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Sclerostin promotes human dental pulp cells senescence

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Background. Senescence-related impairment of proliferation and differentiation limits the use of dental pulp cells for tissue regeneration. Deletion of sclerostin improves the dentinogenesis regeneration, while its role in dental pulp senescence is unclear. We investigated the role of sclerostin in subculture-induced senescence of human dental pulp cells (HDPCs) and in the senescence-related decline of proliferation and odontoblastic differentiation. **Methods.** Immunohistochemical staining and qRT-PCR analyses were performed to examine the expression pattern of sclerostin in young (20- to 30-year-old) and senescent (45- to 80-year-old) dental pulps. HDPCs were serially subcultured until senescence, and the expression of sclerostin was examined by qRT-PCR analysis. HDPCs with sclerostin overexpression and knockdown were constructed to investigate the role of sclerostin in HDPCs senescence and senescence-related impairment of odontoblastic differentiation potential. **Results.** By immunohistochemistry and qRT-PCR, we found a significantly increased expression level of sclerostin in senescent human dental pulp compared with that of young human dental pulp. Additionally, elevated sclerostin expression was found in subculture-induced senescent HDPCs *in vitro*. By sclerostin overexpression and knockdown, we found that sclerostin promoted HDPCs senescence-related decline of proliferation and odontoblastic differentiation potential with increased expression of p16, p53 and p21 and downregulation of the Wnt signaling pathway. **Discussion.** The increased expression of sclerostin is responsible for the decline of proliferation and odontoblastic differentiation potential of HDPCs during cellular senescence. Anti-sclerostin treatment may be beneficial for the maintenance of the proliferation and odontoblastic differentiation potentials of HDPCs.

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9

10 **Abstract**

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Keywords Sclerostin; human dental pulp cell; senescence; p16; p53; p21

Introduction

Dental caries, trauma, abrasion, attrition, erosion and dental treatment lead to tooth tissues destruction, which eventually results in tooth loss. Non-biological treatment strategies for tooth loss, such as bridges, dentures, and implants, may fit poorly or lead to implant rejection (Modino & Sharpe 2005). Dental pulp cells (DPCs), a heterogeneous population of odontoblasts, epithelia, neurocytes, and mesenchymal cells, possess self-renewal and pluripotent differentiation potentials and play a crucial role in maintaining dental pulp homeostasis (Gronthos et al. 2002; Iohara et al. 2004). Engineering tissue regeneration using DPCs as seed cells is a useful strategy to regenerate the dentin-pulp complex, thereby achieving conservation and maintenance of the healthy tooth structure and mechanical strength (Gronthos et al. 2002; Karaoz et al. 2011; Potdar & Jethmalani 2015).

In an aging society, fibrosis, atrophy, loss of cellularity, and degeneration of odontoblasts caused by aging make it more difficult to maintain dental health of older individuals (Nakashima & Iohara 2014). DPC senescence, caused by aging or other cytotoxic factors (e.g., oxidative stress due to oral procedures (Soares et al. 2015) and irradation (Muthna et al. 2010)), is a state of irreversible cellular arrest accompanied by aggregation of intracellular senescence molecules p16 and p53 (Rayess et al. 2012), and changes in secretion of bioactive soluble factors, which lead to the ~~impaired~~ proliferation and differentiation potentials (Iohara et al. 2014). However, little is known about the mechanisms underlying DPC senescence. Previous studies demonstrated that several intracellular factors including p16, p21 and Bmi-1 are involved in the progress of DPC senescence (Choi et al. 2012; Egbuniwe et al. 2011; Mehrazarin et al. 2011). Recently, it was reported that the properties of senescent cells can be reversed by changing the extrinsic microenvironment (Wagner et al. 2008; Nakashima & Iohara 2014). Therefore, identifying specific extrinsic factors involved in DPC senescence is of great importance to biologically based tissue regeneration.

Sclerostin is a 190-amino acid secreted glycoprotein encoded by the *SOST* gene. Its expression is restricted to the great arteries (Zhou et al. 2017), osteocytes (Compton & Lee 2014), chondrocytes (Chan et al. 2011), and cementocytes (Bao et al. 2013). The deletion or down-regulation of sclerostin causes high bone mass diseases such as sclerosteosis (van Lierop et al.

2011) and Van Buchem disease (Loots et al. 2005). As a well-known negative regulator of bone formation (Zhang et al. 2016), sclerostin inhibits the proliferation and differentiation of cementoblasts (Bao et al. 2013) and osteoprogenitor cells including osteosarcoma cells (Zou et al. 2017) and human mesenchymal stem cells (Sutherland et al. 2004). Recently, significantly elevated serum sclerostin was shown in eldly (Modder et al. 2011; Roforth et al. 2014), suggesting a possible role of sclerostin in aging-related bone loss as a result of decreased regenerative potential caused by the accumulation of senescent cells (Farr et al. 2017). In addition, previous studies demonstrated that the increased sclerostin produced by osteoclasts from aged mice led to reduced bone formation (Ota et al. 2013), and anti-sclerostin treatment increased bone mass in aged rats (Li et al. 2009). Moreover, the activator of Sirt1, a critical regulator of aging and longevity (Satoh et al. 2013), rescued ovariectomy-induced bone loss by decreasing sclerostin expression (Artsi et al. 2014). Therefore, it was speculated that sclerostin might impact cellular senescence in terms of differentiation and proliferation.

Similar to the higher level of sclerostin in aged individuals, the expression level of sclerostin varies in embryonic and adult mouse incisors and molars (Naka & Yokose 2011), indicating a possible role of sclerostin in DPC senescence. Additionally, Collignon *et al*. found that sclerostin deficiency increased reparative dentinogenesis in mice (Collignon et al. 2017), implying anti-sclerostin might reverse the deccreased regenerative potential of aged dental pulp. Taken

81 together, we hypothesized a possible correlation between sclerostin and DPC senescence.
82 Therefore, the purpose of our study was to elucidate the role of sclerostin in the process of
83 human dental pulp cell (HDPC) senescence as well as aging-related impairment of HDPC
84 proliferation and odontoblastic differentiation.

85 **Materials and Methods**

86 This work was carried out in accordance with The Code of Ethics of the World Medical
87 Association (Declaration of Helsinki). The protocols and procedures were reviewed and
88 approved by the Ethical Committee of the School and Hospital of Stomatology, Wuhan
89 University, China.

90 **Human dental pulp collection**

91 Healthy and fresh human premolars were extracted from 20- to 80-year-old patients who were
92 under orthodontic or periodontal treatment in the Hospital of Stomatology, Wuhan University.
93 All donors gave their informed consent. The teeth were divided into two groups: the young group
94 contained 30 teeth from 20- to 30-year-old patients and the old group contained 20 teeth from
95 45- to 80-year-old patients.

96 Ten teeth from each group were used for immunohistochemical analysis, and ten teeth from each
97 group for qRT-PCR analysis. The remaining 10 teeth from the young group were used for HDPC
98 culture.

Immunohistochemical analysis

Teeth were fixed with 4% paraformaldehyde at room temperature and decalcified in 10% EDTA solution for more than 6 months. Teeth were cut into 5-μm-thick serial sections, which were collected on poly-L-lysine-coated slides. For immunohistochemical staining, the sections were dewaxed and incubated with rabbit anti-sclerostin (1:200; Abclonal, Boston, USA) at 4°C overnight. The staining was performed using a biotin-streptavidin kit (ZhongShan Biotech, Beijing, China) before the sections were counterstained with hematoxylin.

Real-time PCR analysis

Total RNA was isolated using the RNAiso kit (Takara, Japan). First-strand cDNA syntheses were performed by using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara). Real-time polymerase chain reaction for sclerostin, p16, p53, p21, alkaline phosphatase (ALP), osteocalcin (OCN), osteocalcin (OPN), dentin sialophosphoprotein (DSPP) and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was performed with the SYBR Green Kit (Takara) using a Applied BioSystems 7900HT thermocycler (Applied Biosystems, CA. USA). The primers are listed in *Table 1*. The relative amount or fold change of the target gene was normalized relative to the level of human GAPDH and the control groups.

Cells and cell culture

HDPCs were isolated from the healthy dental pulp of 10 premolars from young group. The dental pulp tissues were digested in a solution containing 4 mg/mL dispase and 3 mg/mL collagenase type I for 1 hour at 37°C and then cultured in α-modified essential medium (α-MEM; HyClone Laboratories, Inc, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, NY, USA) at 37°C under 5% CO_2. Confluent monolayers were dissociated with 0.5% (w/v) trypsin-EDTA for subculture. HDPCs were serially subcultured until the cells spontaneously arrested their replication. For odontoblastic induction, the medium was changed to the odontoblastic induction medium, containing α-MEM, 5% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 10 mmol/L β-glycerophosphate, 50 μg/mL ascorbic acid, and 10 nmol/L dexamethasone (Sigma-Aldrich Co, MO, USA). Cultures were maintained with a medium change every 3 days.

Lentivirus packaging and cell infection

Full human *SOST* cds sequence and a human *SOST* sh-RNA were cloned and inserted into the PCDH-CMV-MCS-EF1-copGFP vector (GenePharma, China) and the GV298 vector (Genechem, China), respectively. Lentiviral particles were produced using three-plasmid systems, including pMD2.G, psPAX2 and the individual vectors, with NEOFECT™ DNA transfection reagent (Neofect, China) according to the manufacturer's instruction. For infection, HDPCs were incubated with lentiviral particles and polybrene (4 μg/mL) in complete medium for 12 hours. Cells with successful infection by pCDH-human-SOST were designated HDPC/SOST, cells

infected by sh-SOST were designated HDPC/sh-SOST, and control cells infected by empty vector were designated HDPC/PCDH and HDPC/sh-Ctrl, respectively. The expression of sclerostin was quantified by real-time PCR and Western blot analysis. The mRNA and protein expression levels were significantly upregulated in HDPC/SOST (P <0.001, Fig. 1E and F), while they were knocked down by over 85% in HDPC/sh-SOST 48 hours after infection (P <.0001, Fig. 1G and H).

Western blot

Cells were lysed in M-PER Mammalian Protein Extraction Reagent (Thermo, IL, USA) combined with a cocktail of protease inhibitors (Roche Molecular Biochemicals, Rotkreuz, Switzerland). Total lysate was loaded and separated by SDS/PAGE. Primary antibodies specific for sclerostin (1:1000, Abclonal), phosphor-β-catenin (p-β-catenin) (1:1000, Cell Signaling Technology, MA, USA), and β-actin (1:5000, Santa Cruz, CA, USA) were used.

Senescence-associated β-galactosidase assay (SA-β-Gal)

To determine senescence of HDPCs, an SA-β-Gal kit (Beyotime, China) was used according to the manufacturer's instructions. In brief, cells seeded on slips were fixed with paraformaldehyde and incubated with SA-β-Gal overnight. Senescent HDPCs were identified by blue-staining under standard light microscopy.

Cell proliferation assay

152 HDPCs were seeded at a density of 2×10^3 cells/well in a 96-well plate and cultured for 24 hours,
153 and then the medium was replaced with fresh medium. On days 1, 2, 3, and 4, the density of
154 viable cells within each well was quantified with the Cell Counting Kit-8 (CCK-8; Dojindo,
155 Japan) according to the manufacturer's protocols. The absorbance at 450 nm was measured to
156 calculate the number of vitable cells in each well. A well with medium and CCK-8 solution but
157 without cells was used as the baseline.

158 **Alkaline phosphatase staining and ALP activity assay**

159 ALP staining was performed according to the manufacturer's instructions (Beyotime). For ALP
160 activity, cells were lysed with 0.1% Triton X-100, and 50 μL lysate was mixed with 100μL p-
161 nitrophenyl phosphate (4mg/mL). The mixture was incubated at 37 °C for 15–20 minutes. The
162 reaction was stopped by the addition of 0.5 N NaOH (100 μL) and read spectrophotometrically at
163 405 nm. The protein concentration of the lysate was determined as described in the
164 manufacturer's instructions of the Pierce BCA protein assay kit (Thermo). The enzyme activity
165 was quantified by a p-nitrophenol standard curve and normalized by protein concentration.

166 **Alizred red staining**

167 Cells were cultured in 12-well cell culture dishes in the odontoblastic induction medium for 14
168 days. Then, cells were fixed with 4% paraformaldehyde and stained with 2% alizarin red. The

169 stain was desorbed with 10% cetylpyridinium chloride for 1 hour and the absorbance was
170 examined at 562 nm.

171 **Statistical analysis**

172 All experiments were repeated at least three times. Quantitative results are expressed as the mean
173 ± standard deviation. Data were analyzed by t-test and one-way analyses of variance using SPSS
174 19.0 software (SPSS Inc, IL, USA). *P* values <0.05 were considered statistically significant.

175 **Results**

176 **Expression of sclerostin is increased in senescent human dental pulp and subculture-**
177 **induced senescent HDPCs**

178 The immunohistochemistry assay showed that the expression of sclerostin in young human pulp
179 was very low, and positive sclerostin staining was only found in odontoblasts of young dental
180 pulp. In contrast, strong staining of sclerostin was observed throughout the senescent human
181 dental pulp, especially in the odontoblasts lining near the pre-dentin (Figure 1A and B). Similarly,
182 qRT-PCR analyses showed a higher expression level of sclerostin mRNA in senescent dental
183 pulp, along with increased expression levels of p16, p53, and p21 (Figure 1C).

184 Rapidly proliferating HDPCs were serially subcultured until the cells spontaneously arrested
185 replication. HDPCs completing 16 and 54 population doublings (PDs) were defined as young
186 and senescent HDPCs, respectively (Mehrazarin et al. 2011). In line with the higher expression

187 levels of sclerostin, p16, p53, and p21 in senescent dental pulp, qRT-PCR analyses showed
188 significantly higher expression levels of sclerostin, p16, p53, and p21 in senescent HDPCs
189 (Figure 1D).

190 **Sclerostin induces HDPCs senescence**

191 To access the role of sclerostin in senescence of HDPCs, sclerostin was overexpressed in early-
192 passaged HDPCs. As shown in Figure 2, sclerostin overexpression significantly increased the
193 number of SA-β-Gal-positive cells. In addition, the CCK-8 assay showed significantly decreased
194 proliferation and higher expression levels of p16, p53, and p21 in sclerostin-overexpressing
195 HDPCs (Figure 2C and D). In contrast, knockdown of sclerostin in late-passaged HDPCs
196 showed fewer SA-β-Gal-positive cells, increased proliferation, and decreased p16, p53 and p21
197 expressions (Figure 2E–H). In conclusion, these results suggest that sclerostin induces HDPCs
198 senescence and inhibits HDPCs proliferation via the p16 and p53 signaling pathways.

199 **Sclerostin inhibits odontoblastic differentiation of HDPCs**

200 To determine the effect of sclerostin on HDPC differentiation, we compared the odontoblastic
201 differentiation ability between early-passaged HDPC/SOST and HDPC/PCDH. As shown in
202 Figure 3, sclerostin overexpression significantly decreased the odontoblastic differentiation of
203 early-passaged HDPCs as shown by the decreased mineralization nodules formation and a
204 decline in ALP activity along with lower expression levels of odontoblastic differentiation

markers such as ALP, OPN, CON, and DSPP (Figure 3A-C). Moreover, knockdown of sclerostin rescued the decreased odontoblastic differentiation of senescent HDPCs (Figure 3D–E). These data imply that the increased expression of sclerostin contributed to the impaired odontoblastic differentiation potential of senescent HDPCs.

The Wnt/*β*-catenin pathway may be involved in the progress of HDPCs senescence related to higher expression of sclerostin

To clarify the mechanisms underlying sclerostin-related HDPCs senescence, we examined the Wnt/*β*-catenin pathway activity. Western blot analysis showed that overexpression of sclerostin suppressed the activity of the Wnt/*β*-catenin pathway by increasing the expression of p-*β*-catenin and sclerostin knockdown significantly increased the activity of the Wnt/*β*-catenin pathway (Figure 4). These results suggest that sclerostin might accelerate senescence of HDPCs in a Wnt signaling pathway-dependent mechanism.

Discussion

The importance of dental pulp cellsin diverse therapeutic application has been increasingly recognized in recent years. However, the impairment of proliferation and differentiation caused by cellular senescence restricts their application in tissue regeneration. So far, little is known about the progress of HDPC senescence. Herein, by sclerotin overexpression in early-passaged HDPCs and knockdown in late-passaged HDPCs, we showed that sclerostin knockdown is beneficial for the maintenance of the proliferation and odontoblastic differentiation potentials of HDPCs during cellular senescence.

In the present study, we found that there was a significantly higher expression of sclerostin in senescent human dental pulp tissues and senescent HDPCs. These data are consistent with the findings that serum sclerostin levels increase with age, which may contribute to age related bone loss (Modder et al. 2011; Zhang et al. 2016). While noteworthy, these results do not provide causality. Therefore, SA-β-Gal staining, a biomarker of cellular senescence (Itahana et al. 2007), was performed in early-passaged HDPCs with sclerostin overexpression and in late-passaged HDPCs with sclerostin knockdown to determine whether the higher expression of sclerostin was the cause of HDPC senescence or not. Sclerostin overexpression accelerated HDPC senescence and sclerostin knockdown decreased senescence of HDPCs *in vitro*. To the best of our knowledge, it was the first study confirming the exact role of sclerostin in cellular senescence. Well-designed studies are now required to determine the role of sclerostin in HDPC senescence *in vivo* and to identify whether this promoting effect of sclerostin on HDPC senescence is universal in other cells or is cell-type specific.

Senescence of cells is mostly due to activation of G1/S cell cycle arrest proteins (Itahana et al. 2004). The p53/p21 and p16/retinoblastoma axes are two important pathways in cellular senescence. The p16 protein mediates cell cycle arrest by inhibiting DNA replication via preventing phosphorylation of the retinoblastoma protein (Rayess et al. 2012). The p53-mediated response to DNA damage, oxidants and hypoxia induces the expression of p21, leading to cellular senescence by inhibiting the activity of cyclin dependent kinases (Muthna et al. 2010; Tonnessen-Murray et al. 2017). Our study showed that senescent human dental pulp and subculture-induced senescent HDPCs exhibited higher expression levels of p16, p53 and p21. These data are in accordance with previous findings that p16, p53 and p21 are highly expressed in senescent dental pulp cells (Mas-Bargues et al. 2017; Muthna et al. 2010). Conversely,

downregulation of p53-p21 decreased senescence of dental pulp stem cells (Choi et al. 2012). Meanwhile, p16 knockdown significantly reduced senescence-related dysfunction of dental pulp mesenchymal stem cells (Feng et al. 2014). Inspired by the similar expression profile of p16, p53, p21 and sclerostin during HDPCs senescence, we examined the effect of sclerostin on the expression of p16, p53 and p21 in HDPCs. Sclerostin overexpression significantly increased the expression levels of p16, p53 and p21, whereas, sclerostin knockdown decreased the expression levels of p16, p53 and p21. Although, there had previously been no reports about the effect of sclerostin on the expression of p16, p53 and p21, it was believed that senescence acted as a tumor suppressor and sclerostin silence significantly increased the proliferation of osteosarcoma cells by promoting the progress of cell cycle in G1/S phase (Zou et al. 2017). Thus, one can conclude that sclerostin may modulate the progression of HDPC senescence via both the p16 and p53 pathways.

Odontoblastic differentiation potential was impaired in senescent HDPCs with decreased expression levels of odontoblastic differentiation markers ALP, OCN, OPN and DSPP, which play key roles in matrix formation and calcification initialization in bone and teeth (Bae et al. 2015; Chen et al. 2005; Kuratate et al. 2008; Ma et al. 2009; Min et al. 2010). In this study, we found that sclerostin significantly inhibited odontoblastic differentiation of early-passaged HDPCs with downregulation of odontoblastic markers ALP, OCN, OPN, and DSPP. Furthermore, knockdown of sclerostin increased odontoblastic differentiation of subculture-induced senescent HDPCs with higher expression levels of these odontoblastic differentiation markers. These results are in line with the finding that mice dental pulp cells with sclerostin deficiency exhibited enhanced *in vitro* mineralization (Mehrazarin et al. 2011). These results

indicate that the increased expression of sclerostin was responsible for impairment of odontoblastic differentiation potential in senescent HDPCs.

It was reported that sclerostin efficiently inhibited Wnt signaling by interrupting the Wnt-Frizzled-LRP5/6 receptor complex *via* binding with the Wnt co-receptor LRP5/6 (Semenov et al. 2005), leading to upregulated the expression of p-*β*-catenin, thereby preventing the nucleus translocation of stabilized *β*-catenin and thus downregulating downstream genes expression (Bae et al. 2015). Wnt signaling plays an essential role in age-related changes in stem cells. Previous studies found that inhibiting Wnt signaling initiated senescence of glioblastoma cells and human WI38 fibroblasts (Lambiv et al. 2011; Ye et al. 2007). Furthermore, Wnt1, an agonist of Wnt signaling, rescued the impaired neurogenic differentiation potential of aged dental pulp stem cells (Feng et al. 2013). In this study, sclerostin overexpression attenuated the activity of Wnt signaling and sclerostin knockdown activated Wnt signaling. These results indicate that sclerostin might accelerate senescence of HDPCs, in part, by decreasing Wnt signaling. However, the exact mechanism of sclerostin-related HDPC senescence requires further study to elucidate.

Conclusion

Taken together, the higher expression of sclerostin might accelerate HDPC senescence and was responsible for the attenuated proliferative and odontoblastic differentiation potentials in senescent HDPCs via p16 and p53 pathways. Sclerostin may serve as a target to delay the progress of HDPCs senescence.

Acknowledgements

We thank M.D.S. Chufang Liao (School and Hospital of Stomatology, Wuhan University) and M.D.S. Yun Wu (School and Hospital of Stomatology, Wuhan University) for HDPC preparation and for their advice on our work.

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Figure 1

Expressions of sclerostin in senescent dental pulp, subculture-induced senescent HDPCs and lentiviral infected HDPCs.

Immunohistochemical staining for (A) sclerostin in young and (B) senescent dental pulp (green arrows point to odontoblasts and red arrows point to dental pulp cells), scale bar = 20μm; (C) qRT-PCR analyses of the expression levels of sclerostin, p16, p53, and p21 in young and senescent dental pulp; (D) qRT-PCR analyses of sclerostin, p16, p53, and p21 expression levels in subculture-induced senescent HDPCs; (E) qRT-PCR and (F) Western blot analyses of sclerostin expression in sclerostin-overexpressing HDPCs; (G) qRT-PCR and (H) Western blot analyses of sclerostin expression in sclerostin knockdown HDPCs. (** p <0.01; *** P <0.001)

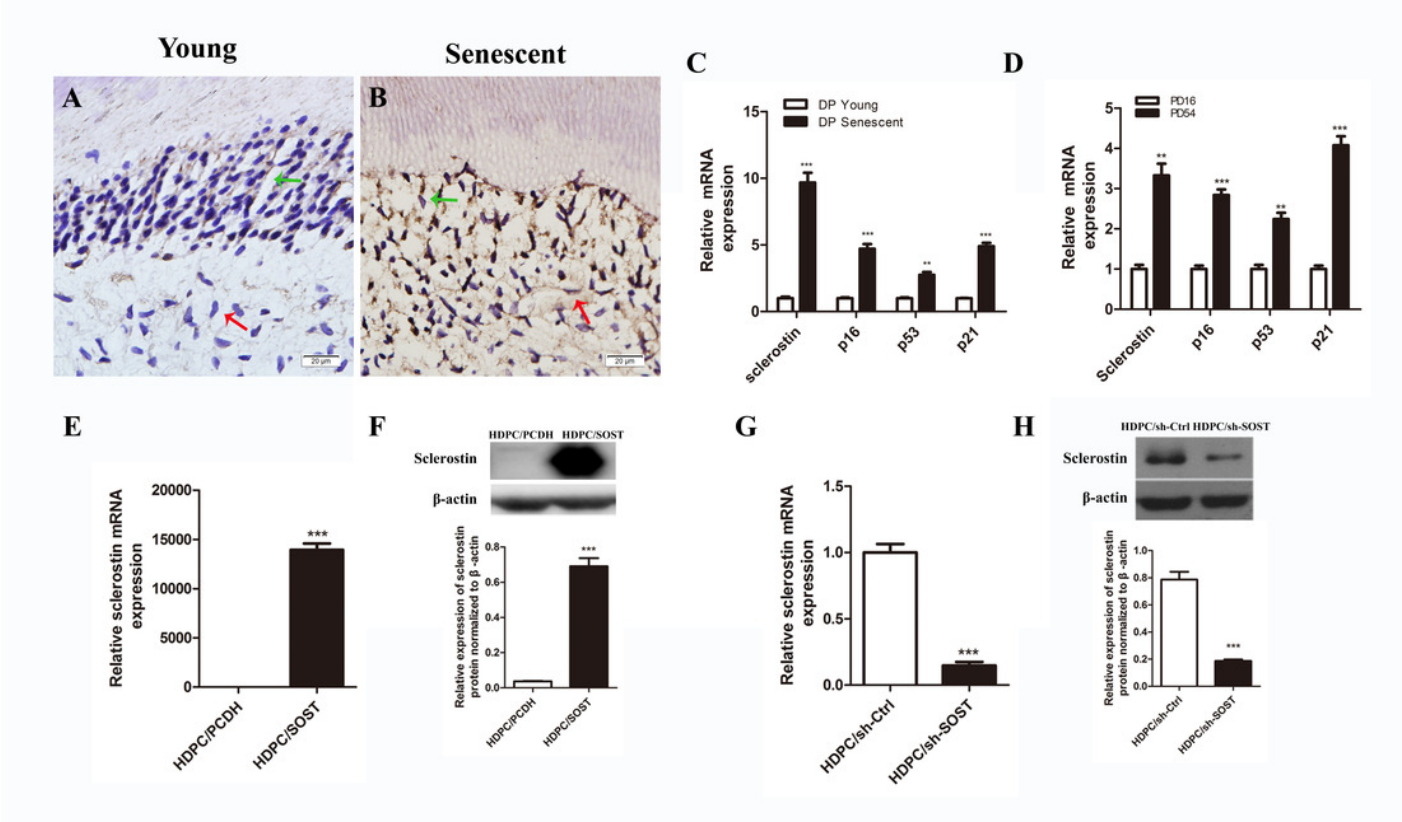

Figure 2

Effects of sclerostin overexpression and knockdown on senescence and proliferation of HDPCs.

(A) SA-β-Gal staining, (B) cell proliferation activity and (C) qRT-PCR analysis of p16, p53, and p21 in HDPCs with sclerostin overexpression; (D) SA-β-Gal staining, (E) cell proliferation activity, and (F) qRT-PCR analysis of p16, p53, and p21 in HDPCs with sclerostin knockdown. (*p <0.05, ** p <0.01; *** P <0.001)

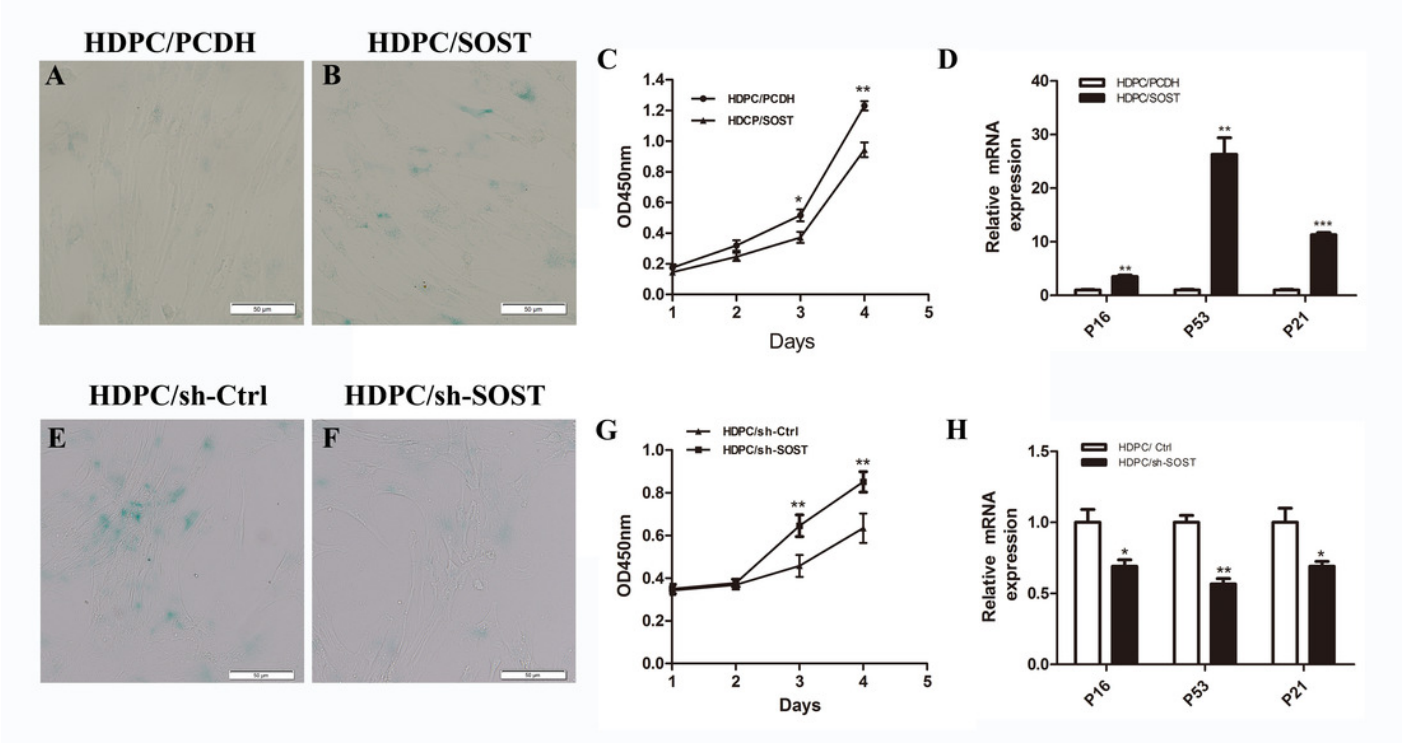

Figure 3

Effects of sclerostin on odontoblastic differentiation of HDPCs.

(A) ALP staining and ALP activity, (B) alizarin red staining for mineral nodule formation, and (C) qRT-PCR analysis of odontoblastic markers in early-passaged HDPCs with sclerostin overexpression; (D) ALP staining and ALP activity, (E) alizarin red staining for mineral nodule formation, and (F) qRT-PCR analysis of odontoblastic markers in late-passaged HDPCs with sclerostin knockdown. (*p <0.05, ** p <0.01; *** P <0.001)

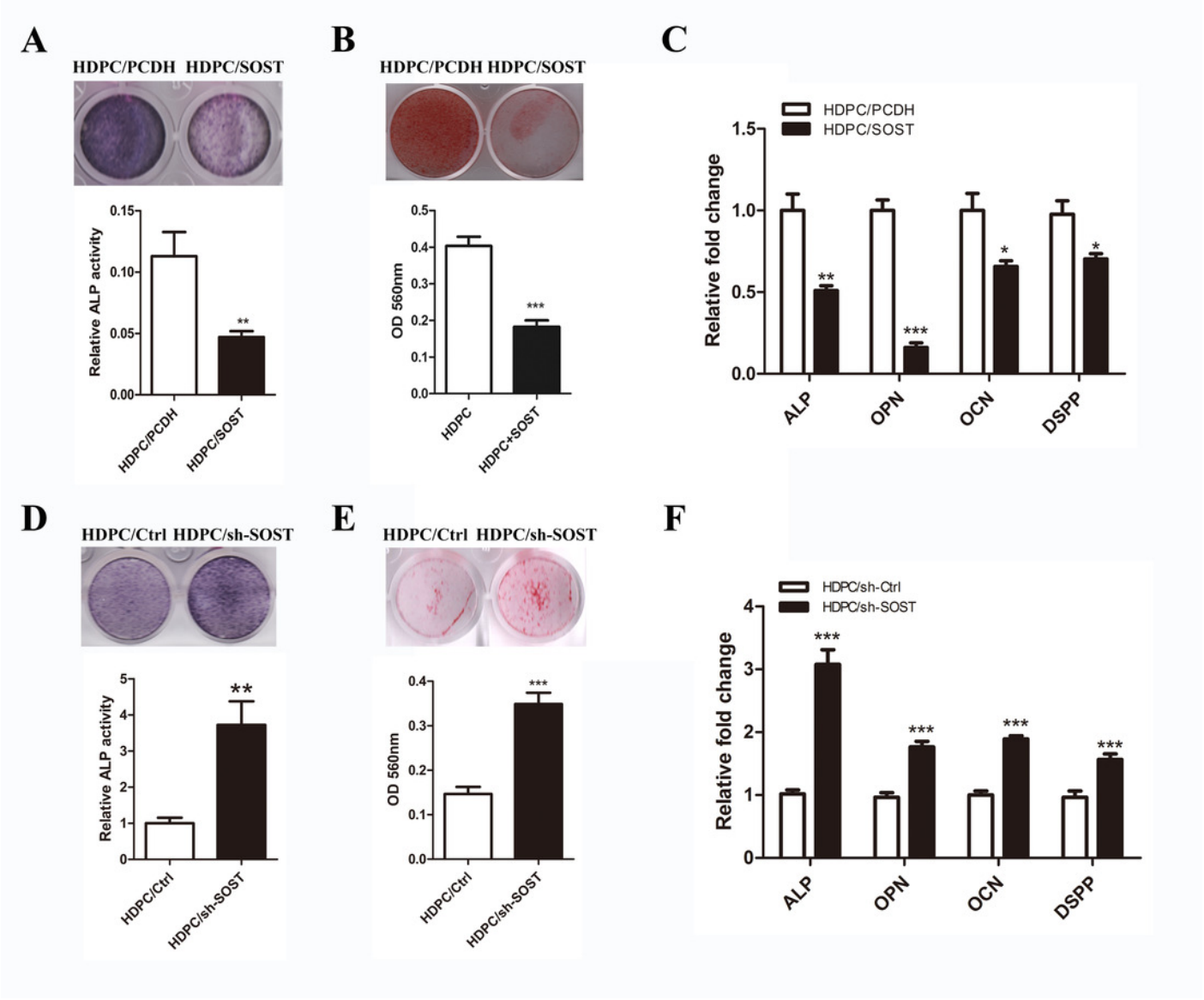

Figure 4

Effect of sclerostin on Wnt/*β*-catenin pathway.

Western blot analysis of p- *β*-catenin expression in (A) early-passaged HDPCs with sclerostin overexpression (B) late-passaged HDPCs with sclerostin knock down. (*p <0.05, ** p <0.01)

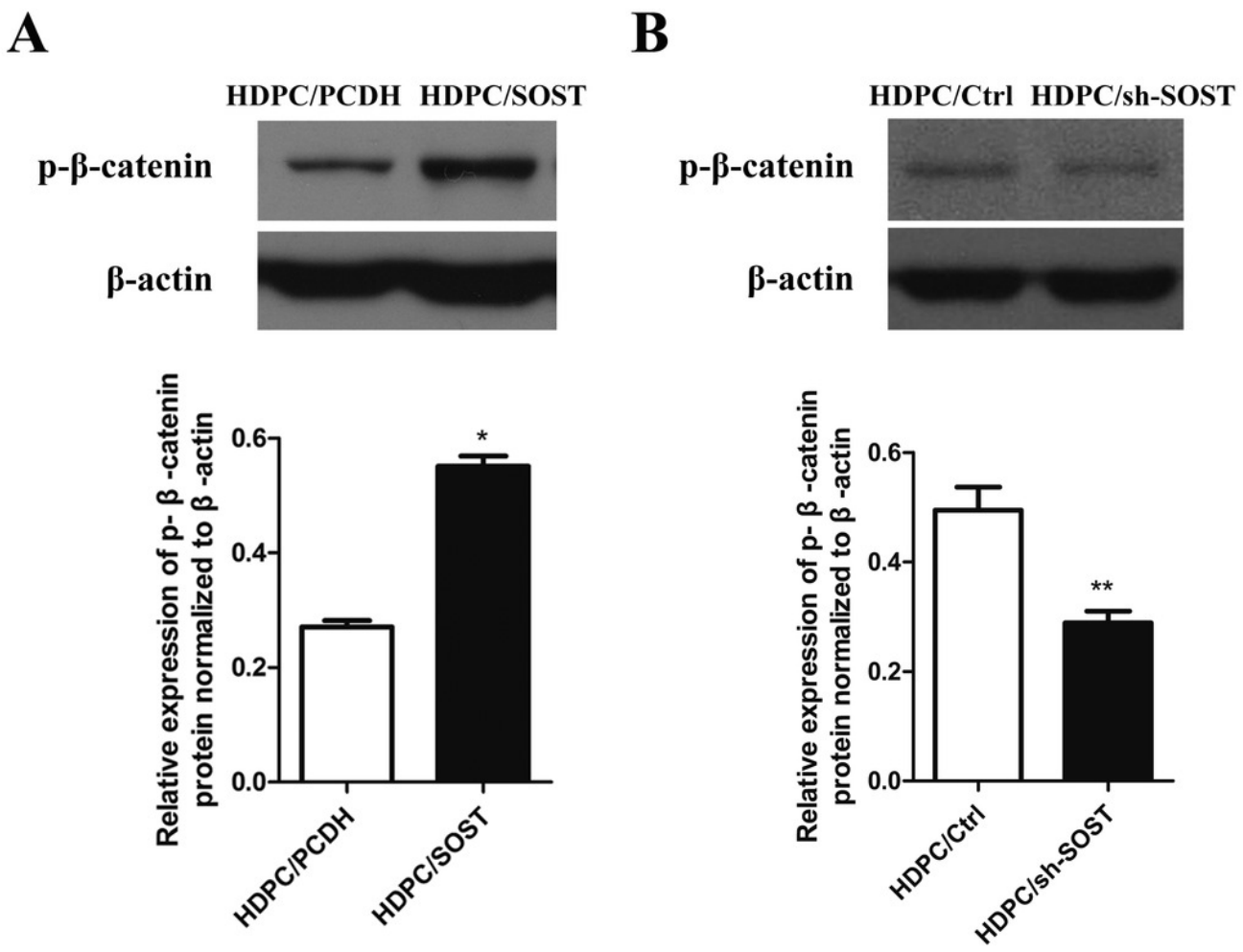

Table 1(on next page)

Primer Sequences Used for Real-time PCR.

1 Table 1 Primer Sequences Used for Real-time PCR

Genes	Forward(5'-3')	Reverse(5'-3')
GAPDH	AACAGCGACACCCACTCCTC	CATACCAGGAAATGAGCTTGACAA
ALP	CGAGATACAAGCACTCCCACTTC	CTGTTCAGCTCGTACTGCATGTC
OPN	GCCGAGGTGATAGTGTGGTT	CAACGGGGATGGCCTTGTAT
OCN	GGTGCAGCCTTTGTGTCCAA	CCTGAAAGCCGATGTGGTCA
DSPP	CAACCATAGAGAAAGCAAACGCG	TTTCTGTTGCCACTGCTGGGAC
Sclerostin	TGGCAGGCGTTCAAGAATGA	GCCCGGTTCATGGTCTTGTT
P16	CCCAACGCACCGAATAGTTAC	CAGCAGCTCCGCCACTC
P53	ACCTATGGAAACTACTTCCTGAAA	CTGGCATTCTGGGAGCTTCA
P21	TCAGGGTCGAAAACGGCG	CCTCTTGGAGAAGATCAGCCG

2