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

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Running Title: PDLMSC and GMSC 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 aimed to compare the *in vitro* regenerative potential of undifferentiated mesenchymal stem cells from either periodontal ligament or gingival origin.

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 osteocytes, chondrocytes 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 mesenchymal stem cells to be used in future periodontal regenerative therapies

Key Words: mesenchymal stem cells; tissue engineering; periodontal ligament; gingival; periodontal regeneration.

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 promoting cell homing in a periodontal defect by placing barrier membranes (guided tissue regeneration) or by using bioactive agents (as enamel matrix derivatives), which promote new cementum formation and periodontal attachment [3-5]. Both approaches have demonstrated efficacy in intrabony periodontal defects, but not in suprabony lesions, which are the most frequently affected. Therefore, these therapies fail most 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 multi potential capacity and possess the ability to differentiate into different mesenchymal cells, such as osteoblasts, adipocytes and chondrocytes, as defined by the International Society fo 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 co-workers, 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).

MATERIAL AND METHODS

Cell Isolation and culture

Tissue specimens

Four retained third molars (3 maxillary and 1 mandibular) from systemically healthy adult individuals (2 men, 2 women) were extracted because of infectious pathology associated with their eruption.

Four teeth from systemically healthy adult patients suffering from periodontitis (2 men, 2 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 100U/ml penicillin, 100µg/ml streptomycin and 50µg/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 micro-centrifuge tube containing collagenase I (3mg/ml, Sigma-Aldrich, St. Louis, MO) and dispase II (4mg/ml, Sigma) in serum-free DMEM:F12 and incubated (30 min, 37°C, 200 rpm). After digestion the samples were centrifuged (7 min, 210 xg, rt). The pellet was re-suspended in complete DMEM:F12 (DMEM:F12 supplemented with 10%FCS, 100U/ml penicillin, 100µg/ml streptomycin, 50µg/ml gentamycin and 2mM L-Glutamine), passed through a sterile cell strainer (70µm, Falcon-BD, Franklin Lakes, NJ) and transferred to a 25cm² tissue culture flask. Media was changed every 3rd-4th day unless otherwise indicated.

Gingival tissue biopsies were obtained from four systemically healthy adult patients (4 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 fibroblasts present in the gingiva. The connective tissue was digested as described above (1hr, 37°C, 200 rpm).

Both PDLMSCs and GMSCs were incubated (37°C, 5%CO₂, 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 (150µm Ø capillary, Roche Diagnostics, Basel, Switzerland) and seeded at 15000 cells/cm² on tissue culture flasks.

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 12h light/12h dark cycles and food and water provided *ad libitum*. All surgical procedures were performed under anesthesia with intraperitoneal injection of a mixture of Ketamine (100mg/kg) and Xilacine (10mg/kg).

For each PDLMSC or GMSC samples, 2.0x10⁶ *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.

In vitro differentiation assays

For osteogenic differentiation, exponentially growing cells ~80% confluence were re-suspended at 3x10⁴ 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.

For adipogenic differentiation, 2.1x10⁴ cells/cm² were plated in complete DMEM:F12 until cells became confluent/post-confluent. Cultures were then subjected to 3 induction/maintenance cycles with Adipogenic-hMSC media (Lonza), which was replaced every 3 days alternating between induction and

1 maintenance media. Lipids produced and accumulated in adipocytes became red when revealed by Oil
2 Red-O staining.

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4 For chondrogenic differentiation we used STEMPRO® Chondrocyte differentiation media (Gibco),
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6 generating micro-mass cultures (3D-chondrules), which were stained with Alcian Blue. Media was
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8 changed every 3rd day for 14 days.
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11 For each differentiation, control cultures were kept in complete DMEM:F12 and revealed with the same
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13 staining protocol than the corresponding experimental sample.
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16 17 **Genomic stability**

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19 Genomic stability was determined by hybridizing genomic DNA from the expanded cells with
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21 GeneChip® Human Mapping 250K Nsp chips (Affymetrix) and comparing the data obtained for each
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23 sample with a known Affymetrix dataset. Signal variations for particular probes determined changes in
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25 copy number of the expanded samples.
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27 28 **Growth rates under suboptimal conditions**

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30 The contaminated and highly inflamed *in vivo* environment of the lesions where PDLMSC or GMSC
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32 cells would be used for periodontal regenerative therapies, was “mimicked” on *in vitro* assays using
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34 suboptimal growth conditions, by renewing the culture media with fresh media every 7 days instead of
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36 the usual every 3-4 days, and determining the growth rate of the cells under these conditions.
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40 41 **Statistical analysis**

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43 Data were analyzed using SPSS 15.0 software (Lead Technologies Inc., Charlotte, NC, USA). Normal
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45 distribution of the samples was determined with the Kolmogorov-Smirnov test. Data were analyzed using
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47 the Student’s t test. The Pearson’s correlation was determined on log-transformed data. Statistical
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49 significance was assumed when $p < 0.05$.
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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-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). 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).

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 700Kb), 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 4 immunodeficient mice (Fig. 3B). The 6

months follow-up, failed to show any sign of tumor growth in any of the samples (Fig. 3C).

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 [29,25,10,30], 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 two. 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 to obtain 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 co-workers that reported that GMSCs displayed fewer inflammation-related changes than PDLMSCs when incubated with pro-inflammatory mediators (TNF- α and IL-1 β) [41]. Similarly, other reports have shown that GMSCs have immune modulatoruy functions that might enhance wound healing [42,31,35].

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 in periodontal regeneration.

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 [32,44,12,45] 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.

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 and 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 Completense 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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FIGURE LEGENDS

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 periodontitis 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 (left column) or periodontitis diagnosed individuals (middle column) was compared with the differentiation potential of Gingiva-derived cells. Adipogenic differentiation was revealed by Oil-Red-O staining (top panels), osteogenic differentiation by alkaline phosphatase staining (middle panels) and chondrogenic differentiation by Alcian Blue staining of the *in vitro* formed chondrules (bottom panels).

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.

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, 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 $\pm$ SD (*, $p < 0.05$).

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Fig. 1

Figure 2
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Fig. 2

C

Tumorigenicity in mice

Patient	sample	months	animals	tumor
1	PDL	6	4	No
2	PDL	6	4	No
3	PDL	6	4	No
4	PDL	6	4	No
5	Gingiva	6	4	No
6	Gingiva	6	4	No
7	Gingiva	6	4	No
8	Gingiva	6	4	No

Figure 4
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Fig. 4