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Long-Term in Vitro Expansion of Stem Cells from the Apical Papilla: Proliferation Capacity and Phenotypic Stability

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Abstract

Stem cells from the apical papilla (SCAP) represent a valuable dental mesenchymal stem cell population with high proliferative and regenerative potential. The present study investigated the effects of long-term in vitro expansion on the proliferative behavior, senescence progression, phenotypic stability, and viability of SCAP. Cells were isolated from the apical papilla of developing human third molars and cultured under standard conditions for up to 40 passages over a period of 177 days. Population doubling (PD) time analysis revealed three distinct growth phases, including an initial adaptation phase, a prolonged period of stable proliferation, and a late phase characterized by significantly increased doubling times indicative of replicative senescence. Flow cytometric analysis demonstrated sustained expression of mesenchymal stem cell markers CD71, CD90, and STRO-1 at both early and late passages, with no statistically significant differences observed. In addition, SCAP preserved high viability following cryopreservation. These findings indicate that SCAP retain their mesenchymal stem cell phenotype and functional stability during extended in vitro cultivation, supporting their suitability for experimental and potential regenerative applications, with mid-passage cells representing the optimal window for use.

Keywords: Stem Cells from Apical Papilla (SCAP), long-term cultivation, stem cells aging, cell senescence, population doubling

Background

Mesenchymal stem cells (MSCs) are recognized as multipotent progenitor cells, capable of self-renewal and differentiation into multiple mesenchymal lineages, including adipocytes, chondrocytes, and osteoblasts. Human MSCs (hMSCs), which originate from mesodermal tissues, possess several properties that make them highly attractive for both research and clinical applications. These include their accessibility from various tissue sources, robust proliferative

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capacity, broad differentiation potential toward terminally specialized cell types, low immunogenicity, and notable immunomodulatory functions (1).

MSCs reside in various tissues as a reservoir of reparative cells that can be stimulated to differentiate in response to injury, disease, or age-related degeneration (2). MSCs derived from dental tissues have attracted increasing attention due to the minimally invasive procedures required for tissue collection, their potential for both autologous and allogeneic applications, and their capacity to differentiate into multiple cell lineages under appropriate in vitro conditions (3,4). Human dental tissues are a rich source of stem cells, with six major subtypes described thus far: 1. dental follicle precursor cells (hDFPCs), 2. dental pulp stem cells (hDPSCs), 3. stem cells isolated from exfoliated deciduous teeth (SHED), 4. apical papilla–derived stem cells (hSCAP), 5. periodontal ligament stem cells (hPDLSCs), and 6. gingiva-derived stem cells (hGMSCs) (5).

The present study focuses on stem cells from the apical papilla (SCAP), which were first isolated by Sonoyama et al. (6), who demonstrated their expression of mesenchymal stem cell markers, high proliferative capacity, and strong osteogenic and dentinogenic differentiation potential. Accumulating evidence highlights the significant regenerative potential of SCAP in the context of dental tissue engineering and repair (7). Notably, their capacity to withstand pathological conditions, including microbial infection and advanced apical periodontitis, further supports their clinical relevance (8). These distinctive biological properties emphasize the need for well-established and physiologically relevant in vitro models to investigate SCAP behavior and therapeutic applications. Nevertheless, concerns have been raised that, as with other MSC types, prolonged in vitro expansion of SCAPs is associated with the onset of replicative senescence, characterized by morphological alterations and a reduced differentiation potential (9). Previous studies have reported spontaneous tumorigenic transformation of stem cells following extended in vitro expansion, demonstrating that long-term cultured MSCs may promote tumor formation upon transplantation into immunodeficient mice (10). Consequently, this remains a subject of ongoing debate, underscoring the need for further investigation to elucidate the underlying mechanisms. In light of these concerns, the present study aimed to evaluate the characteristics of SCAPs expanded in vitro for 40 passages.

Materials and Methods

Stem Cell Isolation

SCAPs were obtained from freshly extracted tooth germs of developing permanent third molars following a previously established protocol (11). Apical papilla tissue was gently separated from the root surface under aseptic conditions and enzymatically digested with 3 mg/mL collagenase type I (Sigma-Aldrich, St. Louis, MO, USA) and 4 mg/mL dispase at 37 °C for 60 minutes. The resulting cell suspension was filtered through a sterile 70 μm cell strainer (Falcon, Corning, NY, USA) to obtain a single-cell suspension and plated in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Eugene, OR, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin.

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Long-Term Cultivation
Cells at 80–90% confluence were detached with 0.025% trypsin–EDTA (Lonza), centrifuged at 3000 rpm for 4 minutes, resuspended in DMEM with 10% FBS, and reseeded at 5×10^3 cells/cm² in new culture flasks (Greiner Bio-One, Frickenhausen, Germany). Cultures were maintained under standard conditions and expanded for long-term experiments. Cells from passages 1–40 were used, and all experiments were performed in triplicate.

Cryopreservation
Upon reaching 80–90% confluence, cells were harvested, counted, and resuspended in freezing medium (60% DMEM, 20% FBS, 20% DMSO; SERVA Electrophoresis GmbH, Heidelberg, Germany) at 1×10^5 cells per cryovial (TPP, Trasadingen, Switzerland). Cryovials were stored at –80 °C for 24 h before transfer to liquid nitrogen for long-term storage.

Cell Proliferation
Population doubling time (DT) was calculated from cell count data obtained at defined time points during the exponential growth phase of each passage using the formula: $DT = (t_2 - t_1) \times \ln(2) / \ln(N_2/N_1)$, where t1 and t2 represent initial and final time points (in hours), and N1 and N2 represent corresponding cell counts. SCAP cells underwent 40 sequential passages over 177 days (February 13 - August 7, 2023), with doubling times calculated for each passage using biological replicate measurements (n=2-4 per passage). Data are presented as mean ± standard deviation. Overall mean population doubling time across all 40 passages was 139.0 ± 32.8 hours (95% CI: 128.2-149.6 hours). Analysis revealed three distinct phases: early passages (P1-10) demonstrated mean DT of 122.4 ± 37.8 hours with high coefficient of variation (CV 30.9%), reflecting population adaptation to culture conditions; mid passages (P11-25) showed stable proliferation with mean DT of 128.1 ± 31.5 hours (CV 24.6%); and late passages (P26-40) exhibited significantly prolonged doubling times of 171.3 ± 18.5 hours (CV 10.8%), indicating replicative senescence. Cumulative population doubling level was estimated to reach approximately 40 doublings by passage 40, with optimal passages for functional studies identified as P12-20 (population doubling level 12-20) (12).

Flow Cytometry
SCAPs at early, intermediate, and late passages were analyzed for MSC marker expression. Cells were harvested with 0.25% trypsin, washed with PBS, and incubated at 4 °C in the dark for 30 min with mouse monoclonal anti-CD71-FITC, anti-STRO-1-APC, and anti-CD90-PE (all Beckman Coulter, Brea, CA, USA). Isotype controls were included. Analysis was performed using a Guava EasyCyte Cytek flow cytometer and GuavaSoft 3.3 software (Luminex, Austin, TX, USA).

Statistical Analysis
Data are presented as mean ± SD of three replicates. Differences between early, intermediate, and late passages were analyzed using one-way ANOVA followed by Tukey's post hoc test; pairwise comparisons used the Student's t-test where appropriate. Normality was confirmed by the Shapiro–Wilk test. Analyses were conducted with SPSS Statistics 28 (IBM, Armonk, NY, USA). A p-value < 0.05 was considered statistically significant.

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Aim

The aim of this study was to characterize the proliferative behaviour and phenotypic stability of stem cells from the apical papilla during long-term in vitro expansion.

Results

Population Doubling Kinetics and Senescence Progression

SCAP cells demonstrated progressive changes in population doubling time across 40 sequential passages spanning 177 days of continuous culture. The overall mean population doubling time across all passages was 139.0 ± 32.8 hours (5.79 ± 1.37 days), with a 95% confidence interval of 128.2-149.6 hours and a range of 88-215 hours (Figure 1).

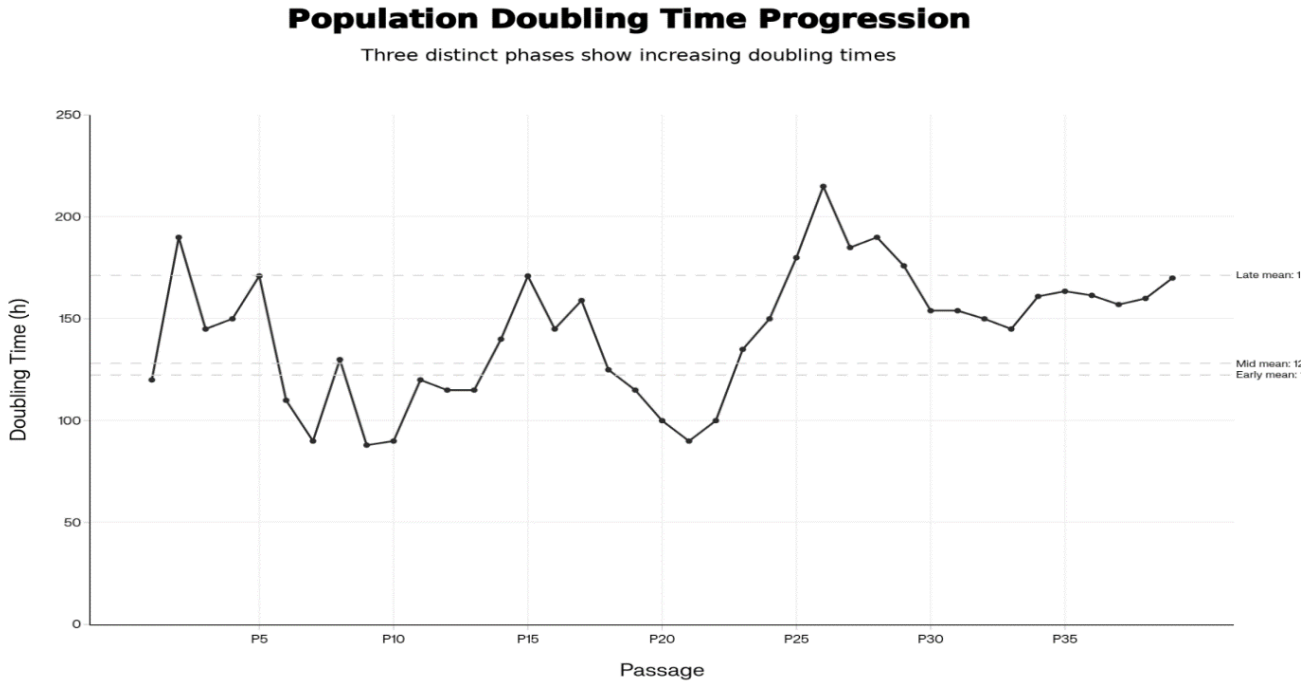

Figure 1: Population doubling time progression across 40 passages showing adaptation, stable growth, and senescence phases

Analysis revealed three distinct phases of growth dynamics with characteristic kinetic parameters (Figure 2).

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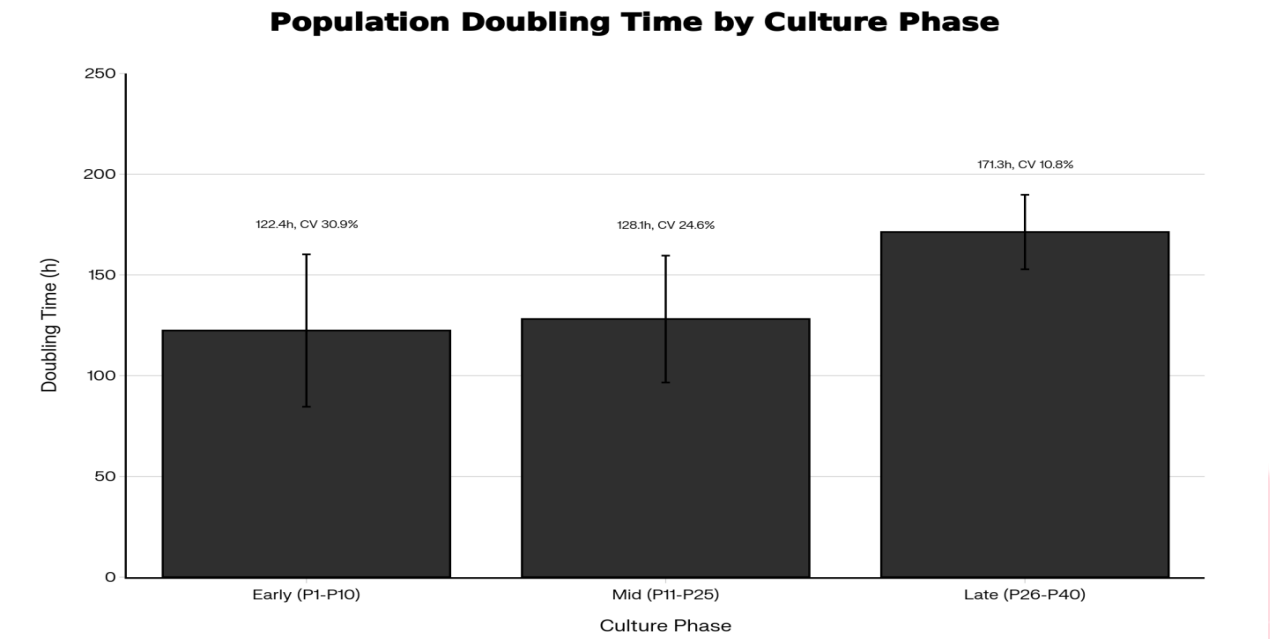

Figure 2: Comparative analysis of population doubling time across three culture phases showing progression toward senescence

Early passages (P1-P10) demonstrated mean DT of 122.4 ± 37.8 hours with high coefficient of variation (CV 30.9%), reflecting population adaptation and heterogeneous growth responses during culture initiation. Peak proliferative capacity was achieved at P9 with a doubling time of 88 hours. Mid passages (P11-P25) showed substantially more consistent growth with mean DT of 128.1 ± 31.5 hours (CV 24.6%), representing the optimal experimental window spanning approximately 50 days (March 20 - May 23, 2023). This phase was characterized by stable, reliable doubling times with normal biological variability. Late passages (P26-P40) exhibited markedly prolonged population doubling times of 171.3 ± 18.5 hours, representing a 34% increase compared to mid-passage values and indicating replicative senescence. Notably, the coefficient of variation dramatically decreased to 10.8%, reflecting synchronized cell cycle lengthening across the population rather than environmental heterogeneity. The maximum doubling time of 215 hours was observed at passage 26, after which late passages remained elevated (>145 hours) with minimal variability. Cumulative population doubling level reached approximately 40 doublings by passage 40, with optimal passages for studies requiring functional stem cell properties identified as P12-20 (population doubling level 12-20) (Figure 3).

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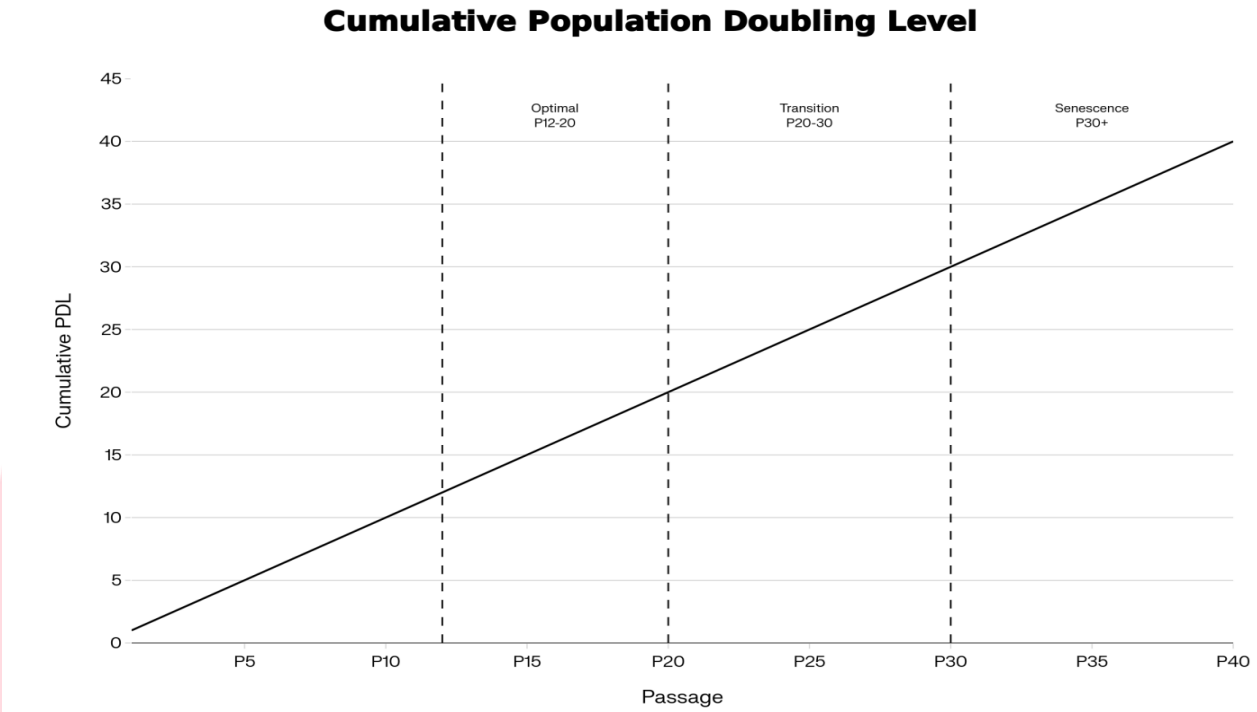

Figure 3: Cumulative population doubling level with critical functional capacity windows marked

Expression of Mesenchymal Stem Cell Markers (Flow Cytometry)

The expression of mesenchymal stem cell (MSC) markers CD71, CD90, and STRO-1 was analyzed in SCAP at early (P1–P5) and late (P35–P40) passages. CD90 was highly expressed in the majority of cells, with mean values of 93.0 ± 3.5% at early passages and 90.7 ± 3.0% at late passages. In contrast, CD71 and STRO-1 were present in smaller subpopulations, with mean expression levels of 23.0 ± 5.5% and 20.0 ± 2.0%, respectively, at early passages, and 21.7 ± 4.0% and 19.0 ± 2.5% at late passages. Representative histograms are shown on Figure 4.

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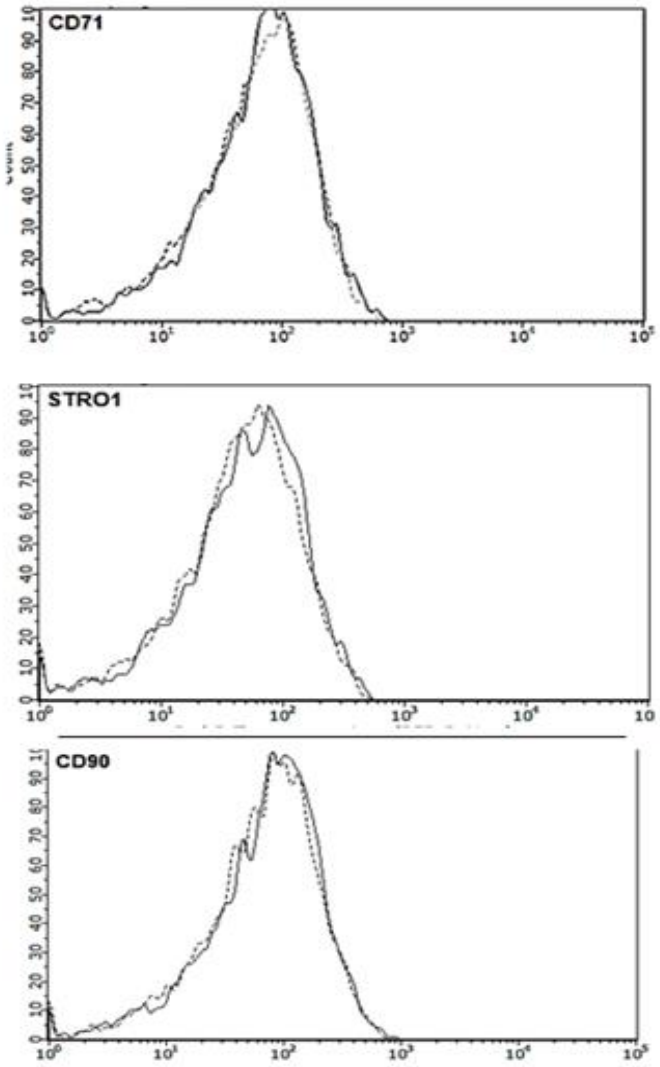
Although a slight decrease in marker expression was observed over long-term culture, statistical analysis revealed no significant differences between early and late passages ($p > 0.05$), indicating that the MSC phenotype of SCAP remained largely stable during prolonged in vitro expansion.



Histograms show the expression of CD71, STRO-1, and CD90 in SCAP at early (P1–P5) and late (P35–P40) passages. Continuous lines represent early passages, and dashed lines represent late passages. The histograms largely overlap, indicating that marker expression remains stable over long-term culture. Data are representative of three independent experiments, and no statistically significant differences were observed between early and late passages ($p > 0.05$).

Cryopreservation and Cell Viability

Stem cells were cryopreserved at passage 3 for future experiments. After thawing, the cell viability was assessed using trypan blue exclusion. Viability of SCAP after cryopreservation was found to be 85% ± 3.2% on average, demonstrating that the cells could be efficiently preserved and maintain their viability for future use in experiments.

Figure 4. Flow cytometric analysis of MSC marker expression in SCAP at early and late passages.

Statistical Analysis

Data are expressed as mean ± standard deviation (SD) for each passage group. All experiments were conducted in triplicate, and statistical analysis was performed using one-way ANOVA with Tukey's post hoc test for multiple comparisons. Differences between early, intermediate, and late passages were considered statistically significant at $p<0.05$. Statistical analysis revealed that there was no-significant decrease in expression of mesenchymal markers between early and late passages ($p < 0.05$).

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Discussion

Oral tissue–derived MSCs constitute an attractive and easily obtainable autologous cell source, positioning them as a viable candidate for use in regenerative therapeutic strategies. However, prolonged in vitro expansion of adult MSCs has been shown to induce replicative senescence, characterized by distinct morphological and functional changes. Wagner et al. (13) described cellular enlargement and flattening, the appearance of prominent nucleoli and cytoplasmic granules, altered differentiation capacity, and progressive telomere shortening associated with advanced passage number. Senescent MSCs can also be identified by positive staining for senescence-associated β-galactosidase (SA-β-gal).

Among dental stem cell niches, SCAPs are of particular interest due to their high potential and ability to support root development even under adverse conditions, such as those resulting from apical periodontitis (8). They appear morphologically small and fibroblast-like or stellate in shape (14). SCAPs demonstrate superior biological characteristics relative to other dental stem cell types, including higher proliferation rate and enhanced regenerative potential in dental tissues (15). They express an array of typical surface antigens found in dental MSCs, alongside the unique marker CD24, which distinguishes them phenotypically (16).

The stability of SCAPs' stem cell characteristics during in vitro expansion has become a subject of increasing concern. Cells isolated from the apical papilla represent a limited and low-yield source, which undergoes senescence upon extended passaging. This process is accompanied by morphological alterations—such as increased cell size and a flattened appearance—as well as a decline in multilineage differentiation capacity (17). Given the existing literature, researchers hold divergent views on the long-term characteristics of stem cells in culture, largely due to concerns about diminished functional capacity and potential acquisition of adverse features, including tumorigenicity.

While some studies have reported morphological alterations in SCAP during prolonged in vitro passaging—such as changes in cell size, shape, or loss of the typical fibroblast-like phenotype (18)—others demonstrate remarkable stability of cellular phenotype across extended culture periods. For instance, RP-89 SCAP maintained consistent expression of MSC markers (CD73, CD90, CD105) through up to 20 passages, with no indication of phenotype loss (19).
In our study, SCAP were expanded for up to 40 passages, and population doubling analysis revealed three distinct phases of growth: an early adaptation phase (P1–P10), a stable proliferation phase (P11–P25), and a late phase characterized by prolonged doubling times, indicating replicative senescence. Importantly, despite these kinetic changes, SCAP retained a consistent fibroblast-like morphology throughout all passages, without detectable alterations in cell size or shape (18). Moreover, flow cytometric analysis demonstrated that expression of MSC markers CD71, STRO-1, and CD90 remained largely unchanged between early and late passages. These findings clearly show that SCAP maintain both their stem cell phenotype and functional potential during extended in vitro cultivation, highlighting passages 12–20 as an optimal window for functional studies and

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experimental applications. This observation strengthens the clinical relevance of SCAP as a reliable and robust cell source.

A comprehensive understanding of senescence-associated alterations is essential for ensuring the functional integrity and therapeutic reliability of expanded MSC populations. Dental MSCs have demonstrated sustained proliferative capacity with minimal signs of senescence up to at least the 15th passage, indicating their suitability for clinical application in regenerative therapies (20). Despite this, SCAPs—like other stem cell populations—are suggested by research teams to show replicative limits in vitro and progressively lose their original phenotypic characteristics during prolonged culture. To address the challenge of limited expansion potential, cell immortalization has emerged as a viable approach, achieved through lentiviral transduction of human telomerase reverse transcriptase (hTERT), which enables extensive propagation while preserving the functional identity of the cells (18). Previous research has shown that dental-derived stem cells exhibit comparable proliferation rates at early (passage 2) and later stages of in vitro expansion (passage 15). Notably, the senescence-associated marker p16 displayed only minor upregulation at higher passages, while p21 expression remained stable. Based on these findings, the authors suggested that continued expression of embryonic and proliferation-related markers, along with minimal activation of senescence pathways, may support the potential clinical application of oral MSCs in regenerative therapies (20).

Maintaining the vitality of the isolated stem cells is of great importance for the success of regenerative therapy (21). Therefore, cell culture conditions should be carefully optimized based on the specific aims of the experimental design and the intended outcomes. In the present study, early-passage SCAP exhibited high levels of stem cell marker expression, and notably, comparable expression was observed in late passages. Together with the population doubling analysis and preserved morphology, these results demonstrate that SCAP retain both their stemness properties and proliferative potential during extended culture, confirming and expanding on previous studies (20).

Conclusion

In conclusion, SCAP can be successfully expanded in vitro over extended periods while maintaining their mesenchymal stem cell phenotype and high post-cryopreservation viability. Although prolonged cultivation leads to replicative senescence, evidenced by increased PD times, this is not accompanied by significant morphological alterations or loss of MSC marker expression. Our study identifies passages 12–20 as an optimal functional window in which SCAP retain both proliferative capacity and stemness characteristics, supporting their reliable use for experimental studies and regenerative dental applications. These findings reinforce the potential of SCAP as a clinically relevant and robust cell source for tissue engineering and regenerative therapies.

Acknowledgments

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