Received: 7 September 2019 | Revised: 11 November 2019 | Accepted: 11 December 2019

DOI: 10.1111/jre.12727

ORIGINAL ARTICLE

Journal of PERIODONTAL RESEARCH WILEY

Uncoupled inflammatory, proliferative, and cytoskeletal responses in senescent human gingival fibroblasts

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Funding information
Fondo Nacional de Desarrollo Científico y Tecnológico, Grant/Award Number: 1170555

Abstract

Background and objective: Aging is characterized by a decline in tissue structure and function that may be explained by the development of cellular senescence. However, the acquisition of specific phenotypic responses in senescent gingival fibroblasts is still poorly understood. Here, we have analyzed whether proliferation of primary cultures of human gingival fibroblasts may affect different cell functions relevant to cellular senescence and tissue deterioration.

Methods: Human gingival fibroblasts from five young donors were expanded until cellular senescence was achieved. Cellular senescence was evaluated by determining modifications in cell size, cell proliferation, p16 and p21 mRNA levels, H2Ax phosphorylation, cell viability, and senescence-associated beta-galactosidase staining. Inflammation was evaluated by analyzing the secretion of cytokines and nuclear translocation of NF-κB. Collagen remodeling was evaluated using a collagen gel contraction assay. Immunofluorescence and confocal microscopy were used to determine changes in the localization of the cytoskeletal proteins. Data analysis was performed to identify changes between cultures of the same donor at early and late passages using the paired sample t test or the Wilcoxon matched-pairs signed-rank test.

Results: Late passage cells showed changes compatible with cellular senescence that included increased cell size, reduced cell proliferation, staining for SA-beta gal, phosphorylated H2Ax, and increased p16 and p21 mRNA levels. Late passage cells showed a decrease in collagen contraction and reduced co-localization between the cytoskeletal proteins actin and vinculin. Importantly, late passage cells neither demonstrated changes in the secretion of inflammatory cytokines nor NF-κB activation.

Conclusion: Our results imply that cytoskeletal changes and inhibition of cell proliferation represent early modifications in the structure and function of senescent gingival fibroblasts that are not coupled with the acquisition of an inflammatory phenotype. Further studies are needed to clarify the impact of different senescence stages during aging of the periodontium.

KEYWORDS

aging, fibroblast, gingiva, senescence

1 | INTRODUCTION

Aging is characterized by progressive tissue deterioration and increased vulnerability to several diseases including cancer, diabetes, cardiovascular disorders, neurological diseases, delayed wound healing, and periodontal diseases.[1-3] Cellular senescence has been proposed as one of the biological responses that may explain cell and tissue aging.[4] Senescence can be initiated by a diverse array of stressors that include continuous cell proliferation, oxidative and genotoxic stress, mitochondrial dysfunction, irradiation, or chemotherapy.[5] During this response, senescent cells undergo cell flattening, increased cell size, modifications in lysosome activity, chromatin remodeling, and induction of several genes and proteins involved in inflammation and tissue remodeling known as the senescence-associated secretory phenotype (SASP).[6] Importantly, it has been proposed that these inflammatory mediators may contribute to tissue deterioration by promoting inflammation and tissue remodeling.[6] Cellular senescence is now recognized as a dynamic multistep process during which different sets of genes and signaling pathways are activated.[5] An irreversible exit from the cell cycle is controlled by p16INK4a,[7] and increased secretion of SASP mediators is coordinated by the NF-κB pathway.[8,9] However, the precise conditions or stressors that activate these signaling pathways are still poorly defined. Despite the importance of cellular senescence in tissue deterioration, little information is available regarding the impact of this phenomenon on the function and organization of the actin cytoskeleton.[10,11] Importantly, defects in gingival wound healing of aged tissues have been linked to altered collagen remodeling and abnormal function of alpha smooth muscle actin (α-SMA).[2]

Replicative senescence can be induced via continuous proliferation of cultured cells. Extensive cell replication is associated with telomere exhaustion that limits cell proliferation inducing cellular senescence.[12-14] In the present study, we analyzed whether cellular senescence, induced by replicative exhaustion, affects functions relevant for fibroblasts including cell proliferation, collagen remodeling, and inflammation.

2 | MATERIAL AND METHODS

2.1 | Cell culture of human gingival fibroblasts and in vitro senescence

Primary cultures of human gingival fibroblasts (HGF) were obtained from clinically healthy gingiva from five young donors during bicuspid extractions (average age 12 years old, two male, three female). All donors signed an informed consent. The Ethical Committee at the Pontificia Universidad Católica de Chile approved the protocol for tissue obtainment (number 160 823 025). Patients reported preexisting medical or drug histories in the previous 6 months. Cells were cultured in DMEM (Gibco BRL, Grand Island, NY), which contains 10% fetal bovine serum (FBS) (Biological Industries) and a mixture of penicillin and streptomycin (Invitrogen) at 37°C in a 5% CO_2 atmosphere. Cells were cultured over a type I collagen film generated by treating cell culture plates with type I collagen (50 μg/mL) prior to seeding cells. Cell cultures were expanded from the tissue explants until passage 4-5. At this time point, cells were considered early passage cells and aliquots were frozen at −80°C. The cells that remained in culture were expanded until passage 30-35 (late passage cells). Cell cultures were considered senescent when they were unable to reach confluence after three weeks in culture from the last passage (1:2) in DMEM supplemented with 10% FBS. To compare between early and late passage cells, an aliquot of early passage cells was defrosted and compared with late passage cells from the same donor.

2.2 | Collagen gel contraction assay

Collagen gel contraction was evaluated as previously described.[2,15] An equivalent number (50 000 cellsperwell) of early and late passage human gingival fibroblasts were seeded over a type I collagen gel (1 mg/mL) previously generated in a 96-well plate. Gels were maintained overnight in the presence of DMEM supplemented with 10% FBS. The day after, gels were gently detached from the borders of the cell culture plate using a pipette point and fixed after 30 minutes with 4% paraformaldehyde. After fixation, gel areas were registered and quantified using ImageJ software. Early (sub-culture 4-5) and late passage (sub-culture 30-35) cells were compared.

2.3 | Antibody array and ELISA

Custom made antibody arrays were obtained from RayBiotech (RayBiotech) by selecting senescence-associated mediators selected from the literature.[6] Early and late passage cells were cultured in DMEM without serum for 48 hours, and conditioned media was analyzed through the antibody arrays following manufacturer instructions. Commercially available ELISA assays (Abcam) were used to quantify IL-6, IL-8, TNF, MMP-3, and TIMP-1 levels in the conditioned culture media of early and late passage cell cultures.

2.4 | Cell proliferation assay

Cell proliferation was evaluated by culturing cells in DMEM supplemented with 10% FBS for 48 hours. Cells were then fixed and processed for immunofluorescence to detect Ki67 and DAPI, comparing early and late passage cells.

2.5 | Cell viability assay/ MTS assay

Early and late passage cells were compared using a cell viability assay based on the reduction in tetrazolium salt, MTS, to form a blue formazan product (Promega). Formazan crystals were solubilized with dimethyl sulfoxide and read at 490 nm.

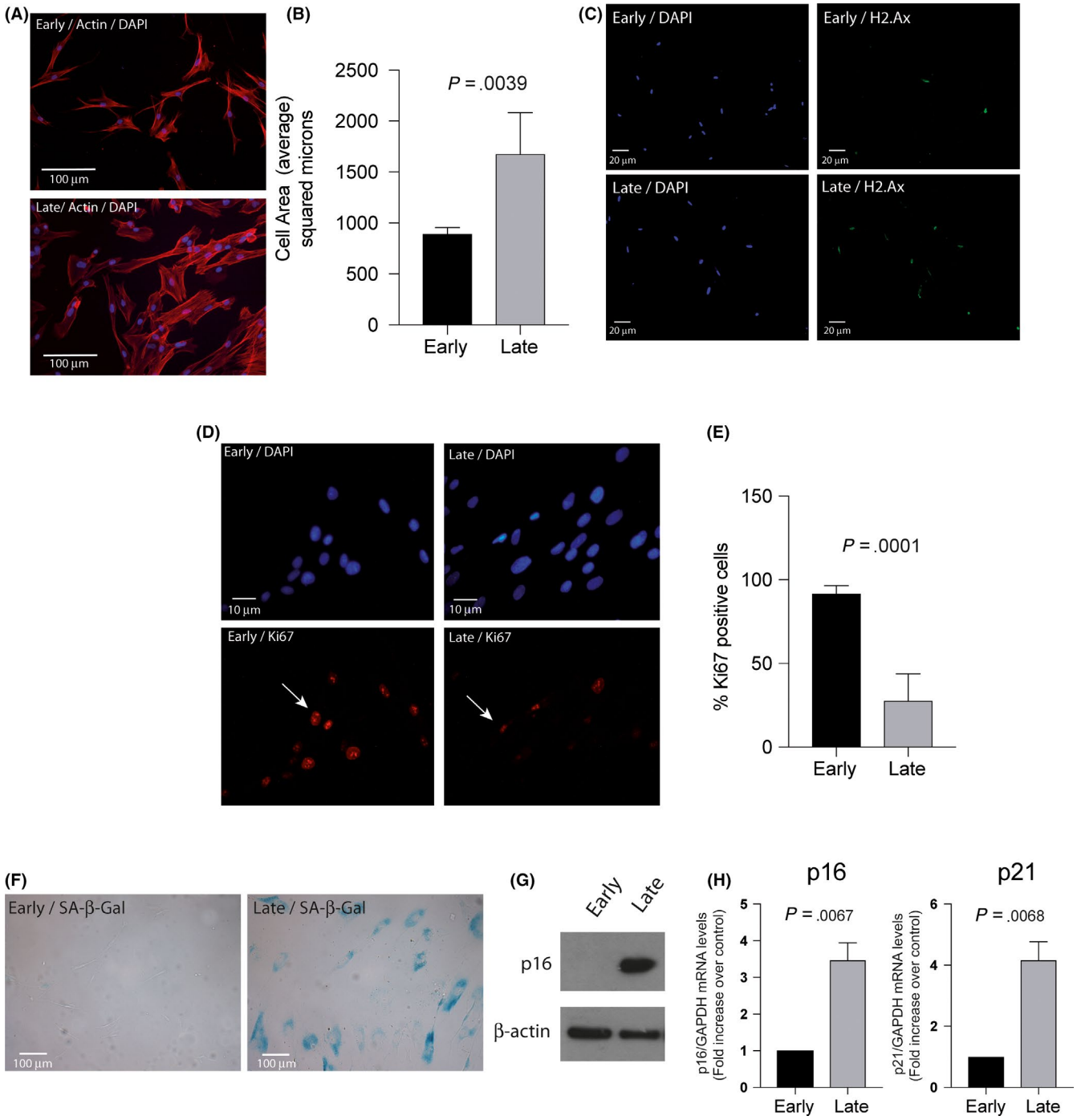

FIGURE 1 Cellular senescence markers are induced by extensive cell passage. Images show early and late passage cells stained with phalloidin and DAPI (A). Graphs show the average and standard error of cell area for early and late passage cells (B). Images show early and late passage cells stained for phosphorylated H2A.x (green), actin (red), and cell nucleus (DAPI) (C). Early and late passage cells stained for DAPI (cell nucleus) and Ki67. Arrows indicate Ki67-positive cells (D). Graphs show the average and standard error of Ki67-positive cells for early and late passage cells (E). Images show early and late passage cells stained for the senescence marker SA-beta gal (F). P16 protein levels analyzed through Western blot of the cell lysate in early and late passage cells (G). Graphs show the average and standard error for p16 and p21 mRNA levels in early and late passage cells (H)

2.6 | Western blot

Cell lysis, protein transfer, and electrophoresis were performed as previously described.[2] Protein levels of p16INK4a were detected in cell lysates of early and late passage cells using a commercial antibody from Abcam.

2.7 | Immunofluorescence

Early and late passage cells were fixed with 4% paraformaldehyde for 10 minutes, permeabilized with 0.25% Triton X-100 for 10 minutes and washed four times with staining buffer (PBS 1X and 4% FBS). Primary antibodies were diluted in SB. The antigen-antibody

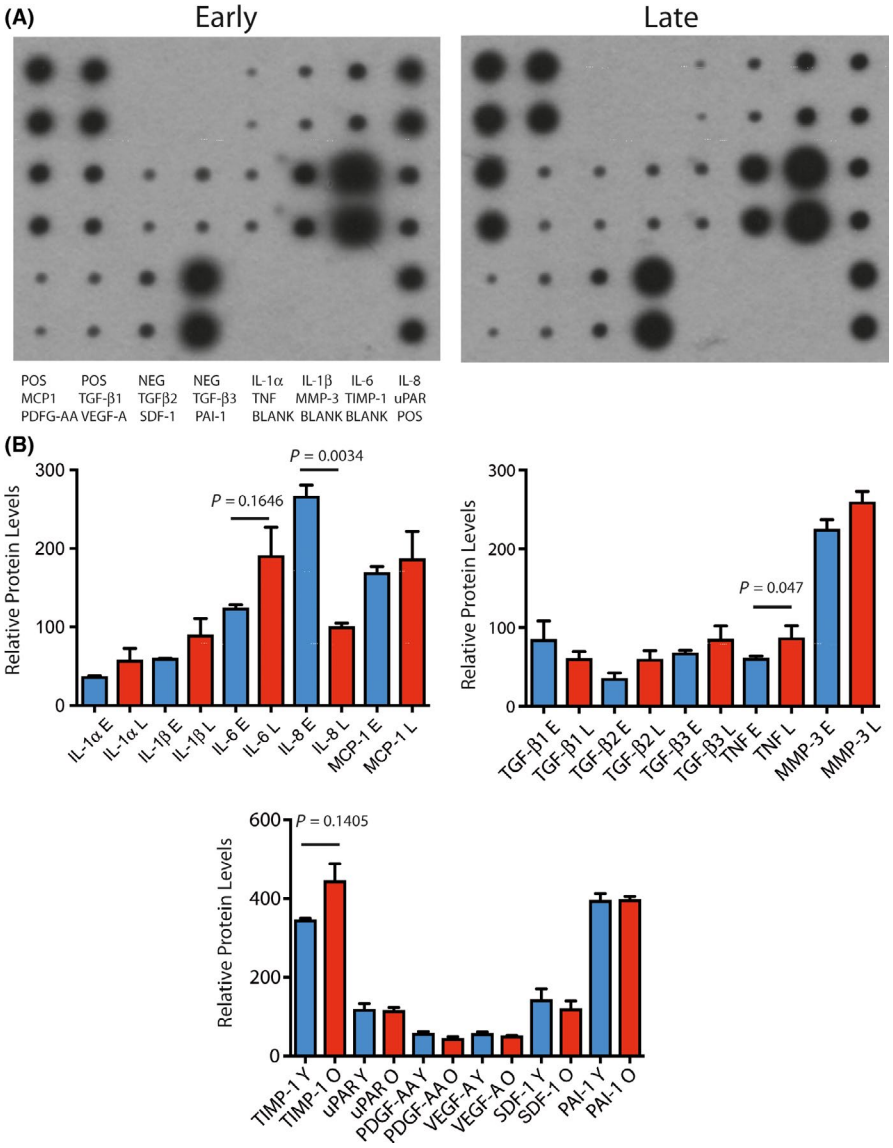

FIGURE 2 Protein array profiling of conditioned media derived from early and late passage cells. Representative protein array membranes exposed to conditioned media derived from early and late passage cells (A). Graphs show the average and standard error derived from the quantified arrays obtained from three independent cell cultures at early and late passage (B)

complex was washed with PBS and incubated with a secondary antibody (1:2500). Nuclei were stained with DAPI (Invitrogen Molecular Probes). Specimens were analyzed using an epifluorescence microscope (Zeiss Axioplan, Göttingen, Germany) or a confocal microscope (Nikon C2 plus) (Melville, NY, USA).

2.8 | Real-time reverse transcription polymerase chain reaction

Early and late passage cells were cultured for 48 hours after cell passage, and total RNA was isolated with Trizol (Ambion Life Technologies, Carlsbad, CA, USA) according to manufacturer instructions. Total RNA (2 μg) was used as a template for RT reactions to synthesize single-stranded cDNA using M-MLV reverse transcriptase and an oligo (dT) primer (Promega, Madison, WI) according to standard procedures. Messenger RNA levels were assessed through real-time quantitative polymerase chain reaction (qPCR) using LightCycler96® equipment (Roche Diagnostics). The reaction was performed using 200 ng of cDNA and LightCycler FastStart DNA Master SYBR Green I kit (Roche) in a final volume of 20 μL. All reactions were performed in duplicate and negative controls were included. The primers used were as follows: p16 (forward), 5′-ACTTCAGGGGTGCCACATTC-3′, p16 (reverse), 5′- CGACCCTGTCCCTCAAATCC-3′, p21 (forward), 5′- AGTCAGTTCCTTGTGGAGCC-3′, p21 (reverse), 5′-CATTAGCGC ATCACAGTCGC-3′, GPDH (forward), 5′-TGCACCACCAACTG CTTAG-3′ and GPDH (reverse), 5′-GTTCAGCTCAGGGATGACC-3′. In every case, mRNA expression was normalized against glyceraldehyde-3-phosphate dehydrogenase as the loading control.

2.9 | Senescence-associated beta-galactosidase staining

Early and late passage cells were stained using a commercial kit to detect senescence-associated beta-galactosidase (Cell Signaling Technology).

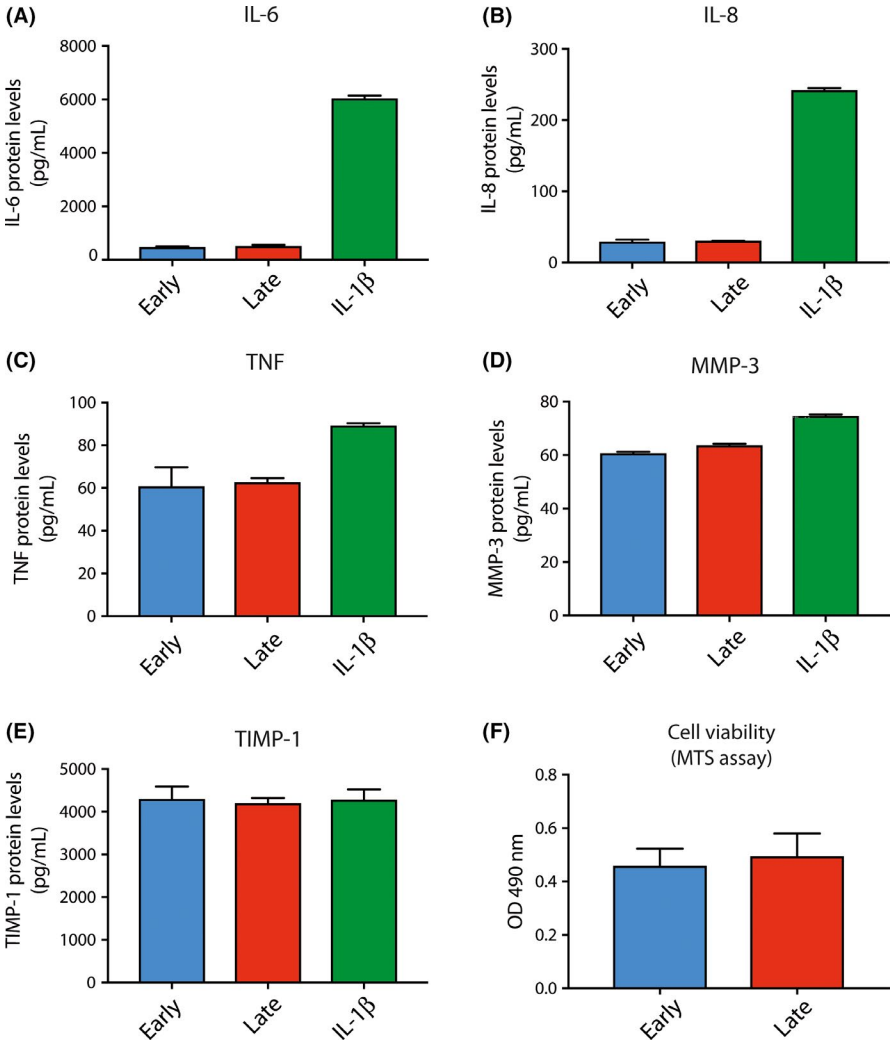

FIGURE 3 Extensive cell passage does not induce an inflammatory phenotype (SASP) in human gingival fibroblasts. Graphs show the average and standard error for protein levels detected in the conditioned media from early and late passage cells determined through ELISA assays for the following molecules: IL-6 (A), IL-8 (B), TNF (C), MMP-3 (D), and TIMP-1 (E). Average and standard error for the MTS assay (mitochondrial metabolism) detected in early and late passage cells (F)

2.10 | Statistical analysis

All experiments were carried out in triplicate. Data points and error bars in the figures represent averages and standard errors. Data distribution was analyzed using the Shapiro-Wilk normality test. Statistical analysis was performed to identify changes between cultures of the same donor at early and late passages using the paired sample t test or the Wilcoxon matched-pairs signed-rank test. Prism 5.0 statistical software was used (GraphPad, Prism). Statistical significance was set at $P < .05$.

3 | RESULTS

3.1 | Cellular senescence in early and late passage cells

Cell cultures reached senescence at passage 30-35. Figure 1 shows the evaluation of cellular senescence of early (4-5) and late (30-35) passages. Evaluation of cell area showed that late passage cells almost doubled the area of early passage cells (Figure 1A,B). Late passage cells also showed several signs of senescence including phosphorylation of H2Ax (DNA damage marker) (Figure 1C). We also observed a significant reduction in the proportion of Ki67 positive cells in late compared to early passage cultures (Figure 1D,E). Increased levels of the senescence marker SA-beta gal (Figure 1F), p16 protein levels (Figure 1G), and p16 and p21 mRNA levels were detected in late compared with early passage cells (Figure 1H).

3.2 | SASP mediators in early and late passage cells

To investigate whether senescence may modulate the secretion of SASP mediators, serum-starved early and late passage cells were cultured for 48 hours and the conditioned media were analyzed using an antibody array system. Figure 2A shows representative antibody arrays tested in cells derived from the same donor at early and late passages. Data derived from protein arrays obtained from 3 independent subjects evaluated at early and late passages are represented in graph 2B. We observed few differences between early and late passage cells. TNF-α showed a significant increase in late passage cells and IL-8 levels were significantly lower in late vs early passage cells.

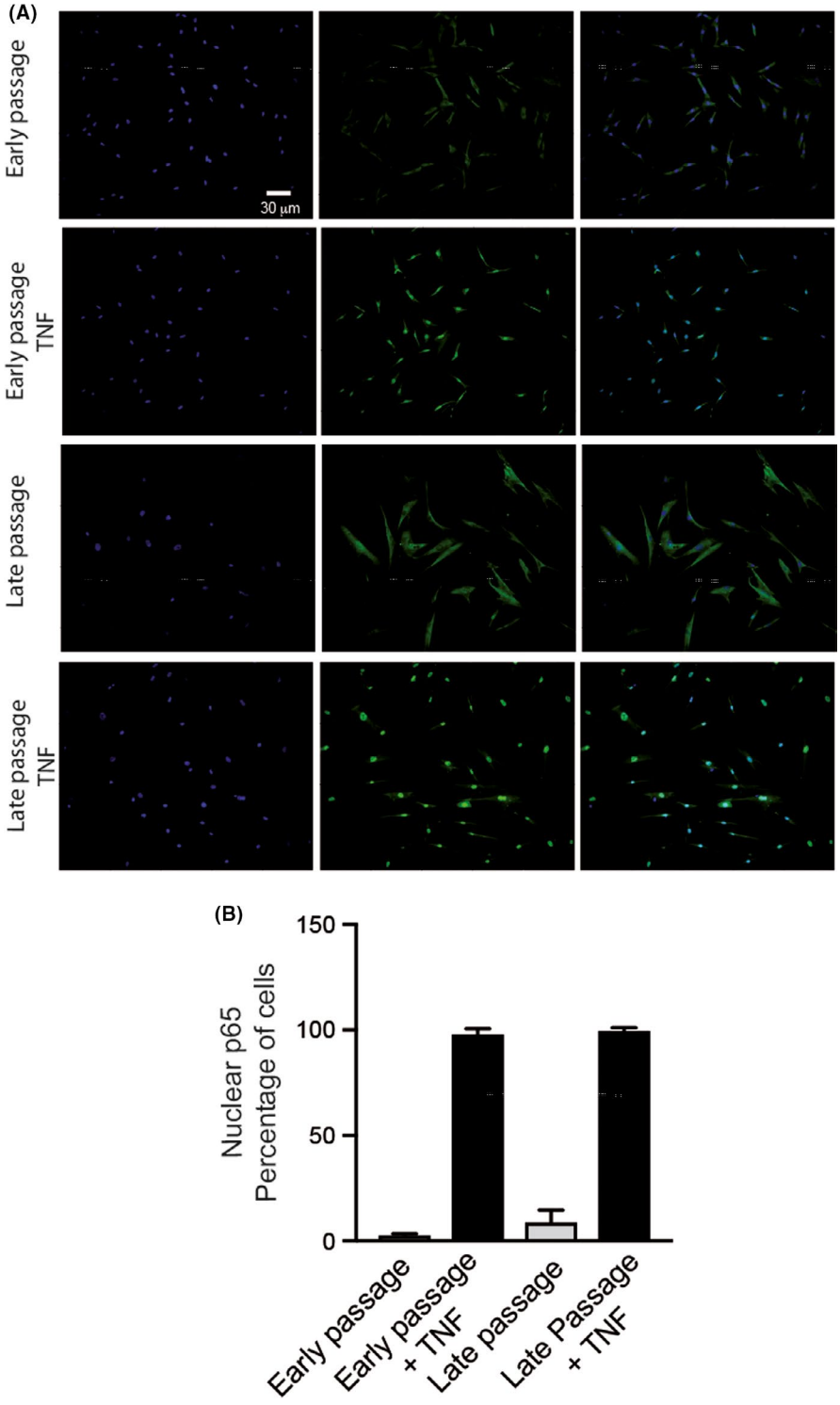

FIGURE 4 Extensive cell proliferation is not associated with NF-κB activation. Localization of the p65 subunit of NF-κB and DAPI was evaluated through immunofluorescence in early and late passage human gingival fibroblasts. As a positive control, cells were stimulated with TNF (A). Graph represents the average and standard error of the percentage of NF-κB localized in the cell nuclei (B)

Higher levels of IL-6, MMP-3, and TIMP-1 in late passage cells were detected; however, these differences did not reach statistical significance. To confirm these results, we performed quantitative tests through ELISA assays to more accurately evaluate the protein levels of TNF-α, IL-8, IL-6, MMP-3, and TIMP-1. As a positive control of inflammation, early passage fibroblasts were stimulated with interleukin-1β for 48 hours in the absence of FBS. These analyses did not show any significant changes in the levels of these mediators in early and late passage cells (Figure 3A-E). We also evaluated cell viability in these cell cultures and did not found any differences as assessed through the MTS assay (Figure 3F).

3.3 | Extensive cell passage does not induce NF-κB activation in gingival fibroblasts

Given that the NF-κB pathway is involved in the regulation of the SASP phenotype in senescent cells, we evaluated the nuclear

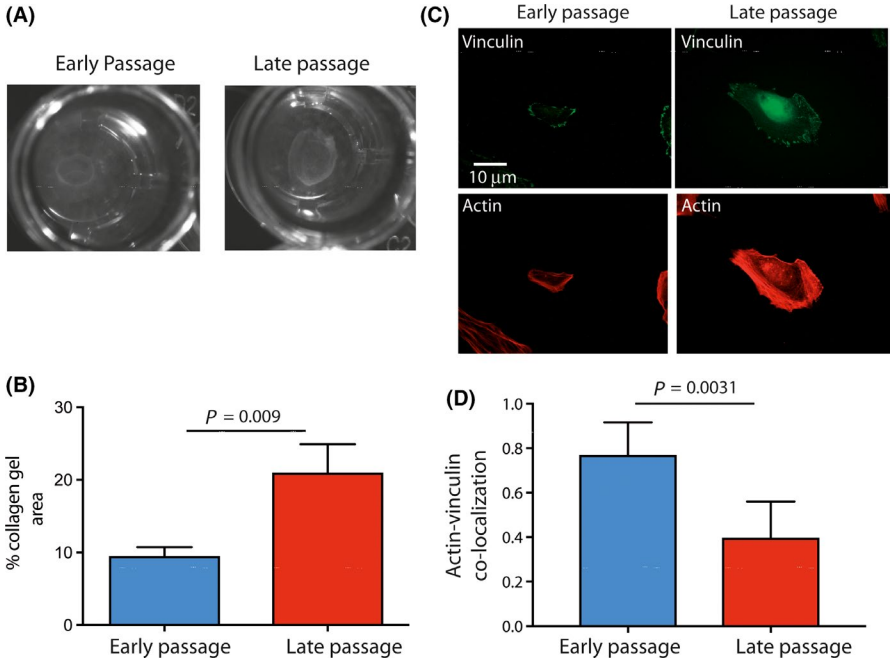

FIGURE 5 Extensive cell passage is associated with altered collagen remodeling. Images are representative of collagen gels from early and late passage cells after 24 h of gel contraction (A). Graphs show the average and standard error for the collagen gel area of early and late passage cells (B). Early and late passage cells were stained for actin and vinculin (C). Actin and vinculin co-localization were analyzed using confocal microscopy. Graphs show the average and standard error for the proportion of co-localization between actin and vinculin in early and late passage cells (D)

translocation of the p65 subunit of NF-κB in serum-starved, early and late passage HGFs. As a positive control of inflammation, both early and late passage cells were stimulated for 1 hour with 5 ng/mL TNF. As shown in Figure 4A,B, no differences were found between early and late passage cells in the localization of the p65 subunit of NF-κB.

3.4 | Late passage cells express defects in collagen remodeling and changes in the distribution of the cytoskeleton proteins actin and vinculin

Given the significant modifications in the size and morphology of late passage cells, we analyzed if cell passage was able to alter matrix remodeling. Using a collagen gel contraction assay, we observed that late passage cells were less efficient in contracting a collagen gel matrix when compared to early passage cells (Figure 5A,B). Using immunofluorescence, we analyzed the distribution of actin and vinculin, two important cytoskeletal proteins involved in cell contraction. In early passage cells, vinculin was mostly distributed in focal contacts. However, in late passage cells, we observed that vinculin was detected in focal contacts and also in a perinuclear location, suggesting that vinculin recruitment into focal adhesions was altered (Figure 5C). Using confocal microscopy, we analyzed the distribution of actin and vinculin. This analysis revealed a significant decrease in actin-vinculin co-localization in late passage cells (Figure 5D). These findings suggest important defects in the organization and functionality of the actin cytoskeleton in late passage HGF.

4 | DISCUSSION

The present study shows that primary cultures of HGF derived from young donors subjected to continuous cell proliferation develop several, but not all signs of cellular senescence. These changes included cell enlargement, irreversible exit from the cell cycle, increased SA-beta gal expression, H2A.x phosphorylation, reduced collagen contraction, and changes in the distribution of the cytoskeletal protein, vinculin. Interestingly, although several signs of cellular senescence were observed, we did not detect the secretion of inflammatory mediators or activation of the NF-κB pathway. We highlight two novel findings from our results. Collagen remodeling has been poorly studied in senescent cells. Our results suggest that the organization and function of vinculin and actin may have important alterations in this cell population. In addition, the present findings suggest that extensive cell passage induces some, but not all, of the cellular changes associated with cellular senescence, suggesting that different phenotypes may be developed during this response.

Senescence was initially interpreted as a static cell fate condition in cells exposed to different stressors. However, it is now identified as a dynamic multistep process in which several states can be recognized.[5,6,16,17] In this regard, the exit from the cell cycle in senescent cells is mainly driven by the activation of the p16INK4A gene.[7] However, development of the SASP phenotype is controlled by a different set of genes that include the transcription factor GATA4 and its downstream target NF-κB.[8,9] Therefore, different sets of genes may control distinct aspects of the senescent phenotype in a coordinated manner.[18] The experimental protocol used in this study exposed cells to continued cell proliferation, a stimulus known to induce replicative senescence. In this process, human fibroblasts develop progressive telomere attrition that exposes an uncapped double-stranded chromosome free end that can initiate the DNA Damage Response (DDR) response.[14] Detection of H2A.x is considered to be a robust marker of DNA damage.[14,19] Although additional features can be detected during DDR, including co-localization of H2Ax with 53BP1 within the nucleus and activation of the DNA damage checkpoint kinases CHK1 and CHK2,[14] cells subjected to

our experimental protocol demonstrated cellular changes compatible with the development of replicative senescence and activation of DDR. Inflammation may contribute to the development of senescence. Interleukin 1 stimulates the development of senescence in skin fibroblasts in a paracrine fashion [20,21] and toll-like receptor 2 may contribute to senescence in IMR-90 fibroblasts.[22] Our study did not detect the secretion of inflammatory mediators (SASP phenotype) or activation of the NF-κB pathway in senescent cells. It is possible that additional factors, like inflammatory mediators as suggested by the abovementioned studies,[20-22] may also be required to initiate the SASP in late passage human gingival fibroblasts. It is also important to consider that the samples used in the present study were originated from healthy gingival tissue obtained from young subjects subjected to a senescent stimulus by continuous culture passage. It is tempting to speculate that fibroblasts derived from elderly subjects might respond differently to extensive cell proliferation. In addition, it has been suggested that senescence may have a physiological role in young subjects, for example, by promoting wound healing in liver and skin.[23,24] However, it is unknown whether senescence in young and elderly subjects has different regulation pathways or may play diverse physiological or pathological roles. Future studies should explore these possibilities.

A key finding of the present study was the identification of cytoskeletal changes in senescent cells that likely alter matrix remodeling. Our findings indicate reduced collagen remodeling as detected by a collagen gel contraction assay and a reduced proportion of vinculin co-localizing with actin. It has been previously demonstrated that vinculin recruitment to focal adhesions plays a central role in cell-matrix adhesions.[25] In the aged heart, it has been shown that changes in vinculin regulation may have important implications in cardiovascular physiology.[26] Previous studies have identified that senescent cells may have significant morphological changes including flattening, enlargement, and vacuolization.[5] Moreover, in elderly mice, cells expressing SA-beta gal experience an increase in size.[27] In recent studies, we have identified that during myofibroblastic differentiation, gingival fibroblasts derived from elderly human donors have important alterations in the localization of the smooth muscle alpha actin isoform that remains mostly out of the actin stress fibers.[2] In addition, HGF derived from elderly donors displayed a defect in collagen remodeling and cell migration.[2] Reduced size of focal adhesion contacts has been described in fibroblasts derived from aged skin in mice [11] and in IMR-90 lung fibroblasts whose senescence was induced by exposure to hydrogen peroxide.[10] Moreover, aged gingival fibroblasts have a reduced ability to bind to type I collagen, affecting the phagocytic pathway of remodeling of this molecule.[28] Beyond these observations, the functional significance of the morphological and cytoskeletal changes associated with senescence remains poorly understood.

In summary, senescent HGF derived from young donors may have important alterations in cell function including cell proliferation, changes in cell morphology, collagen remodeling, and cytoskeletal organization. However, these changes are uncoupled from the inflammatory SASP phenotype.

ACKNOWLEDGEMENTS

This study was financed by a grant from the National Fund for Science and Technology from Chile (FONDECYT, grant number 1170555 to Patricio Smith). Authors declare no conflicts of interest.

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How to cite this article: Páez J, Hernández R, Espinoza J, et al. Uncoupled inflammatory, proliferative, and cytoskeletal responses in senescent human gingival fibroblasts. *J Periodont Res*. 2020;00:1–9. https://doi.org/10.1111/jre.12727