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Impaired odontogenic differentiation of senescent dental mesenchymal stem cells is associated with loss of Bmi-1 expression

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Abstract

Introduction—Dental mesenchymal stem cells (dMSCs) may differentiate into odontoblast-like cells and form mineralized nodules. In the current study, we investigated the effects of senescence on odontogenic differentiation of dMSCs.

Methods—dMSCs were serially subcultured until senescence. Telomere lengths and telomerase activities were determined by quantitative PCR. Expression of genes involved in cell proliferation and differentiation, *e.g.*, Bmi-1, p16^{INK4A}, osteocalcin (OC), dentin sialoprotein (DSP), bone sialoprotein (BSP), and dentin matrix protein-1 (DMP-1) were assayed by Western blotting and quantitative reverse transcription PCR. Exogenous Bmi-1 was expressed in dMSC using retroviral vectors. Odontogenic differentiation was assayed by alkaline phosphatase (ALP) activity.

Results—Subculture-induced replicative senescence of dMSCs led to reduced expression of Bmi-1, OC, DSPP, and BSP compared with rapidly proliferating cells, while p16^{INK4A} level increased. The cells exhibited progressive loss of telomeric DNA during subculture, presumably due to lack of telomerase activity. Bmi-1 transduction did not affect proliferation of cells, but enhanced the expression of OC and DSPP in the late passage cultures. Bmi-1-transduced cells also demonstrated enhanced ALP activity and mineralized nodule formation.

Conclusions—These results indicate that dMSCs lose their odontogenic differentiation potential during senescence, in part, by reduced Bmi-1 expression.

Keywords

Dental pulp stem cell; senescence; cellular aging; odontogenic differentiation; Bmi-1

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The authors deny any conflict of interest.

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INTRODUCTION

Mesenchymal stem cells derived from dental tissues, designated as dMSCs, include dental pulp stem cells (DPSC) isolated from permanent dental pulp tissue, stem cells of exfoliated deciduous teeth (SHED), stem cells of apical papilla (SCAP), and periodontal ligament stem cells (PDLSC) (1–4). These dMSCs share in common their capacity to differentiate into multiple cell types (5). For this reason, recent studies have investigated the potential use of dMSC for tissue engineering purposes. Because dMSCs are readily available in postnatal tissues, these cells provide unique opportunity for regenerative therapies.

Stem cells' self-renewal capacity is determined in part by Bmi-1, a polycomb-group (PcG) protein required for transcriptional repression of its target genes through chromatin remodeling (6,7). Bone marrow reconstitution was impaired in Bmi-1 knockout mice, and there was no detectable self-renewal of adult hematopoietic stem cells (8). Neuronal stem cells (NSC) from Bmi-1 knockout mice showed impaired regeneration, while neural progenitor cell proliferation occurred normally (9). There seems to be a correlation between the regenerative effects of Bmi-1 and p16^{INK4A} repression. The self-renewal defect of NSC in Bmi-1 knockout mice was partially rescued by p16^{INK4A} inactivation (9). Thus, Bmi-1 may play a role in maintaining the differentiation and regenerative capacities of stem cells, including dMSCs.

The purpose of the current study was to investigate the role of Bmi-1 in maintaining the odontogenic differentiation capacity in dMSCs during normal replication of these cells in serial subculture, which leads to replicative senescence or "aging." We found that aging dMSCs lose their replication and odontogenic differentiation potentials, along with loss of Bmi-1 expression. When Bmi-1 level was enhanced by retroviral gene transduction, it partially rescued the dMSCs' differentiation capacity. These findings reveal mechanistic insights underlying the reduced differentiation potential of dMSCs associated with subculture-induced senescence.

MATERIALS AND METHODS

Cells and Cell Culture Methods

DPSC and SCAP were cultured in α -MEM medium (Invitrogen, Carlsbad, CA) supplemented with 10% Fetal Bovine Serum (FBS) (Invitrogen), 5 μ g/ml gentamicin sulfate (Gemini Bio-Products, West Sacramento, CA, USA), and 20 mM L-glutamine (Invitrogen). These cells were obtained at the Center for the Craniofacial Molecular Biology, USC School of Dentistry (Los Angeles, CA), as described previously (1), under approval of appropriate institutional review board. Calcifying condition was induced with 100 μ M L-ascorbic acid 2-phosphate (Sigma, St. Louis, MO), 9 mM KH₂PO₄, and 10 mM β -glycerophosphate, and 9.8 nM dexamethazone (Sigma). Primary normal human keratinocytes (NHK) and normal human fibroblasts (NHF) were cultured as previously described (10). Senescence-associated β -galactosidase (SA β -Gal) activity was measured according to method described earlier (10). Cultured cells were infected with retroviral vectors expressing full-length Bmi-1 (RV-Bmi-1) or the empty vector (RV-B0) as described previously (11).

Western Blotting

Whole cell extracts (WCE) were isolated from the cultured cells, fractionated by SDS-PAGE and transferred to Immobilon membrane (Millipore, Billerica, MA, USA). Antibodies against Bmi-1 (Upstate, Charlottesville, VA), p16^{INK4A} (Santa Cruz Biotechnology, Santa Cruz, CA), ALP (Santa Cruz) and β -actin (Santa Cruz) were used. Chemiluminescence signal was detected using the HyGLO Chemiluminescent HRP antibody detection reagent (Denville Scientific, South Plainfield, NJ).

Quantitative reverse transcription (RT)-qPCR

Total RNA was extracted using RNeasy Plus Mini kit (Qiagen, Valencia, CA). RT was performed with 5 µg RNA using the method described elsewhere (10). qPCR was performed for the relative mRNA expression of Bmi-1, p16^{INK4A}, OC, DSPP, BSP, and DMP-1 using LightCycler 480 (Roche, Basel, Switzerland). The primer sequences and the PCR conditions will be available upon request.

Quantitative measurement of telomeric DNA and telomerase activity

Genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen). DNA was quantified in triplicate using a NanoDrop spectrophotometer (Biolabs). Relative telomere length was determined by using the approach as previously described (12). We also assayed for telomerase activity in cells using the quantitative telomeric repeat amplification protocol (Q-TRAP) assay (13).

Assays of ALP activity and biomineralization

Seven days after cells reached confluence, ALP activity was measured at 405 nm and plotted against a predetermined standard. Also, the cells were stained for ALP activity using the ALP Staining Kit (Sigma). Also, the cells were exposed to the calcifying condition for 28 days and stained with Alizarin Red staining at pH 4.2. Relative Alizarin Red staining density was quantified by destaining in 10% acetylpyridinium chloride (Sigma) and measured at 562 nm.

RESULTS

Rapidly proliferating cultures of DPSC and SCAP were serially subcultured until the cells spontaneously arrested their replication, and their replication kinetics were documented. DPSC and SCAP demonstrated exponential replication for approximately 50 days in culture, completing ~ 40 PDs, then reached the maximum of 64 and 46 PDs, respectively (Fig. 1A). Both cultures demonstrated progressive shortening of telomere DNA during *in vitro* replication (Fig. 1B). When we checked the telomerase enzyme activity, which can synthesize telomeres (14), we found that the dMSCs essentially lacked the enzyme activity (Fig. 1C). This is in contrast to SCC4, an oral cancer cell line which demonstrates high telomerase activity (Fig. 1C). Thus, dMSCs undergo subculture induced senescence through telomere shortening due to lack of telomerase activity.

Replicative senescence occurs with reduced Bmi-1 expression and accumulation of p16^{INK4A} in cultured fibroblasts (6,15). This was also observed in subcultured DPSC, which showed accumulation of p16^{INK4A} and reduced Bmi-1 protein level at higher PDs (Fig. 1D). A similar pattern of *bmi-1* and *Ink4A* gene expression was noted at the mRNA level (Fig. 1E). Senescent cells exhibited flattened and enlarged morphology, while the exponentially replicating cells maintained the spindle shape, typical of mesenchymal cells (Fig. 1F). *In situ* staining for SA β-Gal activity, a marker of senescence (10), revealed accumulation of positively stained cells in senescent culture.

To investigate the effect of senescence on dMSC's odontogenic potential, mRNA expression of OC, DSPP, and BSP were determined by RT-qPCR. Expression levels of these odontogenic markers decreased in cells with higher PDs (Fig. 2A). Next, we assessed the effect of senescence on odontogenic differentiation. DPSC exhibited reduced ALP activity at PD 48 compared with that of PD 13, and this was paralleled by the loss of ALP protein level in the late passage DPSC (Fig. 2B). *In situ* staining for the ALP activity showed almost complete absence of staining in the late passage culture (Fig. 2D). Similarly, late passage

culture of SCAP at PD 44 exhibited loss of ALP activity and the protein expression (Fig. 2C and 2D). These data indicate that dMSCs lose their odontogenic potential during senescence.

We tested whether reduced Bmi-1 level in senescent dMSCs was linked with impaired odontogenic differentiation. Rapidly proliferating DPSC and SCAP were infected with RV-Bmi-1 or RV-B0 (empty vector) and maintained in serial subculture. We examined the effect of Bmi-1 transduction on the odontogenic differentiation and mineralization capacities of cells. DPSC/B0 and DPSC/Bmi-1 were exposed to calcifying condition for seven days. ALP activity and the protein level increased in the calcifying condition and was further enhanced in DPSC/Bmi-1 compared to that of DPSC/B0 (Fig. 3A). Alizarin Red staining revealed mineralization only in cells cultured in the calcifying condition and notable increase of staining in DPSC/Bmi-1 compared with DPSC/B0 (Fig. 3B). Quantification of the Alizarin Red staining confirmed the enhanced mineralization by Bmi-1 transduction in DPSC. ALP activity was also compared between SCAP/B0 and SCAP/Bmi-1 cells, and similar induction of ALP was noted by Bmi-1 transduction in SCAP (Fig. 3C). We then compared the mRNA expression levels of OC, DSPP, BSP, and DMP-1 in DPSC/B0 and DPSC/Bmi-1 at early and late passages (Fig. 3D). Although both DPSC cultures expressed comparable levels of these genes at PD 10, at a later passage (PD 27–28) Bmi-1 transduction significantly enhanced the expression of OC and DSPP. Therefore, the above data indicate that Bmi-1 has positive effect on odontogenic differentiation of dMSCs.

DISCUSSION

We report detailed analyses of altered phenotype in dMSCs during *in vitro* replication. dMSCs underwent spontaneous senescence after serial subculture, as determined by morphological changes, expression of SA β -Gal, and the loss of Bmi-1 expression accompanied with p16^{INK4A} accumulation. This finding is consistent with previous reports showing reduced number of odontoblasts in aged dental pulp of rats and human (16,17). Senescence of dMSCs presumably occurs through the loss of telomeric sequences. Interestingly, DPSC exhibited longer telomere DNA than did SCAP, suggesting different replication and odontogenic differentiation capacities. Compared with SCAP, DPSC underwent approximately 30% more PDs until senescence and demonstrated stronger ALP staining (Fig. 1A and 2D). However, both DPSC and SCAP presumably have similar characteristics in their differentiation and self-renew because they are both derived from the same embryonic source (1,3). Our data also support the findings of Shi *et al.* (18), which showed enhanced mineralization capacity of bone marrow MSCs upon expression of telomerase.

Previous studies showed that MSCs derived from non-dental tissues undergo limited replication through senescence and lose their differentiation capacities. Wagner *et al.* (19) showed that bone marrow MSCs senesced after 7–12 passages and lose their adipogenic differentiation potential. The same study reported increased osteogenic differentiation in senescent bone marrow MSCs. This finding appears to contradict our data, in which senescent dMSCs exhibited diminished odontogenic differentiation. It is possible that MSCs derived from varying tissues may have different propensity of differentiation during senescence. Alternatively, Alizarin Red staining of senescent MSCs may detect non-specific calcification, not involving the regulated expression of the mineralized tissue matrix proteins. Similar reduction of OC mRNA expression was noted in human dental pulp isolated from aged donors (20). Another study showed reduced proliferation and ALP activity in human dental pulp cells of aged donors compared with those of the younger ones (21). One of the clinical features of aged dental pulp is dystrophic calcification that results in eventual obliteration of pulp cavity (22). This process may be distinguished from reparative

dentin formation resulting from physiologic defense mechanism residing in healthy and responsive pulp.

Bmi-1 overexpression has been shown to extend the lifespan of NHK (11) but not in fibroblasts (23). In the current study, Bmi-1 showed no effects on proliferation of dMSCs (data not shown), suggesting that such an effect is cell type-specific. Our data suggest that dMSCs undergo telomere length-dependent senescence, as does fibroblasts (10). Thus, the differential effects of Bmi-1 on cell proliferation may depend on the mechanism underlying senescence in each cell type. Although Bmi-1 transduction did not increase cell proliferation, it is very likely that Bmi-1 is necessary for continued proliferation of cells because the senescent cells accumulate p16^{INK4A}. Thus, increased p16^{INK4A} level in senescent dMSC may be a result of reduced Bmi-1 expression. Also, knockdown of Bmi-1 in younger dMSC culture would lead to premature senescence and loss of odontogenic differentiation potential. This notion needs to be validated experimentally, although there are feasibility issues associated with lack of cell growth after Bmi-1 knockdown. Bmi-1 enhanced the odontogenic differentiation and the mineralization capacities in DPSC. Consistent with this finding, Bmi-1 transduction in DPSC maintained the levels of Notch-1, DMP-1, and DSP proteins, which were diminished in senescent DPSC or the control cells. These data suggest that dMSCs lose their proliferation differentiation capacities during senescence, in part, due to reduced Bmi-1 expression.

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Figure 1. DPSC undergo limited lifespan and replicative senescence upon serial subculture (A) Replication kinetics of DPSC and SCAP were determined and plotted against time in culture. DPSC reached a maximum of PD 64 and SCAP reached a maximum of PD 46. (B) Relative telomere length was determined by q-PCR analysis of 30 ng DNA of SCC4, 16B, NHK, NHF, DPSC (PD 17, 29, 54, >65), SCAP (PD 18, 28, 41, 44). Average telomere versus single copy gene (T/S) ratio was used to determine telomere length. (C) Telomerase activities in SCC4, NHK, DPSC and SCAP were determined in dose dependent manner using SYBR Green Q-TRAP assay. (D) Protein extracts of DPSC at PDs 16, 32, and 54 were analyzed by Western blotting for expression of Bmi-1 and p16^{INK4A}. β-actin was used as a loading control. (E) DPSC at the indicated PDs were assessed for mRNA expression of Bmi-1 and *INK4A* by RT-qPCR. (F) Presenescent (rapidly proliferating) and senescent DPSC were stained for SA β-Gal activity. Original magnification, 100x. Green signal represents the positive SA β-Gal staining.

Figure 2. DPSC and SCAP exhibit abrogation of odontogenic differentiation upon senescence (A) DPSC cultures at varying PD levels were assessed for mRNA expression of OC, DSPP, and BSP by RT-qPCR. (B) Protein extract collected from DPSC at PDs 13 and 48 were assayed seven days post-confluence for the ALP activity and the protein expression level. Protein extracts were analyzed by Western blotting in which β -actin was used as a loading control. (C) ALP activity and the protein expression were assayed in SCAP at PDs 14 and 44 using the same method as shown in panel B. DPSC and SCAP at the indicated PDs were stained for the ALP activity seven days post-confluence. (D) Photographs of the ALP staining are shown for SCAP and DPSC at varying PD levels.

Figure 3. Bmi-1 transduction in DPSC enhances odontogenic differentiation and mineralization capacity

(A) Protein extracts from induced DPSC/B0 and DPSC/Bmi-1 were collected 7 days post-confluence and ALP activity for odontogenic differentiation was measured. Protein extracts from these cultures were also analyzed by Western Blotting for ALP and Bmi-1 protein expression. β -actin was used as a loading control. (B) Infected DPSC were cultured under calcifying conditions for 28 days post-confluence, stained for Alizarin Red and subsequently destained for 1 hour for quantification. (C) ALP activity was determined in SCAP/B0 and SCAP/Bmi-1 cells after 7 days of culture in calcifying condition post-confluence. (D) Odontoblastic markers OC, DSPP, BSP, and DMP-1 mRNA expression of early (PD 11) and late passage DPSC/B0 (PD 27) and DPSC/Bmi-1 (PD 28) were quantified by RT-qPCR.