Title
The Effect of TNF- alpha On The Odontogenic Potential Of Human Dental Stem Cells

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The Effect of TNF- α On The Odontogenic Potential Of Human Dental Stem Cells

A thesis submitted in partial satisfaction

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in Oral Biology

by

Edward Tseng

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ABSTRACT OF THE THESIS

The Effect of TNF- α On The Odontogenic Potential Of Human Dental Stem Cells

By

Edward Tseng

Master of Science in Oral Biology
University of California, Los Angeles, 2013
Professor Cun-Yu Wang, Chair

Tumor necrosis factor- alpha (TNF-α) is a major inflammatory cytokine that stimulates apoptotic signaling pathway and activates the transcription factor nuclear factor kappa B (NF-κB). Its contribution of apoptosis and rate of differentiation in regulating osteoblasts remains controversial. Recently, human mesenchymal stem cells were demonstrated in dental tissues. Human dental stem cells are also multipotent and can be induced to differentiate into different cell lineages. These cells are definitely a key part of achieving the promise of tissue and bone regeneration, along with bone marrow stem cells. In this research study, we wanted to see the effect of TNF- α on odontogenic differentiation of dental stem cells. We treated two different dental stem cell (DSCs) lines – dental pulp stem cells (DPSCs), and apical papilla (SCAPs) with

1ng/mL of TNF- α in different time points. Within 7 days, we could see an early alkaline phosphatase (ALP) expression and activity. In addition, ALP expression and activity were higher with treatment at 1ng/ml. Enhanced matrix mineralization was also observed with Alizarin Red Staining (ARS) after 14 days, and the mineralization was stronger with lower TNF- α concentration treatments. Furthermore, we investigated the effect of TNF- α on transcription factors, RUNX2 and OSX, two critical factors in osteogenic and odontogenic differentiation. The results showed that TNF- α induced the RUNX2 expression in both dental stem cells at different time points (4 hours and 24 hours). However, we observed a decrease in the expression of OSX. In this study, we demonstrated that TNF- α (at a lower concentration) could enhance odontogenic differentiation in dental stem cells. The amount of exposure of TNF- α might be a critical factor in determining its effects on odontoblast lineage commitment of dental stem cells.

This thesis of Edward Tseng is approved.

Shen Hu

Reuben Kim

Cun-Yu Wang, Committee Chair

University of California, Los Angeles

2013

This thesis is dedicated to

Dr. Cun-Yu Wang for all his guidance and support.

My parents, my sister,

all my family members, and all my friends,

who have always supported me.

In addition I would like to dedicate this work to

my beloved Sharon.

Rest in peace.

Much love.

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ACKNOWLEDGEMENTS

I would like to thank all the people in Wang's Lab and all the instructors of the Oral Biology Department

The Effect Of TNF- α On The Osteognic Potential Of Human Dental Stem Cells

Introduction:

The connection between the bone and immune systems is complex, and yet fully understood. The recent studies have revealed that bone metabolism and homeostasis are influenced by immune system. Osteoimmunology is the study of the relationship between bone and immune systems [1-3]. A wide range of molecular and cellular interactions has been studied extensively in osteoimmunolgy (FIG. 1). Studies have revealed that if inflammation continues, deficient of bone formation will continue to occur, and vice versa. Osteoporoses, periodontitis, Rheumatoid arthritis are typical examples of the interaction between immune and bone systems. The abnormality of the immune system leads to high level of osteoclasts, bone-resorbing cells, which cause bone destruction. Evidences have revealed that the immune and bone systems share a number of regulatory molecules such as cytokines, signaling molecules, transcription factors and receptor molecules [3-5]. Major cytokines involved in the inflammatory response during tissue injury include Interleukin (IL)-1, IL-6, IL-11, IL-18, and Tumor necrosis factor-α (TNF-α). These proinflammatory cytokines are well known for their catabolic effects on bones and tissues in inflammatory diseases. Past studies have shown that TNF-α plays a vital role in bone pathologies, which is best known for inducing bone resorption and inhibiting bone formation by promoting osteoclast differentiation [6, 7].

TNF-α is produced by macrophages and other immune cells. It is the endow member of the TNF superfamily. TNF-α has two different receptors- TNF receptor-1 and 2 (TNFR1 & TNFR2). TNFR1 is mostly expressed in all cells. It is also the functional form of the receptor in

bone cells, such as osteoblasts and osteoclasts [8], and TNFR2 regulates inflammation in immune cells [9]. When TNF-α binds to TNFR1 in most cells, they form a complex by TNF

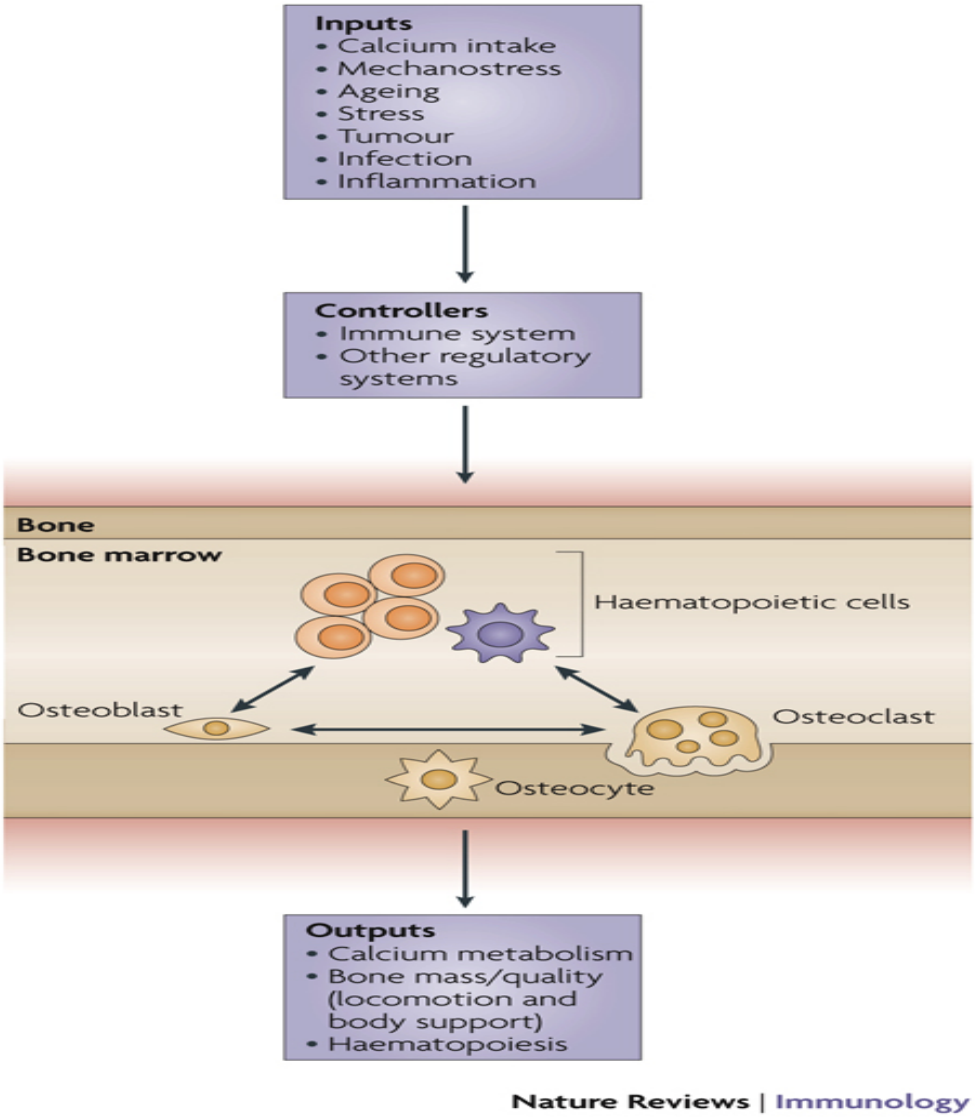

Figure 1. The osteoimmune system
(Source: Takayanagi et al. Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems. *Nature Reviews Immunology* **7**, 292-304 (April 2007) doi:10.1038/nri2062)

receptor-associated protein with death domain (TRADD), receptor-interacting kinase (RIP), and TNFR2, which then activates NF-kB signaling pathway and mitogen activated protein kinase (MAPK), ERK, and JNK [10, 11] (FIG.4). Depending on the specific cell-surface receptor it

binds, TNF-α can either trigger cell death or promote cell survival [12], which is expressed following injury and may affect on bone cells' differentiation, proliferation and apoptosis.

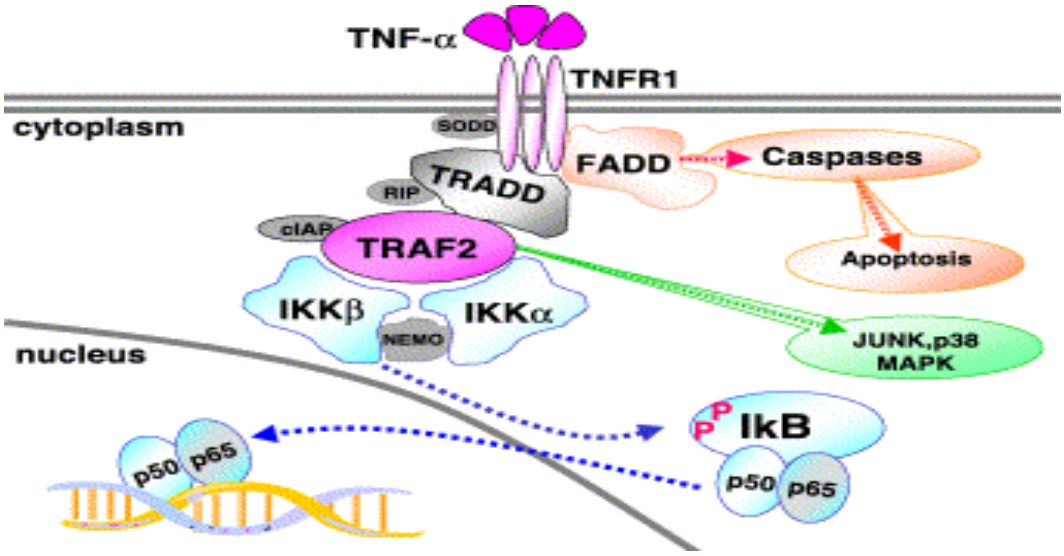

Figure 2. Overview of TNF signaling pathways
(Source: Mark S. Nanes. Tumor necrosis factor-α: molecular and cellular mechanisms in skeletal pathology Gene, Volume 321, 4 December 2003, Pages 1–15 http://dx.doi.org/10.1016/S0378-1119(03)00841-2)

Early studies suggested that TNF-α is a skeletal catabolic agent that stimulates osteoclastogenesis along with receptor activator of NF-κB (RANKL) while hinders osteoblast's bone formative function [8]. As mentioned earlier, TNF-α has a critical role in bone pathophysiology. It suppresses recruitment of osteoblastic cells from progenitor cells, inhibits the expression of bone protein genes, and stimulates gene expressions of osteoclastogenesis [8]. A number of factors have been identified that stimulate differentiation in an osteoblastic direction that include transcription factors RUNX2, and osterix (OSX), and secreted proteins, insulin-like growth factor 1 (IGF-1) and bone morphogenic proteins (BMPs) [8] (FIG. 3). These important differentiating factors are targeted directly or indirectly by TNF-α regulation, which is associated with blocking of IGF-1, RUNX2, and OSX, though not BMP-2, -4, or -6 expression [8, 13]. IGF-1 increase the pre-osteoblast pool though a mitogenic effect and to provide an anti-apoptotic

stimulus, however IGF-1 treatment fails to inhibit TNF-α in past studies [8, 14]. The suppression of RUNX2 by TNF-α could have a critical influence on new bone formation. RUNX2 is an important factor in skeletal development, and many osteogenic genes require RUNX2 for expression [13]. OSX is a Sp-1 family protein that is required downstream of RUNX2 and upregulated by BMP-2 for bone development [8]. TNF-α's effect on the mechanism of OSX inhibition is still unclear, however the inhibition of both RUNX2 and OSX provides a common inhibitory stimulus to osteoblast differentiation [8]. However, TNF-α is also important in the inflammatory response that triggers osteogensis [2]. Recent studies suggest that TNF-α can enhance osteogensis differentiation in MSCs and muscle-derived stromal cells [7, 10, 15-17].

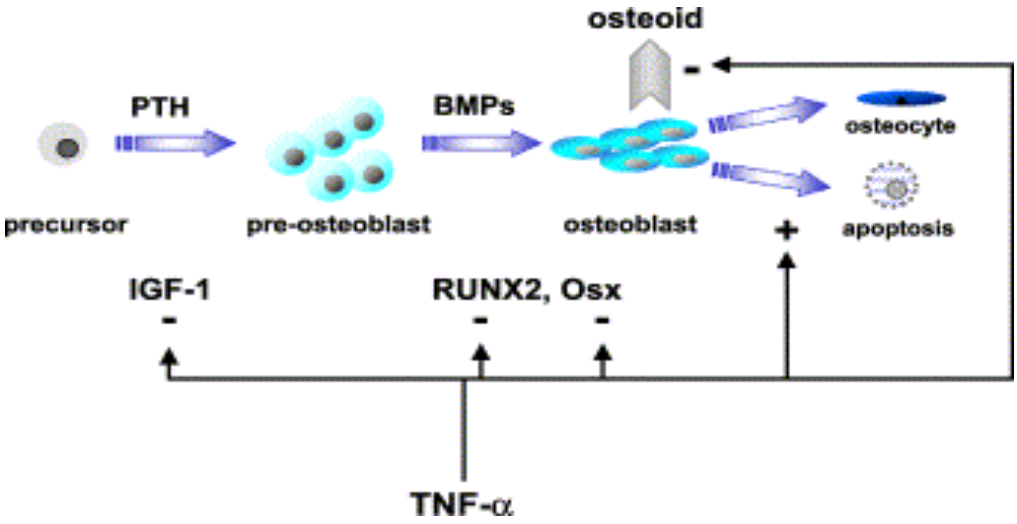

Figure 3. The Effect of TNF on Osteoblast Differentiation
(Source: Mark S. Nanes. Tumor necrosis factor-α: molecular and cellular mechanisms in skeletal pathology Gene, Volume 321, 4 December 2003, Pages 1–15 http://dx.doi.org/10.1016/S0378-1119(03)00841-2)

Bone healing and regeneration is a highly coordinated and complex process responsible for bone resorption and formation. It is also necessary for repairing damaged bone and maintaining bone homeostasis. Osteogenesis is a process mediated by bone formation cells, osteoblasts, which is essential in bone regeneration and treatment of bone disorders. Osteoblasts

have several key roles in bone remodeling, such as production of bone matrix proteins (BMPs), bone mineralization, and expression of osteoclastogenic factors [18]. Osteoblastic cells contain many different population of cells that include mature matrix-producing osteoblasts and immature osteoblast lineage cells [19]. It has shown that mice deficient in osteoblasts are deficient in osteoclasts [20]. However, bone formation decreases in conditional depletion of osteoblasts *in* vivo, while bone resorption by osteoclasts continues [21]. Osteoblasts differentiate from mesenchymal stem cells (MSCs) in a regulated process mediated by different growth factors and cytokines. Runx-2, Sp7, and canonical Wnt signaling control differentiation of osteoblasts, which induce MSCs into pre-osteoblasts then proceed into maturation (FIG. 4).

While in the field of bone tissue engineering has many groundbreaking discoveries and technologies, incorporating stem cells into bone tissues regeneration is one of the most valued advancements. In has been shown in many studies that different stem cell types are capable of osteogenic differentiation, but it is still not clear which stem cells are worthy candidates for generating new bones and tissues. It is important that bone tissue regeneration relies on the type of cell used, and also the characteristics of the culture environment.

Adult stem cells have been isolated from many different distinctive tissues, such as bone marrow, umbilical cord, skeletal muscle, and many more [22]. MSCs are well known and most-used in studies due to their ability to regenerate, and their multilineage potential with appropriate induction [22, 23]. The human bone marrow is the best-characterized source for MSCs, and many researchers use it to compare with other MSCs [24]. The potential and capacity of bone marrow derived-mesenchymal stem cells (BMMSCs) to differentiate into osteoblastic cells have been investigated, compare, and contrasted with other cell types. BMMSCs have higher proliferation rate, minerlization potential, and cell density than osteoblastic cells [25]. However,

isolating and expanding cells from the bone marrow is difficult, and it is also an invasive procedure. Furthermore, the number of MSCs in bone marrow tend to decrease as a human age [26]. BMMSCs may have been studied extensively for many years, but many other more convenient MSCs source have been identified, and dental tissues are one of the sources.

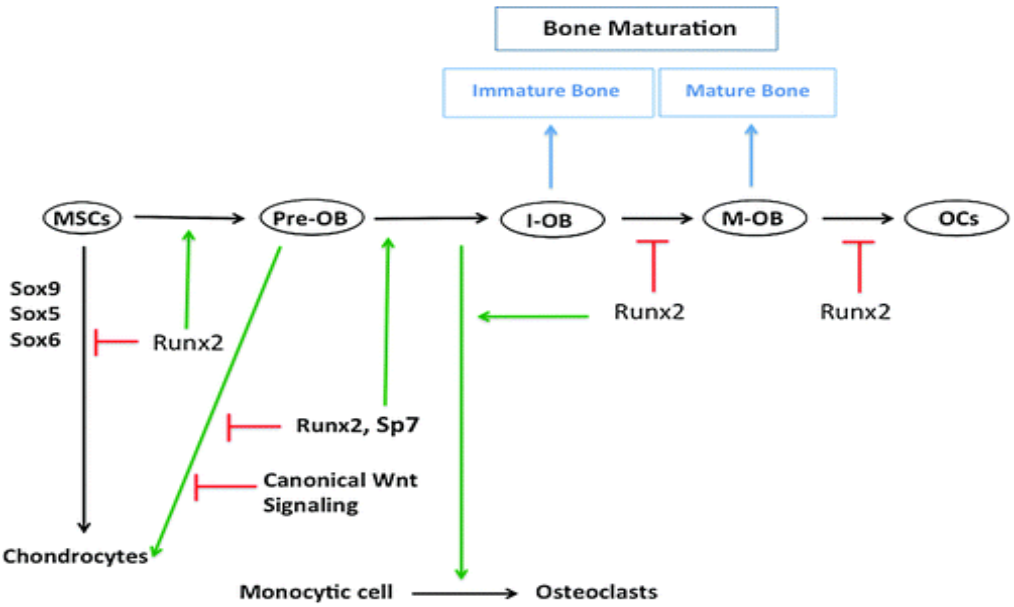

Figure 4. Osteoblast Differentiation
(Source: Caroline Szpalski, Marissa Barbaro, Fabio Sagebin, and Stephen M. Warren. Tissue Engineering Part B: Reviews. August 2012, 18(4): 258-269. doi:10.1089/ten.teb.2011.0440.)

Human dental tissues have been revealed and considered as a candidate source for isolating and expanding MSC-like population [22, 27]. The sources of dental stem cells and their multi-differentiation strategy can be seen in Figure 5. Until now, five different sources of MSCs have been identified: dental pulp (DPSC) [28-30], periodontal ligament (PDLSC) [28, 29], exfoliated deciduous teeth (SHED) [22], dental follicle (DFSC) [31], and root apical papilla (DPPSC, or SCAP) [32]. Cells of postnatal populations from these dental tissues have MSC-like characteristics [22, 33, 34], with capabilities in osteogenic and odontogenic differentiation [35-37]. *In vitro* studies have shown osteogenic differentiation capacity in dental stem cells with

treatment of osteogenic supplements [38]. It has been reported that dental stem cells can be differentiate into osteoblasts in a-MEM containing 20% fetal bovine serum (FBS), ascorbic acid-2-phosphate, and L-glutamine; a strong alkaline phosphatase expression and mineralized nodule formation can also be generated with the presence of ascorbic acid, B-glycerophosphate, and dexamethoasone [38, 39]. Therefore, dental stem cells are a promising tool for the osteogenic differentiation studies and bone tissue regeneration applications.

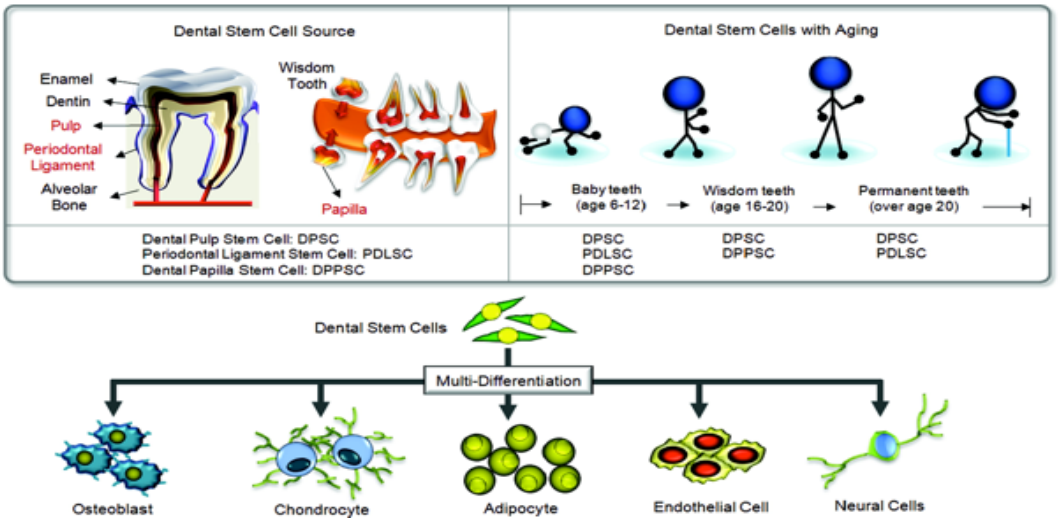

Figure 5. Dental stem cell sources and their multi-lineage differentiation capability
(Source: Byung-Chul Kim, Hojae Bae, Il-Keun Kwon, Eun-Jun Lee, Jae-Hong Park, Ali Khademhosseini, and Yu-Shik Hwang. Tissue Engineering Part B: Reviews. June 2012, 18(3): 235-244. doi:10.1089/ten.teb.2011.0642.)

It is important to note that the efficacy of regenerating bone using dental stem cells has an impact on the efficient *in vitro* differentiation towards the osteoblastic and odontoblastic lineage. As mentioned earlier, bone regeneration is a complex process. Remodeling begins with osteoclasts resorbing bone, removing both matrix and mineral components. Then, osteoblasts come in and start bone formation. During injuries (bone/tissue), osteoblasts travel to the injury site and promote bone formation. In addition, osteoblasts promote bone growth factors, which induce co-migration of MSCs into the repair site and direct their differentiation into the

osteogenic lineages [40]. Inflammation occurs when injuries happen, and it involves recruitment to the injured sites of immune cells and cytokines. It is essential to understand that inflammatory cells play a key role in injury healing and repairing processes, as also stem cell functions can be affected by immune cells and cytokines [41].

Although osteogenic and odontogenic differentiation has been extensively investigated, the unique ways of inflammation that promote negative or positive effects on bone and tissue formation are still not clear and understood. Given the above background, the aim of this study was to determine how the major proinflammatory cytokine, TNF-α, contributes to odontogenic differentiation in dental stem cells. We used two dental cell-lines, DPSCs and SCAPs, in this study. Both dental stem cells have the capacity to undergo osteogenic and odontogenic differentiation [42]. We wanted to test whether TNF-α will increase or decrease differentiation toward odontoblastic phenotype and expression of transcription factors RUNX2 and OSX in these two dental stem cells.

Materials and Methods:

Cell Cultures

Both DPSCs and SCAPs were individually cultured in 100mm tissue culture dishes with α – minimal essential medium (α -MEM ; Invitrogen, Carlsbad, CA, USA), containing 15% fetal bovine serum (FBS; Invitrogen), 100 U/ml penicillin-G, in the presence of 5% CO2 at 37 C. The medium was changed everyday, and cells were detached at 80- 90% confluence by the addition of PBS (Biochrom) containing 10% trypsin-EDTA (Invitrogen) and re-plated.

Osteogenic Induction & TNF- α Treatment

Dental stem cells were detached as mentioned above, counted (2 x 10^5/ mL cells), and re-plated. 6 wells plates for Alizarin red S staining and PCR/Real-Time; 12 wells plates for Alkaline phosphatase activities and staining. After plating, cells are incubated with the medium mentioned above until they are confluence enough for osteogenic induction. The induction medium contained α –MEM, 10% FBS, 50ng/mL ascorbic acid, 10mM β-glyverophosphate, and 5 μM dexamethasone. The induction medium was changed every 2 days.

Three groups were to set up to investigate the effects of TNF-α on both dental stem cells: no induction and TNF-α treatment (negative control), with induction treatment but no TNF-α treatment (positive control), and with induction and TNF-α treatment (1ng/mL). Both dental stem cells are treated 3 and 7 days for Alkaline phosphatase enzyme activity (ALPA) and staining (ALPS); 2 weeks for Alizarin Red S Staining (ARS).

ALP Staining

After 3 and 7 days, removed medium from cells and washed twice with PBS. Then fixed the cells in 70% EtOH at room temperature for 15mins. While waiting, substrate solution was made. Substrate solution consists of two solutions. Solution A was a mixture of 4mg naphtol AS-TR phosphate (SIGMA) with 0.15ml DMF (N, N' –dimethyl formamide). Solution B was 12mg Fast Blue BB-Sal (SIGMA) with 15ml 100mM Tris-HCL (pH 9.6). Both solution were mixed together with added 50ul of 1M MgCl2, and the substrate solution was filtered with 0.2ul filter. After fixation, EtOH was removed and cells were washed twice with distilled water. Then substrate solution was added. The 12-well plate was foiled and incubated at 37C for 30 minutes.

Afterwards solution was removed and preserved in PBS. Pictures were taken with Olympus microscopic camera at magnification of x4.

ALP Activity

Medium was removed from the 12 wells plate. Cells were washed twice with PBS and lysed with Lysis buffer (150 ul/well) containing 0.2% NP-40 and 1mM MgCL2 solution, then incubated at 37C for 15 minutes. After incubation, cells are scraped and transferred into 1.5mL tubes, vortex, and spun down at 4C for 10 minutes. 50ul Alkaline Buffer and 50ul stock substrate solutions are added in each well of 96-well plate. 10ul sample of each cell are added to each well, mixed, and incubated at 37C for 15 minutes. At the end, 110ul 0.5N NaOH was added to each well to stop the reaction. The absorbance was reed at 415nm using a microplate reader (PathTech). ALP activity was calculated from a standard curve after normalizing to the total protein content.

Alizarin Red Staining

After 2 weeks under induction condition and TNF-α treatment, individual dental stem cells from passage (11-15) were rinsed once with DPBS. Then fixed in ethanol for 10-15min at room temperature. After fixation, cells were washed twice with distilled water and stained with 2% Alizarin Red Solution (Millipore). The cells were placed on a shaker for 10 min at room temperature. Afterwards the cells were rinsed three times with distilled water. The results were scanned with a flatbed scanner.

Calcium Quantification Assay

Calcium content in the cell/matrix layer was measured by quantifying the mineralization of the extracellular matrix. Each well was added with 10% w/v cetylpyridinium chloride (CPC) (in 10mM sodium phosphate, pH 7.0) for 30 minutes in room temperature. Aliquots of the ARS extracts were diluted. The concentration was reed and determined at absorbance 540nm on a microplate reader, with an ARS standard curve made in the same solution.

RNA isolation and RT-PCR

Samples of total RNA were extracted from DPSCs and SCAPs cultured in osteogenic induction medium (with/ without TNF-α) as mentioned above for 4 and 24 hours. Total RNA was isolated from MSCs using Trizol reagents (Invitrogen). 2ug aliquots of RNAs were synthesized using random hexamers and reverse transcriptase according to the manufacturer's protocol (Invitrogen). The real-time PCR reactions were performed using the QuantiTect SYBRGreen PCR kit (Qiagen) and the Icycler iQ Multi-color Real-time PCR Detection System. Following primers were used to examine the genic expression of specific osteoblastic markers: RUNX2, fwd: 5' –TGG TTA CTG TCA TGG CGG GTA- 3', RUNX2, rev: 5' – TCT CAG ATC GTT GAA CCT TGC TA- 3', OSX, fwd: 5' - CCC TGC TTG AGG AGG AAG TT- 3', OSX, rev: 5' - CAG GTG AAA GGA GCC CAT TA- 3', GADPH, fwd: 5' ACC CAC TCC TCC ACC TTT GA-3', GADPH, rev: 5' – CTG TTG CTG TAG CCA AAT TCGT-3'. The expression of RUNX-2 and OSX were normalized to the expression of GADPH.

Data Analysis

Data are expressed as the mean ± SEM. The statistical analysis was performed with the student *t* test. Statistical significance was determined using a P value of < 0.05.

Results:

TNF-α increases extracellular matrix mineralization of osteogenic differentiated DPSCs & SCAPs

We investigated the effect of TNF-α on matrix mineralization in DPSCs and SCAPs. Matrix mineralization is essential in osteogenic and odontogenic differentiation. Our aim was to see if small amount of TNF-α could promote mineralization in DPSCs and SCAPs. To evaluate the effect of TNF-α on this process, both DPSCs and SCAPs were treated with 1ng/mL of TNF-α for 14 days during osteogenic differentiation. After differentiation, DPSCS and SCAPs mineralized the deposited matrix (FIG. 6) as visualized by Alizarin Red staining. Treatment with TNF-α during osteogenic differentiation had significant increase in calcium deposition, especially in DPSCs. To quantify mineralization of the matrix, calcium content in the matrix layer was measured using CPC quantification assay (FIG. 7). The calcium content was significantly increased in TNF-α treatment compared to untreated dental stem cells.

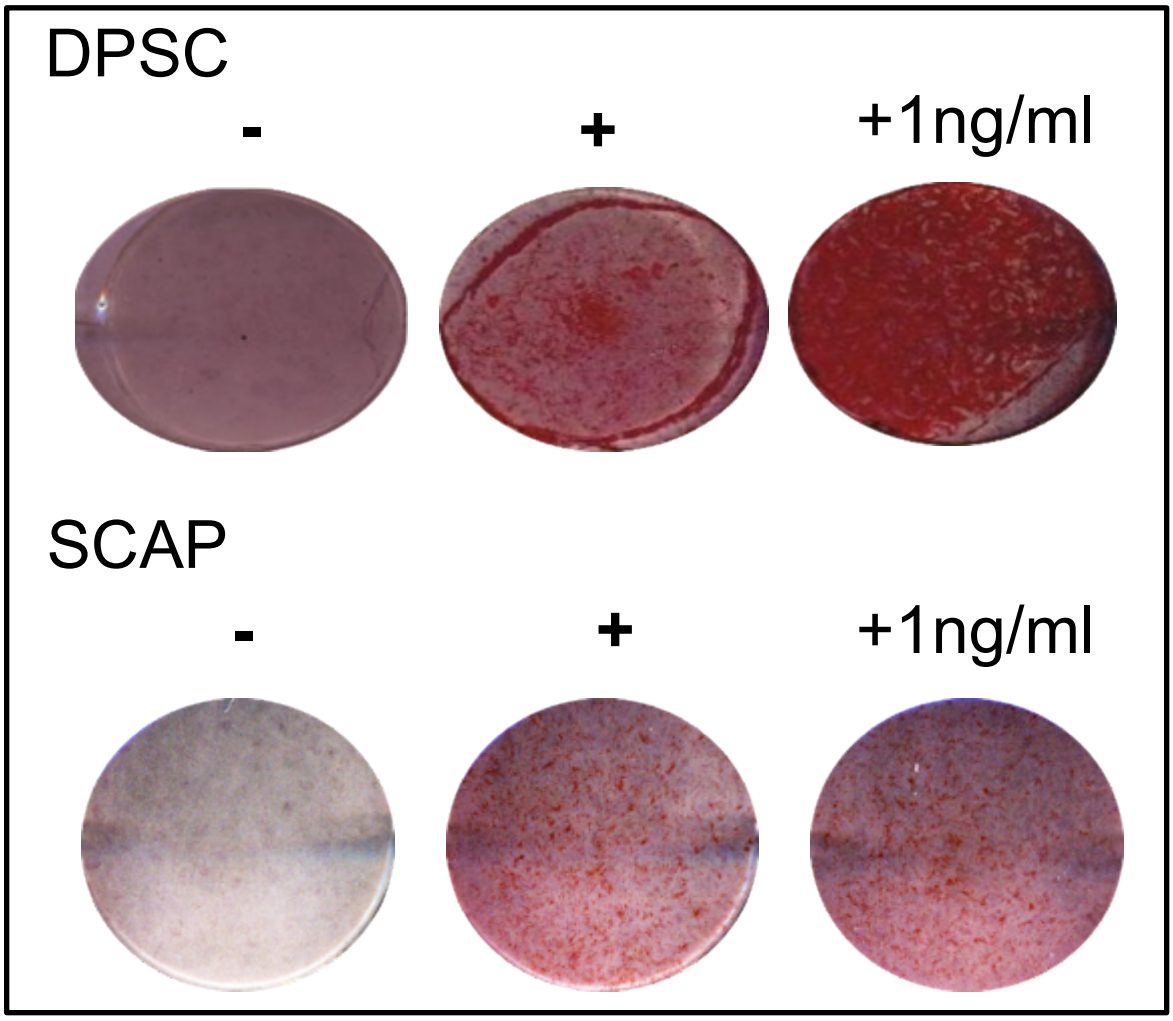

Figure 6. Effect of TNF- α treatment on extracellular matrix mineralization expression in DSCs. DPSCs and SCAPs were induced to differentiate towards osteoblasts with osteogenic induction medium, which were treated with murine TNF-α at concentration of 1ng/ml. Alizarin red staining of mineralized nodules from DPSCs and SCAPs was determined after 14 days of differentiation. (-) is negative control with no osteogenic induction and *TNF-α* treatment. (+) is positive control with osteogenic induction.

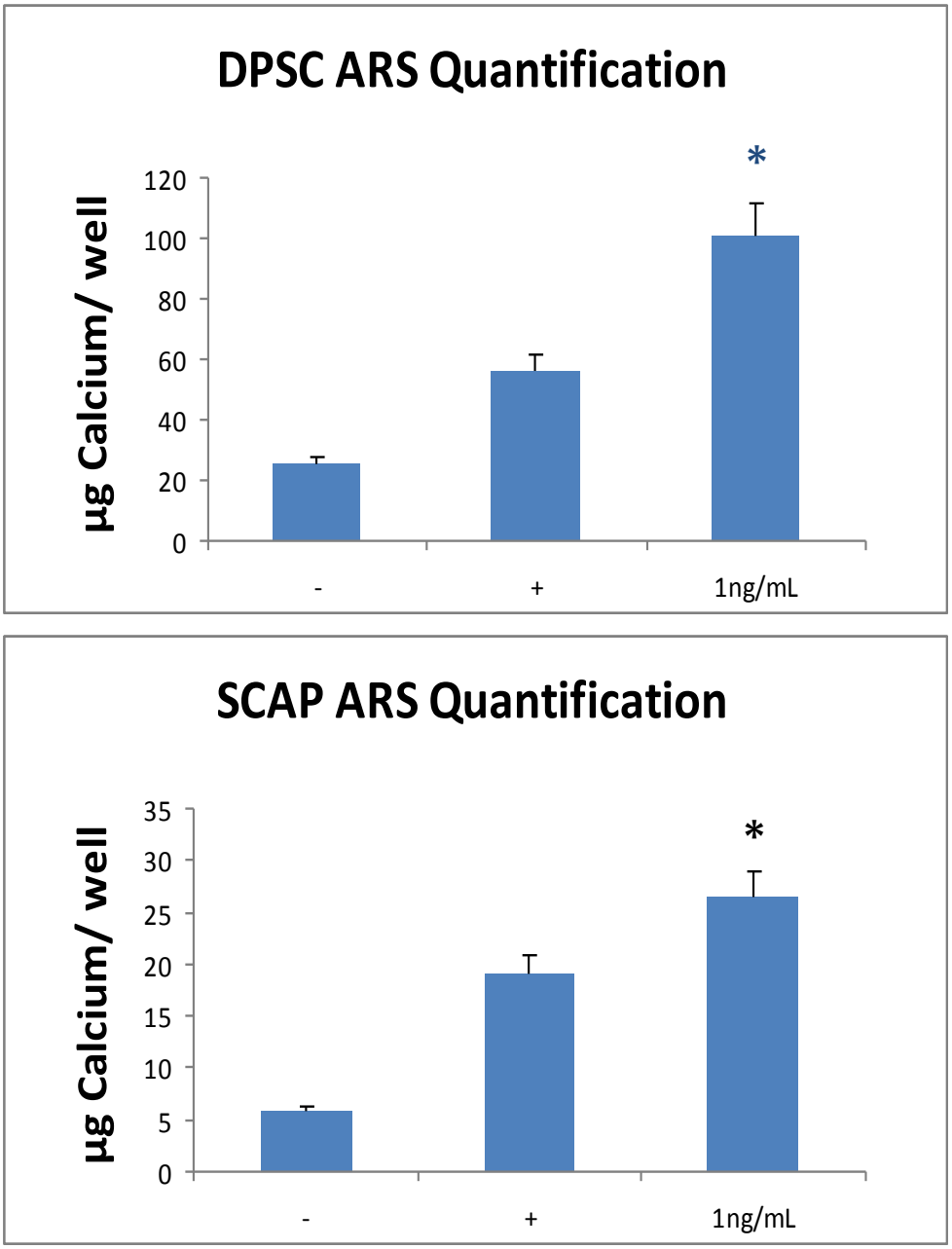

Figure 7. Effect of TNF- α treatment on calcium quantification expression in DSCs. DPSCs and SCAPs were induced to differentiate towards osteoblasts with osteogenic induction medium, which were treated with murine TNF-α at concentration of 1ng/ml. Quantification of positively stained area by Alizarin red in DPSCs and SCAPs with and with no treatment was determined. (-) is negative control with no osteogenic induction and *TNF-α* treatment. (+) is positive control with osteogenic induction. (*P < 0.05, versus cells with induction but no TNF treatment.)

TNF-α increases ALP expression and activity in DPSCs & SCAPs

The influence of TNF-α on osteogenic and odontogenic differentiation of dental stem cells was further investigated. At day 3 and day 7, both dental stem cells were fixed and stained for ALP expression. ALP is a surrogate marker of early osteogenic differentiation. ALP expression significantly increased in both dental stem cells at day 7. DPSCs had weaker ALP expression compared to SCAP (FIG. 8). The ALP activity of both cells was also determined. The results (FIG. 9) showed that dental stem cells with TNF-α treatment had higher ALP activity than the ones without treatment. A significant difference in ALP activity of TNF-α treated and non-treated stem cells at day 7 indicated that TNF-α plays a key role in osteogenic differentiation.

TNF-α increases RUNX-2 expression in DPSCs & SCAPs at different time-point, but decreases OSX expression

We investigated the expression of critical transcription factors in bone formation, RUNX-2 and OSX, using Real Time-PCR. Based on the results obtained from ALP expression and mineral formation in DPSCs and SCAPs, We want to see if TNF-α induces the expression of RUNX-2 and OSX. The results showed that RUNX-2 expression in DPSC was enhanced at 4 hours with treatment and SCAPs was at 24 hours (FIG. 10). However, OSX expression was significantly decreased in both dental stem cells (FIG. 10).

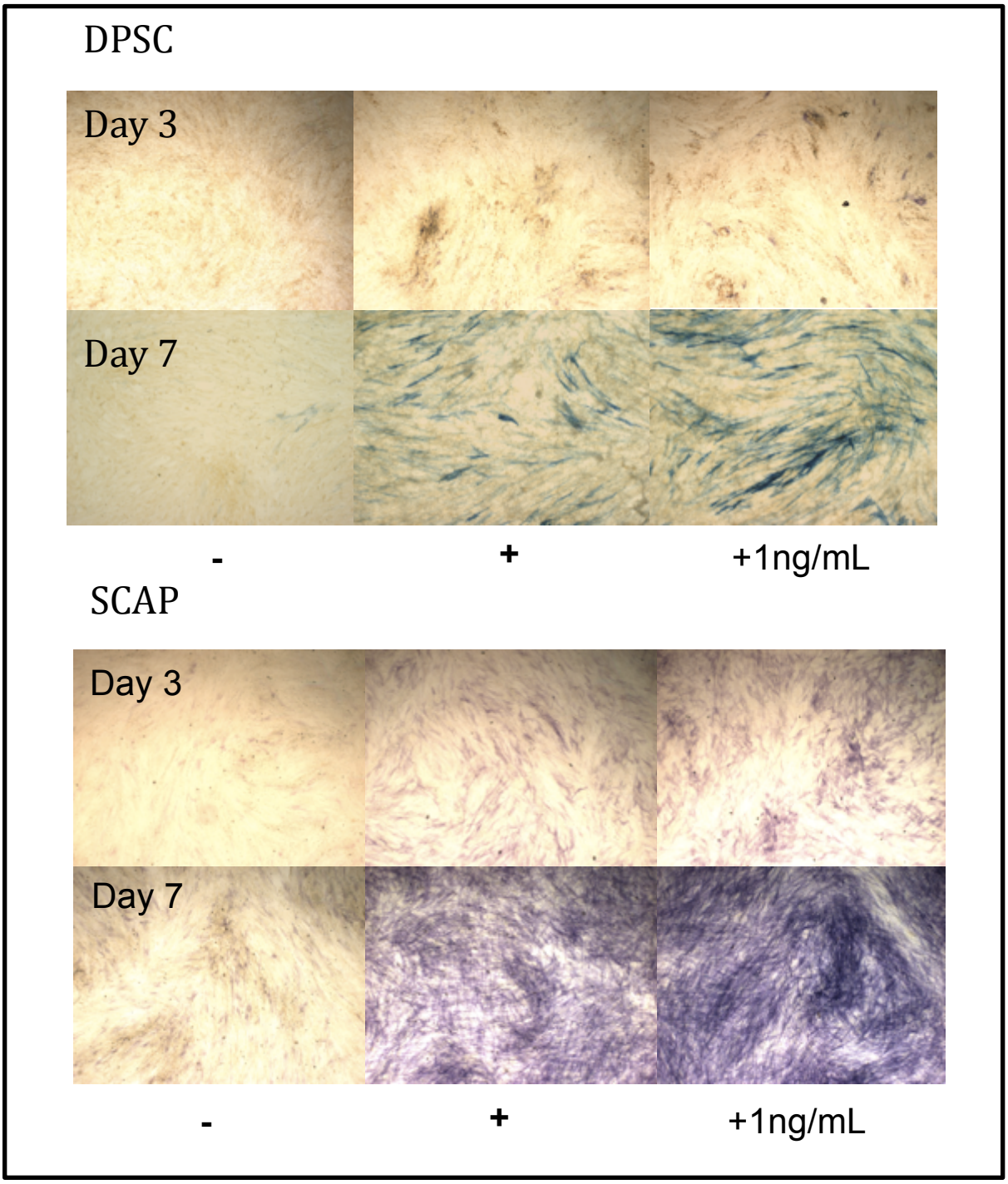

Figure 8. Effect of TNF- α treatment on ALP Expression in DSCs. DPSCs and SCAPs were induced to differentiate towards osteoblasts with osteogenic induction medium, which were treated with murine TNF-α at concentration of 1ng/ml. Staining of ALP expression in DPSCs and SCAPs was determined at day 3 and day 7. (-) is negative control with no osteogenic induction and *TNF-α* treatment. (+) is positive control with osteogenic induction.

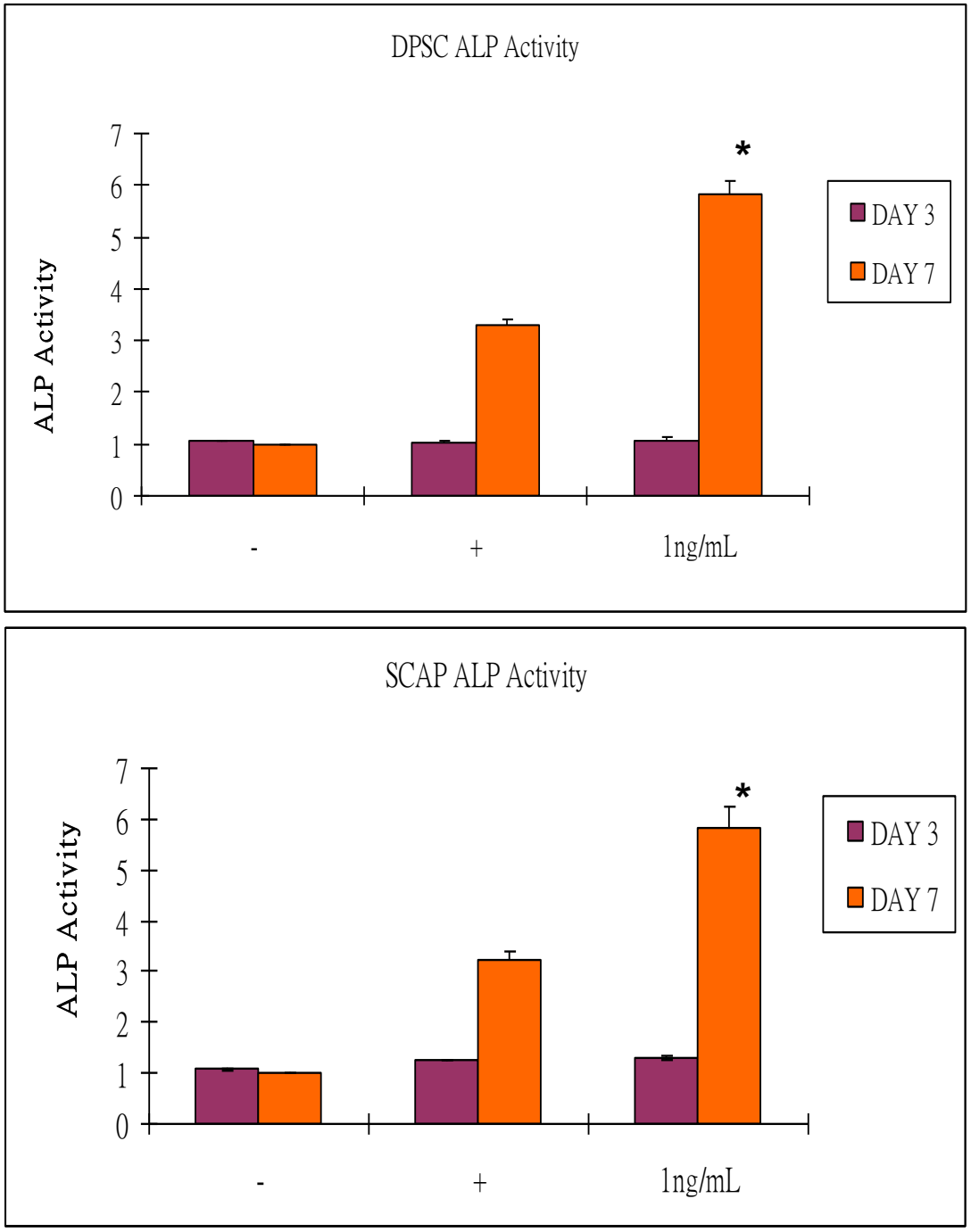

Figure 9. Effect of TNF- α treatment on ALP Activity in DSCs. DPSCs and SCAPs were induced to differentiate towards osteoblasts with osteogenic induction medium, which were treated with murine TNF-α at concentration of 1ng/ml. ALP activity of DPSCs and SCAPs was determined at day 3 and day 7. The data were represented as the

average ± SD (n= 3). (-) is negative control with no osteogenic induction and *TNF-α* treatment. (+) is positive control with osteogenic induction. (*P < 0.05, versus cells with induction but no TNF treatment.)

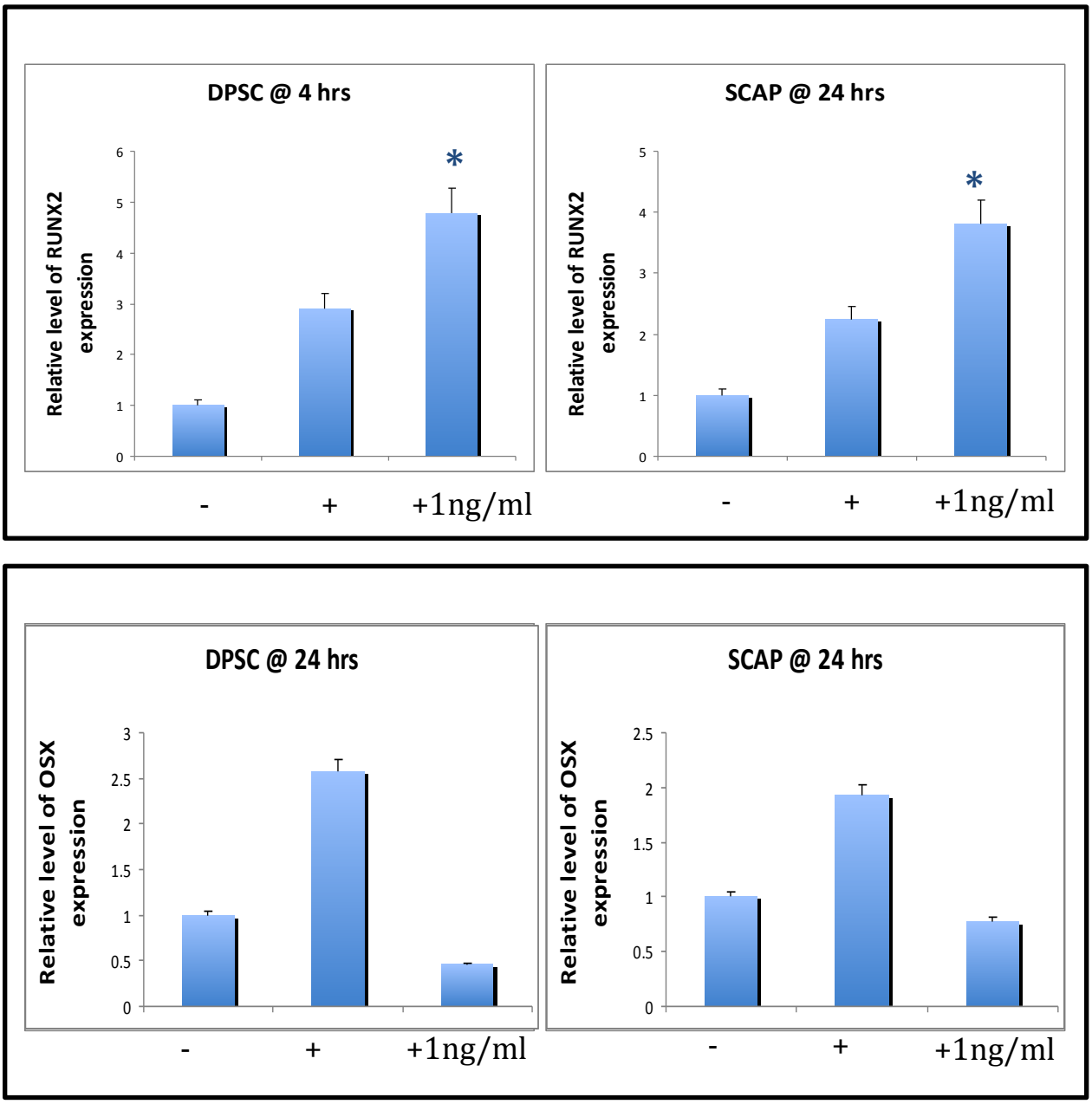

Figure 10. **Effect of TNF- α treatment on RUNX-2 and OSX expression in DSCs.** DPSCs and SCAPs were induced to differentiate towards osteoblasts with osteogenic induction medium, which were treated with murine TNF-α at concentration of 1ng/ml. Four and twenty-four hours of treatment were done, and real time PCR was performed to determine the gene expression. (-) is negative control with no osteogenic induction and *TNF-α* treatment. (+) is positive control with osteogenic induction. (*P < 0.05, versus cells with induction but no TNF treatment.)

Discussion:

The effects of TNF-α on bone and tissue have been extensively explored and investigated. Though the role of TNF-α is controversial in bone formation, recent studies have revealed that TNF-α has the ability to exert opposite effects depending on the condition that it is released, such as promote *in vitro* calcification of vascular cells [6], and osteogenesis in MSCs [7, 10, 15-17]. Here we demonstrated that TNF-α is capable of inducing osteogenic differentiation in dental stem cells (DPSCs and SCAPs). With various studies on different amount of TNF-α used to promote osteogenesis (from 0.1ng/mL to 100ng/mL) [6, 10, 15, 17, 36, 41], and data have showed that small amount of TNF-α could promote osteogenic differentiation. Nevertheless, our data also showed induction of osteogenesis in dental stem cells with TNF-α treatment at 1ng/ml.

In this study we used two dental stem cell lines, DPSCs and SCAPs. Extracted from human pulp, DPSCs have shown to contain expression of osteogenic markers and also response to inductors of osteogenic differentiation. Moreover, DPSCs have the ability to produce more colony-forming units (CFUs) than bone marrow mesenchymal cells (BMMSCs) [40]. They also have high proliferation rate from 80 to 100 times with longer surviving time when compared to BMMSCs [43]. SCAPs are obtained from apical papilla, precursor of the radicular pulp, which are derived from a developing tissue that may represent a population of progenitor and early stem cells [42]. Thus these dental stem cells may be a superior cell source for tissue and bone regeneration. Compare to DPSCs, these cells also have the capacity to differentiate into cells of many different lineages, though SCAPs express lower levels of markers [32].

In this study, our data showed that TNF-α treatment at 1ng/ml significantly enhance expression levels of osteogenic transcription factors and bone marker genes in dental stem cells. In mineralization and alkaline phosphatase expression, with TNF-α treatment at 1ng/ml, both dental stem cells have a significantly stronger osteogenic expression (FIG. 6 & FIG. 8). Our results also showed that TNF-α induced the alkaline phosphatase activity in both dental stem cells at day 7 (FIG. 9). In contrast, studies have shown that at higher concentrations, TNF-α displayed inhibitory effect on osteogenic differentiation [10, 14-17]. It has been reported that the inhibitory effect of TNF-α in higher concentration observed in cell cultures could be reversed by the blocking NF-κB signaling pathway, and the positive effect of TNF-α was not affected with overexpressing IκBα [10]. These findings suggest that cell cultures treated with TNF-α at different concentrations are dominated by different signaling pathways.

In our past study, we have demonstrated that TNF-α activates the nuclear factor- κB (NF-κB) signaling pathway in DPSCs [44]. NF-κB is the critical factor activated by TNF and IL-1 during infection. Heterodimer of p50 and p65/RelA proteins are the most common form of NF-κB, and it is present in an inactive form that is retained in the cytoplasm by IκBα, an inhibitory protein, when is not stimulated [45]. Studies have shown that the IKK complex plays a key role in the activation of NF-κB. It consists two catalytic subunits, IKKα and IKKβ, and a non-catalytic chaperone protein IKKγ [45]. IKKα and IKKβ are activated when stimulated by TNF, or other stimuli, following IKKγ inactivation by ubiquitin by unknown mechanisms [44]. The activated IKK complex starts phosphorylation of the N-terminal region of IκBα at Serines 32 and 36, which then the phosphorylated IκBα is degraded by 26 S proteasome machinery [44]. NF-κB is released from cytoplasm to nucleus by the degradation of IκBα, and it binds to specific elements in the promoter of NF-κB target genes, which activates gene transcription [44, 45].

The role of NF-κB in immune system and bone has been studied extensively in the past decade. NF-κB regulates gene expression during inflammatory and immune responses. Its signaling pathways are also important in regulating bone homeostasis [46]. NF-κB also has an important role in osteoclast differentiation [2, 45]. Studies have shown that mice deficient in functional NF-κB developed osteopetrosis due to lack of osteoclasts, and it affect bone formation through an effect on osteoblastic function [47, 48]. Furthermore, our past data reported that bone mass and bone mineral density in a murine model significantly increased from inhibition of NF-κB [48]. Inflammatory bone destruction in rheumatoid arthritis (RA) patients is related to the activation of NF-κB in synovium and lymphocytes [49], and strong NF-κB transcriptional activity is produced from the induction of arthritis in mice in inflamed joints [50]. Thus, NF-κB has been an important target in the therapeutic treatment of osteoporosis and many inflammatory bone disorders.

It has been suggested that TNF-α treatment activates JNK was transient, due to the fact that JNK activation could be inhibited by NF-κB activation [10]. Furthermore, with the increase of TNF-α concentration, the level of NF-κB dramatically increases as well; however, there was only a moderate increase in the activation level of members of the MAPK family (JNK, ERK and p38) in higher TNF-α concentration [51]. As mentioned earlier of negative role of activated NF-κB signaling pathway in osteogenic differentiation and bone formation, it was reported that ERK, JNK and p38 were able to stimulate differentiation of osteoblasts [10, 52, 53]. It is important identify the signaling pathway(s) that directs DSCs towards osteogenic differentiation. It is possible that our results of enhanced osteogenic differentiation in dental stem cells with TNF-α treatment at 1ng/ml could be mainly related to MAPK family members, since it has positive effects on osteogenic differentiation. Further investigation is needed.

A recent study revealed that TNF-α promotes osteogenic differentiation of mesenchymal stem cells by triggering NF-κB signaling pathway [17]. K. Hess et al. demonstrated that NF-κB induction were monitored by the increased deposition of calcium to the extracellular matrix and enhanced expression of growth factor BMP-2 and osteogenic marker ALP [17]. They mentioned that with the constitutively active IKK2 also enhanced expression of key regulators of osteogenic differentiation, which suggests that NF-κB signaling pathway does have a key role in bone formation process, especially during early osteogenic differentiation [17]. Their results also showed that inducing the NF-κB pathway of human mesenchymal stem cells during initial osteogenic differentiation promotes differentiation with the increase of expression in BMP-2 and osteogenic regulators [17]. It is further supported by a recent study shown that NF-κB facilitates growth plate chondrogenesis by induction of BMP-2 expression and activity [17, 54]. However, osteogenic differentiation is not certainly dependent on NF-κB pathway, as blocking the TNF-α-induced increase of BMP-2 expression does not inhibit mineralization [17].

The important transcriptional regulators of osteoblast differentiation in IKK2-expressing shows that the NF-κB pathway can influence cell fate decisions of mesenchymal stem cells [17]. In this study, we found that TNF-α treatment at 1ng/mL enhanced expression levels of bone marker genes. RUNX-2 is an essential transcription factor for osteoblast differentiation, and it is required for osteoblast cell fate determination [17, 20, 55]. OSX is a zinc-finger transcription factor that is downstream of RUNX2, and it is important to assure osteogenic differentiation [17, 56]. Our data showed that the gene expression of RUNX-2 was enhanced at different time point for both dental stem cells. It could be due to the fact that these two dental stem cells are from different areas of dental tissue, but it is still unclear. However, OSX was decreased in both dental stem cells. It is possible that the delay in induction between RUNX2 and OSX is similar with

published data suggested that OSX is regulated and downstream of RUNX2 [17, 57]. However, these recent findings of TNF-α's effect on mesenchymal stem cells are only *in vitro*. From our past studies of effects from NF-κB on bone, we demonstrated that NF-κB prohibited bone formation of *in vivo* [48]. Further investigation is needed since it is still unclear if the induction of RUNX2, or OSX by NF-κB pathway is indirect or direct.

Lu and his colleagues [7] also demonstrated that TNF-α promotes osteogenic differentiation of human primary osteoblasts (HOBs). They showed that the cytokine stimulates HOBs to secrete soluble factors that foster a microenvironment favoring osteogenic differentiation of adipose tissue-derived mesenchymal stem cells (ASCs) [7]. They suggested that this action is partly dependent on a BMP-2 paracrine loop that is regulated through the p38 MAPK signaling pathway. From Lu's results, exposing TNF-α for a short period of time, the positive loop might overcome its negative effects, as with prolonged exposure of the cytokine, the negative effects dominate [7]. Their proposed explanation for the effect of TNF-α on osteogenic gene expression of osteoblasts/MSCs is that it has direct negative effects on osteogenic differentiation; however it owes the positive effects to activating some pathways that can compensate its negative effects [7].

BMP-2 has a critical role in the early stages of bone remodeling, and it has been widely used for bone regeneration [7, 58, 59]. Interestingly, TNF-α was found to be able to induce BMP-2 production in different cell types, including endothelial cells, chondrocytes, and dental pulp cells [7, 60]. Lu et al proposed that short TNF-α treatment promotes BMP-2 production in HOBs, and the supplementation of BMP-2 inhibitor (Noggin) in the co-culture medium blocked osteogenic gene expression and alp enzyme activity of ASCs after 24 hours of TNF-α treatment [7]. The results suggested that stimulating a paracrine BMP-2 loop in HOBs is one of the

important mechanisms of the positive effects on bone formation mediated by a short period (24 hours) of time with TNF-α treatment [7]. However, the negative effects of TNF-α might overshadow its positive effects on the BMP-2 loop if it is continuously present. Moreover, continuously exposed to TNF-α has been shown to be detrimental to BMP-2-induced osteogenic differentiation through the inhibiting Smad signaling and Ras/Rho-MAPK pathway [7]. They also investigated the mechanism of TNF-α inducing BMP-2 production in osteoblasts. Many different mechanisms and signaling pathways have been suggested to be involved in BMP-2 gene regulation during osteogenic differentiation [7, 61]. P38 MAPK signaling pathway is one of the down-stream signaling pathways of both BMP-2 and TNF-α, which has an important role in regulating bone formation [7]. Lu et al demonstrated that p38 signaling served as the mediator between TNF-α treatment and endogenous BMP-2 production in HOBs. Their hypothesis was further supported by their results where treatment of TNF-α activated the p38 MAPK signaling pathway earlier than BMP-2 induction. The MAPK p38 inhibitor treatment also negated BMP-2 protein expression in HOBs, and later reduced the osteogenic differentiation of ASCs. [7]. Lu et al stated that it is important to note that the positive effects of the TNF-α-p38 MAPK-BMP-2 loop on osteogenic differentiation may only be effective in short-term treatment (24 hours).

In this study with the osteogenic and odontogenic potential dental stem cells, we demonstrated that enhanced osteogenic differentiation could be done in these dental stem cells with TNF-α treatment at a low concentration. For future studies, it would be great to look into the roles of BMP-2 and MAPK in TNF-α-mediated effects on odontogenic differentiation in dental stem cells. It is still a challenge to find the final role of TNF-α in on osteogenic differentiation, odontogenic differentiation, bone formation and tissue regeneration. TNF-α's contribution of apoptosis and rate of differentiation in regulating bone formation remains

controversial. With recent studies demonstrated the potential positive effect of TNF-α in a low concentration, which shows the importance of TNF-α appearance during bone healing and might explains the possible reason for the contradictory effects of inflammation on bone and tissue regeneration. In the future, it may contribute to developing strategies for bone regeneration by controlling amount of inflammatory responses in bone and periodontal diseases.

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