culture periods, the cell viability was compared by using Ez-Cytox cell viability assay kit.

V.11. rhSTC1 protects PDL cells from hypoxia-induced apoptosis

When PDL cells were exposed to hypoxic conditions for 24 h, the number of PI/Annexin V-positive cells significantly increased (Figure 11A&11B). In addition, western blot analysis results also demonstrated that the protein levels of apoptosis markers such as cleaved forms of caspase 3 and caspase 8 and pro-apoptotic protein Bax were significantly increased in response to hypoxia (Figure 11C). However, the levels of anti-apoptotic protein BCL2 were not reduced by hypoxic treatment (Figure 11C). These results suggest that exposure of PDL cells to hypoxic conditions induce apoptotic cell death. Remarkably, the addition of rhSTC1 to culture medium prevented the hypoxia-induced apoptosis (Figure 11): the number of the PI/Annexin V-positive cells and the protein levels of cleaved caspases and BAX were restored to those of cells incubated under normoxic conditions.

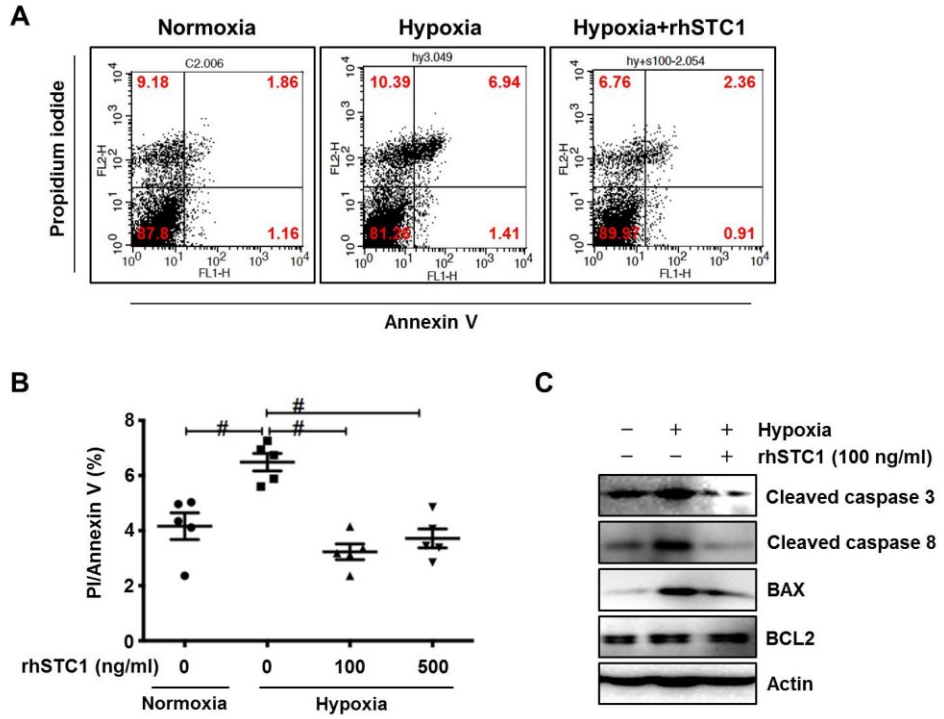

Figure 11. Addition of rhSTC1 to culture medium protected PDL cells from hypoxia-induced apoptotic cell death. PDL cells were incubated for 24 h under normoxic or hypoxic conditions in the presence or absence of rhSTC1 protein. (A, B) The cells were harvested and stained with anti-annexin V-FITC antibody and propidium iodide (PI) and subjected to FACS analysis. (A) The representative FACS analysis results from each group were provided. The number in red indicates the proportion of cells that belong to each square. (B) The proportions of cells in the late apoptotic stage (PI/Annexin V double positive cells) were presented. (C) Cell lysates were prepared and western blot analyses were performed.

V.12. Knockdown of endogenous STC1 increases apoptotic cell death of PDL cells

Because rhSTC1 protected PDL cells from hypoxia-induced cell death, the role of endogenous STC1 in the regulation of cell survival was examined. PDL cells were transiently transfected with control siRNA or STC1 siRNA and incubated for 24 h under normoxic or hypoxic conditions in the presence or absence of rhSTC1. FACS analysis results demonstrated that the addition of rhSTC1 did not significantly change the basal levels of apoptotic cell population when PDL cells were incubated under the normoxic conditions (Figure 12A&12B). Of interest, the addition of rhSTC1 decreased the level of cleaved caspase 3 protein (Figure 12C). However, even under the normoxic conditions, knockdown of endogenous STC1 significantly increased the proportion of apoptotic cells, which was rescued by addition of rhSTC1 (Figure 12A&12B). Consistent with the FACS analysis results, cleaved caspase 3 levels were increased by STC1 knockdown, which was downregulated by rhSTC1 treatment (Figure 12C). When PDL cells were exposed to hypoxic conditions, knockdown of STC1 further increased hypoxia-induced apoptotic cell death, which was also rescued by the addition of rhSTC1 (Figure 12A&12B). Western blotting results also demonstrated that cleaved caspase 3 protein levels were changed in correlation with the proportion of PI/Annexin V-positive cells (Figure 12C).

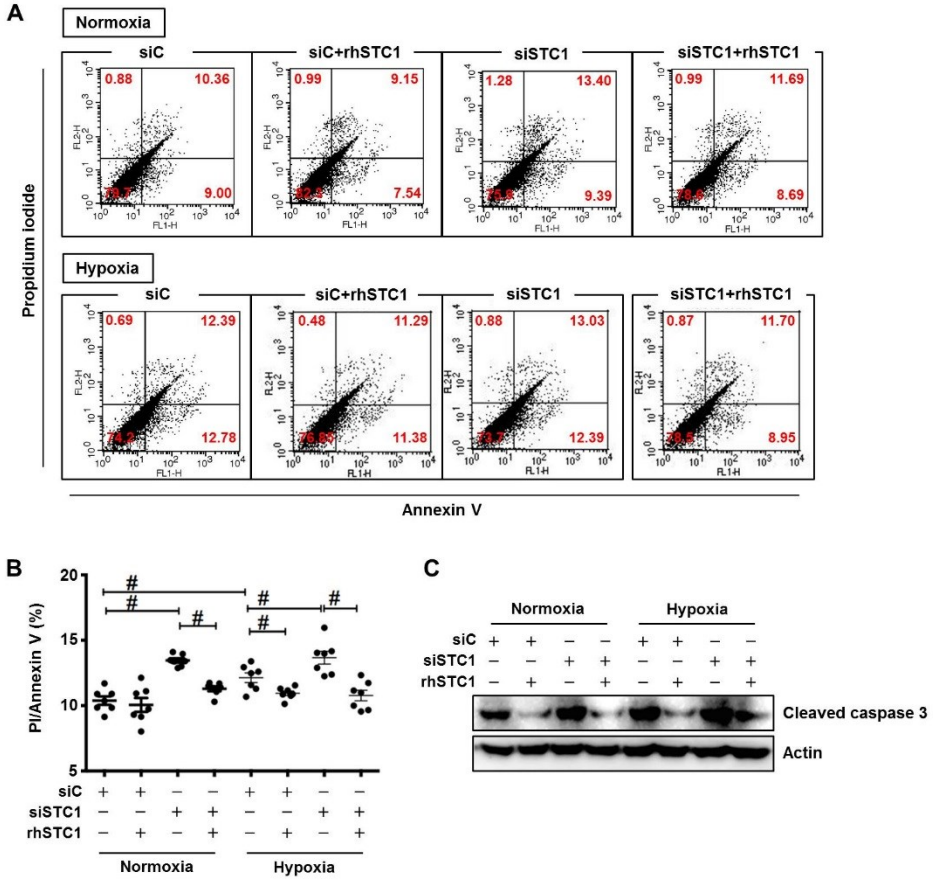

Figure 12. Knockdown of endogenous STC1 increased apoptotic cell death in PDL cells. PDL cells were transiently transfected with control siRNA (siC) or STC1 siRNA (siSTC1) and incubated for 24 h under normoxic or hypoxic conditions in the presence or absence of rhSTC1. (A) The representative FACS analysis results from each group. (B) The proportions of the late apoptotic stage cells were presented. (C) Western blot analyses were

performed using caspase 3 antibody.

V.13. rhSTC1 suppressed osteoclastogenesis

Maintaining the balance between the osteogenesis and the osteoclastogenesis is critical for preventing pathologic bone loss (Tanaka et al., 2005). Therefore, the effect of STC1 on osteoclastogenesis was also examined using RAW264.7 cell line and primary cultured BMM cells. Because TRAP activity assay is generally considered as a cytochemical marker of osteoclasts (Filvaroff et al., 2002), TRAP staining and TRAP activity assays were first performed. Incubation of RAW264.7 and BMM cells with RANKL highly increased TRAP activity and the number of TRAP positive-multinucleated cells, which were significantly suppressed by addition of rhSTC1 (Figure 13). Consistent with TRAP staining data, STC1 significantly downregulated the RANKL-induced expression of osteoclastogenesis related genes, including Nfatc1, Fra1/2, c-Fos, TRAP, CTSK, c-Src and integrin β3 (Figure 14). Among the genes examined, induction of Nfatc1 and Fra2 genes was almost completely blocked by STC1. These results suggest that STC1 exert an inhibitory effect on osteoclast differentiation by suppressing RANKL induction of critical transcription factor such as Nfatc1.

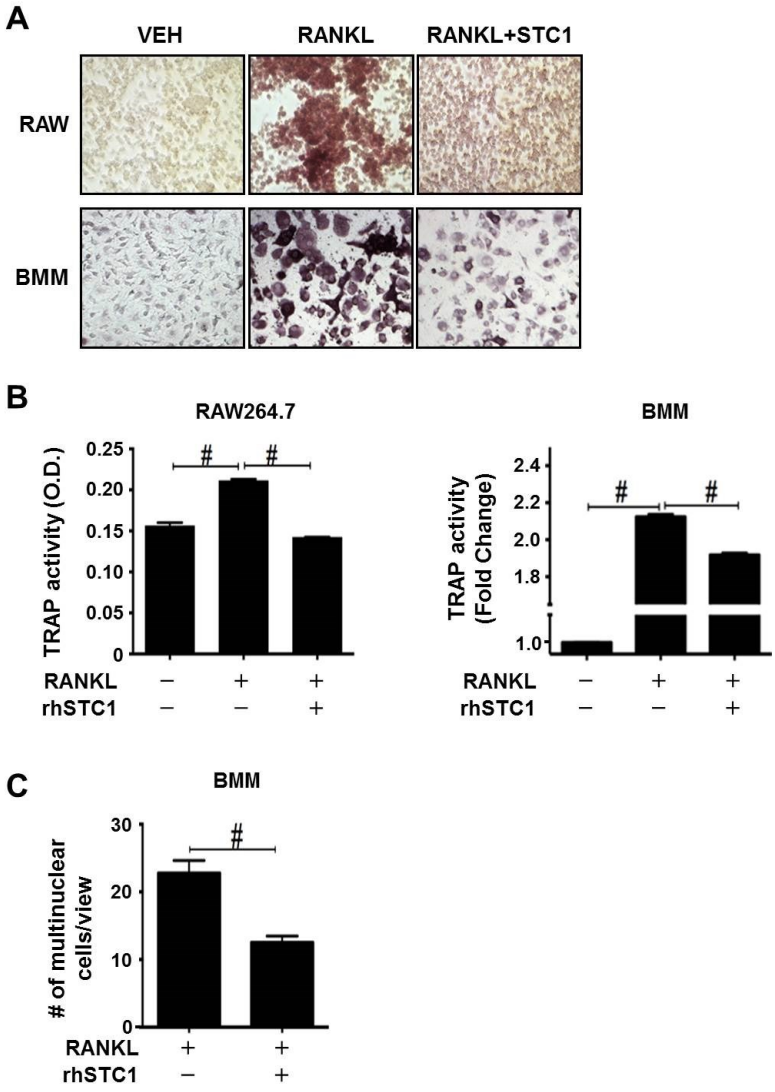

Figure 13. rhSTC1 inhibited RANKL-induced formation of TRAP positive multinucleated cells and TRAP activity. RAW264.7 (RAW) and mouse bone marrow-derived macrophage (BMM) cells were incubated for 4 and 6 days, respectively, in the presence or absence of RANKL and rhSTC1. At the

end of culture period, TRAP staining (A, C) and TRAP activity assay (B) were carried out. (C) The number of TRAP-positive cells which have more than three nuclei was counted under the microscope (x100). The data present the mean of the number of TRAP-positive multinucleated cells counted from four independent fields.

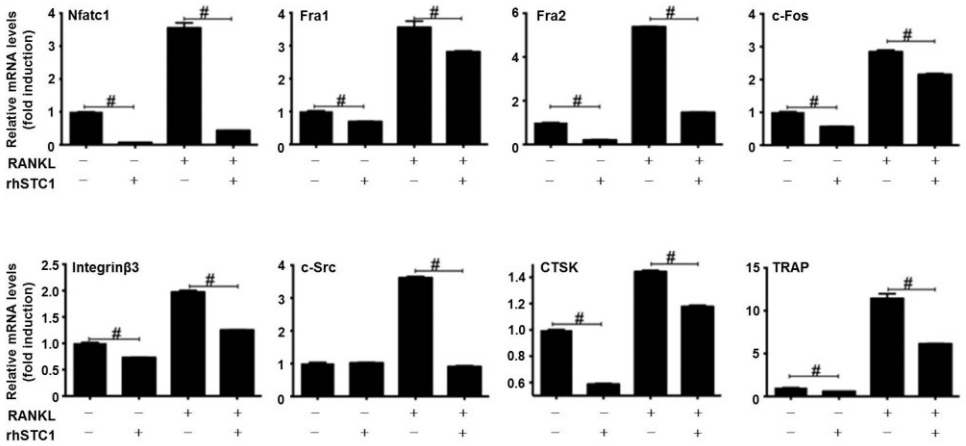

Figure 14. rhSTC1 suppressed RANKL induction of osteoclastogenic marker genes in RAW264.7 cells. RAW264.7 cells were incubated for 4 6 days in the presence or absence of RANKL and rhSTC1. At the end of culture period, mRNA was prepared and quantitative RT-PCR was performed.

VI. DISCUSSION

PART I. The role of STC1 in osteogenic differentiation of PDL cells

Hypoxia has been recognized as one of the most potent stimulants for PDL cells to evoke multiple cellular responses (Tuncay et al., 1994). In clinical situations, the most commonly mentioned hypoxic condition in the PDL tissues is driven by orthodontic force, especially in the compression side, which leads to bone resorption according to the “pressure-tension” theory (Niklas et al., 2013). However, in specific situations such as lower incisor extraction due to severe bone loss, it is commonly found that the low alveolar bone level is restored after space closing (Figure 15A), and sometimes more dense bone is observed even in compression side during setting mesial-angulated teeth upright along with space closure (Figure 15B). If the pressure accompanied with hypoxia just give rise to bone resorption signal, it is hard to explain the reason why bone regeneration is clinically observed in compression side. Therefore, in the present study, it was hypothesized that there should be some locally produced osteogenic signals in the compression side. A report has recently demonstrated that STC1 was significantly up-regulated by compression in 3D-cultured human PDL cells (Li et al., 2013). Under hypoxic conditions, HIF1α upregulates STC1 expression (Yeung et al., 2005). STC1 is involved in various functions including apoptosis, calcium

homeostasis, promoting osteoblastic differentiation and angiogenesis (Yeung et al., 2012). These reports suggest that STC1 is a promising candidate which plays a regulatory role in compression side. In the present study, exposure of PDL cells to hypoxia highly induced STC1 expression. In addition, rhSTC1 significantly enhanced osteogenic differentiation of PDL cells. These results suggest that locally secreted STC1 protein from the PDL cells in compression side contribute to local bone regeneration observed in compression side.

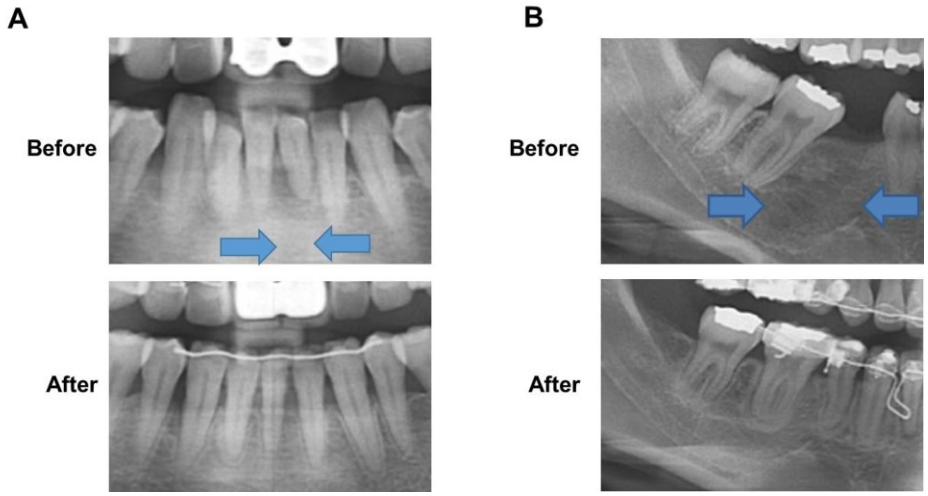

Figure 15. Enhanced bone formation was observed after orthodontic space closure in the area between the two compression sides which faced each other. In clinical perspective, after orthodontic tooth movement, enhanced bone formation is sometimes observed in the places where both compression sides (blue arrows) of teeth with periodontally complicated situations meet together. (A) Lower left central incisor was extracted due to severe bone loss. The alveolar bone is leveled and obvious bone formation was observed after orthodontic space closure. (B) Lower right second molar was uprighted mesially and also protracted. After orthodontic tooth movement, denser bone was observed in the mesial side of lower right second molar tooth.

Of interest, the expression levels of STC1 also increased with the progression of osteogenic differentiation of PDL cells which were cultured in normoxic conditions. Immunohistochemical staining of STC1 in rat periodontal tissues demonstrated that some osteocytic cells and bone matrix surrounding the osteocytic lacuna were positively stained (data not shown). These results were supported by the previous study, which demonstrated that STC1 was expressed during osteogenic differentiation (Yoshiko et al., 2002). Depletion of endogenous STC1 by siRNA or function blocking of STC1 with neutralizing antibody significantly suppressed osteogenic differentiation of PDL cells, suggesting that STC1 plays an important role in osteogenic differentiation of PDL cells. These findings are consistent with a study in the rat calvarial cells showing that rhSTC1 stimulated bone matrix formation and mineralization, whereas retardation of osteogenic differentiation was observed when STC1 antisense oligonucleotides were added (Yoshiko et al., 2003).

Previous studies have indicated that STC1 exerts autocrine and paracrine effect (Filvaroff et al., 2002; Jellinek et al., 2000). In the present study, secreted protein levels of STC1 were significantly up-regulated by osteogenic stimuli in a time dependent manner, and its level reached over 100 ng/ml concentration after 15 days. Considering the results showing that neutralizing antibody to STC1 significantly inhibited osteogenic differentiation

of PDL cells, it is indicated that autocrine/paracrine effect of STC1 is necessary to promote osteogenic differentiation of PDL cells.

Hypoxia would induce the expression and secretion of STC1 in compression side PDL cells, followed by STC1-induced angiogenic and osteogenic responses. Regarding clinical perspectives, the possibility of positive feedback loop in PDL shed a light on the ideals of orthodontic tooth movement and bone remodeling, including minimization of the side effects during orthodontic treatment. STC1 also provides an insight into how bone formation would be enhanced in pressure side shown in Figure 16.

PART II. The involvement of VEGF in STC1-induced osteogenic differentiation of PDL cells

STC1 could stimulate VEGF expression (He et al., 2011). Considering the effect of STC1 on angiogenesis in gastric cancer cells through up-regulation of VEGF (He et al., 2011), it is speculated that STC1 may generate blood vessels to provide oxygen as a protective mechanism in responsive to hypoxia in human PDL. In addition, VEGF is able to enhance not only angiogenesis but also osteogenesis (Liu et al., 2012). The coupling of angiogenesis utilizing VEGF to osteogenesis was also reported (Wang et al., 2007). In line with this, other studies indicated the close relationship between angiogenesis and osteogenesis (Chang et al., 1997; Kusumbe et al., 2014). Based on these reports, it is hypothesized that STC1 may elicit the osteogenic stimulatory effect through the induction of VEGF. As expected, in the present study, rhSTC1 increased expression levels of VEGF in a time-dependent manner. In addition, VEGF levels increased as osteogenic differentiation of PDL cells progressed. Furthermore, knockdown or function blocking of STC1 significantly blocked the induction of VEGF expression by osteogenic stimuli. These results suggest that induction of VEGF expression during osteogenic differentiation depends on STC1 expression, at least in part.

Consistent with the previous report, VEGF clearly enhanced osteogenic differentiation of PDL cells (Lee et al., 2012). Knockdown of VEGF

significantly downregulated STC1-induced osteogenic differentiation of PDL cells, which was partially rescued by the addition of exogenous VEGF. Furthermore, the addition of exogenous VEGF could rescue osteogenic differentiation of PDL cells in which expression of STC1 was silenced. These results indicate that STC1 enhance osteogenic differentiation via inducing VEGF expression in PDL cells.

Although a previous report has shown that VEGF enhances osteoblast differentiation of mesenchymal stem cells via an intracrine mechanism (Liu et al., 2012), in this study, exogenously added VEGF could enhance osteogenic differentiation of PDL cells, which was blocked by addition of neutralizing antibody to VEGF. These results were also consistent with the previous report showing that exogenous VEGF treatment increases osteogenic differentiation of mesenchymal stem cells derived from human PDL (Lee et al., 2012), suggesting that the action mechanism of VEGF differ depending on the tissue and/or species origin of the cells.

PART III. Cell protective effect of STC1 in terms of suppression of cell death in PDL cells

From the perspective of cell death in terms of hypoxia, the degree of hypoxia has been considered as a modulator for cell maintenance, proliferation and death (Choi et al., 2014). Anoxia and severe hypoxia under 2% of oxygen induced significant cell death (Ezashi et al., 2005; Grayson et al., 2006). Consistent with this finding, the present study showed that hypoxia significantly increased PDL cell death and the amount of activated caspases 3 and 8, which were rescued by rhSTC1. Considering the protective role of STC1 against hypoxia-induced cell death in PDL cells, it can be speculated that STC1 may exert a compensatory mechanism of keeping cell viability during orthodontic tooth movement in hypoxic status of PDL cells. Of interest, although exogenously added rhSTC1 did not exert any protective effect on cells cultured in normoxia conditions, knockdown of endogenous STC1 significantly increased the number of apoptotic cells and the levels of cleaved caspase 3 in the cells of normoxic conditions. These results implicate that STC1 exert a protective effect on cell death regardless of whether apoptosis is caused by hypoxia or not.

Although the causal connection between anti-apoptotic and osteogenic effect of STC1 remains unclear, it can be speculated that STC1 promotes osteogenic differentiation by protecting PDL cells from cell death. In fact,

treatment of PDL cells with STC1 for 72 h significantly increased number of viable cells, partially supporting this notion. However, we cannot exclude the alternative possibility that the anti-apoptotic and the osteogenic effect of STC1 are independent processes.

PART IV. STC1 suppresses osteoclastogenesis in RAW264.7 and BMM cells

Balance between osteogenesis and osteoclastogenesis is critical for the orthodontic tooth movement as well as bone health (Baloul et al., 2011). Previous reports have demonstrated that the skull of STC1 transgenic mice exhibited the abnormal distribution of osteoclasts (Filvaroff et al., 2002) and that STC N-terminal fragment, a synthetic STC(1-20) peptide, decreased the formation of TRAP-positive multinucleated cells which were induced by human PTH in mouse bone marrow cells (Yoshiko et al., 1996), suggesting the possible regulatory role of STC1 in osteoclastogenesis. However, comprehensive analysis adopting critical genes regulating osteoclastogenic differentiation has not been reported yet.

The results from the present study demonstrated that addition of exogenous STC1 suppressed the formation of multinucleated TRAP-positive cells in both RAW264.7 and BMM cell systems. Furthermore, analysis of osteoclastogenesis-related genes revealed that STC1 suppressed the induction of NFATc1, which is a critical regulator for osteoclast differentiation. These results suggest that STC1 exert an inhibitory effect on osteoclast differentiation via blocking of NFATc1 induction. Further study, however, is

necessary to clarify the action mechanism of STC1 by which STC1 inhibits osteoclast differentiation.

Although the data were not presented here, addition of STC1 significantly suppressed hypoxia-induced expression of RANK in PDL cells. These results suggest that STC1 inhibits osteoclast formation via inhibition of RANKL expression in PDL cells as well as direct suppression of RANKL-induced osteoclast differentiation in osteoclast precursor cells. Our group has previously reported that hypoxia enhances expression levels of RANKL in PDL cells via inducing HIF1α (Park et al., 2011). Therefore, it is implicated that hypoxia-induced STC1 in PDL cells plays a role to keep alveolar bone and tooth root from excessive resorption by attenuating osteoclastogenesis.

VII. CONCLUSION

The present study demonstrated that hypoxia increased the production of STC1 in PDL cells. STC1 protein exhibited the protective effect on the PDL cells from hypoxia-induced cell death. In addition, STC1 enhanced osteogenic differentiation of PDL cells while inhibiting osteoclast differentiation of bone marrow precursor cells. Osteogenic stimuli induced the expression of STC1 in PDL cells, and depletion of STC1 significantly downregulated osteogenic differentiation. The autocrine/paracrine effect of STC1 further provides the positive feedback resulting in osteogenic differentiation in PDL. VEGF is induced by STC1 and mediates the osteogenic effect of STC1 in PDL cells. The regulatory function of STC1 in bone remodeling of periodontal tissue is schematically illustrated in Figure 16. These results provide the insight on the function of STC1 in the regulation of periodontal bone remodeling, which was elicited by applied force during orthodontic tooth movement.

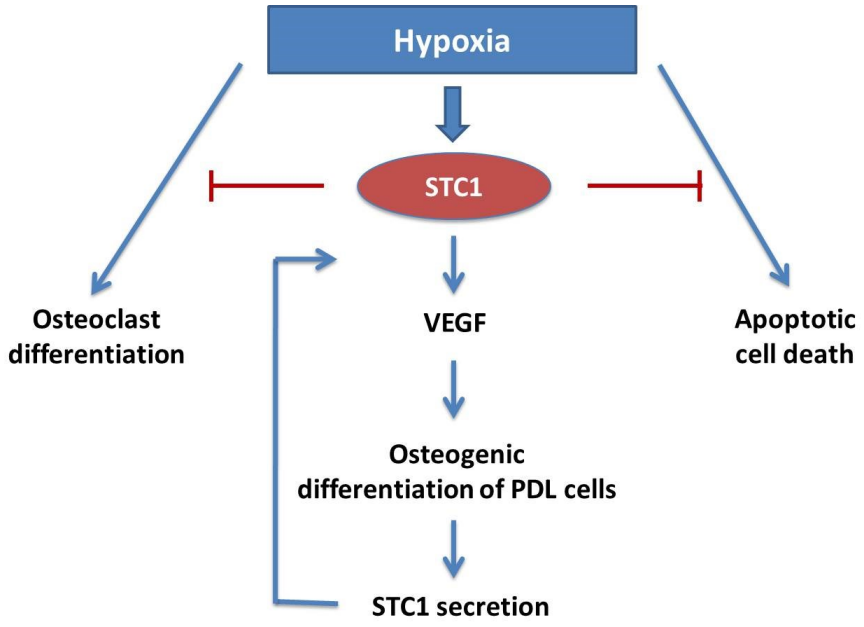

Figure 16. Schematic illustration of the functional role of STC1 in the perspective of regulation of periodontal bone remodeling

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IX. KOREAN ABSTRACT

조골세포와 파골세포 분화에 미치는 Stanniocalcin 1의 조절 작용

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채 화 성

교정력을 가할 때 치아를 이동 기전을 설명 하는 이론 중 가장 유력하게 받아들여 지고 있는 압박-인장 이론에 의하면, 교정력에 의해 압박측에서 가장 먼저 생리학적으로 일어나는 현상은 치주인대의 저산소 상태이다. 예비실험에서 사람 치주인대섬유모 세포를 저산소 상태에서 24시간 배양한 후 microarray 분석을 시행한 결과 대조군과 비교하여 저산소 상태에서 mRNA 발현수준이 가장 크게 증가한 유전자는 Stanniocalcin1(STC1)으로 확인되었다. 따라서 본 연구에서는 사람 치주인대섬유모 세포에서 분비된 STC1의 역할을 알아보고자 하였다.

일차배양된 사람 치주인대섬유모 세포를 ATCC에서 구입하여 사용하였고, 저산소상태를 유도하기 위해 Gaspak을 사용하였다. 조골세포 분화에 미치는 영향을 알아보기 위해 STC1 단백질 처치 또는 STC1 과발현

을 유도하고 조골세포 분화 유도 배지에 사람 치주인대섬유모 세포를 배양한 후 조골세포 분화표지 유전자 발현 수준, alkaline phosphatse(ALP) 활성, 기질석회화에 미치는 영향을 분석하였다. 세포에서 발현되는 STC1의 양은 정량적 역전사효소연쇄중합반응, Western blot 분석, ELISA 분석을 통해 확인하였다. 저산소 조건 및 STC1이 세포사멸에 미치는 영향을 확인하기 위하여 세포에 propidium iodide/Annexin V 형광염색을 시행하여 양성으로 염색된 세포수를 flow cytometer로 계수하였고, 세포사멸 마커에 대한 Western blot 분석을 실행하였다. 내인성 STC1의 역할을 확인하기 위해 STC1에 대한 small interfering RNA 및 STC1 중화항체를 사용하였다. 파골세포 분화에 미치는 영향을 알아보기 위해 RAW264.7 생쥐대식세포주와 일차배양된 생쥐골수유래대식세포에 RANKL을 처리하여 파골세포 분화를 유도하였고, 파골세포 분화관련 유전자에 대한 역전사효소연쇄중합반응, tartrate-resistant acid phosphatase (TRAP) 염색을 시행하여 파골세포 생성을 관찰하였다.

연구 결과, Gaspak으로 저산소상태를 유도했을 때 사람 치주인대섬유모 세포에서 STC1의 유의한 증가가 관찰되었다. Gaspak으로 유도한 저산소증은 사람 치주인대섬유모세포에서 propidium iodide/Anexin V에 모두 양성인 세포수를 유의하게 증가시켜 세포사멸을 촉진시키는 효과가 있었다. 정상산소상태에서 배양한 세포에서 STC1 발현을 감소시켰을 때

세포사멸이 촉진되었고, 저산소증 상태에 STC1 단백질을 추가했을 때 저산소증에 의한 세포사멸이 억제되었으며, 세포사멸 수와 세포사멸 표지 단백인 caspase 3의 활성화가 감소되었다. 한편 치주인대섬유모 세포를 조골세포 분화배지에서 배양하였을 때 시간이 경과함에 따라 조골세포 분화 표지 유전자뿐 아니라 STC1의 발현도 증가하였고 배지로 분비되는 STC1 단백질 양도 증가하였다. 세포에 STC1의 과발현을 유도하거나 재조합 STC1 단백질을 배지에 첨가한 경우 조골세포 분화 표지 유전자 발현, ALP 활성, 기질 석회화가 유의하게 증가하였다. STC1에 대한 중화항체를 처리하거나 STC1 발현을 저해하였을 때 치주인대섬유모세포의 조골세포 분화가 감소하였다. 세포에 STC1을 처리하거나 조골세포 분화를 유도하면 vascular endothelial growth factor(VEGF) 발현이 증가하였고, STC1 발현 저해나 중화항체 사용시 VEGF 발현도 감소하였다. 또한 VEGF short interfering RNA처치시 STC1에 의해 발현이 증가된 조골세포 분화 표지 유전자 및 ALP 활성이 유의성 있게 감소하였고, VEGF 단백질의 처치로 회복 되었다. STC1은 TRAP 양성 다핵세포의 생성을 유의하게 저해하였고, *Nfatc1* 같은 파골세포 분화 관련 유전자의 발현을 유의하게 감소시켰다.

이상의 결과는 교정력이 가해진 치아의 압박측에서 저산소상태가 유도되었을 때 발현 증가되는 STC1은 치주인대섬유모세포의 세포사멸을

감소시키고, 조골세포 분화 촉진과 파골세포 분화 억제를 통해 과도한 치조골 소실 및 치근 흡수를 최소화하는데 기여할 수 있음을 시사하였다.

주요어: 사람 치주인대섬유모세포, Stanniocalcin 1, 저산소증, 조골세포 분화, 세포사멸, 파골세포 분화

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