Decellularized adipose tissue solid foams for hDPSC differentiation to osteogenic and adipogenic cells in 3D culture

Iratxe Madarieta*[1], Jon Luzuriaga[2], Patricia García-Gallastegui[4], Nerea García-Urkia[1], Igor Irastorza[2], Francisco-Javier Fernandez-San-Argimiro[1], Verónica Uribe-Etxebarria[2], Lucía Jiménez-Rojo[2], Fernando Unda[2], Beatriz Olalde[1], Jose Ramon Pineda[2,3] and Gaskon Ibarretxe*[2]

[1] TECNALIA, Basque Research and Technology Alliance (BRTA), E20009 Donostia-San Sebastian, Spain.

[2] Department of Cell Biology and Histology, Faculty of Medicine and Nursing, University of the Basque Country (UPV/EHU), 48940 Leioa, Spain.

3 Achucarro Basque Center for Neuroscience Fundazioa, 48940 Leioa, Spain.

4 Department of Physiology, Faculty of Medicine and Nursing, University of the Basque Country (UPV/EHU), 48940 Leioa, Spain.

* Correspondence to:

Iratxe Madarieta: iratxe.madarieta@tecnalia.com

Gaskon Ibarretxe: gaskon.ibarretxe@ehu.eus

Abstract

Human Dental Pulp Stem Cells (hDPSCs) constitute one very interesting stem cell source for tissue engineering and regeneration, owing to their high multi-lineage differentiation ability, the absence of ethical issues as they are considered a biological waste from dental clinics, their high immune-tolerance upon *in vivo* transplantation, and their ample possibilities of implementation in personalized medicine and autologous tissue engineering therapies. Natural extracellular matrix (ECM)-based materials constitute very promising scaffolds for *in vitro* cell culture, enhancing the attachment and differentiation of cells as well as mimicking the *in vivo* environment more accurately. Among others, adipose tissue represents a potentially abundant source of human and animal origin ECM which can be easily obtained in large quantities by liposuction surgery. The combination of hDPSCs with decellularized adipose ECM-derived materials offers great possibilities for the development of novel 3D culture platforms for the tailored (re)generation of diverse tissues like bone and adipose tissue. In this chapter, we describe different 3D culture methodologies of hDPSCs on biologic scaffold materials composed of decellularized porcine and human adipose tissue. These 3D culture systems offer a much preferable alternative to traditional tissue culture treated cell culture plates in 2D and/or single protein composed biomaterials because, by better preserving the bioinductive signals that participate in the interaction between the cells and the extracellular matrix in native tissues, they reproduce a much closer situation to *in vivo* conditions.

Keywords

Human Dental Pulp Stem Cells, 3D culture, Adipose tissue, Decellularization, Extracellular matrix, Solid foam, Osteogenesis, Adipogenesis, Cell Differentiation

1. Introduction

Human dental pieces are often extracted in dental clinics because of orthodontic or diverse oral health reasons. Taking into account that millions of human teeth worldwide are immediately disposed of after their extraction every year, this constitutes a great waste of a source of high quality stem cells. Since their discovery in the year 2000 [1], hDPSCs have attracted considerable attention due to their high capacity to differentiate to many mature cell lineages, including not only the classical mesenchymal cell-related lineages (i.e. bone, cartilage, adipose) but also non-mesenchymal ones [2–4]. Particularly, these cells have shown a high capacity to generate adult neural cells (i.e. glia and neuron-like cells), which opens the door to their application on therapy of central and peripheral nerve system lesions [5, 6]. HDPSCs can be cultured both under serum-containing and serum-devoid conditions. Conventional culture methodology involves the addition of Fetal Bovine Serum (FBS), which favors the acquisition of a plastic-adherent mesenchymal cell phenotype of hDPSC cultures, and ultimately their osteo/odontoblastic differentiation [7, 8]. On the contrary, when hDPSCs are cultured in FBS-free media on low-binding plastic flasks, they form floating cell aggregates, called dentospheres, which preserve much better the neural crest progenitor characteristics of these cells [5].

The design of new 3D culture systems that better mimic the interactions that occur between cells and the extracellular matrix (ECM) in live tissues is a long-standing demand of the tissue engineering and pharmaceutical industry. Many pharmaceutical compounds yielding a promising biological activity in conventional *in vitro* 2D cultures fail to reproduce those results when they are applied *in vivo* [9]. A more cost-efficient alternative would be to first test those compounds under 3D culture conditions, containing bioscaffolds that more accurately mimic the natural composition and 3D

structure of tissues. Specifically, the biologic scaffold materials composed of ECM have been successfully applied in both preclinical and clinical studies [10]. Adipose tissue represents an abundant source of human origin ECM which can be obtained by liposuction [11]. Interestingly, the adipose tissue ECM composition includes a high abundance of components of the Basement Membrane (BM) [12]. Our research group had previously described the processing of human and porcine adipose tissues to obtain decellularized adipose tissue (DAT) scaffolds which may be implemented to 3D culture systems [13].

The assessment of biological activity of a pharmacological reagent under 3D culture conditions could provide very useful information before switching to *in vivo* studies and clinical trials. Life science research and industry are increasingly aware of the potential of the 3D culture system for the better monitoring of cell behavior [14]. Furthermore, if both the cells and the scaffold were derived from tissues of the same patient, it would be possible to make a completely autologous 3D culture, paving the way for strictly personalized medicine applications.

Modern decellularization technologies allow the isolation of ECM components from a great variety of tissues [15]. DATs are very promising biocompatible scaffold for 3D cell culture, owing to their high concentration of bioactive molecules and adhesion glycoproteins, which favor the adhesion and survival of the seeded cells [13, 16–18]. This material can be formulated in a variety of textures and incorporated to different cell culture plates and devices. In this work, we will describe the methodology of hDPSC 3D culture using DAT bioscaffolds of both porcine (pDAT) and human (hDAT) origin, which are incorporated to conventional cell culture plates. We use this system to generate *in vitro* reconstructions of bone-like and adipose-like tissues.

2. Materials

2.1. Disposables.

All the disposables must be sterile or autoclaved before their use.

- 24 well plates, to incorporate DAT solid foams on them (cat# 142475, Nunc, Roskilde, Denmark).
- 48-well plates, to incorporate solid foams on them (cat# 150687, Nunc, Roskilde, Denmark)
- Falcon tubes 15ml (cat# 339650, Nunc, Roskilde, Denmark).
- Glass slides, to mount detached solid foams for optic microscopy analysis (cat# 11562203, Fisherbrand™, Loughborough, United Kingdom).
- Micropipette tips (10µL cat# 9400300; 1000µL cat# 9401030; 200µL cat#16693952, Fisher Scientific, Waltham, MA, United States).
- Self-sealing sterilization pouches paper/film, to introduce dental pieces to the bench clamp (cat# KMNSF-057130, Anqing Kangmingna Packaging Co Ltd, Anhui, China).
- T-25 cell culture flask for adherent cells, to amplify the initial hDPSC cultures (cat# 83.3910.002, Sarstedt, Nümbrecht, Germany).

2.2. Reagents.

For the isolation of hDPSCs and preparation of culture media (all the reagents must be sterile and filtered before their use):

- Amphotericin B solution (cat# A2942-100ML, Sigma Aldrich. St. Louis USA), to add to the tooth collecting tube, together with Penicillin and Streptomycin.

- Ascorbic acid (cat# A4544, Sigma Aldrich. St. Louis USA), as a component of the osteogenic differentiation medium, together with Dexamethasone and Beta glycerol phosphate.
- Beta glycerol phosphate (cat# 50020-100G, Sigma Aldrich. St. Louis USA).
- Collagenase (cat# 17018-029, Thermo Fisher Scientific, Waltham, MA, United States). To digest the dental pulp, together with Dispase.
- Dexamethasone (cat# D-085, Supelco Analytical/Merck KGaA, Darmstadt, Germany).
- Dispase (cat# 17105-041, Thermo Fisher Scientific, Waltham, MA, United States).
- Dulbecco's Modified Eagle Medium (DMEM), as the main basal medium for hDPSC culture (cat# 12-733, Lonza Basel, Switzerland). It must be supplemented with FBS, Glutamine and Penicillin/Streptomycin.
- Foetal Bovine Serum (FBS) (cat# SV30160.03, Hyclone, GE Healthcare Life Sciences, Logan, UT, United States).
- Hank’s Balanced Salt Solution (HBBS) (cat# 14175-095, Thermo Fisher Scientific, Waltham, MA, United States), as the basal medium for the Collagenase/Dispase enzymatic solution.
- 3-Isobutyl-1-methylxanthine IBMX (cat #I5879-250MG, Sigma Aldrich. St. Louis USA), as a component of the adipogenic differentiation medium, together with Dexamethasone and Insulin.
- Insulin (cat# I0516-5ML, Sigma Aldrich. St. Louis USA).
- L-glutamine (cat# G7513, Sigma, St. Louis, MO, United States).
- Penicillin and streptomycin antibiotics (cat# 15140-122, Gibco).

- Phosphate Buffered Saline (PBS) (cat# P5493-1L, Sigma Aldrich. St. Louis USA).
- Trypan Blue Solution (cat# T8154, Sigma Aldrich. St. Louis USA), to assess cell viability upon passaging.
- Trypsin-EDTA (cat# 59417C-500ML, Sigma Aldrich. St. Louis USA), to detach adherent hDPSCs from cell culture plates.

For the preparation of DAT scaffolds:

- 2-propanol Emprove essential (cat# 1009951000, Merck Life Science, Darmstadt, Germany).
- Acetic acid Glacial (99%) (cat# 11375890, Fisher chemical). Dissolution at 0.5M in ultrapure water.
- ACK Lysing Buffer (cat# 11375890, Thermo Fisher Scientific, Waltham, MA, United States).
- Ammonium hydroxide, solution 28.0-30.0% NH_3 basis (Sigma Aldrich. St. Louis USA). Dissolution at 0.1% (v/v) in PBS-A.
- Antibiotics and protease inhibitors (PBS-B): PBS-A with 1% (v/v) antibiotic-antimycotic solution (Sigma Aldrich. St. Louis USA) and 1 % (v/v) protease inhibitor cocktail set III EDTA-free (Calbiochem/Sigma Aldrich. St. Louis USA).
- Phosphate buffer saline (PBS-A): Di-sodium hydrogen phosphate dihydrate (Merck KGaA, Darmstadt, Germany) 0.89 g/L and Sodium dihydrogen phosphate dihydrate (cat# 1.06342, Merck KGaA, Darmstadt, Germany) 1.29 g/L.
- Triton®X-100 Emprove Bio (cat# 1.08643, Merck KGaA, Darmstadt, Germany). 1% (v/v) dissolution in sterile ultrapure water.

- Water: Milli-Q purification system (cat# ZR0Q008WW, Millipore). Sterilized in autoclave (Selecta) 20 min at 121ºC and 1atm.

For histochemical (HC) and immunohistochemical (IHC) staining:

- Alizarin Red (cat#A3882, Acros organics, Sigma Aldrich. St. Louis USA).
- NTBI ALP substrate (5-Bromo-4-chloro-3-indolyl phosphate/Nitro Blue tetrazolium (cat# B3804; Sigma, MO, St. Louis USA).
- Oil Red. (cat# O-0625, Sigma Aldrich. St. Louis USA).
- Paraformaldehyde (PFA) (cat# 158127-500G, Sigma Aldrich. St. Louis USA).

For electron microscopy sample preparation:

- Cacodylate buffer (cat# C0250, Sigma-Aldrich, Spain).
- Ethanol (cat# 32221-1L-M, Sigma-Aldrich, Spain).
- Glutaraldehyde (cat# 50-262-19, Fisher Scientific, Pittsburgh, USA).
- Hexamethyldisilazane (cat# 440191, Sigma-Aldrich, Spain).
- Isosmolar sucrose (cat# S7903, Sigma-Aldrich, Spain).
- Osmium tetraoxide (OsO_4) (cat# O5500, Sigma-Aldrich, Spain).

2.3. Equipment.

- Analytical Balance (BP221S, Sartorius, Göttingen, Germany).
- Automatic Cell Counter (BioRad TC20, California, USA).
- Cell culture hood (Cultek TCA 60, Madrid, Spain).
- Centrifuge (Function Line, Heraeus Instruments, Hanau, Germany).
- Confocal microscope (Zeiss LSM880, Jena, Germany).
- Scanning Electron Microscope (SEM; S-3400N, Hitachi, Tokio, Japan).
- Incubator (Hera Cell 150, Heraeus Instruments, Hanau, Germany).

- Micropipettes 200-1000µl.
- Shaker (Polymax 1040, Heidolp Instruments, Schwabach, Germany).
- Swivel bench Clamp, to break dental pieces (cat# 7431H60, Ferretería Domingo, Binéfar, Spain).
- Vacuum oven (Vaciotem-TV, J.P. Selecta, Abrera, Spain).
- Polytron® Inmersion disperser PT3100 with PT-DA3012/EC and PT-DA-12/2EC-F154 aggregates and Dispersing vessel (Kinematica).
- Lyophilizer: Christ. Epsilon 2-4LSCplus.
- Mixer mill. Retsch MM400.

3. Methods

3.1 Preparation of initial hDPSC cultures in plastic flasks.

- The extraction of hDPSCs requires breaking the dental piece to gain access to the pulp chamber. Teeth are collected from a dental clinic (*see* **Note 1**) and harvested in a sterile medium solution containing antibiotics and antifungals (*see* **Note 2**).
- Upon arrival atto the laboratory, teeth are taken out of the collecting tube inside the cell culture hood and placed into autoclaved sterilization pouches. The pouches are then folded carefully and brought to a swivel bench clamp, previously cleaned with ethanol 70%. The handle must be turned firmly to break the teeth, while avoiding piercing the bags (*see* **Note 3**).
- Once the dental pulp is removed from the fractured teeth, it is placed in an enzymatic solution containing 3 mg/ml Collagenase and 4 mg/ml Dispase in HBSS containing neither Ca^{2+} nor Mg^{2+} (*see* **Note 4**).

- The dental pulp is digested in an incubator at 37 ºC, 5% CO_2 for 45 min-1 h (*see* **Note 5**).
- After the enzymatic digestion, the cell suspension is mixed thoroughly up with a 1000 µl micropipette and centrifuged at 150g for 5 min. The supernatant is discarded and the pellet is resuspended in 1 mL of fresh culture medium containing FBS (*see* **Note 6**).
- The cells are then dissociated mechanically using a 1000 µL micropipette (*see* **Note 7**).
- After getting a homogeneous cell suspension, 10 µL of the sample are mixed with Trypan blue and placed into the cell counting slide (*see* **Note 8**). Cells are then seeded into the culture flasks at the desired density.
- Very often, the cell suspension of a single dental pulp is placed on a T25 flask for further cell selection and amplification, in a variable time that can last from one week to one month, depending on the patient and the amount of the initially collected dental pulp material (*see* **Note 9**).
- Initial hDPSCs cultures are established in 2D using conventional tissue culture treated flasks. The isolated cells are cultured in a DMEM solution supplemented with 10% of heat-inactivated FBS, 2 mM L-glutamine, 100 U/mL penicillin and 150 mg/mL streptomycin (*see* **Note 10).**
- At the beginning cells are maintained in a T25 flask until they reach confluence. Cell culture media is changed every 3-4 days. (*see* **Note 11**). As early as 3-5 days *in vitro*, clonogenic colonies of adherent cells can be observed.
- Cell colonies will expand until they eventually bring the flask to full confluence (*see* **Note 12**). When the culture is nearly confluent, cells are ready for passing them to a bigger flask to keep amplifying the population. At this stage, the

collected cells can also be plated to initiate the experiments, or frozen for later use [19] (*see* **Note 13**).

- For cell passage, after removing the culture medium, PBS is added to the flask to wash media leftovers before harvesting the cells (*see* **Note 14**).
- Then 1.5 mL (T25) or 3 mL (T75) of 0.5% Trypsin-EDTA are added, and the flask is incubated for 5 minutes at 37ºC. Cells will detach from the bottom of the flasks. Thereafter, the same volume of FBS 10%-DMEM is added to the flask to inactivate the effect of the Trypsin-EDTA. Once the cells are detached, and the whole volume of the flask is collected and centrifuged at 150g for 5 minutes. Then the supernatant is removed and the cell pellet is resuspended in culture medium for its passage to another flask or a well plate.
- HDPSC cultures under these conditions will eventually acquire a typical mesenchymal cell -like plastic adherent phenotype, where almost 100% of cells are positive for the mesenchymal stem cell markers CD90, CD105 and CD73 [20–22]. HDPSCs in these conditions may differentiate to mesenchyme-derived mature cell lineages, such as osteocytes, chondrocytes or adipocytes (see **Note 15**).

3.2 Decellularization of Adipose Tissue.

- Tissue harvesting: Human AT is obtained from Biopredict International, according to the French Ministry of Higher Education and Research (permission AC-2013-1754). Porcine AT is obtained from the local meat industry (JAUCHA S.L.). Both tissues are transported and conserved at -20 ºC.
- Tissue cleaning and creaming: Human AT is cleaned of connective tissue and blood vessels with scalpel, followed by serial washes with water. Porcine AT is

cleaned and freed of blood components. Both porcine and human AT are creamed using a beater (Taurus BAPI 1200W), and stored at -20 ºC in 500 mL surgical specimen containers (Labolan) until the next step (see **Note 16**).

- Tissues are defrosted at 4 ºC overnight and at 37 ºC until a pourable tissue is obtained. Tissues are poured into dispersing vessels (30-100 mL volume approximately) and homogenized on cold water first with PT-DA3012/EC and secondly with the PT-DA-12/2EC-F154 aggregates at 12,000 rpm for 5 min (see **Note 17**).
- The homogenized tissues are placed on centrifugation containers or 50 mL conical centrifuge tubes. The same volume of water is added and vigorously mixed manually or using a vortex. Homogenized tissues are centrifuged at 900g for 5 min for lipid separation. The supernatant lipids are manually discarded, and the protein pellets are maintained at the bottom of the tubes.
- Protein pellets from 10-20 tubes are collected in 50 mL conical centrifuge tubes and washed with water by centrifugation (900g, 5 min) for three times. At this stage, pellets can be kept at -20 ºC or treated to continue with the decellularization protocol.
- Decellularization: Fresh or defrosted pellets are treated with 30 mL isopropanol. To improve reagent penetration apply vacuum to each tube through a vacuum pump during 5 min. Maintain the tubes in an orbital shaker for 18 h at room temperature (see **Note 18**).
- Wash thoroughly by centrifugation 7 times with PBS-A, 1 time with PBS-B (see Reagent section 2.2) and 1 time with water.
- Add 30 mL triton x-100 1% solution and 0.1% (v/v) ammonium hydroxide to each pellet, apply vacuum to each tube through a vacuum pump during 5 min for

reagent penetration and maintain in an orbital shaker for 44 h at room temperature (see **Note 19**).

- Wash thoroughly by centrifugation 7 times with PBS-A, 1 time with PBS-B and 1 time with water.
- Freeze at -20 ºC and lyophilize at (0.63 mbar and -10 ºC) for 24-48 h to completely dry the decellularized ECM-derived material (see **Note 20**).
- Mill by a mixer mill (30 frequency for 2 min) previously freezing in liquid nitrogen (2-3 min.) Repeat the process two times (see **Note 21**).
- Pour in cleaned vials, identifyied the batch, and preserve at 4 ºC under vacuum desiccator (see **Note 22**).
- Decellularization criteria (see **Note 23**).

3.3 Preparation of DAT solid foam biological scaffolds.

- Dissolve the decellularized material in 0.5 M acetic acid solution (5 mg/mL) by magnetic stirring for 48 h. Pour in a desired mould (e.g. multiwell plate) a specific quantity of acetic dissolved material (see **Note 24**).
- Freeze the mould at -20 ºC overnight and lyophilize at 0.63 mbar and -10 ºC) for 24-72 h, to obtain the solid foam (see **Note 25**).
- Visualize the structure of the solid foam by SEM. The dried samples were mounted, pulse coated with gold and visualized by SEM with an accelerating voltage of 10 kV (see **Figure 1**).
- Plates are sealed with medical packaging sterilization flat roll, sterilized with ethylene oxide (Esterilización SL, Barcelona, Spain) and preserved at 4ºC under vacuum desiccator until use for cell culture (see **Note 26**).

3.4 Culture of hDPSCs and differentiation to osteoblasts in DAT solid foams: Generation of bone-like tissue in 3D.

- HDPSCs are collected from plastic cell culture flasks as shown in section 3.1. After Trypsin-EDTA dissociation, cells are resuspended in standard DMEM +10% FBS cell culture medium, and seeded on the solid foams which are incorporated to the bottom of cell culture plates (**see Note 27**).
- After cellular adhesion, replace the initial medium with osteogenic differentiation medium. The osteogenic differentiation medium consists of regular culture medium supplemented with 50 µM ascorbic acid (A4403; Sigma, MO, USA), 20 mM β-Glycerolphosphate (50020; Sigma, MO, USA) and 10 nM dexamethasone (D4902; Sigma, MO, USA). Keep the other half of the cell culture wells in standard culture medium.
- Assessment of Alkaline Phosphatase (ALP) activity: ALP is an important marker of osteoprogenitor cells. After 2 weeks of hDPSC culture on solid foams, fix the samples briefly (see **Note 28**) using 4 % paraformaldehyde (PFA) for 1 min and wash them with 0.05 % Tween 20-PBS. ALP staining is performed using NTBI as substrate, checking the staining process every 3 min. After the incubation, the samples are post-fixed with PFA for 10 min and washed three times with PBS for 5 min.
- Osteoprogenitor cells within the foams are identified as ALP+, stained with a dark blue color (see **Figure 2**).
- Assessment of foam calcification by Alizarin Red staining: When osteoprogenitor cells differentiate to mature osteoblasts, they induce the mineralization of the surrounding ECM, which can be assessed by Alizarin Red staining after 4 weeks in culture.

- After fixation with 4% paraformaldehyde for 10 min, wash samples with distilled water and stain them using 2 % Alizarin Red at pH 4.1-4.3 for 45 min in the dark at room temperature. Thereafter, thoroughly remove the remaining staining solution with several washes of distilled water (see **Note 29**).
- Calcified parts of the foam are identified by their red color (see **Figure 3**).

3.4 Culture of hDPSCs and differentiation to adipocytes in DAT solid foams: Generation of adipose-like tissue in 3D.

- HDPSCs are collected from plastic cell culture flasks as shown in section 3.1. After Trypsin-EDTA dissociation, cells are resuspended in standard DMEM +10% FBS cell culture medium, and seeded on the solid foams which are incorporated to the bottom of cell culture plates (**see Note 27**).
- After cellular adhesion, replace the initial medium with adipogenic differentiation medium, which consists of regular culture medium supplemented with 0.5 mM IBMX (I5879, Sigma), 1 µg/mL insulin (91077C, SAFC Biosciences, St. Louis, Massachusetts, USA) and 1 µM dexamethasone (D4902, Sigma). Keep the other half of the cell culture wells in standard culture medium.
- Assessment of lipid droplet production by Oil Red staining: Adipogenic differentiation of hDPSCs can be assessed by the generation of lipids on the solid foams after 4 weeks.
- Samples are fixed with 10% PFA for 10 min and then washed with Polyethylene glycol (PEG). Lipid droplets were detected by incubation with a solution containing 0.5% Oil Red in PEG.
- Incubate the samples with Oil Red for 15 min, then wash them with a solution of 60% PEG in distilled water, and finally with distilled water (3 times for 1 min

each), before leaving them in PBS. Spherical shaped lipid droplets in the sample are stained in red color (see **Figure 4**).

4. Notes

- 1: Tooth samples are considered as a biological waste, and they are extracted for external reasons to the research project.
- 2: Tooth samples are placed in a tube containing sterile basal DMEM medium with antifungals and antibiotics, and stored at 4ºC until use. HDPSCs can be kept with good viability for a period up to 8 h after dental extraction.
- 3: Press the dental piece using the rotary handle, until the tooth crown breaks. If it does not, change its orientation to search for the best breaking position with the lowest resistance.
- 4: Place each pulp in 1 mL of enzymatic digestion solution. Use Hank's Balanced Salt Solution (HBSS) as solvent. It is possible to mix different dental pulps from the same patient, but mixing pulp material of different genetic origin must be avoided.
- 5: Shake the tube manually every 15 minutes.
- 6: FBS will inactivate any remains of enzymatic activity in the cell pellet.
- 7: Make passages very gently and avoid making bubbles, because this would compromise cell viability.
- 8: Automatic cell counter devices are practical to obtain a fast count of total live cells, but it is also possible to estimate this cell count manually, with a hemocytometer.
- 9: A long culture period favours the selection of hDPSCs over other non stem cell types of the dental pulp. It is important to plate the cells at a sufficient initial density, to ensure long-term culture viability.
- 10: Cells are cultured in an appropriate tissue culture treated flask for adherent cells, for example the 83.3910.002 model of Sarstedt.

- 11: Culture media should be changed every two days if the culture is almost under confluence or if it contains a high number of cells.
- 12: HDPSCs have a fibroblast-like morphology when they are cultured in standard DMEM + FBS medium, but they highly express specific stem cell markers like Nestin and Oct4 [20]. When cultures reach confluence, a plastic adherent cell monolayer will be observed at the bottom of the flask.
- 13: In case of freezing the cells, two million cells are usually fast frozen with 5-10% DMSO per vial, in a liquid nitrogen container.
- 14: Trypsin-EDTA is normally used to detach and dissociate the cells. It is important to wash the culture flasks first with sterile PBS, to remove traces of FBS that would inactivate Trypsin.
- 15: For the generation of other non-mesenchymal lineages like neural cells, hDPSCs should be cultured in the total absence of FBS.
- 16: For human AT, firstly, use a scalpel to discard the blood vessels and connective tissue and after pour the tissue (approximately 100 mL) in a container with high quantity of ultra-pure water (approximately 150 mL). Shake vigorously, wait for separation and discard the water with a pipette. Repeat this step at least three times. For Porcine AT, it is important to cut in small pieces (0.5 cm x 0.5 cm) prior to beat for at least 2 min at maximum power.
- 17: Homogenize in cold water (water with ice) during the process and make manual orbital shaking of the vessel during the homogenization. It is also recommendable to use specific dispersing vessels (Kinematika) and to clean the aggregates after each homogenization.

- 18: To apply vacuum, put a septum to each 50 mL conical centrifuge tube and connect to a vacuum pump through a needle and plastic tube. Maintain at least 5 min and tap genly to ensure that the material is completely immersed in liquid.
- 19: Due to foam formation, it is recommended to introduce a little part of the needle to apply vacuum as described in note 18.
- 20: For lyophilization, the material needs to be completely frozen. Open the tubes, cover them with some parafilm and make holes on them with a needle.
- 21: Take liquid nitrogen in a resistant container, put a small amount of the material into the grinding jars, include grinding balls and close the jars. Immerse the jars in liquid nitrogen for 2-3 minutes. Program the cycle on the mill (30 frequency, 2 min.). Repeat the process without opening the jars. Finally, open the jar and take the milled material out. Clean the jar with ethanol.
- 22: The obtained DAT is a yellowish fine powder with easy handling.
- 23: pDAT and hDAT decellularization criteria. The pDAT and hDAT meet the tissue decellularization criteria established by the research community such as: i) Remnant DNA $\leq$ 50ng/mg, ii) absence of cell nuclei. In addition, delipidation is a critical parameter in adipose tissue decellularization, and with this protocol remnant lipids are kept at $\leq$ 10%. For more information see [13].
- 24: Dissolve at controlled temperature and stirring. Solid foams can be produced in any moulds and in any thickness. We typically apply 200 µL/well in 24 well plates, or 100 µl/well in 96 well plates. It is recommendable to rework the edges with a tip.
- 25: The solid foams are thin and joined by the edges being easy to manage in cell culture techniques. In some instances solid foams could be partially

detached from the bottom of the plate, but even then it is possible to mount them in glass slides for microscopy and histology techniques.

- 26: The sterilization was carried out maintaining the samples for 270 min at 38 ºC, Relative humidity 40%, 2 vacuums, aeration in chamber for 45 min and aeration in room for 48 h.
- 27: Allow adhesion of cells to the foams for a minimum of 24-48 hours, before changing the culture medium.
- 28: The initial fixation of the sample has to be very brief, because longer times would lead to enzyme denaturation and loss of activity.
- 29: Control non-calcified foams will also retain some amount of Alizarin Red stain. Sometimes it is even necessary to leave the samples washing overnight at room temperature to minimize background staining.

Figure captions

Figure 1. Ultrastructure of DAT solid foams: SEM images of hDAT and pDAT solid foams showing the highly interconnected pore microstructure in 3D. Scale bars: 100 µm (top) and 50 µm (bottom).

Figure 2. ALP staining of hDPSC cultures over glass coverslips and DAT: Cultures were processed in parallel and stained after 2 weeks. Note the differences in ALP staining between cultures grown in normal medium (top panel; A) and osteogenic differentiation medium (bottom panel; B). Cell nuclei were counterstained with DAPI. Scale bar: 100 µm.

Figure 3. Alizarin Red staining of hDPSC cultures on DAT: Cells were grown on pDAT and stained after 4 weeks. Note the difference in Alizarin Red staining in cultures grown in normal culture medium (control), and osteogenic differentiation medium (Osteo). Cell nuclei were counterstained with DAPI. Scale bar: 50 µm.

Figure 4. Oil Red staining of hDPSC cultures on DAT: Cells were grown on hDAT and stained after 4 weeks. Note the difference in Oil Red staining in cultures grown in normal culture medium (control), and adipogenic differentiation medium (Adipo). Cell nuclei were counterstained with DAPI. Scale bar: 20 µm.

Acknowledgements

This research was supported by the Basque Government (ELKARTEK program PLAKA KK-2019-00093 & Health Department RIS3, 2021333012), by the Spanish Ministry of Science and Innovation MCIN/AEI/10.13039/501100011033: grants RYC-2013-13450 & PID2019-104766RB-C21, by the European Social Fund “ESF investing in your future”, and by the University of the Basque Country UPV/EHU (PPGA20/22).

References

1. Gronthos S, Mankani M, Brahim J, et al (2000) Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc Natl Acad Sci U S A 97:13625–13630. https://doi.org/10.1073/pnas.240309797

2. Huang GT-J, Gronthos S, Shi S (2009) Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine. J Dent Res 88:792–806. https://doi.org/10.1177/0022034509340867

3. Ibarretxe G, Crende O, Aurrekoetxea M, et al (2012) Neural crest stem cells from dental tissues: a new hope for dental and neural regeneration. Stem Cells Int 2012:103503. https://doi.org/10.1155/2012/103503

4. Tirino V, Paino F, De Rosa A, Papaccio G (2012) Identification, isolation, characterization, and banking of human dental pulp stem cells. Methods Mol Biol 879:443–463. https://doi.org/10.1007/978-1-61779-815-3_26

5. Luzuriaga J, Polo Y, Pastor-Alonso O, et al (2021) Advances and Perspectives in Dental Pulp Stem Cell Based Neuroregeneration Therapies. Int J Mol Sci 22:. https://doi.org/10.3390/ijms22073546

6. Luo L, He Y, Wang X, et al (2018) Potential Roles of Dental Pulp Stem Cells in Neural Regeneration and Repair. Stem Cells Int 2018:1731289. https://doi.org/10.1155/2018/1731289

7. Yu J, He H, Tang C, et al (2010) Differentiation potential of STRO-1+ dental pulp stem cells changes during cell passaging. BMC Cell Biol 11:32. https://doi.org/10.1186/1471-2121-11-32

8. Pisciotta A, Riccio M, Carnevale G, et al (2012) Human serum promotes osteogenic differentiation of human dental pulp stem cells in vitro and in vivo. PLoS One 7:e50542. https://doi.org/10.1371/journal.pone.0050542

9. Horvath P, Aulner N, Bickle M, et al (2016) Screening out irrelevant cell-based models of disease. Nat Rev Drug Discov 15:751–769. https://doi.org/10.1038/nrd.2016.175

10. Badylak SF (2007) The extracellular matrix as a biologic scaffold material. Biomaterials 28:3587–3593. https://doi.org/10.1016/j.biomaterials.2007.04.043

11. Fraser JK, Wulur I, Alfonso Z, Hedrick MH (2006) Fat tissue: an underappreciated source of stem cells for biotechnology. Trends Biotechnol 24:150–154. https://doi.org/10.1016/j.tibtech.2006.01.010

12. Mariman ECM, Wang P (2010) Adipocyte extracellular matrix composition, dynamics and role in obesity. Cell Mol Life Sci 67:1277–1292. https://doi.org/10.1007/s00018-010-0263-4

13. Cicuéndez M, Casarrubios L, Feito MJ, et al (2021) Effects of Human and Porcine Adipose Extracellular Matrices Decellularized by Enzymatic or Chemical Methods

on Macrophage Polarization and Immunocompetence. Int J Mol Sci 22:3847. https://doi.org/10.3390/ijms22083847

14. Keller PJ, Pampaloni F, Stelzer EH (2006) Life sciences require the third dimension. Curr Opin Cell Biol 18:117–124. https://doi.org/10.1016/j.ceb.2005.12.012

15. Dzobo K, Motaung KSCM, Adesida A (2019) Recent Trends in Decellularized Extracellular Matrix Bioinks for 3D Printing: An Updated Review. Int J Mol Sci 20:. https://doi.org/10.3390/ijms20184628

16. Brown BN, Freund JM, Han L, et al (2011) Comparison of three methods for the derivation of a biologic scaffold composed of adipose tissue extracellular matrix. Tissue Eng Part C Methods 17:411–421. https://doi.org/10.1089/ten.TEC.2010.0342

17. Yu C, Bianco J, Brown C, et al (2013) Porous decellularized adipose tissue foams for soft tissue regeneration. Biomaterials 34:3290–3302. https://doi.org/10.1016/j.biomaterials.2013.01.056

18. Yang J-Z, Qiu L-H, Xiong S-H, et al (2020) Decellularized adipose matrix provides an inductive microenvironment for stem cells in tissue regeneration. World J Stem Cells 12:585–603. https://doi.org/10.4252/wjsc.v12.i7.585

19. Raik S, Kumar A, Rattan V, et al (2020) Assessment of Post-thaw Quality of Dental Mesenchymal Stromal Cells After Long-Term Cryopreservation by Uncontrolled Freezing. Appl Biochem Biotechnol 191:728–743. https://doi.org/10.1007/s12010-019-03216-6

20. Luzuriaga J, Pineda JR, Irastorza I, et al (2019) BDNF and NT3 Reprogram Human Ectomesenchymal Dental Pulp Stem Cells to Neurogenic and Gliogenic Neural Crest Progenitors Cultured in Serum-Free Medium. Cell Physiol Biochem 52:1361–1380. https://doi.org/10.33594/000000096

21. Irastorza I, Luzuriaga J, Martinez-Conde R, et al (2019) Adhesion, integration and osteogenesis of human dental pulp stem cells on biomimetic implant surfaces combined with plasma derived products. Eur Cell Mater 38:201–214. https://doi.org/10.22203/eCM.v038a14

22. Karamzadeh R, Eslaminejad MB, Aflatoonian R (2012) Isolation, characterization and comparative differentiation of human dental pulp stem cells derived from permanent teeth by using two different methods. J Vis Exp 4372. https://doi.org/10.3791/4372

Figure 1

hDAT solid foams

pDAT solid foams

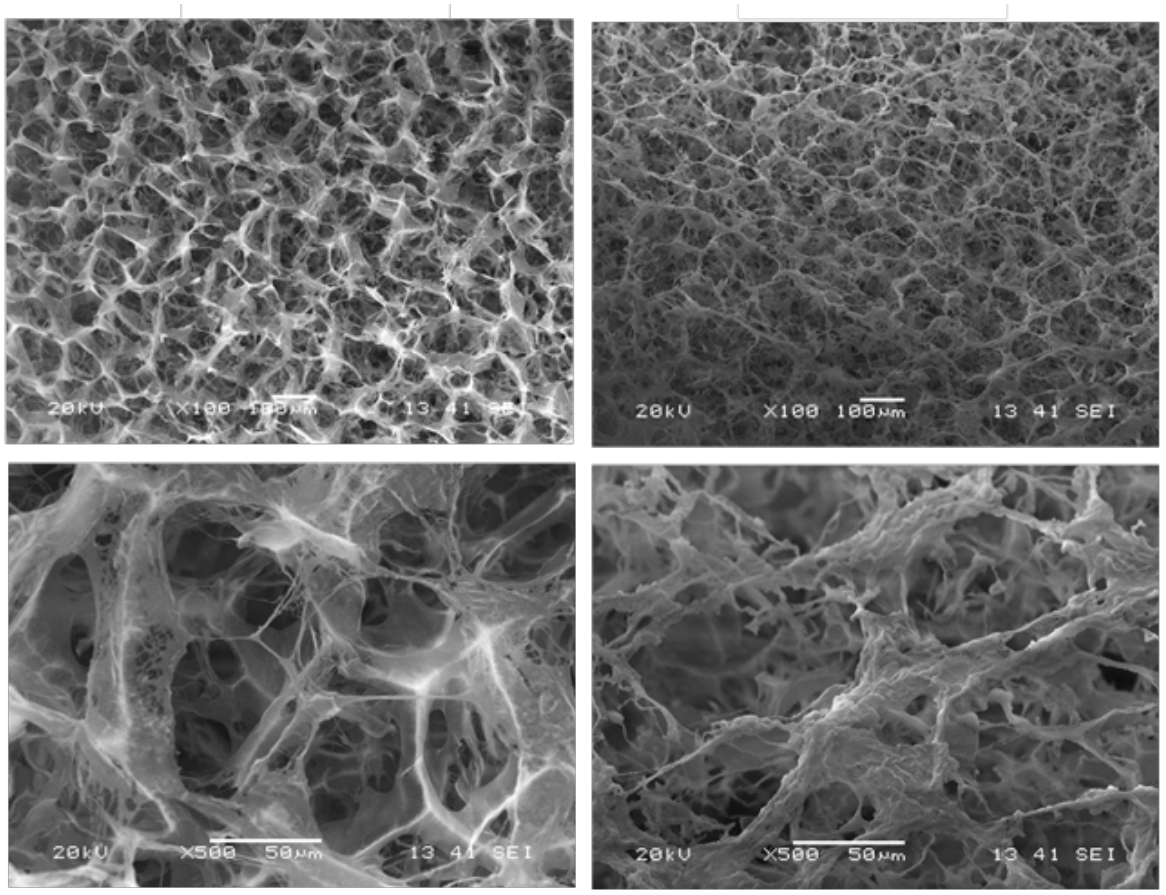

Figure 2

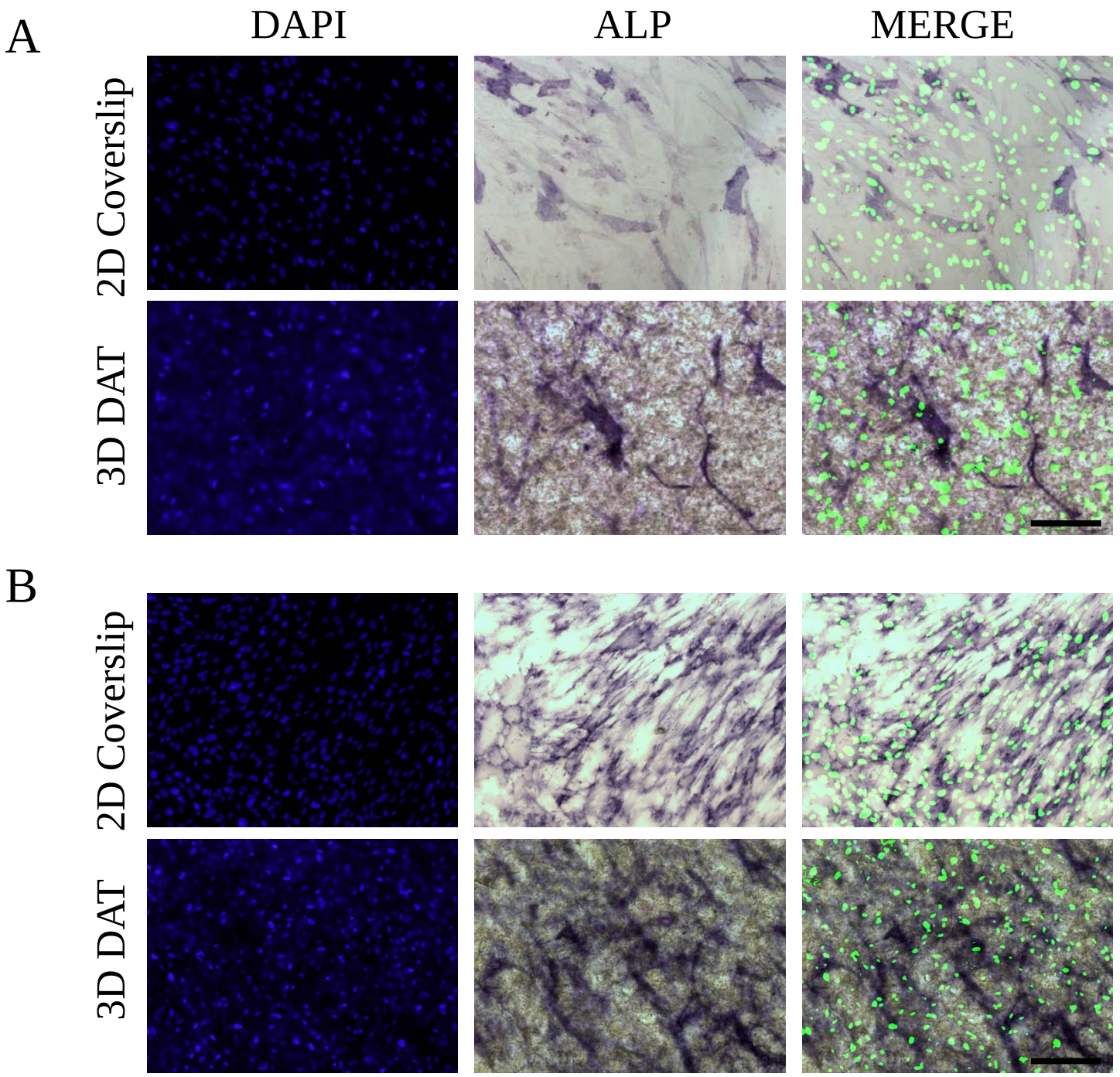

Figure 3

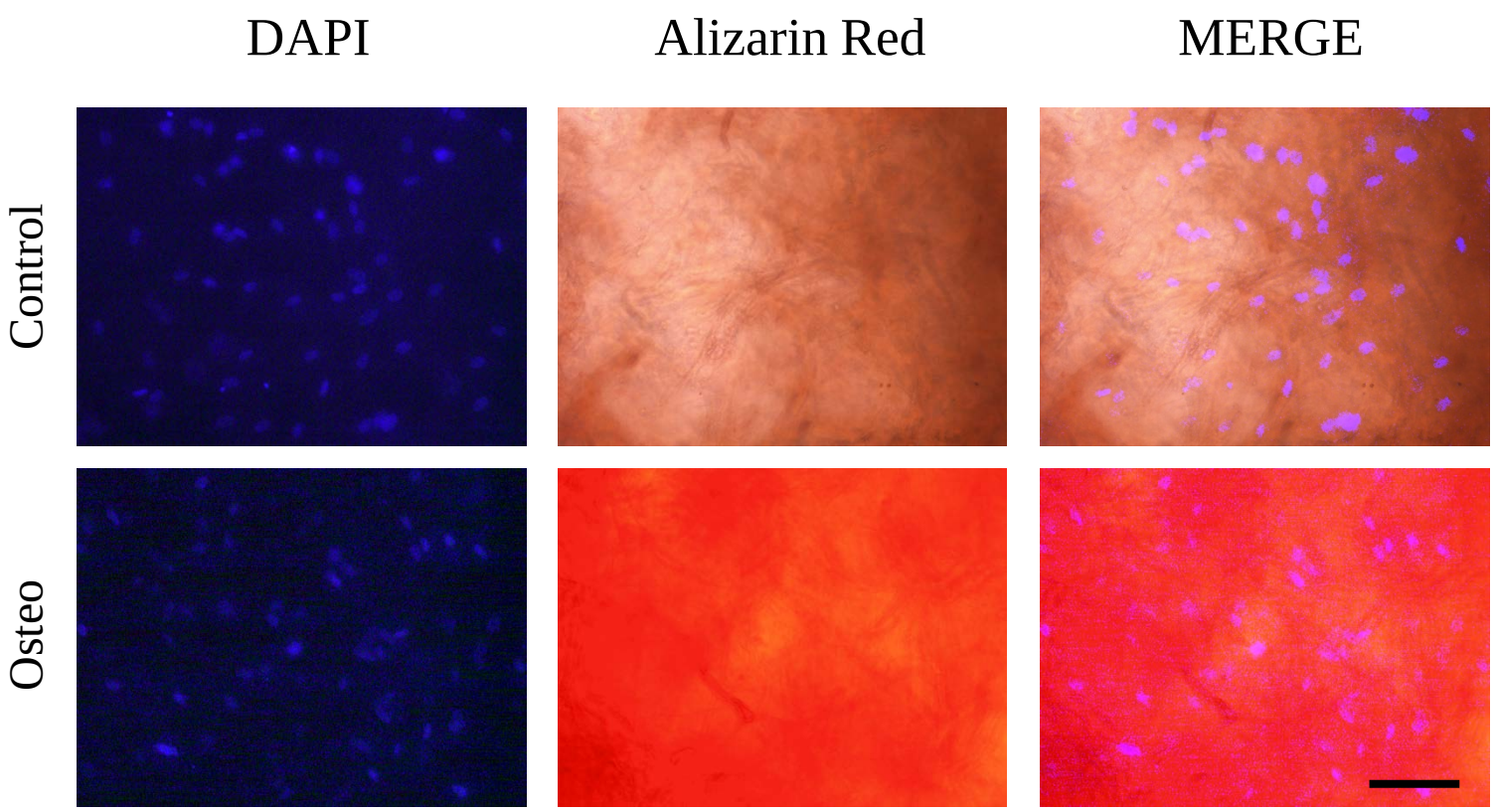

Figure 4