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Senescence and odontoblastic differentiation of dental pulp cells

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A running head: differentiation of dental pulp cells

Abstract

Cellular senescence has been suggested to be involved in physiological changes of cytokine production. Previous studies showed that the concentration of tumor necrosis factor-α (TNF-α) is higher in the blood of aged people compared with that of young people. So far, the precise effects of TNF-α on odontoblastic differentiation of pulp cells has been controversial. Therefore, we aimed to clarify how this cytokine affected pulp cells during aging. Human dental pulp cells (HDPCs) were cultured until reaching the plateau of their growth, and the cells were isolated at actively (yHDPCs) or inactively (sHDPCs) proliferating stages. sHDPCs expressed senescence-related molecules while yHDPCs did not. When these HDPCs were cultured in odontoblast-inductive medium, both young and senescent cells showed mineralization, but mineralization in sHDPCs was lower compared with yHDPCs. However, the administration of TNF-α to this culture medium altered these responses: yHDPCs showed downregulated mineralization, while sHDPCs exhibited significantly increased mineralization. Furthermore, the expression of TNFR1, a receptor of TNF-α, was significantly upregulated in sHDPCs compared with yHDPCs. Downregulation of *TNFR1* expression led to decreased mineralization of TNF-α-treated sHDPCs, whereas restored the reduction in TNF-α-treated yHDPCs. These results suggested that sHDPCs preserved the odontoblastic differentiation capacity, and

TNF-α promoted odontoblastic differentiation of HDPCs with progress of their population doublings through increased expression of *TNFR1*. Thus, TNF-α might exert a different effect on odontoblastic differentiation of HDPCs depending on their proliferating activity. In addition, the calcification of pulp chamber with age may be related with increased reactivity of pulp cells to TNF-α.

Introduction

Dental pulp cells have a high repair ability but do not mineralize under physiological conditions. However, under pathologic conditions, the protective response, such as reparative dentin formation, is induced by inflammatory stimuli during pulp wound healing (Goldberg et al., 2008) or mechanical stimuli. In this protective event, odontoblast-like cells are differentiated from the progenitor cells in the pulp tissue and secrete dentin matrices (Dimitrova-Nakov et al., 2014). These matrices include extracellular matrix proteins, such as bone sialoprotein (BSP), dentin sialophosphoprotein (DSPP), and osteocalcin (OCN), which are involved in odontoblastic differentiation (Chen et al., 1992; Suzuki et al., 2012; Wei et al., 2007). In addition, the expression of Nestin, which forms type VI intermediate filament proteins, is also related to

odontoblastic differentiation (Lee et al., 2012).

The protective response of dental pulp is mediated by inflammatory molecules such as tumor necrosis factor-α (TNF-α) derived from immune cells (Jontell et al., 1998). Several studies have examined the involvement of TNF-α in odontoblastic differentiation. One study reported that TNF-α suppressed mineralization in an odontoblast-like cell line (Nakayama et al., 2016), whereas another study showed that TNF-α promoted osteogenic differentiation of human dental pulp cells (Paula-Silva et al., 2009). Thus, whether TNF-α can induce odontoblastic differentiation of dental pulp cells remains unclear.

Tumor suppressors, such as p16, p21 and p53, increase in gene expression with age and are known senescence-related genes (Bringold and Serrano, 2000; Johmura et al., 2014). Cellular senescence is also characterized by an accumulation of senescence-associated β-galactosidase (SA-β-gal) activity (Dimri et al., 1995). Senescent cells secrete higher levels of proteins with a senescence-associated secretory phenotype, such as IL-6, TNF-α, MMPs, monocyte chemoattractant protein-1, and IGF binding proteins, in multiple tissues with age (Coppe et al., 2008). A relationship between aging and chronic inflammation has been demonstrated (Franceschi et al., 2000), and the concentration of the TNF-α inflammatory molecule was shown to be increased in blood in older people compared with that in the young (Paolisso et al., 1998b). However, whether dental pulp

cells show a different response to TNF-α depending on senescence remains unknown.

In this study, we hypothesized that odontoblastic differentiation of dental pulp cells might be promoted by TNF-α depending on their senescent stage. Therefore, the aim of this study was to investigate how the population doubling of dental pulp cells affected their reactivity against TNF-α on their odontoblastic differentiation and to examine the possible underlying mechanism.

Materials and methods

Cell culture

Three lines of human dental pulp cells (HDPCs) were isolated from healthy human premolars of a 24-year-old male (HDPC-3R), a 21-year-old female (HDPC-3Q), and a 23-year-old male (HDPC-3S) who visited Kyushu University hospital for tooth extraction, with informed consent. HDPCs were cultured in α-minimal essential medium (α-MEM; Gibco-BRL, Grand Island, NY) containing 10% fetal bovine serum (FBS; Biowest, Nuaille, France) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. All procedures were performed in compliance with the Research Ethics Committee, Kyushu

University (No.20A-3).

Determination of population doublings

Population doublings (PDs) of HDPCs were determined until cells reached a plateau in proliferation. PD was calculated by the following formula: $[\log10N_h - \log10N_s] / \log10[2]$, in which N_h and N_s indicate the cell number at harvest and at seeding, respectively. PD was calculated by adding the PD of the previous passage to the PD calculated at each passage.

Senescence assay

SA-β-gal activity was measured by the β-Galactosidase Staining Kit (Cell Signaling Technology, Inc., Danvers, MA) following the manufacturer's protocol. Briefly, cells were fixed in 2% formaldehyde and 0.2% glutaraldehyde. After cells were washed, they were incubated overnight in the staining solution (1 mg/mL 5-bromo-4-chloro-3-indolyl h-D-galactosidase, 40 mM citric acid, pH 6.0, 40 mM sodium phosphate, pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM sodium chloride, and 2 mM magnesium chloride) at 37°C. Under a microscope (Keyence, Osaka, Japan), the

senescent cells appeared blue.

Odontoblastic differentiation of HDPCs

HDPCs were cultured using three different medium conditions: 10% FBS/α-MEM as control culture medium (CM), CM containing 2 mM $CaCl_2$ as differentiation medium (DM), or DM containing TNF-α (DM+TNF-α). Our recent studies showed that DM promoted odontoblastic differentiation of HDPCs (Mizumachi et al., 2017; Serita et al., 2017). After culturing, the cells were subjected to Alizarin red S staining and PCR analysis.

Alizarin red S staining

HDPCs were fixed for 1 h with 10% formalin. After washing with sterile water, the cells were incubated with Alizarin red S solution (pH 4.1–4.3) (Sigma-Aldrich, Tokyo, Japan) for 1 h. Each Alizarin red-positive region was evaluated using a microscope (Keyence).

Quantitative RT-PCR

Total cellular RNA was harvested using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. First-strand cDNA was synthesized from 1 mg total RNA using an ExScript RT Reagent kit (Takara Bio Inc., Shiga, Japan). Quantitative RT-PCR was performed with a SYBR Green II RT-PCR kit (Takara Bio Inc.) using a Thermal Cycler Dice Real Time System (Takara Bio Inc.) under the following conditions: 95 °C for 10 s and then 40 cycles at 95 °C for 5 s and 60 °C for 30 s, followed by a dissociation program at 95 °C for 15 s, 60 °C for 30 s and 60 °C for 30 s and 95 °C for 15 s. Specific primer sequences, annealing temperatures, and product sizes for each gene are listed in a TABLE. β-actin served as an internal control. Expression levels of the target genes were calculated using ΔΔCt values.

siRNA transfection

HDPCs were transfected with TNFR1 siRNA (MISSION siRNA; Hs_TNFRSF1A_3459; Sigma) or control siRNA (MISSION siRNA Universal Negative Control #1; SIC-001-10; Sigma) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. Briefly, cells at 80% confluence were cultured in Opti-MEM I (Invitrogen)

containing 10% FBS. A siRNA–lipid complex, comprising 10 pmol siRNA and 3 µl of Lipofectamine RNAiMAX in 50 µl of Opti-MEM, was prepared. After incubation for 5 min at room temperature, the complex was added to the cells and cells were incubated for 24 h.

Statistical analysis

All data are presented as the mean ± SD. Statistical analyses were performed by one-way ANOVA followed by Tukey's test for multiple comparisons. Student's unpaired t-test was performed for comparisons of two mean values. $P < 0.05$ was considered statistically significant.

Results

Determination of PDs of HDPCs and the expression of aging-related markers

PDs of HDPCs cultured in 10% FBS/α-MEM were determined by culturing until the cell proliferation reached a plateau (Fig. 1A and Supplementary Fig. 1A, E). In HDPC-3R

cells, the cell proliferation activity after PD25 was diminished (Fig. 1A). The cells

isolated at PD6 showed active proliferation, while the cells at PD28 had almost reached

a plateau of growth (Fig. 1A). To verify if the cells at PD28 were senescent, SA-β-gal

activity was examined and the results demonstrated that PD28 cells showed positive SA-

β-gal activity whereas PD6 cells did not (Fig. 1B). In addition, RT-PCR analysis of aging-

related markers, *p16*, *p21*, and *p53* showed that the expression levels of these genes in

HDPC-3R cells at PD28 were significantly upregulated compared with the cells at PD6

(Fig. 2C). The other two HDPC lines, HDPCs-3Q and -3S, also showed the same results;

HDPC-3Q at PD6 and HDPC-3S at PD5 showed active proliferation while HDPC-3Q at

PD27 and HDPC-3S at PD26 exhibited downregulated proliferation and upregulated

senescence markers (Supplementary Fig. 1A–F). Based on these data, HDPCs at actively

and inactively proliferating stages were used as young HDPCs (yHDPCs) and senescent

HDPCs (sHDPCs), respectively, in the following analyses.

Expression of odontoblast-related genes in yHDPCs and sHDPCs

The expression of odontoblast-related genes, such as *BSP*, *DSPP*, *Nestin*, and *OCN* was

investigated in yHDPCs and sHDPCs cultured in 10% FBS/α-MEM (Fig. 2 and

Supplementary Fig. 2). The results showed that the expression levels of these genes were significantly higher in sHDPCs than in yHDPCs.

Effects of TNF-α on odontoblastic differentiation of HDPCs

We recently showed that DM promoted odontoblastic differentiation of HDPCs (Mizumachi et al., 2017; Serita et al., 2017). To examine the effects of TNF-α on odontoblastic differentiation of yHDPCs and sHDPCs, the cells were cultured in CM or DM with or without TNF-α for 7 days. Both yHDPCs and sHDPCs cultured in CM alone formed few alizarin red S-positive mineralized deposits, while both cells cultured in DM alone showed increased mineralized deposits (Fig. 3A, D and Supplementary Fig. 3A, D, G, J). However, sHDPCs treated with TNF-α revealed enhanced mineralization upon differentiation with DM, whereas TNF-α-treated yHDPCs showed reduced mineralization compared with the cultures in DM (Fig. 3A, B, D, E and Supplementary Fig. 3A, B, D, E, G, H, J, K). Furthermore, the expression of odontoblast-related genes, such as *BSP, DSPP, Nestin*, and *OCN*, in both yHDPCs and sHDPCs cultured under the same conditions also showed similar results as the mineralization data (Fig. 3C, F and Supplementary Fig. 3C, F, I, L).

Comparison of the mineralization ratio between yHDPCs and sHDPCs

The mineralization area in yHDPCs and sHDPCs cultured in CM or DM with or without TNF-α was first determined and then the cell number in each condition was counted. From these data, the mineralization ratio per cell was calculated. The results showed that sHDPCs demonstrated significantly higher mineralization activity per cell under TNF-α treatment compared with yHDPCs (Fig. 4 and Supplementary Fig. 4A, B).

Contribution of TNFR1 to TNF-α-induced odontoblastic features in sHDPCs.

Gene expression of a TNF-α receptor, TNFR1, was examined in yHDPCs and sHDPCs (Fig. 5A and Supplementary Fig. 5A, F). sHDPCs were found to express *TNFR1* at a higher level compared with yHDPCs (Fig. 5A and Supplementary Fig. 5A, F). To further examine whether the effect of TNF-α on the odontoblastic differentiation of sHDPCs was through the TNFR1 receptor, the cells were transduced with *TNFR1* siRNA or control siRNA for 24 h. The suppression of *TNFR1* in the transduced sHDPCs was first confirmed by quantitative RT-PCR assays (Fig. 5B and Supplementary Fig. 5B, G). Then, the

transduced sHDPCs were cultured in CM or DM with or without TNF-α for 9 days. sHDPCs cultured in DM showed increased alizarin red S-positive deposits, whereas the cells cultured in CM showed no positive reaction (Fig. 5C, D and Supplementary Fig. 5C, D, H, I). Control and TNFR1 siRNA-transduced sHDPCs in DM without TNF-α showed similar mineralization levels (Fig. 5C, D and Supplementary Fig. 5C, D, H, I). However, when cultured in DM with TNF-α, siTNFR1-transduced sHDPCs showed significantly reduced mineralization compared with control siRNA-transduced cells (Fig. 5C, D and Supplementary Fig. 5C, D, H, I). The expression of odontoblast-related genes, such as *BSP, DSPP, Nestin*, and *OCN*, in sHDPCs cultured under the same conditions also showed similar results (Fig. 5E and Supplementary Fig. 5E, J).

Contribution of TNFR1 to TNF-α-reduced odontoblastic features in yHDPCs.

The effect of TNF-α on the odontoblastic differentiation of yHDPCs through the TNFR1 was also investigated by the same methods using the transduction with TNFR1 siRNA or control siRNA as described above. Downregulation of *TNFR1* in the transduced yHDPCs was verified by quantitative RT-PCR assays (Fig. 6A and Supplementary Fig. 6A, E). Although yHDPCs cultured in CM showed no alizarin red S-positive reaction, the cells

cultured in DM showed increased mineralization (Fig. 6B, C and Supplementary Fig 6B, C, F, G). Control and TNFR1 siRNA-transduced yHDPCs showed similar degree of mineralization levels in DM without TNF-α (Fig. 6B, C and Supplementary Fig 6B, C, F, G). However, when cultured in DM with TNF-α, control siRNA-transduced yHDPCs reduced mineralization, whereas the transduction of siTNFR1 restored it (Fig. 6B, C and Supplementary Fig 6B, C, F, G). *BSP, DSPP, Nestin*, and *OCN*, in yHDPCs cultured under the same conditions also exhibited corresponding results (Fig. 6D and Supplementary Fig. 6D, H).

Discussion

In the present study, we first clarified that HDPCs showed odontoblastic features with increasing PDs, and furthermore that the reactivity of these cells against TNF-α in terms of odontoblastic differentiation with TNF-α was promoted through increased expression of TNFR1 and its altered signaling pathway, depending on their PD. Our results indicated that HDPCs form the calcified bodies such as denticles and pulp stones in the pulp chamber with age.

A recent study demonstrated that normal human diploid fibroblasts exposed to various

senescence-inducing stimuli showed the same senescent features as nevus cells that were

oncogene-induced senescent cells in vivo (Johmura et al., 2014). Thus, to obtain sHDPCs,

we performed a long-term culture and found that HDPCs whose proliferation ratio was

decreased accumulated SA-β-gal activity and gene expression of aging-related markers.

A previous report showed that dental pulp cells of aged rats showed increased

mineralization and ALP activity compared with those of young rats. (Ma et al., 2009).

However, the mechanism was unclear. Our present study revealed that sHDPCs showed

enhanced expression of odontoblast-related genes compared with yHDPCs, suggesting

that HDPCs might acquire odontoblastic features with aging.

 Several studies demonstrated that the TNF-α concentration in human plasma was

positively associated with advancing age (Michaud et al., 2013; Paolisso et al., 1998a).

Furthermore, a recent study demonstrated that TNF-α promoted the senescence of

nucleus pulposus cells (Li et al., 2017). Interestingly, we found that the gene expression

of TNF-α in sHDPCs was upregulated compared with that in yHDPCs, indicating the

association of TNF-α with aging of HDPCs (Supplementary Fig. 7).

 Several studies had previously investigated the roles of TNF-α in the odontoblastic

differentiation of dental pulp cells, however the results were controversial. For example,

one study showed that TNF-α suppressed mineralization in an odontoblast-like cell line

(Nakayama et al., 2016), whereas another report stated that TNF-α promoted osteogenic differentiation of HDPCs (Paula-Silva et al., 2009). Although the discrepancy between these studies might depend on the PD and differentiation stages of the cells, the mechanism remained unclear. In our study, sHDPCs treated with TNF-α further promoted odontoblastic differentiation whereas TNF-α treatment reduced it in yHDPCs. A recent study revealed that young and old dental pulp cells under low-grade inflammation present similar levels of ALP activity, whereas in irreversible pulpitis, the old pulp cells showed significantly upregulated ALP activity compared with young cells (Aslantas et al., 2016). These data could argue the possible involvement of different signaling pathways in TNF-α-inducing events depending on the cell aging stage, which might cause different cell responses.

We further focused on what contributed to the increased reactivity of sHDPCs against TNF-α, compared with yHDPCs. Our present results demonstrated that HDPCs showed increased TNFR1 expression with increasing PDs and that enhanced TNFR1 expression was involved in TNF-α-induced odontoblastic differentiation of sHDPCs. These findings suggested that the increased expression of TNFR1 may play pivotal roles in promoting odontoblastic differentiation of dental pulp cells of aged people. On the other hand, although the expression of TNFR1 in yHDPCs was lower than that of sHDPCs, TNFR1

was also involved in TNF-α-reduced odontoblastic differentiation in yHDPCS. This paradoxical result suggested the different signaling pathways of TNF-α via TNFR1 between yHDPCs and sHDPCs. A previous study reported that human fibroblasts from aged individuals showed increased levels of NF-κB activation and increased inflammatory gene expression when compared with cells derived from young individuals (Adler et al., 2007). In addition, NF-κB/p65 DNA binding increased with age in several tissues including skin, liver, kidney, cardiac muscle, and gastric mucosa (Helenius et al., 1996b;Xiao and Majumdar, 2000; Helenius et al., 1996a). Thus such pathway might contribute to TNF-α-treated HDPCs.

In conclusion, this study revealed that sHDPCs showed increased odontoblastic features and that TNF-α promoted odontoblastic differentiation of sHDPCs via the increased expression of TNFR1 and its altered signaling pathway that were associated with their senescence.

These results provide further understanding of the detailed mechanism of odontoblastic differentiation with aging and will provide beneficial information for the development of various preservative treatments of dental pulp tissue. Further studies are needed to clarify the age-dependent signaling pathways of dental pulp cells.

Acknowledgments

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Figure legends

Fig. 1. Senescence of cultured human dental pulp cells (HDPCs). **(A)** HDPCs were cultured until reaching a plateau in proliferation and their population doublings (PDs) were determined. HDPCs at PD6 showed active proliferation and HDPCs at PD28 just reached the plateau in proliferation. **(B)** SA-β-gal activity in HDPCs at PD6 and PD28 was examined. Only senescent cells were observed to be blue. Bars: 100 μm. **(C)** Quantitative RT-PCR assays of *p16*, *p21*, and *p53* in HDPCs at PD6 and PD28. Data are shown as mean ± standard deviation (n = 3). *$p < 0.05$, **$p < 0.01$.

Fig. 2. Comparison of the expression of odontoblast-related genes in young human dental pulp cells (HDPCs) and senescent HDPCs. Quantitative RT-PCR results of the expression of odontoblast-related genes *BSP*, *DSPP*, *Nestin*, and *OCN* are shown. Data are shown as mean ± standard deviation (n = 3). **$p < 0.01$.

Fig. 3. Effects of TNF-α on odontoblastic differentiation of human dental pulp cells

(HDPCs.) **(A, D)** Alizarin red S staining of young HDPCs (yHDPCs) **(A)** and senescent

HDPCs (sHDPCs) **(B)** cultured in control culture medium (CM) or differentiation

medium (DM) with or without TNF-α (10 ng/ml) for 7 days. **(B, E)** The graphs show the

quantification of the Alizarin red-S positive areas in yHDPCs **(B)** and sHDPCs **(E)**. **(C,**

F) Quantitative RT-PCR was performed to examine the expression of odontoblast-related

genes *BSP*, *DSPP*, *Nestin*, and *OCN* in yHDPCs **(C)** and sHDPCs **(F)** cultured in CM or

DM with or without TNF-α (10 ng/ml) for 5 days. Data are shown as mean ± standard

deviation (n = 3). *$p < 0.05$, **$p < 0.01$.

Fig. 4. Comparison of mineralization ratio between yHDPCs and sHDPCs. Alizarin red

S positive areas in yHDPCs and sHDPCs cultured in control culture medium (CM) or

differentiation medium (DM) with or without TNF-α for 7 days were determined and

mineralization ratio per cell was calculated. Data are shown as mean ± standard deviation

(n = 3). **$p < 0.01$.

Fig. 5. Contribution of TNFR1 to odontoblastic differentiation of sHDPCs treated with

TNF-α. **(A)** Quantitative RT-PCR of *TNFR1* in yHDPCs and sHDPCs. **(B)** sHDPCs were

transduced with TNFR1 siRNA (siTNFR1) or control siRNA (Cont), and suppression of

TNFR1 was confirmed by quantitative RT-PCR assays. **(C)** Alizarin red S staining of the transduced sHDPCs cultured in control culture medium (CM) or differentiation medium (DM) with or without TNF-α (10 ng/ml) for 9 days. **(D)** The graph shows the quantification of the Alizarin red S positive areas. **(E)** Quantitative RT-PCR of the expression of odontoblast-related genes *BSP*, *DSPP*, *Nestin*, and *OCN* in the transduced sHDPC-3R in CM or DM with or without TNF-α (10 ng/ml) for 5 days. Data are shown as mean ± standard deviation (n = 3). *$p < 0.05$, **$p < 0.01$.

Fig. 6. Contribution of TNFR1 to odontoblastic differentiation of yHDPCs treated with TNF-α. **(A)** yHDPCs were transduced with TNFR1 siRNA (siTNFR1) or control siRNA (Cont), and suppression of *TNFR1* was confirmed by quantitative RT-PCR assays. **(B)** Alizarin red S staining of the transduced yHDPCs cultured in control culture medium (CM) or differentiation medium (DM) with or without TNF-α (10 ng/ml) for 9 days. **(C)** The graph shows the quantification of the Alizarin red S positive areas. **(D)** Quantitative RT-PCR of the expression of odontoblast-related genes *BSP*, *DSPP*, *Nestin*, and *OCN* in the transduced yHDPC-3R in CM or DM with or without TNF-α (10 ng/ml) for 5 days. Data are shown as mean ± standard deviation (n = 3). *$p < 0.05$, **$p < 0.01$.
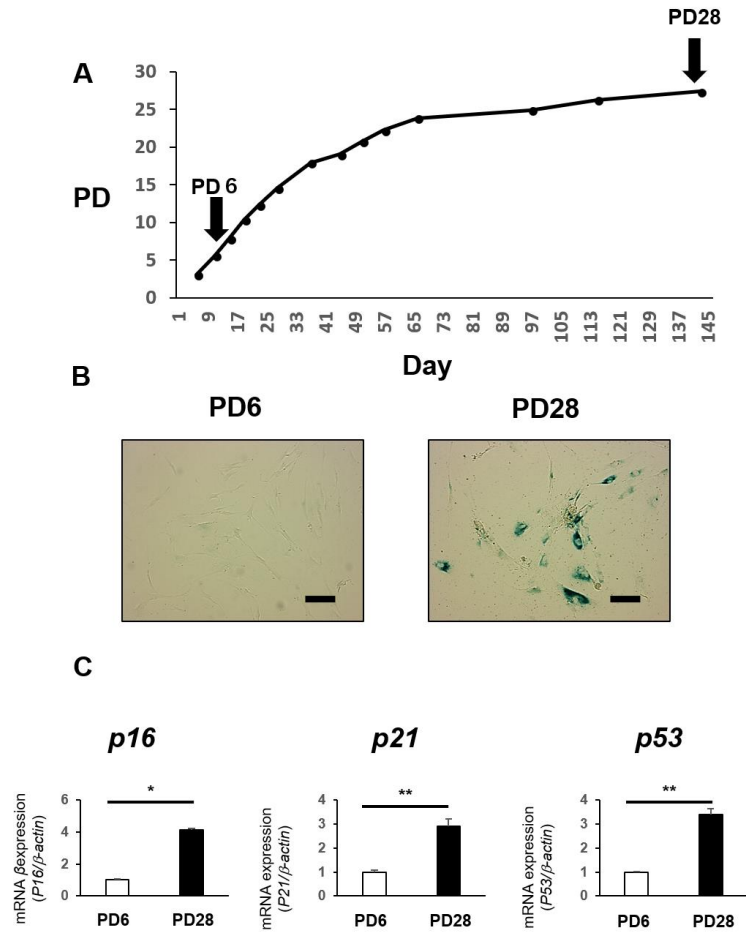
Fig. 1. Senescence of cultured human dental pulp cells (HDPCs). (A) HDPCs were cultured until reaching a plateau in proliferation and their population doublings (PDs) were determined. HDPCs at PD6 showed active proliferation and HDPCs at PD28 just reached the plateau in proliferation. (B) SA-β-gal activity in HDPCs at PD6 and PD28 was examined. Only senescent cells were observed to be blue. Bars: 100 μm. (C) Quantitative RT-PCR assays of *p16*, *p21*, and *p53* in HDPCs at PD6 and PD28. Data are shown as mean ± standard deviation (n = 3). *$p < 0.05$, **$p < 0.01$.

Fig. 2. Comparison of the expression of odontoblast-related genes in young human dental pulp cells (HDPCs) and senescent HDPCs. Quantitative RT-PCR results of the expression of odontoblast-related genes *BSP*, *DSPP*, *Nestin*, and *OCN* are shown. Data are shown as mean ± standard deviation (n = 3). **$p < 0.01$.
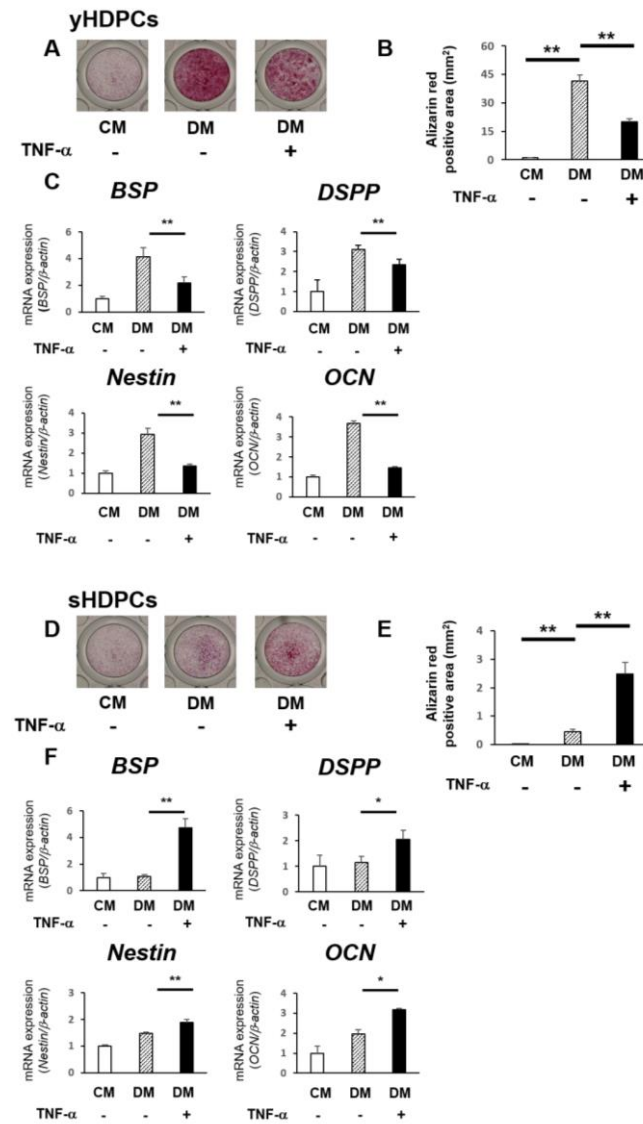
Fig. 3. Effects of TNF-α on odontoblastic differentiation of human dental pulp cells (HDPCs.) **(A, D)** Alizarin red S staining of young HDPCs (yHDPCs) **(A)** and senescent HDPCs (sHDPCs) **(B)** cultured in control culture medium (CM) or differentiation medium (DM) with or without TNF-α (10 ng/ml) for 7 days. **(B, E)** The graphs show the quantification of the Alizarin red-S positive areas in yHDPCs **(B)** and sHDPCs **(E)**. **(C, F)** Quantitative RT-PCR was performed to examine the expression of odontoblast-related genes *BSP*, *DSPP*, *Nestin*, and *OCN* in yHDPCs **(C)** and sHDPCs **(F)** cultured in CM or DM with or without TNF-α (10 ng/ml) for 5 days. Data are shown as mean ± standard deviation (n = 3). *$p < 0.05$, **$p < 0.01$.
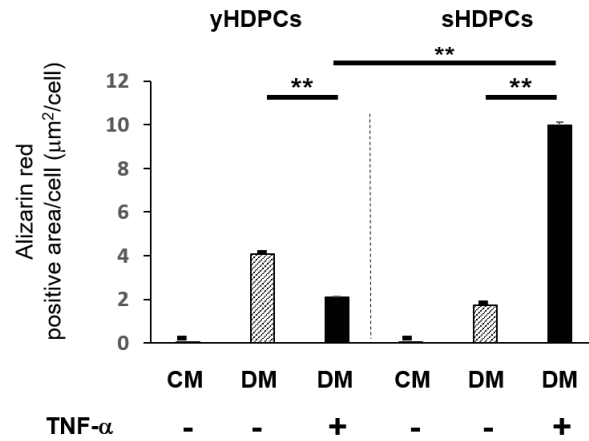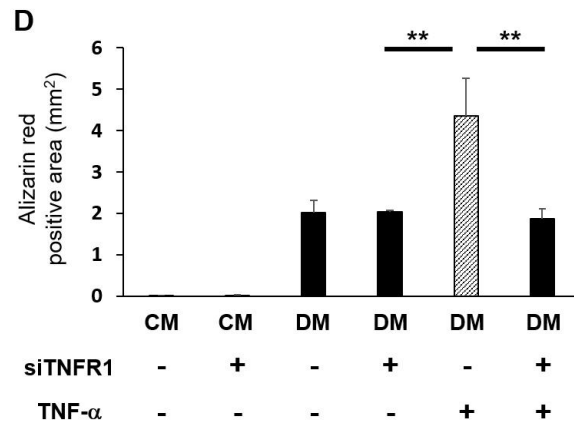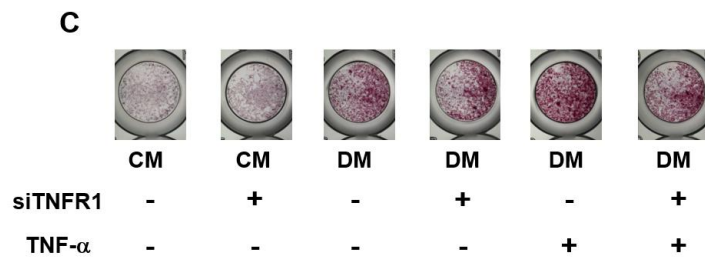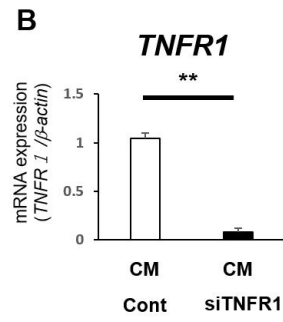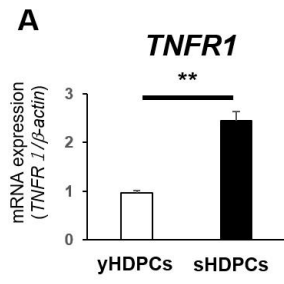
Fig. 4. Comparison of mineralization ratio between yHDPCs and sHDPCs. Alizarin red S positive areas in yHDPCs and sHDPCs cultured in control culture medium (CM) or differentiation medium (DM) with or without TNF-α for 7 days were determined and mineralization ratio per cell was calculated. Data are shown as mean ± standard deviation (n = 3). **$p < 0.01$.

A

TNFR1

mRNA expression
(*TNFR 1 /β-actin*)

**

yHDPCs   sHDPCs

B

TNFR1

mRNA expression
(*TNFR 1 /β-actin*)

**

CM   CM
Cont   siTNFR1

C

	CM	CM	DM	DM	DM	DM
siTNFR1	-	+	-	+	-	+
TNF-α	-	-	-	-	+	+

D

Alizarin red
positive area (mm²)

**   **

	CM	CM	DM	DM	DM	DM
siTNFR1	-	+	-	+	-	+
TNF-α	-	-	-	-	+	+

E
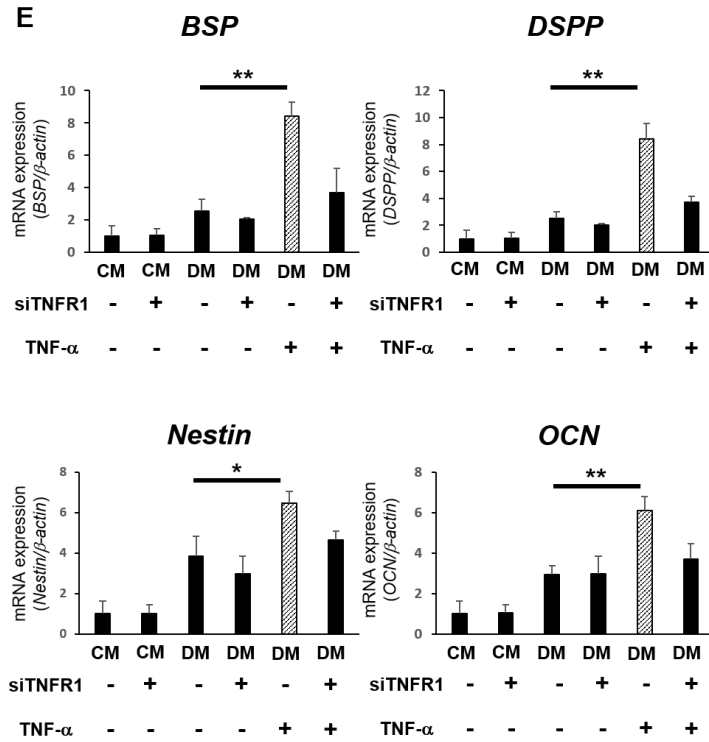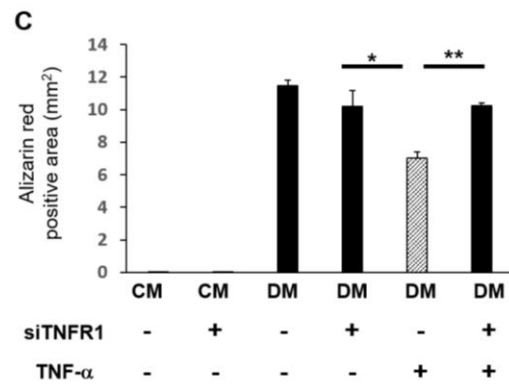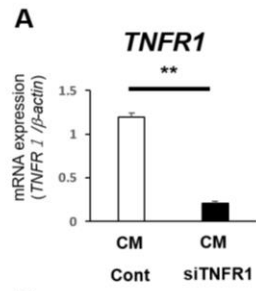
BSP

DSPP

Nestin

OCN

Fig. 5. Contribution of TNFR1 to odontoblastic differentiation of sHDPCs treated with TNF-α. **(A)** Quantitative RT-PCR of *TNFR1* in yHDPCs and sHDPCs. **(B)** sHDPCs were transduced with TNFR1 siRNA (siTNFR1) or control siRNA (Cont), and suppression of *TNFR1* was confirmed by quantitative RT-PCR assays. **(C)** Alizarin red S staining of the transduced sHDPCs cultured in control culture medium (CM) or differentiation medium (DM) with or without TNF-α (10 ng/ml) for 9 days. **(D)** The graph shows the quantification of the Alizarin red S positive areas. **(E)** Quantitative RT-PCR of the expression of odontoblast-related genes *BSP*, *DSPP*, *Nestin*, and *OCN* in the transduced sHDPC-3R in CM or DM with or without TNF-α (10 ng/ml) for 5 days. Data are shown as mean ± standard deviation (n = 3). *$p < 0.05$, **$p < 0.01$.

A

TNFR1

B

	CM	CM	DM	DM	DM	DM
siTNFR1	-	+	-	+	-	+
TNF-α	-	-	-	-	+	+

C

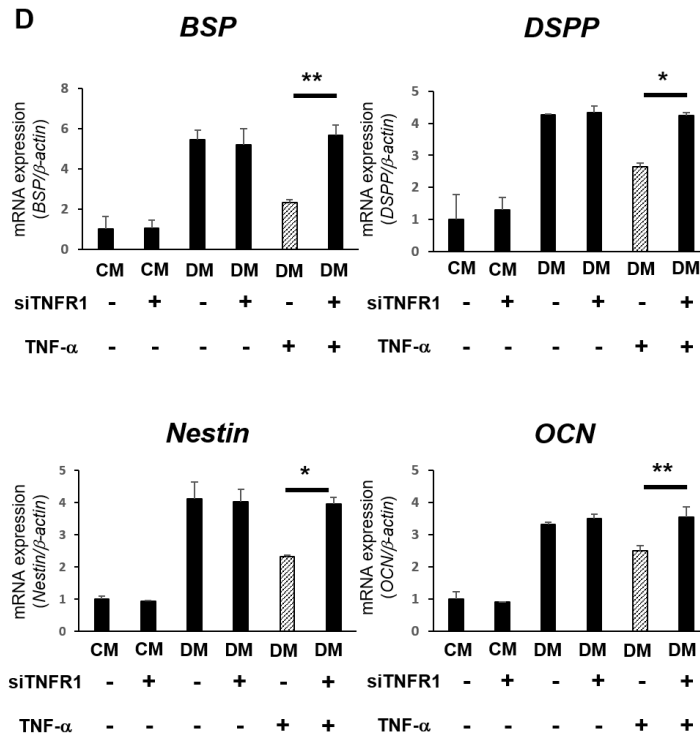|	CM	CM	DM	DM	DM	DM
siTNFR1	-	+	-	+	-	+
TNF-α	-	-	-	-	+	+

Fig. 6. Contribution of TNFR1 to odontoblastic differentiation of yHDPCs treated with TNF-α. **(A)** yHDPCs were transduced with TNFR1 siRNA (siTNFR1) or control siRNA (Cont), and suppression of *TNFR1* was confirmed by quantitative RT-PCR assays. **(B)** Alizarin red S staining of the transduced yHDPCs cultured in control culture medium (CM) or differentiation medium (DM) with or without TNF-α (10 ng/ml) for 9 days. **(C)** The graph shows the quantification of the Alizarin red S positive areas. **(D)** Quantitative RT-PCR of the expression of odontoblast-related genes *BSP*, *DSPP*, *Nestin*, and *OCN* in the transduced yHDPC-3R in CM or DM with or without TNF-α (10 ng/ml) for 5 days. Data are shown as mean ± standard deviation (n = 3). *$p < 0.05$, **$p < 0.01$.

TABLE: Specific primer sequences, annealing temperature, cycle numbers, product sizes, and sequence IDs for quantitative RT-PCR
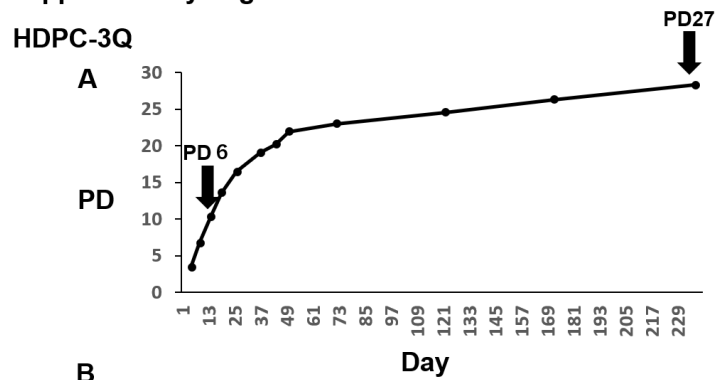
Target gene (abbreviation)	Forward (top) and reverse (bottom) primer sequences	Size of amplified products (bp)	Annealing temperature(℃)	Cycles	Sequence ID
P16	CAACGCACCGAATAGTTACG ACCAGCGTGTCCAGGAAG	171	60	40	XM_011517676.2
P21	GTCTTGTACCCTTGTGCCTC GGCGTTTGGAGTGGTAGAAA	122	60	40	NM_001291549.1
P53	AGGAAATTTGCGTGTGGAGT AGTGGATGGTTGTACAGTCA	107	60	40	NM_001126118.1
BSP	ACTGGTGCCGTTTATGCCTTG CTGGCACAGGGTATACAGGGTTAG	182	60	40	NM_004967.3
DSPP	ATATTGAGGGCTGGAATGGGGA TTTGTGGCTCCAGCATTGTCA	136	60	40	NM_014208.3
OCN	CCCAGGCGCTACCTGTATCAA GGTCAGCCAACTCGTCACAGTC	112	60	40	NM_199173.5
Nestin	TGGCCACGTACAGGACCCTCC AGATCCAAGACGCCGGCCCT	143	60	40	NM_006617.1
TNFR1	ATGCCGGTACTGGTTCTTCC TGCCGAAAGGAAATGGGTCA	90	60	40	NM_001346091.1
TNF-α	CCTGCTGCACTTTGGAGTGA GAGGGTTTGCTACAACATGGG	143	60	40	NM_000594.3
β-actin	ATTGCCGACAGGATGCAGA GAGTACTTGCGCTCAGGAGGA	89	60	40	NM_001101.3

Senescence and odontoblastic differentiation of dental pulp cells
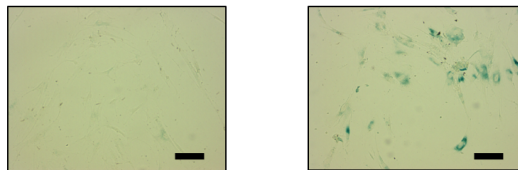
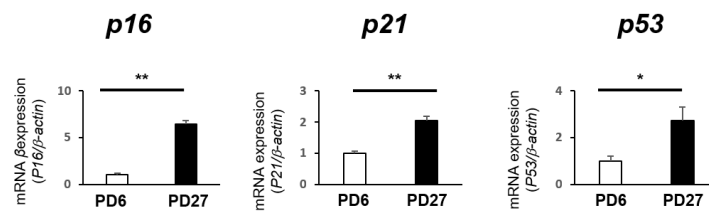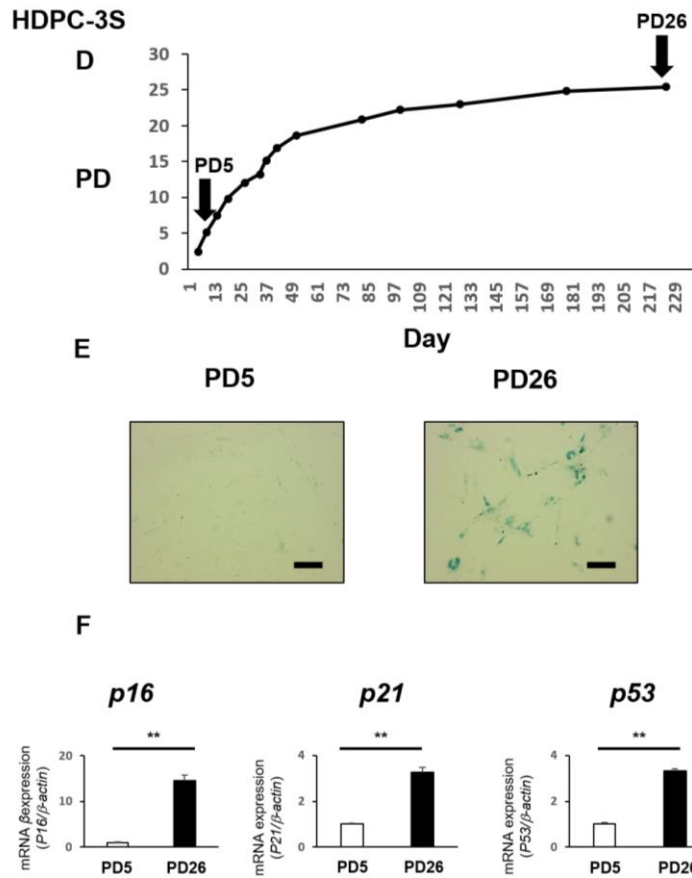Supplementary Fig.1-7

Supplementary Fig. 1

Supplementary Fig. 1

HDPC-3S

**D** (graph, y-axis "PD" 0–30, x-axis "Day" 1–229, with arrows marking PD5 and PD26)

E PD5 PD26

F p16 p21 p53

Supplementary Figure 1. Senescence of cultured human dental pulp cells (HDPCs). **(A, D)** HDPC-3Q **(A)** and HDPC-3S cells **(D)** were cultured until reaching the plateau in proliferation and their population doublings (PDs) were determined. **(B, E)** SA-β-gal activity in HDPC-3Q **(B)** and HDPC-3S cells **(E)** was examined. Only senescent cells were observed to be blue. Bars: 100 µm. **(C, F)** The expression of aging-related genes *p16, p21*, and *p53* in HDPC-3Q **(C)** and HDPC-3S cells **(F)** was examined by quantitative RT-PCR assays. Data are shown as mean ± standard deviation (n = 3). *$p < 0.05$, **$p < 0.01$.
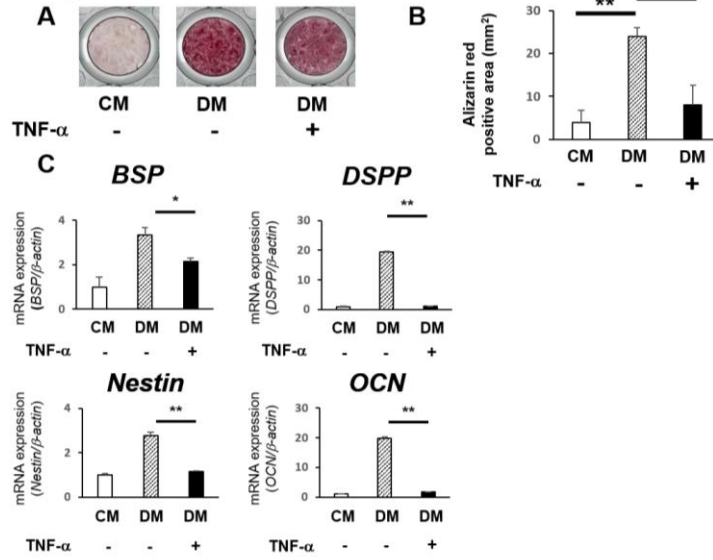
Supplementary Fig. 2

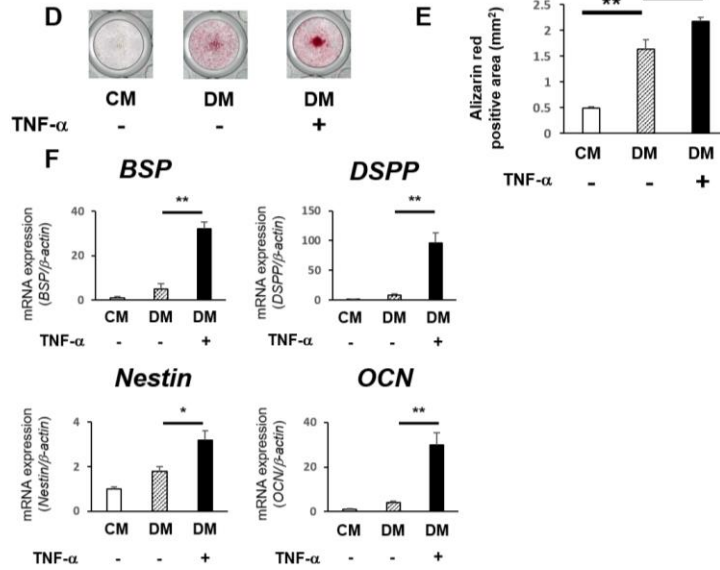**HDPC-3Q**
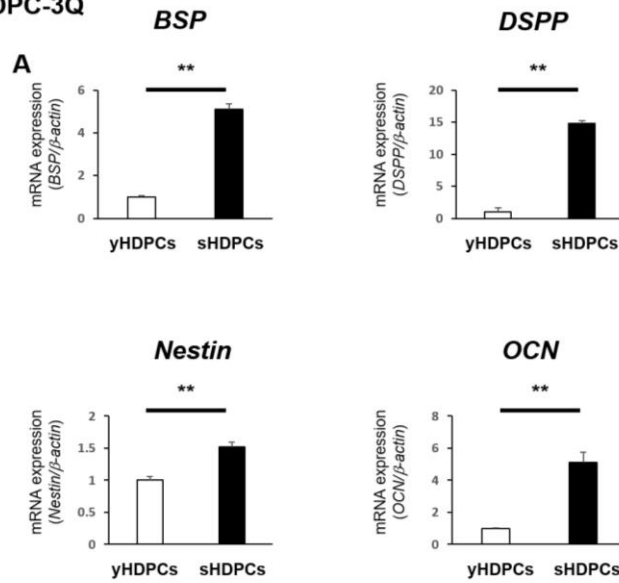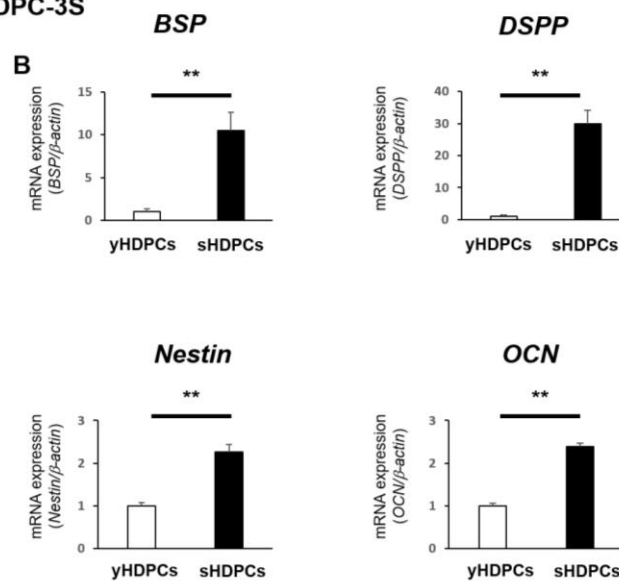
Supplementary Figure 2. Comparison of the expression of odontoblast-related genes in young human dental pulp cells (yHDPCs) and senescent HDPCs (sHDPCs). **(A, B)** Quantitative RT-PCR for the expression of odontoblast-related genes *BSP*, *DSPP*, *Nestin*, and *OCN* in HDPC-3Q **(A)** and HDPC-3S cells **(B)**. Data are shown as mean ± standard deviation (n = 3). **$p < 0.01$.
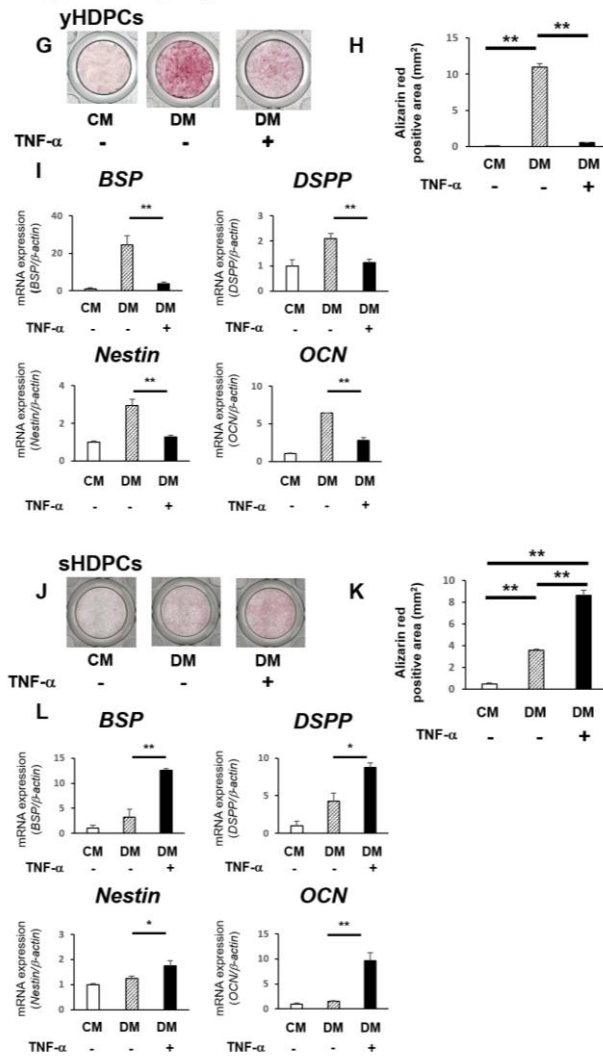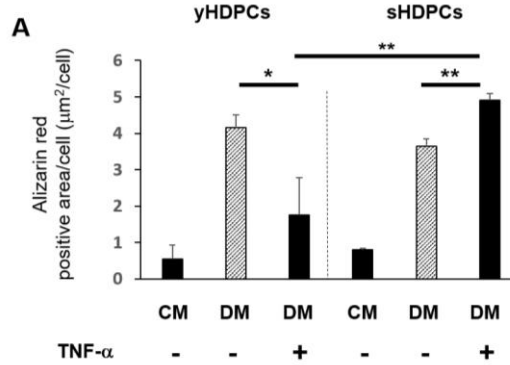
Supplementary Fig. 3 HDPC-3Q

yHDPCs

A

CM   DM   DM

TNF-α   -   -   +

B

Alizarin red positive area (mm²)

CM   DM   DM

TNF-α   -   -   +

C

BSP

mRNA expression (*BSP/β-actin*)

CM   DM   DM

TNF-α   -   -   +

DSPP

mRNA expression (*DSPP/β-actin*)

CM   DM   DM

TNF-α   -   -   +

Nestin

mRNA expression (*Nestin/β-actin*)

CM   DM   DM

TNF-α   -   -   +

OCN

mRNA expression (*OCN/β-actin*)

CM   DM   DM

TNF-α   -   -   +

sHDPCs

D

CM   DM   DM

TNF-α   -   -   +

E

Alizarin red positive area (mm²)

CM   DM   DM

TNF-α   -   -   +

F

BSP

mRNA expression (*BSP/β-actin*)

CM   DM   DM

TNF-α   -   -   +

DSPP

mRNA expression (*DSPP/β-actin*)

CM   DM   DM

TNF-α   -   -   +

Nestin

mRNA expression (*Nestin/β-actin*)

CM   DM   DM

TNF-α   -   -   +

OCN

mRNA expression (*OCN/β-actin*)

CM   DM   DM

TNF-α   -   -   +

Supplementary Figure 3. Effects of TNF-α on odontoblastic differentiation of human dental pulp cells (HDPCs). **(A, D, G, J)** Alizarin red S staining of yHDPC-3Q **(A)**, sHDPC-3Q **(D)**, yHDPC-3S **(G)** and sHDPC-3S cells **(J)** cultured in control culture medium (CM) or differentiation medium (DM) with or without TNF-α (10 ng/ml) for 7 days. **(B, E, H, K)** The graphs show the quantification of the Alizarin red-S positive areas in yHDPC-3Q **(B)**, sHDPC-3Q **(E)**, yHDPC-3S **(H)** and sHDPC-3S cells **(K)**. **(C, F, I, L)** Quantitative RT-PCR for the expression of odontoblast-related genes *BSP*, *DSPP*, *Nestin*, and *OCN* in yHDPC-3Q **(C)**, sHDPC-3Q **(F)**, yHDPC-3S **(I)** and sHDPC-3S cells **(L)** cultured in CM or DM with or without TNF-α (10 ng/ml) for 5 days. Data are shown as mean ± standard deviation (n = 3). *$p < 0.05$, **$p < 0.01$. sHDPC = senescent HDPC; yHDPC = young human dental pulp cell.
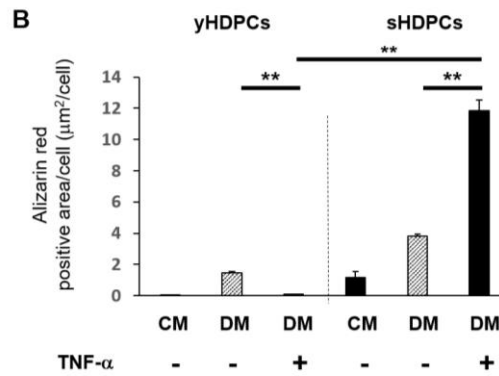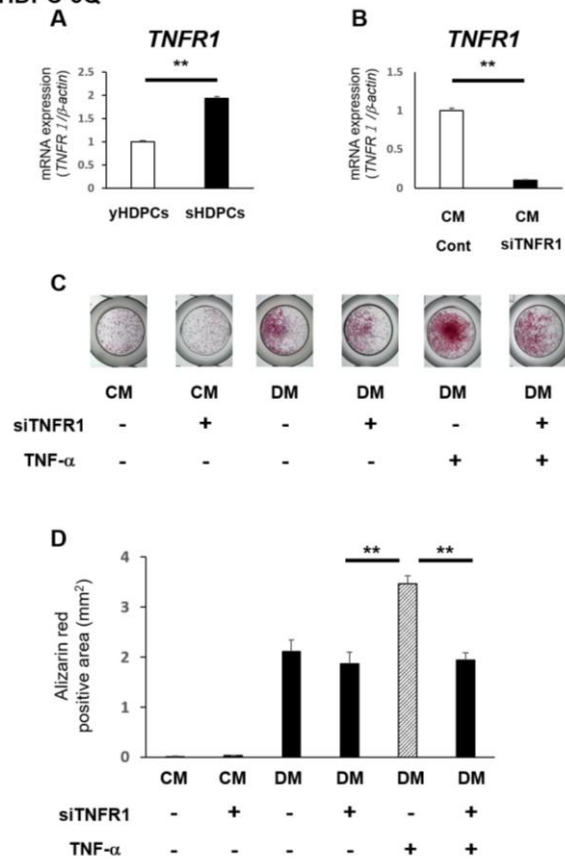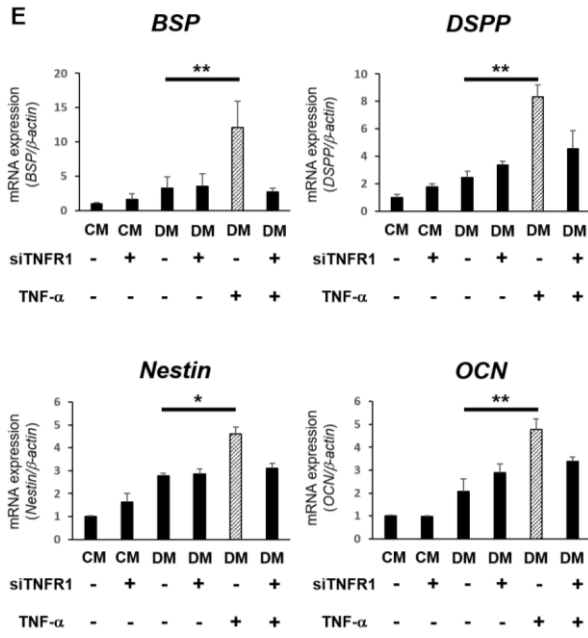
Supplementary Figure 4. Comparison of mineralization ratio between young human dental pulp cells (yHDPCs) and senescent HDPCs (sHDPCs). **(A, B)** Alizarin red S positive areas in yHDPC-3Q **(A)**, sHDPC-3Q **(A)**, yHDPC-3S **(B)** and sHDPC-3S **(B)** cells cultured in control culture medium (CM) or differentiation medium (DM) with or without TNF-α for 7 days were determined and the mineralization ratio per cell was then calculated. Data are shown as mean ± standard deviation (n = 3). *$p < 0.05$, **$p < 0.01$.

Supplementary Fig. 5

HDPC-3Q

A

TNFR1

B

TNFR1

C

	CM	CM	DM	DM	DM	DM
siTNFR1	-	+	-	+	-	+
TNF-α	-	-	-	-	+	+

D

	CM	CM	DM	DM	DM	DM
siTNFR1	-	+	-	+	-	+
TNF-α	-	-	-	-	+	+

E

BSP

DSPP

Nestin

OCN

Supplementary Fig. 5

HDPC-3S

F

TNFR1

**

mRNA expression
(*TNFR 1/β-actin*)

yHDPCs   sHDPCs

G

TNFR1

**

mRNA expression
(*TNFR 1/β-actin*)

CM   CM
Cont   siTNFR1

H

	CM	CM	DM	DM	DM	DM
siTNFR1	-	+	-	+	-	+
TNF-α	-	-	-	-	+	+

I

Alizarin red
positive area (mm²)

**   **

	CM	CM	DM	DM	DM	DM
siTNFR1	-	+	-	+	-	+
TNF-α	-	-	-	-	+	+
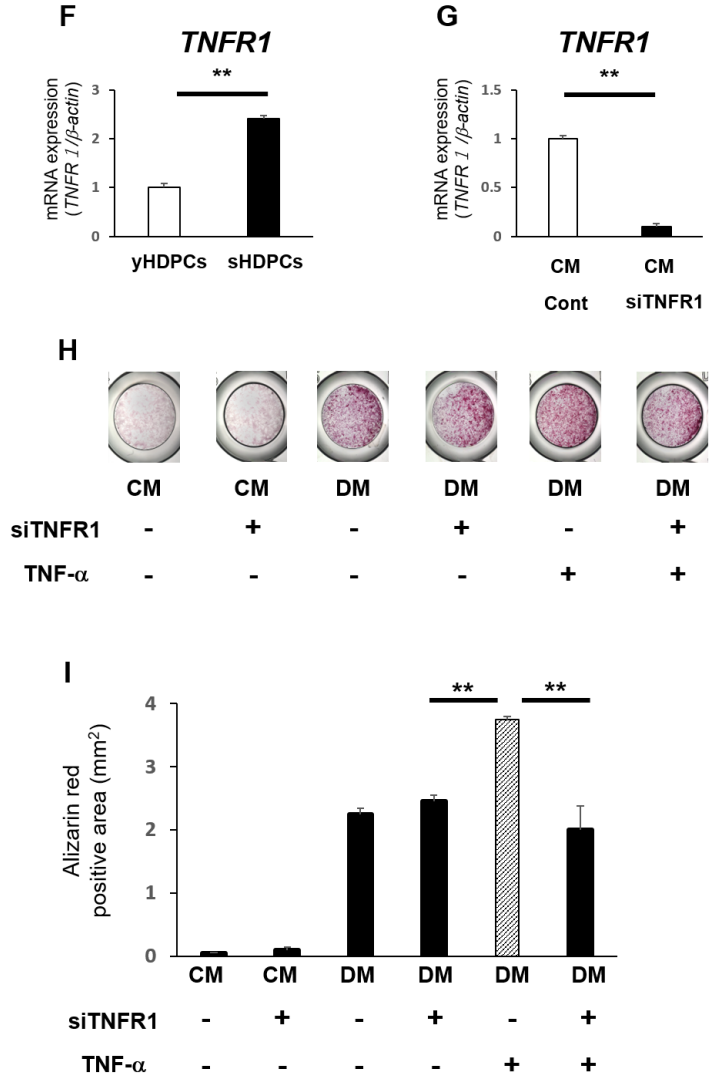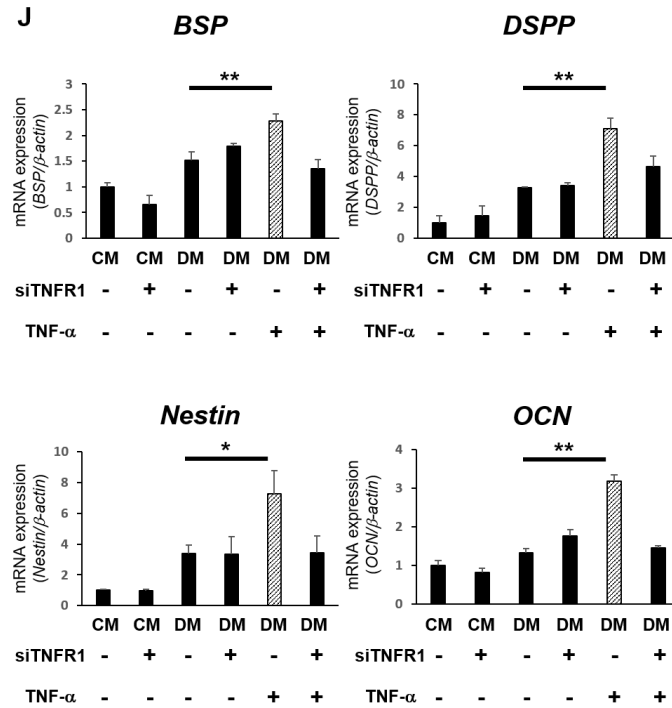
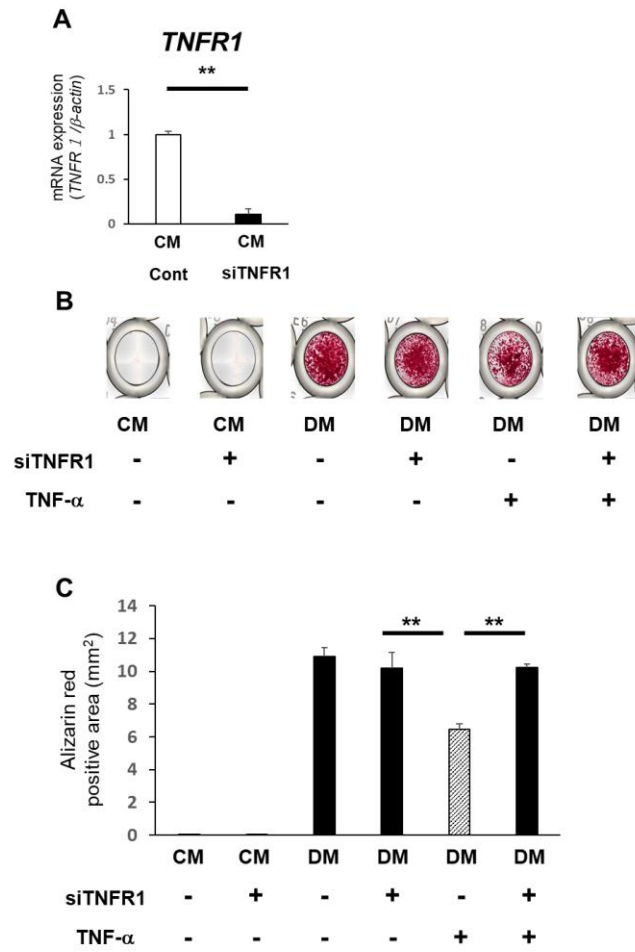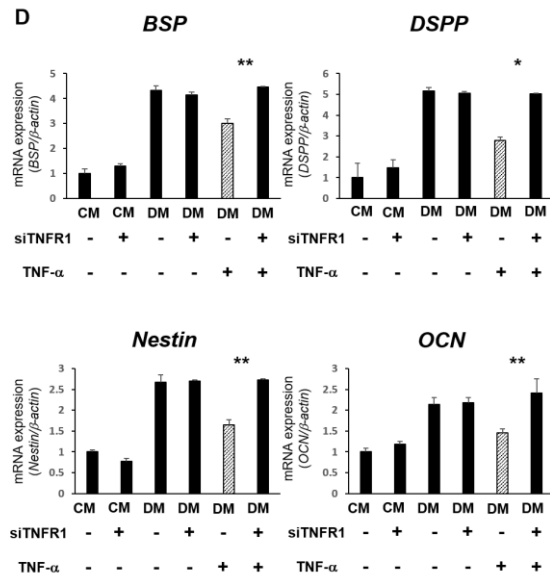**Supplementary Figure 5.** Contribution of TNFR1 to odontoblastic differentiation of sHDPCs treated with TNF-α. **(A, F)** Quantitative RT-PCR for the gene expression of *TNFR1* in yHDPC-3Q **(A)**, sHDPC-3Q **(A)**, yHDPC-3S **(F)** and sHDPC-3S cells **(F)**. **(B, G)** sHDPC-3Q **(B)** and sHDPC-3S **(G)** cells were transduced with TNFR1 siRNA (siTNFR1) or control siRNA (Cont), and suppression of *TNFR1* was confirmed by quantitative RT-PCR assays. **(C, H)** Alizarin red S staining of the transduced sHDPC-3Q **(C)** and sHDPC-3S **(H)** cells cultured in control culture medium (CM) or differentiation medium (DM) with or without TNF-α (10 ng/ml) for 9 days. **(D, I)** The graph shows the quantification of the Alizarin red S positive areas. **(E, J)** Quantitative RT-PCR for the expression of odontoblast-related genes *BSP*, *DSPP*, *Nestin*, and *OCN* in the transduced sHDPC-3Q **(E)** and sHDPC-3S cells **(J)** in CM or DM with or without TNF-α (10 ng/ml) for 5 days. Data are shown as mean ± standard deviation (n = 3). *$p < 0.05$, **$p < 0.01$. sHDPC = senescent HDPC; yHDPC = young human dental pulp cell.
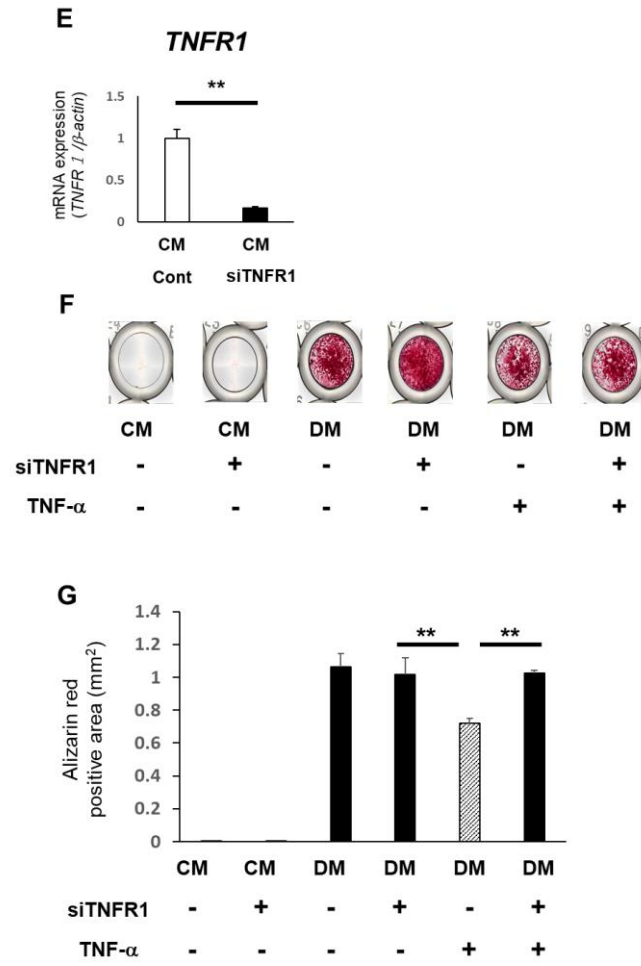
Supplementary Fig. 6   yHDPC-3Q

A

TNFR1

** (significance bar)

mRNA expression
(*TNFR 1 /β-actin*)

1.5

1

0.5

0

CM	CM
Cont	siTNFR1

B

	CM	CM	DM	DM	DM	DM
siTNFR1	-	+	-	+	-	+
TNF-α	-	-	-	-	+	+

C

Alizarin red
positive area (mm²)

14
12
10
8
6
4
2
0

** **

	CM	CM	DM	DM	DM	DM
siTNFR1	-	+	-	+	-	+
TNF-α	-	-	-	-	+	+

D

BSP

DSPP

Nestin

OCN

Supplementary Fig. 6   yHDPC-3S

E

TNFR1

F

	CM	CM	DM	DM	DM	DM
siTNFR1	-	+	-	+	-	+
TNF-α	-	-	-	-	+	+

G

|	CM	CM	DM	DM	DM	DM
siTNFR1	-	+	-	+	-	+
TNF-α	-	-	-	-	+	+
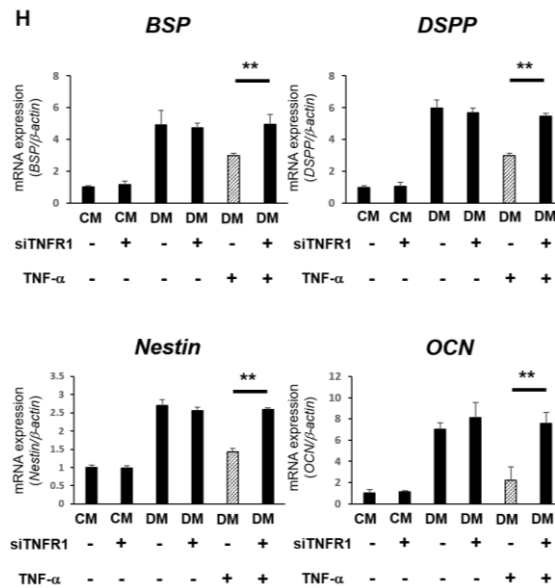
Supplementary Figure 6. Contribution of TNFR1 to odontoblastic differentiation of yHDPCs treated with TNF-α. **(A, E)** yHDPC-3Q **(A)** and sHDPC-3S **(E)** cells were transduced with TNFR1 siRNA (siTNFR1) or control siRNA (Cont), and suppression of *TNFR1* was confirmed by quantitative RT-PCR assays. **(B, F)** Alizarin red S staining of the transduced yHDPC-3Q **(B)** and yHDPC-3S **(F)** cells cultured in control culture medium (CM) or differentiation medium (DM) with or without TNF-α (10 ng/ml) for 9 days. **(C, G)** The graph shows the quantification of the Alizarin red S positive areas. **(D, H)** Quantitative RT-PCR for the expression of odontoblast-related genes *BSP*, *DSPP*, *Nestin*, and *OCN* in the transduced yHDPC-3Q **(D)** and yHDPC-3S cells **(H)** in CM or DM with or without TNF-α (10 ng/ml) for 5 days. Data are shown as mean ± standard deviation (n = 3). *$p < 0.05$, **$p < 0.01$. sHDPC = senescent HDPC; yHDPC = young human dental pulp cell.

Supplementary Fig. 7
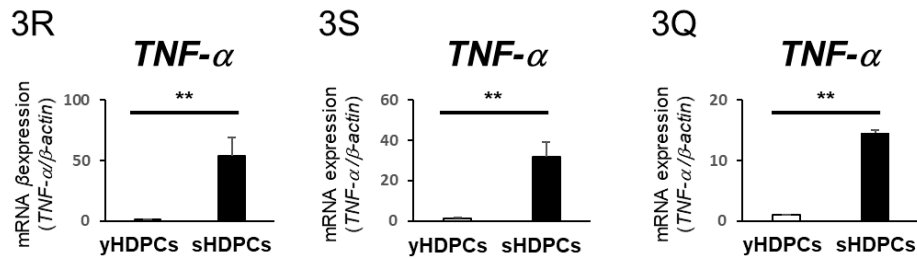
Supplementary Figure 7. Comparison of the expression of TNF-α in young human dental pulp cells (yHDPCs) and senescent HDPCs (sHDPCs). Quantitative RT-PCR for the expression of *TNF-α* in HDPC-3R, HDPC-3Q and HDPC-3S cells. Data are shown as mean ± standard deviation (n = 3). **$p < 0.01$.