


Generation of a novel model of bioengineered human oral mucosa with increased vascularization potential

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Abstract

Objective: The aim of this study was to generate novel models of bioartificial human oral mucosa with increased vascularization potential for future use as an advanced therapies medicinal product, by using different vascular and mesenchymal stem cell sources.

Background: Oral mucosa substitutes could contribute to the clinical treatment of complex diseases affecting the oral cavity. Although several models of artificial oral mucosa have been described, biointegration is a major issue that could be favored by the generation of novel substitutes with increased vascularization potential once grafted *in vivo*.

Methods: Three types of mesenchymal stem cells (MSCs) were obtained from adipose tissue, bone marrow, and dental pulp, and their *in vitro* potential was evaluated by inducing differentiation to the endothelial lineage using conditioning media. Then, 3D models of human artificial oral mucosa were generated using biocompatible fibrin-agarose biomaterials combined with human oral mucosa fibroblasts and each type of MSC before and after induction to the endothelial lineage, using human umbilical vein endothelial cells (HUVEC) as controls. The vascularization potential of each oral mucosa substitute was assessed *in vitro* and *in vivo* in nude mice.

Results: *In vitro* induction of MSCs kept in culture was able to increase the expression of VEGF, CD31, and vWF endothelial markers, especially in bone marrow and dental pulp-MSCs, and numerous proteins with a role in vasculogenesis become over-expressed. Then, *in vivo* grafting resulted in a significant increase in blood vessels formation at the interface area between the graft and the host tissues, with significantly positive expression of VEGF, CD31, vWF, and CD34 as compared to negative controls, especially when pre-differentiated MSCs derived from bone marrow and dental pulp were used. In addition, a significantly higher number of cells committed

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to the endothelial lineage expressing the same endothelial markers were found within the bioartificial tissue.

Conclusion: Our results suggest that the use of pre-differentiated MSCs could contribute to a rapid generation of a vascular network that may favor *in vivo* biointegration of bioengineered human oral mucosa substitutes.

KEYWORDS

mesenchymal stem cells, oral mucosa, tissue engineering, vascularization

1 | INTRODUCTION

Oral disorders are very prevalent conditions affecting more than 3 billion people worldwide, with oral cancer, trauma, and severe periodontal disease representing the most prevalent and severe cases.¹ In most of these cases, treatment is highly dependent on the availability of healthy human oral mucosa for replacement therapy. In this regard, several models of three-dimensional tissue-engineered human oral mucosa have been developed, optimized, and characterized using different cells, biomaterials, and signaling molecules.^{2,3} Major applications of bioartificial oral mucosa include clinical transplantation in maxillofacial surgery, periodontics, and other related therapies.⁴ Although previously described models showed potential usefulness, one of the main clinical limitations of bioartificial tissues is the lack of vascularization, which could lead to tissue hypoxia once grafted *in vivo*, which could hinder biointegration.⁵ In this context, recent studies demonstrated the importance of generating bioartificial tissues containing a prevascular network capable of promoting an efficient supply of nutrients and oxygen after implantation.⁶

Different strategies have been developed to promote blood vessels formation in bioengineered tissues, including the incorporation of endothelial cells⁷, enriching biomaterials with endothelial growth factors⁸ or the direct injection of endothelial cells at the implantation site⁷, although none of these strategies demonstrated to be fully effective. Due to their demonstrated pro-vasculogenic properties⁵, mesenchymal stem cells (MSCs) have been proposed as a promising strategy for vascularization of artificial tissues.⁹ Interestingly, MSCs have been previously used in tissue engineering due to their immunosuppressive and anti-apoptotic properties able to favor regeneration of damaged tissues¹⁰ and their capability to act as potent regulators of the immune response.¹¹ In addition, it has been shown that MSCs are able to express several angiogenic factors such as VEGF, which could facilitate a rapid vascularization of the tissue after implantation.^{12,13} Furthermore, it is well known that different types of MSCs of the human body are highly heterogeneous.¹⁴ For this reason, specific studies capable of determining the *in vitro* and *in vivo* vascularization potential of different MSCs sources used in bioengineered oral mucosa are in need. In addition, the pro-vasculogenic role of human MSCs has not been previously demonstrated in maxillofacial and periodontal scenarios in which the use of MSCs for the generation of a human oral mucosa substitutes with improved vascularization potential could be an interesting approach.

In this work, we have developed novel models of human artificial oral mucosa based on fibrin-agarose biomaterials combined with MSCs able to differentiate to the endothelial cell lineage, in order to assess the possibility of inducing rapid vascularization and biointegration after *in vivo* implantation.

2 | MATERIALS AND METHODS

2.1 | Primary cell cultures

2.1.1 | Human oral mucosa fibroblasts (HFOM)

To generate primary cell cultures of human oral mucosa fibroblasts (HFOM), small biopsies of human oral mucosa were obtained from healthy donors subjected to minor oral surgery with local anesthesia. In brief, human oral mucosa (HOM) fibroblasts were obtained by enzymatic digestion of the HOM stroma using 2 mg/ml solution of *Clostridium histolyticum* type I collagenase (Gibco BRL) at 37°C for 6 hours with agitation. Isolated HOM fibroblasts were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich/Merck) supplemented with 10% fetal bovine serum (Sigma-Aldrich/Merck) and 1% antibiotics and antimycotics (100 U/ml penicillin G, 100 mg/ml streptomycin, and 0.25 mg/ml amphotericin B) (Sigma-Aldrich/Merck) using 75 cm² culture flasks with filter caps (Sarstedt, Nümbrecht, Germany), as previously described by the research group.^{15,16}

2.1.2 | Mesenchymal stem cells (MSCs)

To generate primary cultures of three types of human MSCs, we obtained small biopsies from adipose tissue, bone marrow, and dental pulp following previously described protocols.^{17,18,19,16} All tissue types were washed in phosphate-buffered saline (PBS) containing antibiotics and antimycotics and enzymatically digested for 6 h at 37°C using a 2 mg/ml solution of *Clostridium histolyticum* type I collagenase (Gibco BRL). Isolated MSCs from adipose tissue (ADSC), bone marrow (BMSC), and dental pulp (DPSC) were cultured in 75 cm² culture flasks with filter caps (Sarstedt) using DMEM supplemented with 10% fetal bovine serum and 1% antibiotics/antimycotics. In all cases, cells were cultured at 37°C in a humidified incubator with 5% CO₂ using standard cell culture

conditions. The culture medium was changed every 2–3 days. Once the cells reached 70% confluence, cells were dissociated with 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich/Merck).

2.1.3 | Human umbilical vein endothelial cells (HUVEC)

Human umbilical vein endothelial cells (HUVEC) were commercially obtained from the American Type Culture Collection (ATCC). HUVEC were cultured in Endothelial Growth Medium (Sigma-Aldrich/Merck) using 75 cm² culture flasks with filter caps (Sarstedt). Cells were cultured at 37°C in a humidified incubator with 5% CO₂, and the culture medium was changed every 2–3 days.

2.2 | MSCs characterization

Multilineage differentiation potential of isolated MSCs was confirmed by subculturing MSCs on chamber slides and inducing differentiation to adipogenic, chondrogenic, and osteogenic cell lineages using specific conditioning media. Histochemical analysis was performed by oil red O, alizarin red S, and alcian blue as previously described.²⁰ In addition, ADSC, BMSC, and DPSC were characterized to confirm their stemness profile before endothelial induction. For this, expression of typical MSCs markers was evaluated by flow cytometry using a Human MSC Analysis BD Stemflow™ kit (BD Biosciences). In brief, 5 × 10⁵ cells corresponding to ADSC, BMSC, and DPSC were placed in flow cytometry tubes and washed with 2 ml of staining buffer (R&D Systems Inc). Then, Fc receptors were blocked by incubating the cells for 5 min with 2 ml of PBS containing 0.1% bovine serum albumin and 0.1% FBS. Next, cells were stained with a positive cocktail (FITC CD90, PerCP-Cy CD105, and APC CD73) and a negative MSC cocktail (PE CD45, PE CD34, PE CD11b, PE CD19, and PE HLA-DR) and incubated for 45 min at 4°C in darkness. Once cells were labeled, cells were placed in a staining buffer and analyzed using a FACSCalibur flow cytometer (BD Biosciences).

2.3 | Endothelial differentiation of MSCs

To induce the endothelial differentiation of non-differentiated ADSC, BMSC, and DPSC (called ndADSC, ndBMSC, and ndDPSC, respectively), these cells were cultured for 21 days in an endothelial differentiation medium composed by 199 medium (Sigma-Aldrich/Merck) supplemented with 20% FBS, 1.5% endothelial growth supplement from bovine neural tissue, and 1.5% antibiotics and antimycotics (100 U/ml penicillin G, 100 mg/ml streptomycin, and 0.25 mg/ml amphotericin B) as previously reported.²¹ The culture medium was changed three times a week, and subconfluent cells were dissociated using 0.25% EDTA (all reagents, from Sigma-Aldrich/Merck). Cells induced with endothelial differentiation medium for 21 days

were called differentiated cells (dADSC, dBMSC, and dDPSC, respectively).

2.4 | In vitro immunofluorescence analysis

To identify the expression of relevant endothelial differentiation markers in ADSC, BMSC, and DPSC, we carried out immunofluorescence analyses using specific antibodies anti-vascular endothelial growth factor (VEGF), anti-CD31 (PECAM-1), anti-von Willebrand Factor (vWF), and anti-CD34. These analyses were carried out on native, non-differentiated, and differentiated MSCs. HUVEC was used as a control group. First, 10⁴ cells were cultured in chamber slides (Nunc™ Lab-Tek™ Thermo Fisher Scientific), fixed with 70% ethanol, washed in PBS, and blocked for 30 min with normal horse serum and casein (Vector Laboratories). Then, samples were incubated for 1 h with primary antibodies with a dilution 1:100 for anti-VEGF (Abcam), 1:500 for anti-CD31 (PECAM-1; Abcam), 1:100 for anti-vWF (Abcam), and 1:2500 for anti-CD34 (Abcam), washed in PBS, and incubated with secondary antibodies for 1 h at room temperature using FITC-conjugated anti-mouse (Sigma-Aldrich/Merck) or CY3-conjugated anti-rabbit antibodies (Sigma-Aldrich/Merck). All study samples and controls were counterstained with DAPI (Vector Laboratories) and analyzed using an Eclipse 90i microscope (Nikon). Results were evaluated by three independent histologists using a semiquantitative method scoring the signal as strongly positive (+++), positive (++), mildly positive (+), slightly positive (+/-), or negative (-).

2.5 | Expression analysis of vascularization-related proteins

Expression of relevant proteins playing a role in promoting vascularization was assessed in primary cell cultures of non-differentiated and differentiated ADSC, BMSC, and DPSC using a human angiogenesis array kit (R&D Systems Inc.). First, 10⁶ cells of each type were harvested and mixed with 1.0 ml of MCL1 mammalian cell lysis extraction buffer (Sigma-Aldrich/Merck) containing 20% of Tris-EDTA, NaCl, SDS (lauryl sulfate), DOC (deoxycholic acid), Igepal CA, and 1% of protease inhibitor cocktail. Cell lysates were then centrifuged at 10,000 g for 10 min at 4°C, and the supernatant was collected. Protein concentration was measured using a Pierce BCA-200 Assay Kit (Thermo Fisher Scientific). Then, the protein array membranes were blocked with 2.0 ml of the buffer included in the kit, and 1.0 ml of protein extract corresponding to each type of sample was added to the membrane at final concentration of 300 µg/ml. After this, all samples were treated with 15 µl of reconstituted detection antibody cocktail, washed, and incubated with 2.0 ml of Streptavidin-HRP. Finally, samples were washed and Chemi Reagent Mix was spread all over the membranes and incubated for one minute at room temperature prior to X-ray film exposition for 10 min. Films were scanned,

and signal intensity was quantified at each spot using Image J software (Wayne Rasband, NIH). Analyses were carried out using triplicates ($n = 3$).

2.6 | Generation of novel models of human oral mucosa stroma with pro-vascularization potential

To determine the vascularization potential of each cell type in a three-dimensional tissue substitute generated by tissue engineering, we generated novel models of human oral mucosa stroma (HOM) based on the previously described fibrin-agarose biomaterial. In brief, hydrogels were developed with a mixture of human plasma combined with type VII agarose and cultured cells, as previously described.^{22,15} To prevent gel fibrinolysis, the mixture was supplemented with tranexamic acid Amchafibrin™ (Fides Ecopharma), and 1% calcium chloride was added at the final step to induce polymerization. This mixture was aliquoted on 6-well plates with 24 mm Transwell Permeable Supports (Corning Life Sciences). Four different models were generated: 1) artificial oral mucosa stroma containing 5×10^5 oral mucosa fibroblasts (HOM-HFOM). This is the previously developed bioartificial tissue and was used as negative control, 2) artificial oral mucosa stroma containing 2.5×10^5 oral mucosa fibroblasts and 2.5×10^5 HUVEC (HOM-HUVEC). This tissue substitute was used as positive control; 3) artificial oral mucosa stroma containing 2.5×10^5 oral mucosa fibroblasts and 2.5×10^5 non-differentiated MSCs (HOM-ndADSC, HOM-ndBMSC, or HOM-ndDPSC); and 4) artificial oral mucosa stroma containing 2.5×10^5 fibroblasts and 2.5×10^5 differentiated MSCs (HOM-dADSC, HOM-dBMSC, or HOM-dDPSC) (Figure 1).

2.7 | In vivo evaluation of bioengineered human oral mucosa models

To determine the *in vivo* vascularization potential of each model of bioengineered oral mucosa stroma, HOM models were grafted on immune-deficient *Foxn1^{nu-/-}* athymic mice. Shortly, mice were anesthetized using a mixture of 0.001 mg/g of body weight

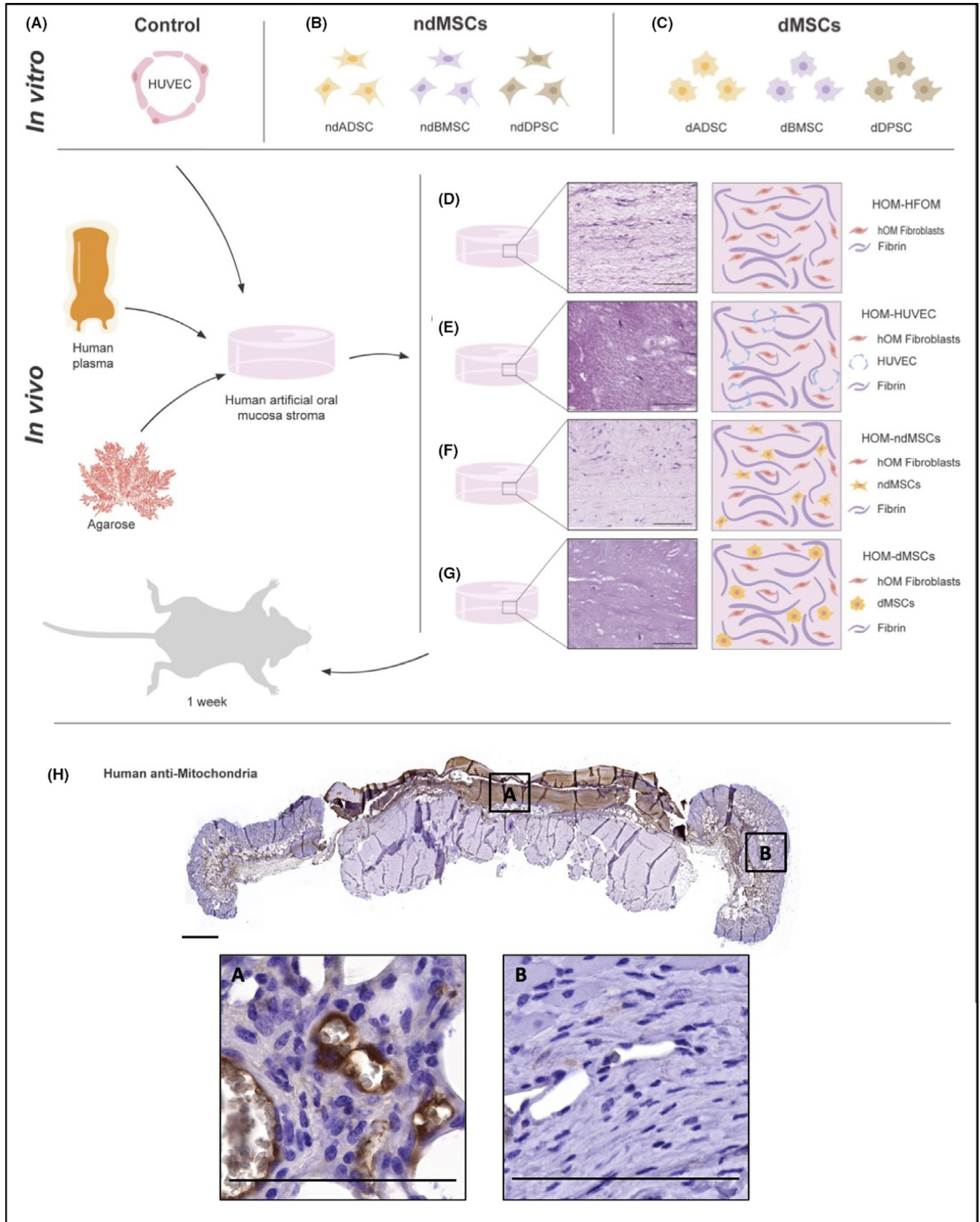
of acepromazine—Calmo-Neosan™—(Boehringer Ingelheim,) and 0.15 mg/g of body weight of ketamine—Imalgene 1000—(Merial labs). Then, a skin area of 24 mm² was surgically removed from the interscapular area of each animal and the bioengineered oral mucosa stroma models were trimmed to the same diameter. Samples were grafted at the injury site using absorbable suture material, and a plastic ring was implanted and sutured at the injury borders to prevent border contraction. All mice were euthanized one week after implantation of the bioengineered tissue for histological and immunohistochemical analyses.

2.8 | Histology and immunohistochemistry

Tissues were fixed in 4% formaldehyde and embedded in paraffin in order to obtain 5 μm-thick histological sections. Tissue sections were dewaxed, rehydrated, and stained with hematoxylin and eosin (HE) for histological analysis.

First, the localization and distribution of the human cells grafted in the animals were determined by immunohistochemistry for the anti-human mitochondria-specific antigen using anti-Mitochondria clone 113-1 antibodies (Sigma-Aldrich/Merck), using a 1:80 dilution of the primary antibody. Then, to evaluate the *in vivo* vascularization potential of each HOM model, an immunohistochemical analysis was performed using the well-established markers of endothelial cells and blood vessels^{23,24} anti-VEGF (Abcam) with 1:100 dilution, anti-CD31 (PECAM-1, Abcam) with 1:500 dilution, anti-vWF (Abcam) with 1:100 dilution, and anti-CD34 with 1:2500 dilution as were previously used for *in vitro* analysis. In all cases, tissue sections were deparaffinized using xylene and rehydrated through a graded series of alcohols (100%, 96%, 70%, 50%, and water) before antigen retrieval with 0.01 M citrate buffer, pH 6.0, and 0.01 M EDTA buffer, pH 8.0, at 95°C. Then, samples were prehybridized for 30 min with normal horse serum and casein (Vector Laboratories). Then, samples were incubated overnight with primary antibodies at 4°C, washed with PBS, and incubated with secondary antibodies for 1 h at room temperature. The complex epitope-antibody was detected using 3,3'-diaminobenzidine -DAB- (Vector Laboratories), and tissue sections were counterstained for 20 s using Harry's hematoxylin. In all cases, images were obtained using a Panoramic® DESK II DW scanner (3D Histotech).

FIGURE 1 Schematic representation of the experimental groups used in the present work. (A) *In vitro* control group of human umbilical vein endothelial cells (HUVEC) kept in culture. (B) *In vitro* non-differentiated MSCs (ndMSCs), including adipose stem cells (ndADSC), bone marrow stem cells (ndBMSC), and dental pulp stem cells (ndDPSC) kept in culture. (C) *In vitro* differentiated MSCs (dMSCs), including adipose stem cells (dADSC), bone marrow stem cells (dBMSC), and dental pulp stem cells (dDPSC) kept in culture. (D) Generation of negative control bioartificial human oral mucosa substitutes (HOM-HFOM) containing oral mucosa fibroblasts (hOM Fibroblasts). (E) Generation of positive control bioartificial human oral mucosa substitutes (HOM-HUVEC) containing human umbilical vein endothelial cells (HUVEC) and hOM Fibroblasts. (F) Generation of bioartificial human oral mucosa substitutes (HOM-ndMSCs) containing non-differentiated MSCs and hOM Fibroblasts. (G) Generation of bioartificial human oral mucosa substitutes (HOM-dMSCs) containing differentiated MSCs and hOM Fibroblasts. (H) Immunohistochemical analysis of anti-human Mitochondria in bioartificial human oral mucosa substitutes implanted on athymic mice. Peripheral areas of the injury site corresponding to mouse host tissue are squared in green. Human oral mucosa substitutes implanted on the athymic mice are squared in orange. Higher magnification images showing human origin cells (in brown) and host mouse cells (in blue), along with blood vessels showing positive signal (white arrows) and negative signal (black arrows) are shown in panels to the right. Scale bars: 100 μm



For each vascular marker, microvessel density (MVD) was measured by quantifying the number of blood vessels per mm^2 of tissue surface at the vascular interphase area of each sample type. In

addition, the number of cells showing positive signal was also quantified at the cellular area of the grafted tissues to determine the presence of cells committed to the endothelial lineage.

2.9 | Statistical analysis

To analyze the protein expression results obtained with the human angiogenesis array kit, we first determined the average expression found for each type of sample and calculated the fold-change value of differentiated cells vs. non-differentiated cells of the same cell type (for instance, dADSC vs. ndADSC). Then, a rank test was applied to compare the results obtained for differentiated cells vs. non-differentiated cells of the same cell type (for instance, dADSC vs. ndADSC). Proteins showing 1) an increase of at least 20% expression upon differentiation (fold-change >1.20) and 2) a *p* value below 0.05 for the rank test were selected as statistically significant.

For the analysis of vascular markers, quantification results of blood vessels or cells showing positive signal for each marker were compared with negative control HOM-HFOM and positive control HOM-HUVEC using the Pairwise Mann-Whitney exact test, since samples did not fulfill normality criteria. *p* values below 0.05 were considered statistically significant, and tests were performed double-tailed. These comparisons were carried out using the Real Statistics Resource Pack software (Release 7.2) available at www.real-statistics.com.

2.10 | Research committee approval and ethics statement

This study and the donation of the tissues used in this work (oral mucosa and tissues rich in MSC) were approved by the Research and Ethics Committee in Biomedical Research of Granada (*Comité Coordinador de Ética de la Investigación Biomédica*, ref. 0116-N-19, approval date 29/05/2019). All tissue donors provided informed consent. Animal experimentation was approved by the Animal Experimentation Ethics Committee of Granada (*Comité de Ética y Experimentación Animal*, CEEA) and *Consejería de Agricultura, Ganadería, Pesca y Desarrollo Sostenible*, Junta de Andalucía, Spain, protocol code 08/07/2019/123, date of approval January, 10/09/2019.

3 | RESULTS

3.1 | Phenotype characterization of human MSCs

Histological evaluation of MSCs cultures (ADSC, BMSC, and DPSC) demonstrated that these cells were able to adhere to the culture flask surface, and the typical mesenchymal spindle-shape, elongated morphology. Specific conditioning media revealed that MSCs used in this study were able to differentiate into adipogenic, chondrogenic, and osteogenic cell lineages, as determined by alizarin red S, alcian blue, and oil red O staining (Figure 2). Flow cytometry characterization of native MSCs cultures demonstrated that ndADSC, ndBMSC, and ndDPSC were positive for CD73, CD90, and CD105 markers. The analysis of CD73 marker showed 99.7% positive cells in ADSC,

95.4% in BMSC, and 99.3% in DPSC, whereas CD90 was positive in 85.2% of ndADSC, 87.0% of ndBMSC, and 97.8% of ndDPSC, and CD105 was positive in 84.8% ndADSC, 89.7% ndBMSC, and 97.3% ndDPSC. Negative cocktail markers (CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA-DR) were absent in all mesenchymal stem cell types (Figure 2).

3.2 | In vitro differentiation of human MSCs

3.2.1 | Immunofluorescence assay

Semiquantitative analysis of several markers of endothelial differentiation revealed that HUVEC expressed VEGF, CD31, and vWF, but were negative for CD34. In the case of non-differentiated MSCs, most markers were negative, except for ndBMSC, which had mildly positive expression of VEGF and vWF. Moreover, ndDPSC revealed slightly positive vWF expression. When MSCs were induced *in vitro*, we found an increase in the expression of VEGF, CD31, and vWF. For VEGF, we found a mildly positive signal in dADSC and a positive signal in dBMSC and dDPSC, whereas CD31 was slightly positive in dBMSC, and dDPSC and vWF showed strongly positive signal in dADSC and positive signal in dBMSC and dDPSC, and CD34 was negative in all cell types (Figure 3; Table 1).

3.2.2 | Protein expression analysis

Several proteins became upregulated in cells subjected to *in vitro* induction. As shown in Figure 4, 11 out of the 55 proteins analyzed (20%) were upregulated in dDPSC, including Amphiregulin, Artemin, EG-VEGF, Endoglin, FGF basic, HGF, IGFBP-2, IGFBP-3, LAP (TGF- β 1), Platelet Factor 4 (PF4), and VEGF, with three of these proteins (Amphiregulin, PF4, and VEGF) showing more than 100-fold expression upon differentiation. Eight proteins (14.5%) were significantly upregulated in dBMSC, including CXCL16, Coagulation Factor III, EGF, FGF-4, LAP (TGF- β 1), Serpin F1, TIMP-1, and TIMP-4. Interestingly, 6 of the 8 proteins upregulated in dBMSC were also found to be overexpressed in differentiated dADSC, which showed 10 proteins (18.2%) upregulated upon induction, including CXCL16, EGF, FGF-4, IL-1 β , PF4, Serpin F1, Thrombospondin-2, TIMP-1, TIMP-4, and Vasohibin.

3.3 | In vivo evaluation of the vascularization potential of the novel models of HOM

3.3.1 | Histological and immunohistochemical analysis

All artificial HOM models grafted *in vivo* were properly biointegrated in the host animal, and we did not find any microscopical signs of hemorrhage, tumorigenesis, rejection, infection, or other side effects linked to the grafted tissues.

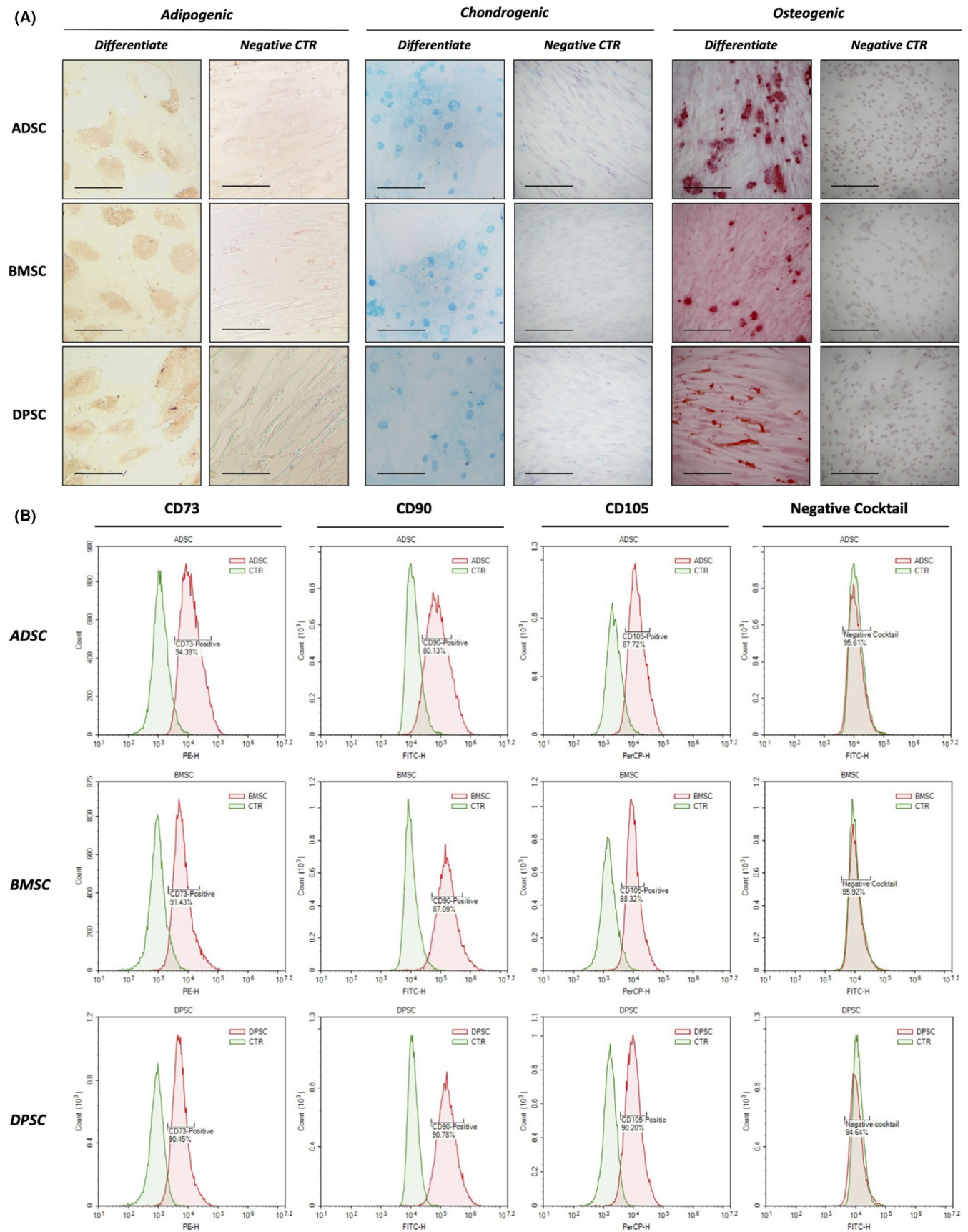


FIGURE 2 Phenotype characterization of human MSCs. (A) Multilineage differentiation capabilities of ADSC, BMSC, DPSC after adipogenic, chondrogenic, and osteogenic induction Scale bars: 100 μ m. (B) Evaluation of the stemness profile of MSCs by the detection of CD73, CD90, and CD105 markers by flow cytometry

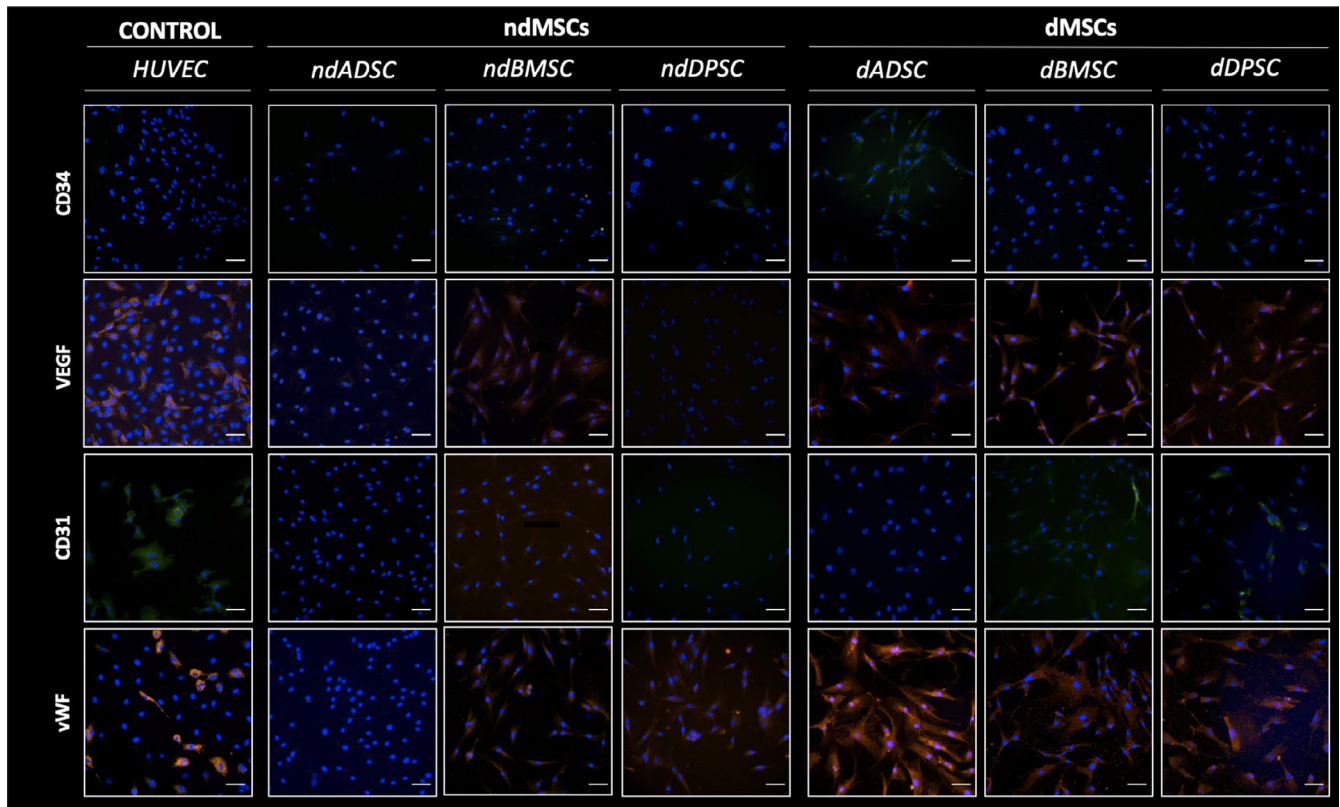


FIGURE 3 *In vitro* characterization of human umbilical vein endothelial cells (HUVEC), non-differentiated MSCs (ndMSCs) and differentiated MSCs (dMSCs) using CD34, VEGF, CD31 and vWF immunofluorescence. ndADSC: non-differentiated adipose stem cells; ndBMSC: non-differentiated bone marrow stem cells; ndDPSC: non-differentiated dental pulp stem cells; dADSC: differentiated adipose stem cells; dBMSC: differentiated bone marrow stem cells; dDPSC differentiated dental pulp stem cells. Scale bars: 50 μ m

TABLE 1 Semiquantitative analysis of relevant vascular markers in human umbilical vein endothelial cells (HUVEC), non-differentiated MSCs (ndMSCs), and MSCs differentiated *in vitro* (dMSCs). Expression was classified as strongly positive (+++), positive (++) , mildly positive (+), slightly positive (+/-), or negative (-)

	HUVEC	ndADSC	ndBMSC	ndDPSC	dADSC	dBMSC	dDPSC
VEGF	+	-	+	-	+	++	++
CD31	+	-	-	-	-	+/-	+/-
vWF	+++	-	+	+/-	+++	++	++
CD34	-	-	-	-	-	-	-

As shown in Figure 5, histological analysis of grafted HOM revealed the presence of three histological zones at the grafting site (zones A, B, and C). Zone A corresponded to the remaining artificial oral mucosa stroma substitute implanted in the animals. Zone B corresponded to the rest of the grafted HOM containing abundant cells of the bioartificial tissue. Finally, zone C represented the interface area between the native tissues belonging to the host animals and the grafted HOM, and contained numerous blood vessels. Interestingly, cells tended to concentrate at specific zones, especially at zone B, followed by zone C, while very few cells were found in zone A.

In order to confirm the human origin of HFOM, HUVEC, and MSCs implanted in mice, samples were analyzed using anti-human mitochondria antigen antibodies. Results revealed a positive

expression in the grafted tissue, whereas peripheral host tissues showed negative signal (Figure 1 and supplementary Figure S1).

3.3.2 | Analysis of vascular markers by Immunohistochemistry

To determine the vascularization potential of the novel models of HOM, we analyzed the presence of structures showing positive signal for each specific marker in each tissue type grafted *in vivo* (Figures 6-10). In general, zone A was negative for the analyzed markers, whereas zone B contained numerous positive cells and zone C was rich in vessels showing positive immunohistochemical staining and was used to determine MVD. In addition, the use of

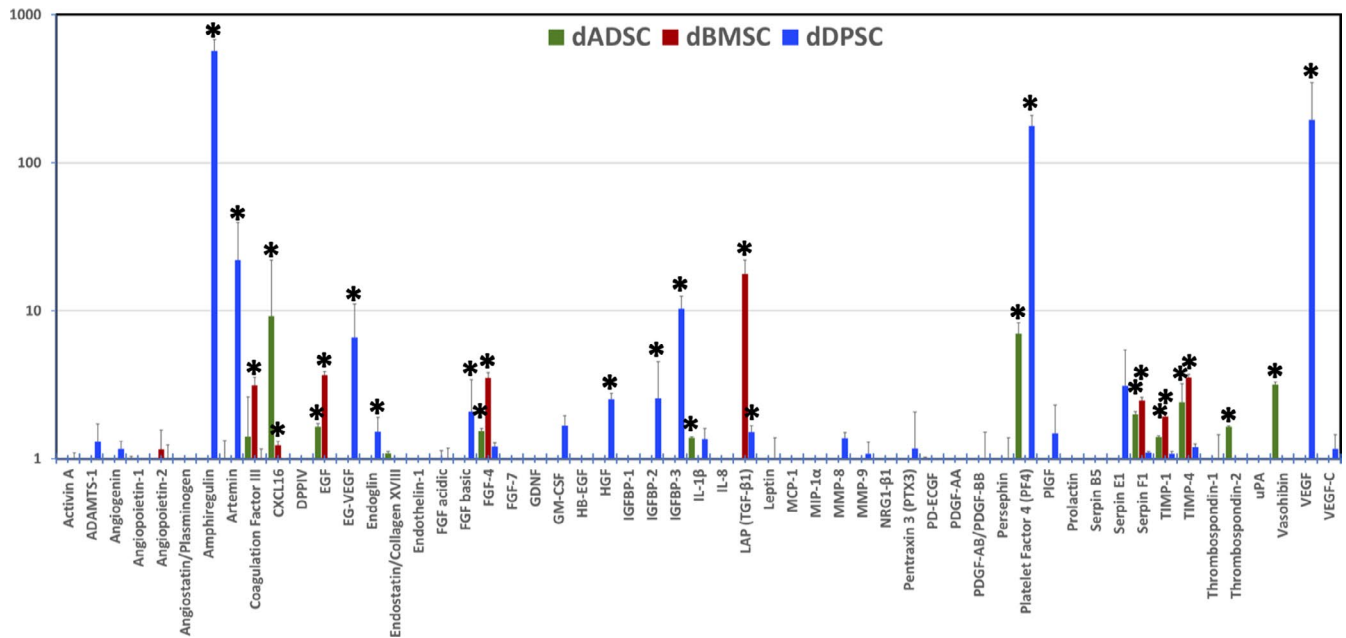


FIGURE 4 Proteins upregulated in dADSC, dBMSC, and dDPSC subjected to *in vitro* endothelial differentiation using inductive endothelial culture media. For each protein, the average fold-change expression of non-differentiated ndMSCs versus differentiated dMSCs is shown for each cell type. Error bars correspond to standard deviations. Statistically significant differences of non-differentiated MSCs versus differentiated MSCs are highlighted with asterisks (*)

anti-human mitochondria antigen antibodies allowed us to find that some of these blood vessels, approximately half of them, were of human origin, whereas other vessels consisted of host mouse cells (Figure 1).

First, analysis of MVD in zone C using VEGF markers (Figures 6 and 10) showed that the number of vessels found in HOM-HUVEC positive controls was significantly higher than HOM-HFOM negative controls ($p = 0.0188$). For HOM generated with non-differentiated MSCs, only HOM-ndBMSC showed lower MVD than positive controls ($p = 0.0106$), while HOM-ndDPSC was significantly higher than negative controls ($p = 0.0244$). HOM-ndADSC, HOM-ndDPSC, and all HOM tissues generated with differentiated cells were comparable to positive controls ($p > 0.05$ for all of them). Interestingly, MVD was significantly higher in HOM-dBMSC as compared to HOM-ndBMSC ($p = 0.0188$) and in HOM-dDPSC as compared to HOM-ndDPSC ($p = 0.0244$). When VEGF-positive cells were quantified in zone B, we found very few positive cells in HOM-HFOM negative controls and in HOM-ndDPSC and very high number in HOM-HUVEC positive controls, with differences being statistically significant ($p < 0.0001$ for HOM-HFOM vs. HOM-HUVEC and $p = 0.0001$ for HOM-ndDPSC vs. HOM-HUVEC). HOM generated with non-differentiated MSCs showed significant differences with positive controls ($p = 0.0019$ for HOM-ndADSC, $p < 0.0001$ for HOM-ndBMSC and $p = 0.0001$ for HOM-ndDPSC), and HOM-ndADSC ($p = 0.0019$) and HOM-ndBMSC ($p = 0.0142$) were also significantly higher than negative controls. Finally, HOM containing differentiated MSCs showed statistical differences with negative controls ($p < 0.0001$ for HOM-dADSC and HOM-dDPSC and $p = 0.0019$ for HOM-dBMSC), but were comparable to positive controls ($p > 0.05$) and were significantly higher than

HOM containing non-differentiated MSCs ($p = 0.0019$ for HOM-ndADSC vs HOM-dADSC and for HOM-ndBMSC vs HOM-dBMSC, and $p < 0.0001$ for HOM-ndDPSC vs. HOM-dDPSC).

For CD31 (Figures 7 and 10), we found that the MVD in zone C was significantly lower in negative controls ($p = 0.0002$) and HOM generated with non-differentiated MSCs ($p < 0.0001$ for HOM-ndADSC, $p = 0.0008$ for HOM-ndBMSC, and $p = 0.0002$ for HOM-ndDPSC) than in positive controls, with non-significant differences among these samples. For HOM containing differentiated MSCs, HOM-dADSC ($p = 0.0028$) and HOM-dDPSC ($p = 0.0106$) contained significantly lower number of vessels than positive controls, although HOM-dBMSC was similar to positive controls ($p > 0.05$). HOM-dBMSC and HOM-dDPSC were significantly higher than negative controls ($p = 0.0106$ and $p = 0.0142$, respectively). HOM-dADSC and HOM-dDPSC showed significantly higher MVD than HOM-ndADSC ($p = 0.0003$) and HOM-ndDPSC ($p = 0.0188$). At zone B, the number of CD31-positive cells was significantly higher in positive controls than in negative controls ($p < 0.0001$). HOM containing non-differentiated MSCs had significantly fewer cells than positive controls ($p < 0.0001$ for all comparisons), although HOM-ndBMSC and HOM-ndDPSC were significantly higher than negative controls ($p < 0.0001$). For differentiated HOM, we found a significant increase in positive cells, and HOM-dBMSC and HOM-dDPSC became comparable to positive controls ($p > 0.05$).

Analysis of MVD as determined by vWF immunostaining of vessels found in zone C (Figures 8 and 10) revealed again that the number of vWF-positive cells was higher in positive controls as compared to negative controls ($p < 0.0001$). HOM with non-differentiated MSCs had lower number of vessels than positive controls ($p < 0.0001$ for

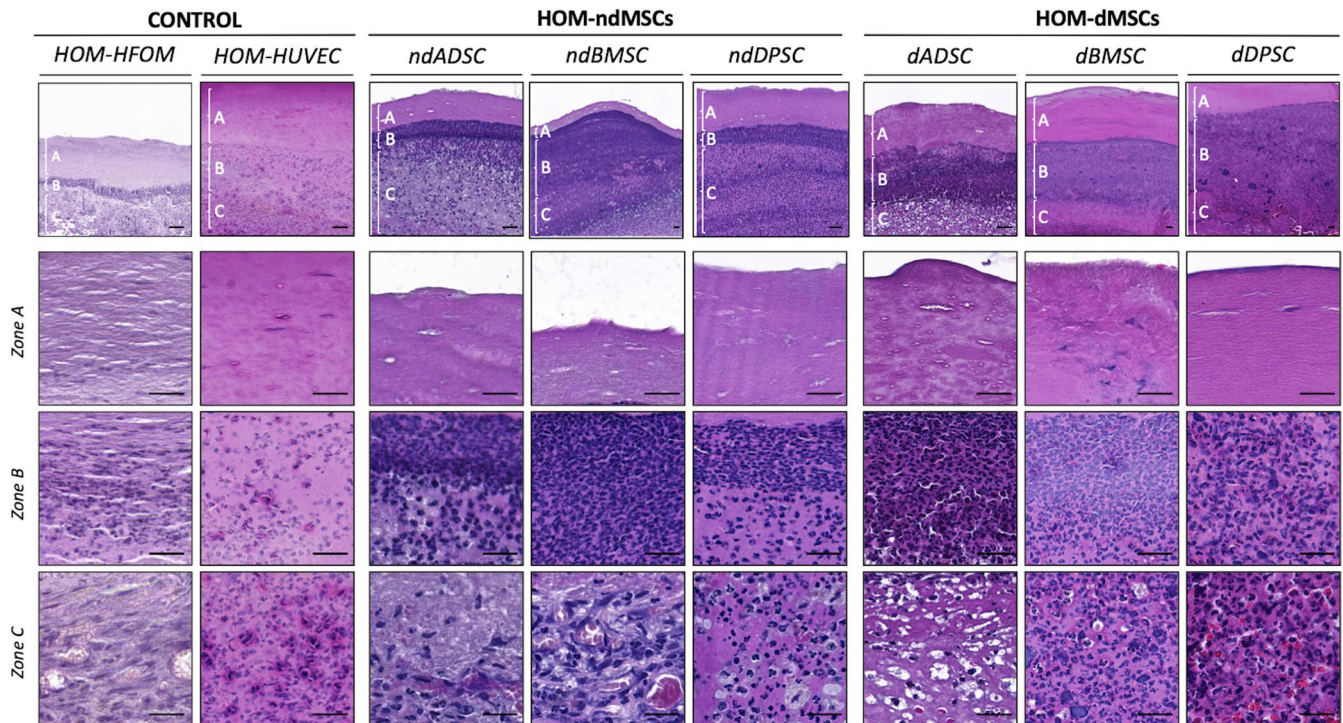


FIGURE 5 Histological analysis of the different bioartificial human oral mucosa models generated in the present work after one week of *in vivo* follow-up using hematoxylin and eosin staining (HE). For each sample, low-magnification images are shown on top, and the three zones (A, B, and C) identified in each sample are shown at higher magnification in the lower panels. HOM-HFOM: negative control group containing human oral mucosa fibroblasts; HOM-HUVEC: positive control group containing human umbilical vein endothelial cells and oral mucosa fibroblasts; HOM-ndMSCs: human oral mucosa substitutes containing non-differentiated MSCs and oral mucosa fibroblasts, including non-differentiated adipose stem cells (ndADSC), non-differentiated bone marrow stem cells (ndBMSC) and non-differentiated dental pulp stem cells (ndDPSC); HOM-dMSCs: human oral mucosa substitutes containing differentiated MSCs and oral mucosa fibroblasts, including differentiated adipose stem cells (dADSC), differentiated bone marrow stem cells (dBMSC), and differentiated dental pulp stem cells (dDPSC). Scale bars: 20 μ m

HOM-ndADSC, $p = 0.0142$ for HOM-ndBMSC $p = 0.0315$ for HOM-ndDPSC), and HOM-ndBMSC and HOM-ndDPSC were significantly higher than negative controls ($p = 0.0244$ and $p = 0.0001$, respectively). Upon differentiation, we found a significant increase of MVD in HOM-dADSC ($p = 0.0005$) and HOM-dBMSC ($p = 0.0244$), and HOM-dBMSC and HOM-dDPSC became comparable to positive controls ($p > 0.05$). Regarding the number of vWF-positive cells found at zone B, positive controls were significantly higher than negative controls ($p < 0.0001$), and HOM with non-differentiated MSCs showed significantly fewer vWF-positive cells than positive controls ($p < 0.0001$ for HOM-ndADSC, $p = 0.0004$ for HOM-ndBMSC $p = 0.0048$ for HOM-ndDPSC) and significantly more positive cells than negative controls ($p = 0.0400$ for HOM-ndADSC, $p = 0.0004$ for HOM-ndBMSC $p = 0.0004$ for HOM-ndDPSC). For HOM containing differentiated cells, we found a significant increase of vWF-positive cells in HOM-dDPSC as compared to HOM-ndDPSC ($p = 0.0004$), and HOM-dBMSC and HOM-dDPSC became statistically similar to positive controls ($p > 0.05$).

Finally, each type of sample was evaluated using CD34 markers (Figures 9 and 10 and Supplementary Figure S1). Results showed that MVD at zone C was very high in positive controls and very low in negative controls ($p = 0.0001$ for the comparison of both controls). The number of CD34-positive vessels found in HOM with

non-differentiated cells was significantly higher than negative controls ($p < 0.0001$ for all types of HOM with non-differentiated cells), but significantly lower than positive controls ($p = 0.0152$ for HOM-ndADSC, $p = 0.0055$ for HOM-ndBMSC $p = 0.0206$ for HOM-ndDPSC). When differentiated cells were used, HOM-dBMSC and HOM-dDPSC became similar to positive controls ($p > 0.05$), but HOM-dADSC was lower than positive controls ($p = 0.0360$). At zone B, positive controls had more CD34-positive cells than negative controls ($p = 0.0004$). When HOM containing non-differentiated cells were analyzed, we found very few positive cells in HOM-ndADSC ($p = 0.0008$ vs. positive controls), but the number of CD34-positive cells found in HOM-ndBMSC and HOM-ndDPSC was not significantly lower than positive controls ($p > 0.05$). Then, HOM generated with differentiated MSCs showed a trend to show a higher number of CD34-positive cells than positive control, which reached statistical significance in the case of HOM-dADSC ($p = 0.0238$).

4 | DISCUSSION

Biofabrication methods applied to tissue engineering have been significantly improved over the past few years. However, vascularization is still one of the main limitations of bioengineered tissues.

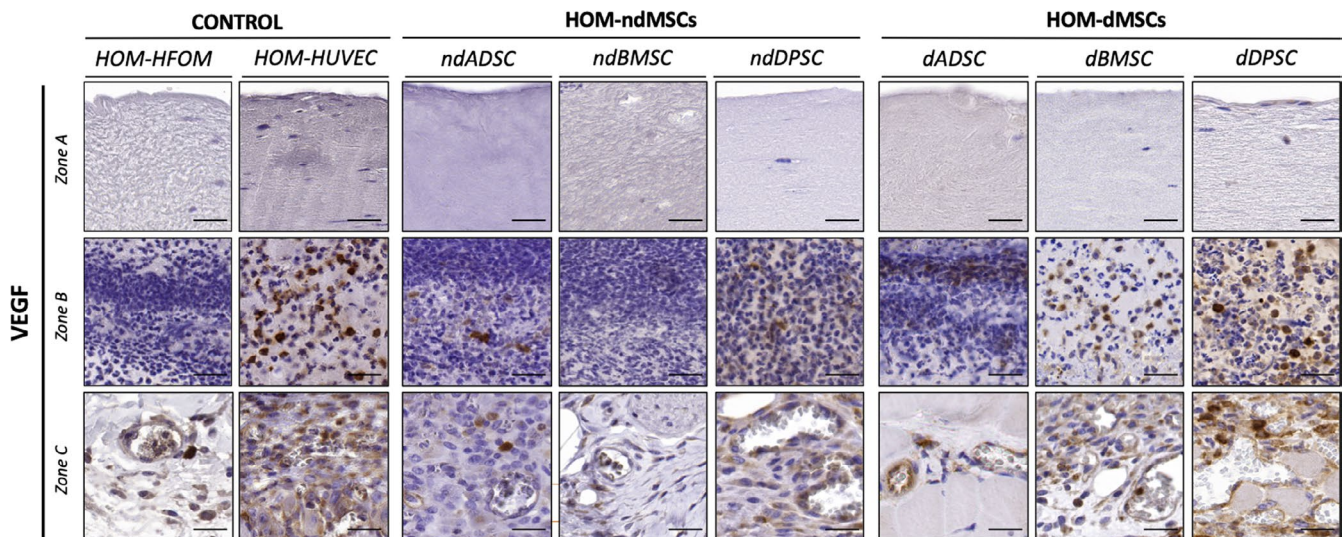


FIGURE 6 Immunohistochemical analysis of VEGF expression in each sample grafted in laboratory animals for one week. HOM-HFOM: negative control group containing human oral mucosa fibroblasts; HOM-HUVEC: positive control group containing human umbilical vein endothelial cells and oral mucosa fibroblasts; HOM-ndMSCs: human oral mucosa substitutes containing non-differentiated MSCs and oral mucosa fibroblasts, including non-differentiated adipose stem cells (ndADSC), non-differentiated bone marrow stem cells (ndBMSC) and non-differentiated dental pulp stem cells (ndDPSC); HOM-dMSCs: human oral mucosa substitutes containing differentiated MSCs and oral mucosa fibroblasts, including differentiated adipose stem cells (dADSC), differentiated bone marrow stem cells (dBMSC), and differentiated dental pulp stem cells (dDPSC). Scale bars: 20 μ m

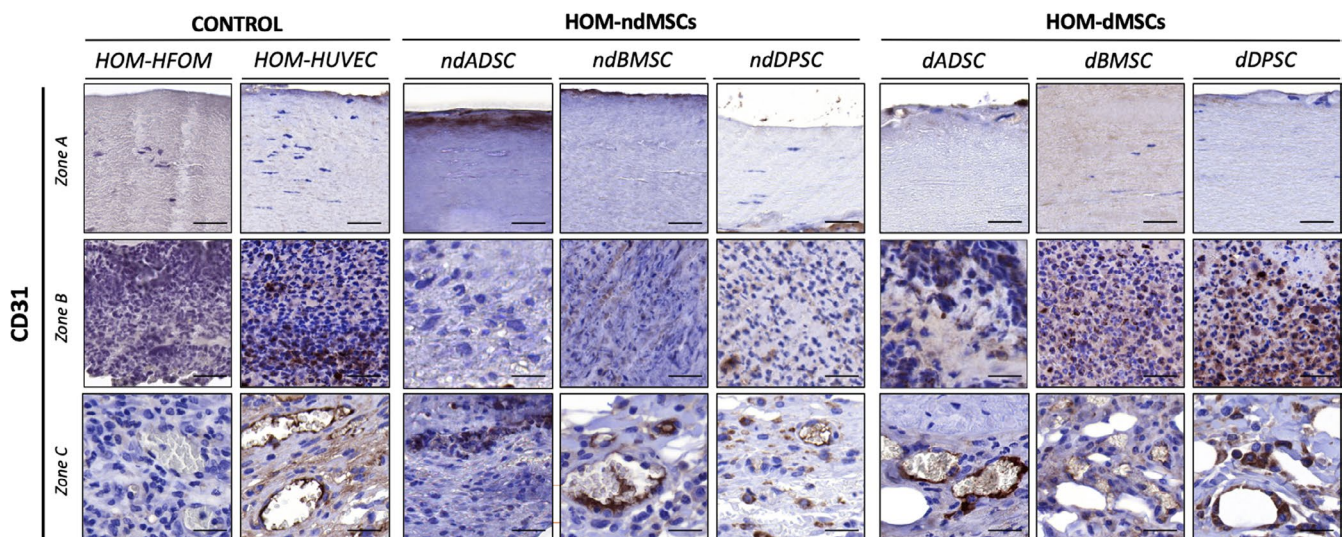


FIGURE 7 Immunohistochemical analysis of CD31 expression in each sample grafted in laboratory animals for 1 week. HOM-HFOM: negative control group containing human oral mucosa fibroblasts; HOM-HUVEC: positive control group containing human umbilical vein endothelial cells and oral mucosa fibroblasts; HOM-ndMSCs: human oral mucosa substitutes containing non-differentiated MSCs and oral mucosa fibroblasts, including non-differentiated adipose stem cells (ndADSC), non-differentiated bone marrow stem cells (ndBMSC), and non-differentiated dental pulp stem cells (ndDPSC); HOM-dMSCs: human oral mucosa substitutes containing differentiated MSCs and oral mucosa fibroblasts, including differentiated adipose stem cells (dADSC), differentiated bone marrow stem cells (dBMSC), and differentiated dental pulp stem cells (dDPSC). Scale bars: 20 μ m

It is well known that human tumors cannot grow more than 3 mm without the formation of novel vessels by angiogenesis.²⁴ In tissue engineering, vessel formation is necessary for the grafted tissue to receive nutrients and oxygen,²⁵ and vascularization is a crucial step for tissue biocompatibility and viability at the receptor niche. *In vivo* formation of blood vessels can be achieved either by vasculogenesis

or by angiogenesis. Vasculogenesis refers to differentiation of precursor cells into endothelial cells able to form blood vessels *de novo*, whereas angiogenesis is a process by which preexisting blood vessels spread into tissues by sprouting or intussusception.²⁶

Numerous models of human bioengineered oral mucosa have been described, with some of them showing promising clinical results

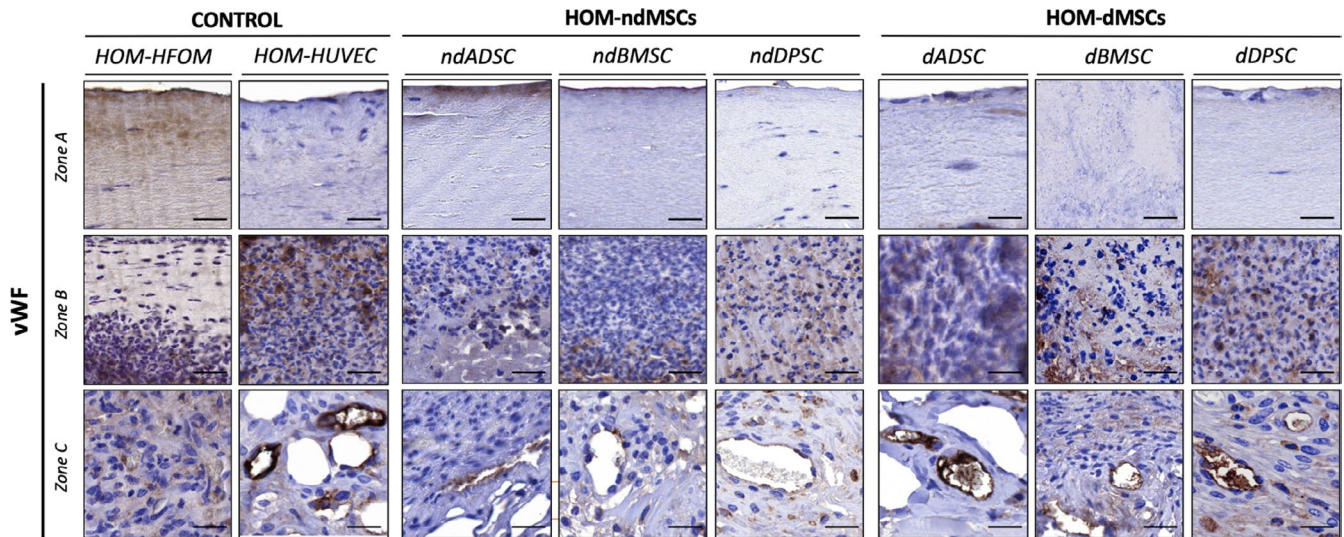


FIGURE 8 Immunohistochemical analysis of vWF expression in each sample grafted in laboratory animals for one week. HOM-HFOM: negative control group containing human oral mucosa fibroblasts; HOM-HUVEC: positive control group containing human umbilical vein endothelial cells and oral mucosa fibroblasts; HOM-ndMSCs: human oral mucosa substitutes containing non-differentiated MSCs and oral mucosa fibroblasts, including non-differentiated adipose stem cells (ndADSC), non-differentiated bone marrow stem cells (ndBMSC), and non-differentiated dental pulp stem cells (ndDPSC); HOM-dMSCs: human oral mucosa substitutes containing differentiated MSCs and oral mucosa fibroblasts, including differentiated adipose stem cells (dADSC), differentiated bone marrow stem cells (dBMSC), and differentiated dental pulp stem cells (dDPSC). Scale bars: 20 μ m

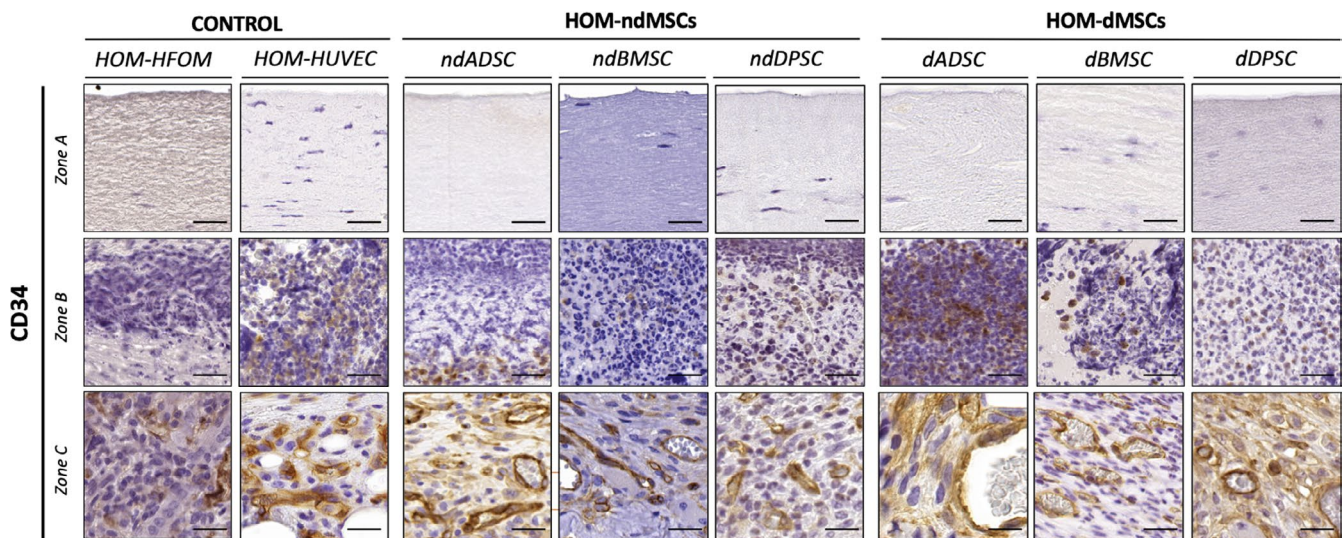


FIGURE 9 Immunohistochemical analysis of CD34 expression in each sample grafted in laboratory animals for one week. HOM-HFOM: negative control group containing human oral mucosa fibroblasts; HOM-HUVEC: positive control group containing human umbilical vein endothelial cells and oral mucosa fibroblasts; HOM-ndMSCs: human oral mucosa substitutes containing non-differentiated MSCs and oral mucosa fibroblasts, including non-differentiated adipose stem cells (ndADSC), non-differentiated bone marrow stem cells (ndBMSC) and non-differentiated dental pulp stem cells (ndDPSC); HOM-dMSCs: human oral mucosa substitutes containing differentiated MSCs and oral mucosa fibroblasts, including differentiated adipose stem cells (dADSC), differentiated bone marrow stem cells (dBMSC), and differentiated dental pulp stem cells (dDPSC). Scale bars: 20 μ m

in patients.^{27,28} However, a fully functional oral mucosa model able to generate an efficient vascular network once grafted *in vivo* is in need. In this regard, interesting recent reports demonstrated the usefulness of cultured human endothelial cells to generate a prevascularized oral mucosa.²⁵ These authors used HUVEC isolated from human umbilical cord combined with extracellular matrix nanofilms

biomaterials and demonstrated that these cells were able to differentiate *in situ* and form blood capillaries in the oral mucosa stroma. Although this method is promising, the use of HUVEC cells is subjected to several limitations such as the low *in vitro* proliferation capability of these cells or the high likelihood of immunogenic rejection if the cells are used allogeneically.^{29,30}

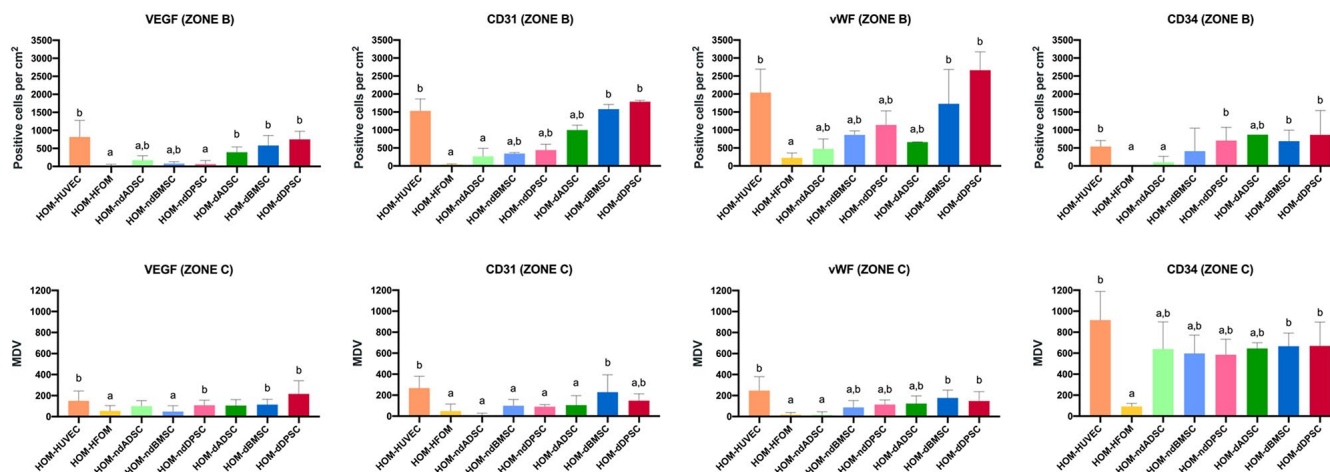


FIGURE 10 Quantification of the number of cells and blood vessels showing positive signal for each endothelial cell marker (VEGF, CD31, vWF, and CD34) on each type of sample grafted *in vivo* in laboratory animals. In zone B, the number of positive cells per mm² is shown, whereas the microvessel density (MVD) corresponding to the number of positive vessels found per mm² is shown for zone C. HOM-HUVEC: artificial oral mucosa stroma containing HUVEC; HOM-HFOM: artificial oral mucosa stroma containing only oral mucosa fibroblasts; HOM-ndADSC, HOM-ndBMSC, and HOM-ndDPSC: artificial oral mucosa stroma containing each type of non-differentiated MSC; HOM-dADSC, HOM-dBMSC, and HOM-dDPSC: artificial oral mucosa stroma containing differentiated MSCs. Significant values of the comparison between all study groups vs HOM-HUVEC control cells were indicated with superscript a. Significant values of the comparison between all study groups vs HOM-HFOM negative control group were indicated with superscript b

A possible alternative to highly differentiated cells such as HUVEC is the use of MSCs, due to their low immunogenicity, high proliferation rate, and high differentiation potential.^{16,31} In fact, MSCs have been previously used for vascularization strategies.^{32,33,34,35} However, most of these approaches are focused on skin, bone, and spinal cord regeneration, and there is no evidence for the use of MSCs for the generation of prevascularized oral mucosa substitutes. In this work, we developed novel human artificial oral mucosa stroma models using three different MSCs sources to determine the vascularization potential of each MSC type and assess the possibility of inducing rapid vascularization after *in vivo* implantation.

First, we demonstrated that the three types of MSCs used in this work had intrinsic endothelial differentiation potential. Once isolated, ADSC, BMSC, and DPSC showed multilineage differentiation capability and high expression of the main undifferentiation markers that are associated with multipotentiality, such as CD73, CD90, and CD105.³²

Interestingly, ndBMSC and ndDPSC showed some native expression of vWF and VEGF, which were present in HUVEC, although differentiation induction was able to increase this expression not only in these cell types, but also in ndADSC, and induced CD31 expression. These results confirm the endothelial differentiation potential of these MSCs types, as previously demonstrated by several authors.^{36,31,37}

Although the genetic and molecular signals associated with endothelial differentiation of these cells need further characterization in future studies, we were able to demonstrate that a number of key proteins involved in angiogenesis and vasculogenesis became upregulated in differentiated MSCs using a protein array. According to these results, DPSC could have the highest *in vitro* endothelial

differentiation potential, as dDPSC overexpressed 10 pro-angiogenic factors, with the highest expression corresponding to Amphiregulin and VEGF. Previous reports demonstrated that the elevated expression of Amphiregulin may act as an inducer of VEGF.³⁸ The concomitant expression of the anti-angiogenesis factor PF4 may suggest that the *in vitro* endothelial differentiation process could be upregulated by an intricate network often overlapping signaling pathways involving pro- and anti-angiogenic proteins³⁹, in which PF4 could act as a regulator of the vascularization process.⁴⁰

After *in vitro* characterization, we determined the vascularization potential of each cell type in a three-dimensional model of bio-artificial oral mucosa stroma grafted *in vivo* on athymic mice. This model was previously used by our group for the *in vivo* evaluation of different models of human oral mucosa due to the incapability of nude mice to reject human xenografts.^{22,41,16} Results confirmed that HOM was fully biocompatible *in vivo*, and animals grafted with the different types of HOM were free from any significant complications or side effects. Since HOM grafted *in vivo* was analyzed at very early follow-up times (7 days), histological analysis showed three well-differentiated zones, with the most external zone A corresponding to rests of the grafted biomaterial, zone B corresponding to a cell-rich area in which the grafted HOM is actively remodeled and zone C corresponding to an interphase between the host niche containing blood vessels and the grafted bioartificial tissue, and our analyses demonstrated that part of the newly formed vessels contained grafted human cells.

Most likely, these three areas can only be found at very early stages of *in vivo* follow-up, since previous works carried out by our research group showed that human oral mucosa substitutes grafted in nude mice for longer periods of time became fully integrated, and

these three zones could not be identified.⁴² The fact that zone A contained very few cells could be explained by the direct contact with air, which could induce cells to migrate to deeper zones, as previously demonstrated.⁴³

To determine the capability of each type of HOM to induce blood vessels formation, we performed a microvessel density (MVD) analysis in zone C of each type of HOM grafted *in vivo*, since this area corresponded to the limit between the host tissue and the grafted HOM. Along with the analysis of MVD, we used a combination of four different endothelial cell markers to assess the presence of blood vessels at each sample type.²³ The reason for this is that endothelial cells are highly heterogeneous, and there is a lack of a fully sensitive and specific trouble-free endothelial marker.^{23,44} Although all these markers are able to identify blood vessels, it has been demonstrated that the strongest expression of CD34 is found in small capillaries and acts as a marker of endothelial progenitor cells committed to develop small blood vessels⁴⁵, whereas vWF is positive in higher-caliber vessels, being negative or weakly positive in capillaries.⁴⁵ CD31 (PECAM-1) is a transmembrane glycoprotein, member of immunoglobulin superfamily, expressed on early and mature vascular endothelial cells, and can therefore be used to identify all types of blood vessels, although this marker has been especially characterized in veins.^{46,24} Finally, VEGF is considered to function as a panendothelial cell marker, although it is preferentially expressed upon arterial differentiation in association with the Notch signaling pathway.⁴⁷ VEGF also has a direct effect on endothelial proliferation and migration⁴⁸, and its overexpression is associated with endothelial differentiation.⁴⁹ By using a combinatorial analysis with these four endothelial markers, we were able to evaluate the number of vessels being formed at zone C, and to contribute to characterize the type of vessels that develop in each sample type.

Results of these analyses showed that the use of HUVEC significantly increased MVD at zone C, confirming previous reports demonstrating the vascularization potential of these cells.²⁵ Although further specific studies should be carried out in this sense, our results suggest that most blood vessels found at zone C corresponded to CD34-positive capillaries, with a lower number of vessels being positive for vWF, CD31, and VEGF. These results are plausible, since it is expected that low-size vessels are formed in the first place, followed by arteries and veins. Strikingly, we found that the use of MSC in HOM was able to increase MVD as compared to control HOM-HFOM, especially for HOM-dBMSC and HOM-dDPSC, which showed a very similar number of blood vessels that HOM-HUVEC. As in the previous case, most vessels were CD34-positive, again suggesting that the grafted tissue is initially occupied by a network of capillaries, and arteries and veins were less abundant at this stage of analysis. Future studies should be carried out at longer follow-up periods to determine the formation of larger size vessels. An interesting finding of the present work is that non-differentiated MSC also showed certain capability to induce blood vessel formation, although at a lower level than differentiated cells. These results are in agreement with our *in vitro* analyses showing that non-differentiated MSCs do express some endothelial cell markers in culture, which is in accordance with the pluripotential capability of these cells.^{50,51}

After analyzing the vascular interphase of tissues grafted at zone C, we studied the area found at zone B corresponding to a region of *in vivo* remodeling of the implanted HOM. First, we found that no blood vessels were identified in this area, which is not surprising given the short follow-up time used for this analysis (one week). In this regard, we previously described that human bioartificial oral mucosa grafted in nude mice is able to develop abundant blood vessels after 4 weeks of *in vivo* implantation.⁴² Then, our analyses reveal that cells found at this zone are able to express relevant endothelial markers, suggesting that these cells could be in an early phase of differentiation to the endothelial cell lineage. As expected, cells found in HOM-HFOM samples were virtually negative for all analyzed markers, whereas cells corresponding to HOM-HUVEC samples expressed high amounts of VEGF, CD31, vWF, and CD34, which is in agreement with the endothelial differentiation phenotype of HUVEC cells. As described for the blood vessels found at zone C, we found that HOM-dBMSC and HOM-dDPSC showed very high amounts of cells with positive signal for these four markers, with no differences with control HOM-HUVEC samples, while HOM-dADSC and HOM containing non-differentiated MSC showed a trend to improve the results found with control HOM-HFOM, but were only comparable or even higher than HOM-HUVEC for HOM-dADSC analyzed with VEGF and for HOM-ndBMSC, HOM-ndDPSC, and HOM-dADSC with CD34. Again, these results may imply that the use of specific cell types in HOM could be associated with an active process of cell differentiation toward the endothelial cell lineage, which could generate mature blood vessels in a more advanced stage of differentiation at increased follow-up times. We may also hypothesize that differentiating cells could be migrating to zone C to contribute to blood vessel formation in this area. Future works should confirm or dismiss these hypotheses.

The fact that cell density was very high at zone B could also favor endothelial differentiation. In this regard, it has been demonstrated that endothelial cells undergoing vascular differentiation tend to migrate and form dense cell clusters, since a close cell-cell interaction is necessary to form well-established vascular networks.^{52,53}

An unsolved question is the mechanism by which cells are able to increase MVD in HOM. One possibility is that most blood vessels found at zone C are formed by angiogenesis by cell sprouting from host vessels, whereas cells undergoing endothelial differentiation at zone B could correspond to HOM cells that will migrate and join the vascular network at zone C. However, it is also possible that the increased number of vessels found at zone C of certain groups of samples (especially, HOM-dBMSC and HOM-dDPSC) is a consequence of a vasculogenesis process in which grafted cells form *de novo* vascular networks that interconnect and integrate with the host vessels. Most likely, a combined mechanism including an active process of angiogenesis and vasculogenesis may occur in these cases. This hypothesis is in agreement with the results found for the immune detection of human cells demonstrating that vessels consisting of human cells along with vessels composed by non-human cells were present at the graft site. Previous studies demonstrated that the anti-human mitochondria antigen antibody used here is specific for human cells and allows the identification of human cells grafted in mice.^{54,55}

The present study has several limitations. In the first place, *in vitro* angiogenesis assays, such as tube formation experiments, should be performed to demonstrate the *in vitro* angiogenesis potential of each type of MSC. In the second place, future studies should determine with higher accuracy the origin of the vessels found at the implanting area and the exact percentage of vessels derived from the grafted tissue and from host vessels.

In summary, our results suggest that the use of certain types of MSC in HOM, especially HOM-dBMSC and HOM-dDPSC, is associated with a significant increase in the formation of blood vessels once grafted *in vivo*. These results open the door to the future use of pre-differentiated MSC to induce the rapid formation of a vascular network after oral mucosa implantation in patients, especially in cases when the implantation site is affected by associated conditions altering tissue grafting. In addition, future studies should be carried out to determine whether the results obtained for the human oral mucosa are also applicable to the generation of other types of human tissues and organs by tissue engineering with increased vascularization potential, such as the human skin and palate.

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DATA AVAILABILITY STATEMENT

The data presented in this study are available upon request from the corresponding authors.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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