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ORIGINAL ARTICLE

Comparison of periodontal ligament and gingiva-derived mesenchymal stem cells for regenerative therapies

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Abstract
Objectives Tissue-engineering therapies using undifferentiated mesenchymal cells (MSCs) from intra-oral origin have been tested in experimental animals. This experimental study compared the characteristics of undifferentiated mesenchymal stem cells from either periodontal ligament or gingival origin, aiming to establish the basis for the future use of these cells on regenerative therapies.
Materials and methods Gingiva-derived mesenchymal stem cells (GMSCs) were obtained from de-epithelialized gingival biopsies, enzymatically digested and expanded in conditions of exponential growth. Their growth characteristics, phenotype, and differentiation ability were compared with those of periodontal ligament-derived mesenchymal stem cells (PDLMSCs).
Results Both periodontal ligament- and gingiva-derived cells displayed a MSC-like phenotype and were able to differentiate into osteoblasts, chondroblasts, and adipocytes. These cells were genetically stable following in vitro expansion and did not generate tumors when implanted in immunocompromised mice. Furthermore, under suboptimal growth conditions, GMSCs proliferated with higher rates than PDLMSCs.
Conclusions Stem cells derived from gingival biopsies represent bona fide MSCs and have demonstrated genetic stability and lack of tumorigenicity.
Clinical relevance Gingiva-derived MSCs may represent an accessible source of messenchymal stem cells to be used in future periodontal regenerative therapies.

Keywords Mesenchymal stem cells · Tissue engineering · Periodontal ligament · Gingival · Periodontal regeneration

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Introduction

Periodontitis is a chronic inflammatory disease of bacterial etiology, affecting a high percentage of the worldwide adult population [1]. This disease initiates and progresses in susceptible individuals by periodontal tissue destruction, as a consequence of the chronic host inflammatory immune response against pathogenic bacteria residing in the dental biofilm. Current treatment is based on bacterial biofilm removal. With these therapies, the disease process is arrested and the long-term maintenance of periodontal health can be achieved, but re-establishment of the original anatomy of the periodontal apparatus is, however, unlikely to occur [2]. Regenerative approaches are based on either using bioactive agents (as enamel matrix derivatives), which promote new cementum formation and periodontal attachment [3–5], or by placing barrier membranes to prevent overgrowth of epithelial cells from populating bone/PDL spaces (guided tissue regeneration). Both approaches have demonstrated efficacy in the regeneration of intrabony periodontal defects, but not in suprabony lesions, which are the most frequently affected. Therefore, these therapies fail most of the time to achieve a true regenerative outcome. This underlies the demand for more effective therapies in the management of this chronic inflammatory condition and allows envisaging the successful use in a near future of tissue-engineering approaches using

mesenchymal stem cells (MSCs), in combination with appropriate scaffolds, to achieve an efficient regeneration of tissues.

It has been clearly demonstrated that adult tissues of mesenchymal origin retain a small fraction of pluripotent mesenchymal cells able to regenerate mesenchymal tissues in case of injury or disease [6]. Although initially isolated from bone marrow (BMSC) [7, 8], MSCs have also been isolated from tissues within the oral cavity, such as periodontal ligament (PDL) [9, 10], dental pulp [11, 12], deciduous teeth [13], dental follicle [14], apical papilla [15], and gingival tissues [16, 17]. Although there is a lack of specific surface markers identifying MSCs, all of these fibroblast-like cells have shown in vitro, to fulfill the minimal criteria for MSCs, to attach to plastic surfaces, to express unique cell surface antigens, being able to self-renew and maintain their multipotential capacity, and possess the ability to differentiate into different mesenchymal cells, such as osteoblasts, adipocytes, and chondrocyes, as defined by the International Society for Cellular Therapy (ISCT) [18].

Human gingival MSCs (GMSC) in comparison to other sources of MSCs from the oral cavity have the clear advantages due to their easy accessibility and rapid wound healing of the donor area [17, 19]. It is however unknown whether GMSCs have the same growth ability and safety when compared with other intra-oral MSCs such as periodontal ligament-derived mesenchymal cells (PDLMSCs).

Similarly to experimental in vitro studies, experimental in vivo studies on the periodontal regenerative potential of these cells have shown that adipose tissue-derived mesenchymal stem cells (ADMSCs) were able to regenerate periodontum on degree III furcation lesions and intrabony defects [20, 21]. In vitro expanded PDLMSCs, from either autologous or allogeneic origin, were able to regenerate lost periodontal tissue either alone or combined with three-dimensional scaffolds [10, 22]. These cells have shown their potential to form a cementum/PDL-like structure in immunocompromised rodents [9], being their donor origin traced with appropriate markers [23, 24]. Similarly, in periodontitis models in minipigs and in beagle dogs, autologous PDLMSCs were able to regenerate surgically created periodontal defects when implanted with porous ceramic scaffolds [25, 26]. Seo and coworkers also demonstrated that PDLMSCs had the ability to develop a new periodontal ligament, including cementum, alveolar bone, and periodontal fibers [9].

It has been debated, however, whether other MSCs derived from different intraoral sources might have the same potential to regenerate a functional periodontal ligament as PDLMSCs.

It was, therefore, the purpose of this experimental investigation, to compare the safety and differentiation capabilities of human cells derived from the gingival (GMSC) with those derived from the periodontal ligament (PDLMSC).

Materials and methods

Cell isolation and culture

Tissue specimens

Four retained third molars (three maxillary and one mandibular) from systemically healthy adult individuals (two men, two women) were extracted because of infectious pathology associated with their eruption.

Four teeth from systemically healthy adult patients suffering from periodontitis (two men, two women) were extracted because of root fracture or hopeless periodontal prognosis, but with the enough periodontal attachment to allow for harvesting periodontal ligament.

Immediately after extraction, the teeth were immersed in DMEM:F12 media (Lonza, Basel, Switzerland) containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamycin (Gibco, Life Technologies, Grand Island, NY). After thorough washing of the teeth with PBS, the periodontal ligament was scrapped from the root surface with a curette (#4R4L Columbia Curette, EverEdge #9 Handle), placed in a microcentrifuge tube containing collagenase I (3 mg/ml, Sigma-Aldrich, St. Louis, MO) and dispase II (4 mg/ml, Sigma) in serum-free DMEM:F12 and incubated (30 min, 37 °C, 200 rpm). After digestion, the samples were centrifuged (7 min, 210×*g*, rt). The pellet was resuspended in complete DMEM:F12 (DMEM:F12 supplemented with 10%FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamycin, and 2 mM L-glutamine), passed through a sterile cell strainer (70 μm, Falcon-BD, Franklin Lakes, NJ) and transferred to a 25-cm^2 tissue culture flask. Media was changed every third to fourth day unless otherwise indicated.

Gingival tissue biopsies were obtained from four systemically healthy adult patients (four women with ages between 18 and 60) within the course of prescribed periodontal surgery in the palatal maxilla. These gingival specimens were completely de-epithelialized with a scalpel, which allowed the exclusion of most of the keratinocytes present in the gingiva. The connective tissue was digested as described above (1 h, 37 °C, 200 rpm).

Both PDLMSCs and GMSCs were incubated (37 °C, 5%CO_2, 95 % humidity) until they reached 80–90 % confluence. At this time, they were trypsinized (0.05 % trypsin-EDTA, Gibco) (5 min, 37 °C). The enzymatic activity was then inhibited with an excess of complete media. Cells were counted with a TTC model CASY® cell counter (150 μm Ø capillary, Roche Diagnostics, Basel, Switzerland) and seeded at 15,000 cells/cm^2 on tissue culture flasks.

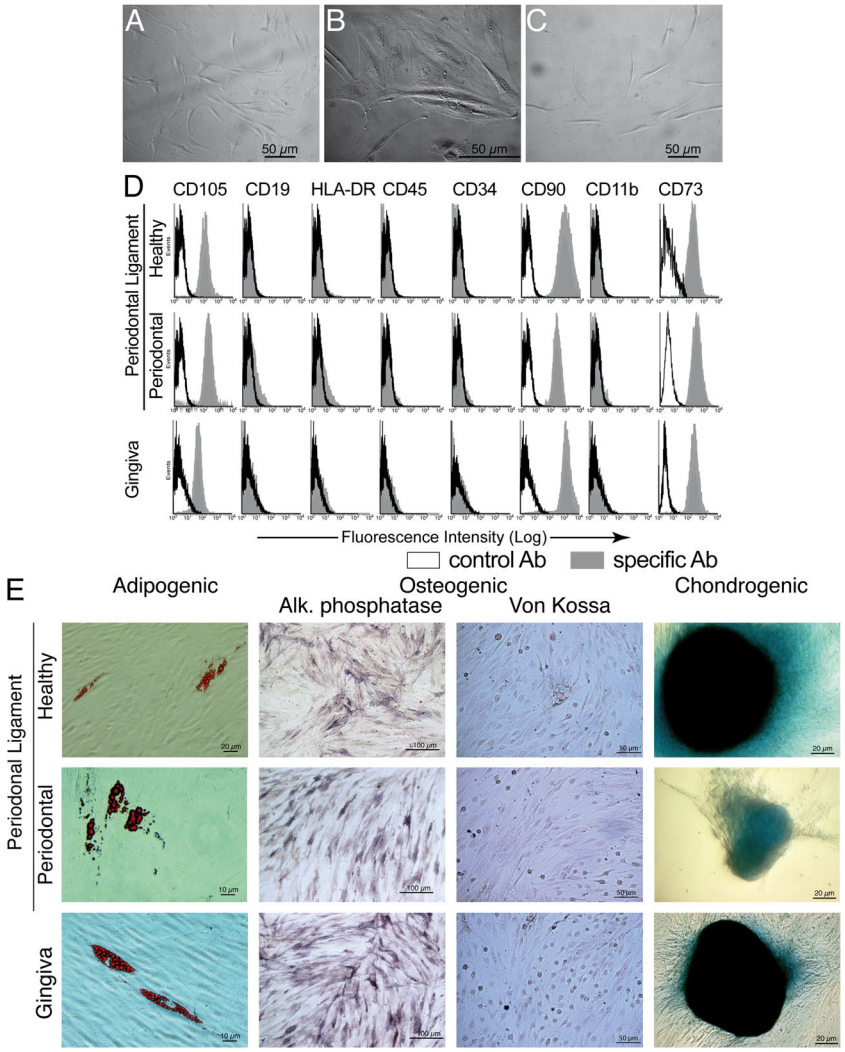

Fig. 1 Gingiva-derived and PDL-derived cells represent bona fide MSCs. Phase-contrast microscopy showing that gingiva-derived (**a**) and periodontal ligament-derived (**b**, **c**) cells attach to plastic. In the periodontal ligament-derived cells, this is true for both normal (**b**) and periodontitis-affected individuals (**c**). **d** Flow cytometry analyses of periodontal ligament-derived cells from healthy and periodontal diagnosed individuals as well as from gingiva-derived cells. Cells stained with a control isotypic antibody were used to determine cell autofluorescence (control Ab, empty histograms), whereas the staining with the specific antibodies is shown in *gray* (specific Ab). **e** The differentiation potential of periodontal ligament-derived cells from healthy (*top row*) or periodontitis-diagnosed individuals (*middle row*) was compared with the differentiation potential of gingiva-derived cells (*bottom row*). Adipogenic differentiation was revealed by Oil-Red-O staining (*left panels*), osteogenic differentiation by alkaline phosphatase staining or Von Kossa staining (*middle panels*), and chondrogenic differentiation by Alcian Blue staining of the in vitro formed chondrules (*right panels*)

Flow cytometry

After Fc-receptor blocking with 10 % goat serum (10 min, rt), single cell suspensions were labeled (20 min, 4 °C) with the following anti-human monoclonal antibodies: FITC-CD11b, FITC-CD19, FITC-CD34, FITC-CD45, APC-CD73, FITC-CD90, FITC-HLADR (Becton Dickinson), and mouse anti-CD105 [27] (mAb P4A4, a kind gift from C. Bernabeu, CIB-CSIC, Madrid, Spain). The P4A4 mAb was revealed with an Alexa488-anti-mouse IgG (Invitrogen). After two washes with PBS, flow cytometry analyses were carried out gating the living cells on a FL500 flow cytometer (Expo32 software, Coulter, Miami, FL).

In vivo tumorigenicity

Immunocompromised $Rag2^{-/-}$ mice were bred and housed at the CIB animal facility on controlled rooms (22 ± 2 °C, 40–60 % humidity), with 12 h light/12 h dark cycles and food and water provided ad libitum. All surgical procedures were performed under anesthesia with intraperitoneal injection of a mixture of ketamine (100 mg/kg) and xilacine (10 mg/kg).

For each PDLMSC or GMSC samples, 2.0×10^6 in vitro-expanded cells were transplanted subcutaneously in the back of four animals. The control group was injected with the same volume of PBS. Animals were checked at weekly intervals (6 months) to determine tumorigenicity.

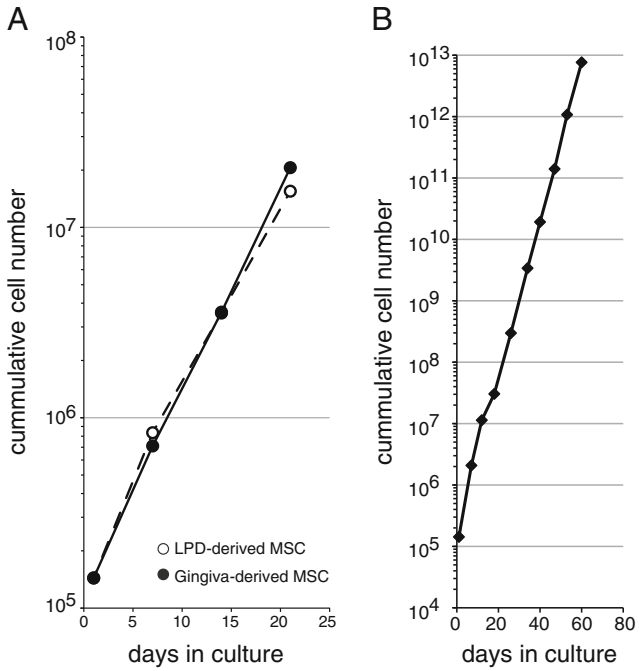

Fig. 2 Proliferation rates of PDLMSCs and GMSCs. The proliferation ability of PDLMSCs and GMSCs was determined by analyzing the cumulative cell number over time in culture (**a**). **b** Extended analysis demonstrating that, over a period of 70 days, the GMSCs maintain their proliferation rate. Cell numbers were determined by triplicate counting using a Casy® TTC cell counter; for all samples, the variability was <5 %

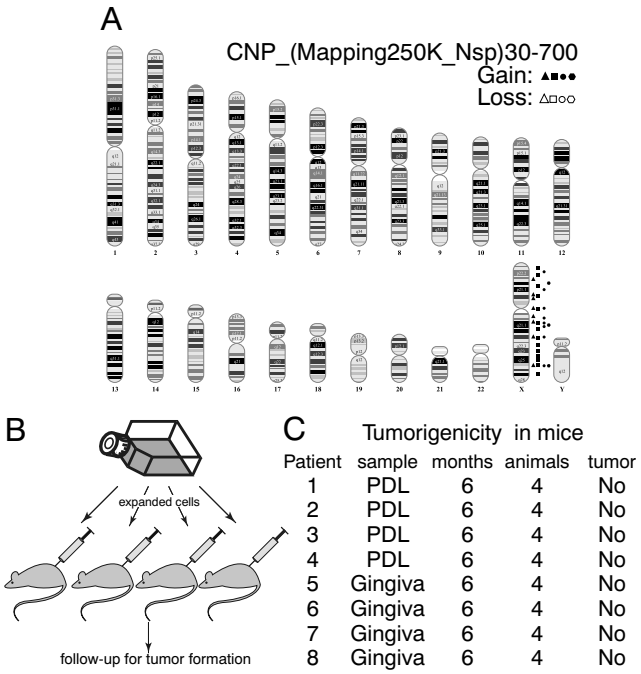

Fig. 3 Genomic stability and tumorigenicity analyses of expanded GMSCs. **a** Genomic stability of in vitro expanded GMSCs, represented as gain or loss of genomic material on a schematic representation of the human karyotype, as compared to a reference set. **b** Schematic representation of the tumorigenicity assays, where from each patient, 2×10^6 expanded cells were injected subcutaneously in immunodeficient (Rag $2^{-/-}$) mice ($n = 4$). Tumorigenicity was determined for the following six months. **c** Tabulation of the experimental results

In vitro differentiation assays

For osteogenic differentiation, exponentially growing cells ~80 % confluence were re-suspended at 3×10^4 cells/ml in NH-OsteoDiff Medium (Miltenyi Biotec), with media changes every third day. After 10 days, dishes were stained with SIGMAFAST BCIP/NBT alkaline phosphatase, osteoblasts are able to process this substrate and become dark purple. Von Kossa staining confirmed the mineralization potential of the differentiated cells.

For adipogenic differentiation, 2.1×10^4 cells/cm^2 were plated in complete DMEM:F12 until cells became confluent/post-confluent. Cultures were then subjected to three induction/maintenance cycles with Adipogenic-hMSC media (Lonza), which was replaced every 3 days alternating between induction and maintenance media. Lipids produced and accumulated in adipocytes became red when revealed by Oil Red-O staining.

For chondrogenic differentiation, we used STEMPRO® Chondrocyte differentiation media (Gibco), generating micro-mass cultures (3D chondrules), which were stained with Alcian Blue. Media was changed every third day for 14 days.

For each differentiation, control cultures were kept in complete DMEM:F12 and revealed with the same staining protocol than the corresponding experimental sample.

Genomic stability

Genomic stability was determined by hybridizing genomic DNA from the expanded cells with GeneChip® Human Mapping 250KNsp chips (Affymetrix) and comparing the data obtained for each sample with a known Affymetrix dataset. Signal variations for particular probes determined changes in copy number of the expanded samples.

Growth rates under suboptimal conditions

The contaminated and highly inflamed in vivo environment of the lesions where PDLMSC or GMSC cells would be used for periodontal regenerative therapies, was “mimicked” on in vitro assays using suboptimal growth conditions, by renewing the culture media with fresh media every 7 days instead of the usual every 3–4 days and determining the growth rate of the cells under these conditions.

Statistical analysis

Data were analyzed using SPSS 15.0 software (Lead Technologies Inc., Charlotte, NC, USA). Normal distribution of the samples was determined with the Kolgomorov-Smirnov

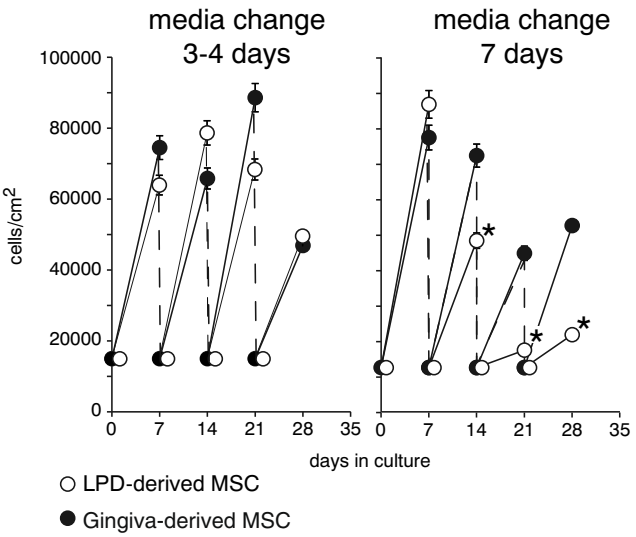

Fig. 4 Proliferation rates of PDLMSCs and GMSCs under suboptimal growth conditions. PDLMSCs and GMSCs displayed similar proliferation rates with DMEM:F12 supplemented with 10 % FCS when media changes were carried out every 3–4 days (*left panel*). However, under suboptimal growth conditions, when media changes were carried out every 7 days, the proliferation rate of GMSCs was significantly higher (days 14, 21, and 28) than PDLMSCs. Cell numbers were determined by triplicate counting using a Casy® TTC cell counter. For all samples, the data is shown as mean ± SD (*$p < 0.05$)

test. Data were analyzed using the Student's *t* test. The Pearson's correlation was determined on log-transformed data. Statistical significance was assumed when $p < 0.05$.

Results

The gingiva-derived cells were obtained by enzymatic digestion of the biopsies, rather than by explant growth [28]. Cell adherence to plastic was confirmed on the first passage. There were no detectable differences when comparing gingiva-derived or PDL-derived cells, nor when comparing cells from healthy and periodontitis-diagnosed patients (Fig. 1a–c).

Expression analyses for the phenotypic markers were carried out on passage 2, demonstrating that cells derived from gingiva or PDL had both the following phenotype: $CD73^+CD90^+CD105^+CD34^-CD45^-HLA\text{-}DR^-CD11b^-CD19^-$, irrespective whether the donors were healthy or periodontitis affected subjects (Fig. 1d). More than 97 % of the cells expressed the appropriate phenotype even at early passages, thus demonstrating that these represent phenotypically homogeneous populations.

In addition, both cells derived from gingiva or PDL from passage 3 or higher demonstrated differentiation potential into osteogenic, chondrogenic, or adipogenic lineages, failing to show relevant differences, neither on the fraction of differentiated cells nor on signal intensity (Fig. 1e). Undifferentiated controls did not stain with any differentiation marker (not shown). This differentiation potential allowed their differentiation from fibroblasts. Taken together, these data show that both cells derived from gingiva or PDL fulfilled the minimal requirements for MSCs, and therefore were referred to as PDLMSC and GMSC, respectively.

Furthermore, under optimal growth conditions, GMSCs and PDLMSCs showed a similar proliferation rate (Fig. 2a), maintaining an exponential growth for at least 70 days, as determined by cumulative proliferation indexes (Fig. 2b). These data indicated that there was no Hayflick effect on the cultures, suggesting that the cells did not get senescent (even after additional growth, data not shown).

Using GeneChip® Human Mapping 250K-Nsp (Affymetrix), we determined the GMSCs expanded cells genomic stability by comparing the results from our samples with a reference set (48 individuals, Affymetrix). With the resolution used (30 changes in a minimum of 700 kb), the only differences detected were restricted to the X chromosome (gain in all cases), due to the fact that all donors were females and the reference set contained 50 % men and 50 % women. We did not detect any duplications or deletions in other chromosomes (Fig. 3a), suggesting genomic stability in the expanded GMSCs.

The lack of tumorigenicity of the expanded cell populations was demonstrated in four samples from each GMSC and PDLMSC, each was injected subcutaneously in four immunodeficient mice (Fig. 3b). The 6-month follow-up failed to show any sign of tumor growth in any of the samples (Fig. 3c), and hMSCs were found on the scaffolds, but not in organs such as lung, spleen, liver, testis, or ovaries.

Under conditions with a limited supply of media and growth factors present in the sera, both GMSC and PDLMSC proliferated less than with the usual media changes every 3–4 days. Interestingly, unlike growth under optimal conditions where GMSCs and PDLMSCs showed the same proliferation index (Fig. 2a), under conditions with a limited supply of fresh media and growth factors, GMSCs showed a significant higher proliferation rate than PDLMSCs ($p < 0.05$) (Fig. 4).

Discussion

In this investigation, we have demonstrated that both periodontal ligament-derived cells and de-epithelialized gingiva-derived cells fulfill all the ISCT criteria characterizing MSCs [18]. Furthermore, we have shown that these MSCs have genomic stability and lack of tumorigenicity and were both able to proliferate both in optimal as well as in suboptimal conditions.

These results are in agreement with other recent investigations demonstrating the differentiation potential of cells from the periodontal ligament compartment [10, 25, 29, 30], the

dental pulp [11, 12], or from less specialized intra-oral connective tissue compartments, such as the gingiva [13, 15, 31–37].

Some authors have suggested that the differentiation potential of MSCs might depend on their origin, and their isolation for therapeutic purposes must take into account the tissue source [14, 38]. The majority of MSCs for therapeutic purposes have been obtained from either bone marrow or adipose tissue [8, 21, 39]; we have shown, however, in this study that both PDLMSCs and GMSCs demonstrate good proliferation rates in both optimal and suboptimal conditions for therapeutic purposes. The GMSCs have the additional advantage of being a very accessible donor tissue and not needing the tooth extraction for harvesting the donor cells.

It is worth to note that both PDLMSCs and GMSCs were phenotypically very homogeneous populations as early as in passage 2. The proliferation rates and cumulative cell numbers using optimal growth conditions indicated that PDLMSCs and GMSCs had similar proliferation rates, obtaining sufficient cell numbers amenable for therapeutic usage, even from small gingival biopsies. In the case of GMSCs, this might be due to the fact that in this investigation, we prepared a cell suspension by mechanical and enzymatic digestion of the biopsy, rather than using explants, as proposed by Mitrano and colleagues [17]. This technique allowed obtaining a relative large cell number, maximizing the probability of the biopsy-derived cells to attach to the plate and start proliferating.

Although we did not detect any differences in the differentiation potential of GMSCs and PDLMSCs, irrespective whether the donors had periodontal health or disease, we cannot discard differences in the frequency of stem/early progenitors between PDLMSCs and GMSCs, which need to be ascertained by appropriate clonal differentiation analyses. Furthermore, in terms of safety, both PDLMSCs and GMSCs demonstrated genetic stability and lack of tumorigenicity in immune-compromised animals, clearly indicating their amenability for therapeutic usage. These results agree with previous studies that also reported a stable genomic behavior of ex vivo expanded PDLMSCs and GMSCs [19, 40].

It is worth to note, however, that under suboptimal proliferation conditions, GMSCs displayed a higher proliferation potential than PDLMSCs, suggesting a better response of GMSCs to unfavorable culture conditions what may be implied as an increased adaptability to suboptimal conditions and may represent an advantage for therapeutic purposes.

These results are in agreement with the study from Yang and coworkers that reported that GMSCs displayed fewer inflammation-related changes than PDLMSCs when incubated with pro-inflammatory mediators (TNF-α and IL-1β) [41]. Similarly, other reports have shown that GMSCs have immunomodulatory functions that might enhance would healing [31, 35, 42].

In summary, we have shown that gingiva-derived cells represent bona fide MSC and have a similar proliferative potential than periodontal ligament-derived cells. This, together with their genetic stability and lack of tumorigenicity in immune-compromised animals suggest their potentiality for therapeutic use. Moreover, GMSCs have shown a differential capability of growth under suboptimal conditions, which together with their clear advantage in terms of accessibility and decreased morbidity for harvesting suggest the potential use of these cells for therapeutic purposes, not only in periodontal regeneration or within the oral cavity but also for regenerative medicine in general, since obtaining gingival biopsies is quite easy and with few complications, and from each biopsy, sufficient number of cells can be obtained, after expansion, for most MSC-based therapies.

There are still questions and uncertainties to be unraveled. First, periodontal tissue regeneration requires the formation of root cementum, alveolar bone, and periodontal ligament. Although there is an experimental study that confirmed histologically the ability of GMSCs to generate these tissues in vivo [43], more studies are needed to provide a higher level of evidence. Second, periodontal regeneration must reconstruct both the hard and soft tissues lost during periodontal disease; this requires the use of appropriate scaffolds providing space maintenance in a heavily contaminated oral environment. Although the use of cell therapies with MSCs may favor their biological potential in a contaminated environment due to their proven immune-suppressive [12, 32, 44, 45] and anti-inflammatory properties [32, 46], these cells must be able to seed and proliferate within the scaffold and being protected under the gingival tissues for adequate wound healing. This would require delicate surgical techniques and optimal hygienic wound healing.

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Compliance with ethical standards

Conflict of interest Author Silvia Santamaria declares that she has no conflict of interest. Author Nerea Sanchez declares that she has no conflict of interest. Author Mariano Sanz declares that he has no conflict of interest. Author Jose A. Garcia-Sanz declares that he has no conflict of interest. All the authors declare that they had full control of all primary data and agree to allow the Journal to review it if requested.

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Ethical approval All human studies have been approved by the Universidad Complutense de Madrid ethics committee for Clinical Research and have been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its latter amendments. The Universidad Complutense de Madrid bioethics committee approved the experimental protocols involving animals. The experiments involving animals have been carried following the appropriate EU and Spanish national laws.

Informed consent All persons gave their informed consent prior to their inclusion in the study by signing an IRB-approved informed consent.

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