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ABSTRACT

In recent decades, two-dimensional (2D) cell cultures have been the experimental model of choice in biological and biomedical research. This simple, inexpensive and versatile system is easily reproducible and has enabled us to understand mechanisms and identify pathways. However, 2D models have significant limitations, including unnatural morphology, a lack of extracellular matrix and tissue architecture, and reduced complexity, which makes it difficult to fully understand cellular behaviour. For this reason, interest has increasingly shifted towards 3D cell models — systems in which cells organise themselves into complex structures, such as organoids. However, organoids themselves have significant drawbacks, such as higher cost and difficulty in standardisation. Currently, there is no unified protocol for generating organoids. The primary aim of this study has been to generate organoids from dental pulp stem cells (DPSCs) to create a useful model for translational studies. These stem/mesenchymal cells are of interest due to their high regenerative potential and ease of recovery. The cells were subjected to conditions that allowed osteogenic differentiation using a specific differentiation medium. Cell viability was assessed using the CCK-8 assay and trypan blue staining, while osteogenic morphology and mineralisation were evaluated via Alizarin Red analysis and Raman microspectroscopy. Finally, immunofluorescence analysis was used to evaluate the expression of odonto/osteogenic markers such as RUNX and OSX. Alizarin Red analysis revealed a greater presence of calcium phosphate deposits compared to the control groups (CTR), a finding that was confirmed by Raman spectral profiles attributable to a hydroxyapatite-based biomaterial. Immunofluorescence analysis also revealed increased expression of odonto/osteogenic markers (RUNX and OSX), alongside reduced expression of stemness markers.

In summary, the organoids derived from dental pulp stem cells (DPSCs) exhibited highly effective osteogenic differentiation. This was evidenced by the organised deposition of a mineralised extracellular matrix rich in hydroxyapatite, which is the primary component of native bone and dentin tissue, as revealed by colorimetric and spectral analyses. This phenotypic maturation was accompanied by the coordinated activation of specific markers of mature cell lines. These results suggest that dental pulp organoids could be used as a valuable model for studying mineralization and a potential platform for drug screening and regenerative medicine applications..

1 INTRODUCTION

1.1 From 2D monolayers to 3D organoid models

Traditional two-dimensional (2D) cell cultures have long been the cornerstone of in vitro biological research. The recent emergence and rapid development of 3D culture technologies, especially organoid systems, has completely transformed in vitro culture tools for biomedical research. These new technologies have overcome the key limitations of traditional 2D cell culture systems, which are unable to replicate the intricate spatial organization, cell-to-matrix interactions, and the complex biochemical/biophysical gradients of the in vivo microenvironment (Clevers, 2016; Dutta et al., 2017; Lancaster and Knoblich, 2014; Marconi et al., 2025). This has led to the development of an organoid technology with an immense potential diseases modeling and drug screening. Organoids derived from pluripotent stem cells (PSCs) or adult stem cells (ASCs), including those obtained from dental pulp stem cells (DPSCs), have become valuable biomimetic platforms for studying infectious diseases, genetic disorders, and translational oncology (Takebe and Wells, 2019; Zhu et al., 2023). While two-dimensional (2D) monolayer cell cultures remain standard due to their simplicity, they fail to reproduce tissue architecture, cell-to-cell and cell-to-matrix interactions adequately, or the chemical and physical gradients that regulate complex processes such as differentiation, morphogenesis, and drug response (Jensen and Teng, 2020). In contrast, 3D systems, particularly organoids, allow the reconstruction of more physiologically relevant microenvironments, improving predictability compared with 2D models and animal models (Zhao et al., 2022; Zhu et al., 2023).

Despite their widespread use, terminology in the field remains inconsistent: terms such as “spheroid”, “multicellular aggregate”, and “organoid” are often used interchangeably, even though they describe biological entities that differ in terms of complexity, tissue organization, and capacity for self-organization (Ikeda et al., 2016; Ius et al., 2020). Spheroids are primarily formed through cell-to-cell adhesion. They are relatively simple, often monocultural clusters (e.g., tumor spheroids) that lack a defined tissue architecture or regenerative capacity. Organoids are defined by their capacity for self-organization and differentiation. Derived from stem cells (PSCs or adult ASCs), organoids recapitulate the complex microanatomy of the target organ—including multiple cell lineages, specialized functions, and spatial patterning

(e.g., the formation of a central lumen or crypt-villus structures) (Chasman et al., 2016; Clevers, 2016; Kretzschmar and Clevers, 2016).

A critical limitation of many 3D models is their poor geometric uniformity and limited reproducibility between batches and laboratories. These characteristics are essential prerequisites for quantitative, comparable drug screening (Dominietto and Capobianco, 2016; Zhang et al., 2009). The physiology of organoids is closely linked to the dynamics of nutrient, oxygen, and waste metabolite diffusion. Structures exceeding 400–500 μm in diameter tend to develop a necrotic or quiescent “core” due to limited oxygen diffusion and the accumulation of catabolites, a phenomenon widely described in multicellular tumor spheroids and other 3D models (Tseng et al., 2018). However, although hypoxia is a limiting factor in regenerative applications, it can be exploited for modelling purposes in oncology, allowing a more accurate reproduction of the microenvironment of solid tumors and the study of drug responses under oxygen-gradient conditions (Boucherit et al., 2020; Däster et al., 2017).

Moreover, the transition from a simple aggregate to a functional organoid depends strictly on the supply of nutrients and the support provided by natural or synthetic extracellular matrices (ECMs), such as Matrigel or defined hydrogels, which convey mechano-transduction signals of adhesion, structural polarity, and biochemical signaling that instructs stem cells to differentiate in specific lineages (Gjorevski et al., 2016; Priesnitz and Becker, 2018; Stolz and Dikic, 2018).

1.2 Stem Cell Classification and Plasticity

Stem cells are primitive, non-specialized cells with the dual ability to sustain their own population over time and to produce differentiated progeny. These characteristics rely on two core properties: self-renewal, the ability to generate, at each cell division, daughter cells that retain the same phenotypic and functional characteristics as the parent cell; and differentiation potential, the capacity to give rise to one or more mature cell types, thereby contributing to development, tissue homeostasis, and repair processes (Morrison et al., 1997; Seita and Weissman, 2010; Weissman, 2000).

Stem cells can be classified according to two main criteria: the degree of differentiation potential (or plasticity) and the tissue of origin (Poliwoda et al., 2022; Singh et al., 2016). Based on their differentiation potential, they are traditionally divided into:

- **Totipotent stem cells** that can generate all cell types of the organism, including both embryonic and extraembryonic tissues (for example, the tissues forming fetal annexes such as the trophoblast). They correspond to the cells of the earliest pre-implantation stages, particularly embryos at the 4–8 cell stage, during the first 1–3 days after fertilization, when each blastomere still retains the ability to give rise to a complete organism (Bindu A and B, 2011).
- **Pluripotent stem cells** are typically derived from the inner cell mass of the blastocyst, between days 4 and 14 after fertilization. Although they are no longer able to form extraembryonic tissues, they can differentiate into all embryonic tissues organized into the three germ layers (ectoderm, mesoderm, and endoderm), and therefore represent an extremely versatile resource for studying developmental and differentiation processes (Poliwoda et al., 2022; Singh et al., 2016).
- **Multipotent stem cells** that can proliferate and be maintained in culture for extended periods, but they do not self-renew indefinitely, unlike pluripotent cells. They can differentiate into several distinct cell types, although they are generally restricted to derivatives of a single germ layer. Adult stem cells, which are resident in many tissues, belong to this category and support physiological turnover and local tissue repair (Can, 2008; Poliwoda et al., 2022; Singh et al., 2016).
- **Unipotent stem cells** are found in adult tissues and are highly specialized and organ specific. While retaining self-renewal capacity, they can differentiate exclusively into the single cell type characteristic of their tissue of origin, thereby contributing to the structural and functional maintenance of that tissue and to its repair following damage (Can, 2008; Singh et al., 2016).

Within intratissue regenerative compartments, the overall multipotentiality of the system is ensured by the coordinated presence of adult stem cell populations with different degrees of “stemness”, which collectively support the renewal of specialized tissues (Young et al., 2004)

Stem cells are also commonly distinguished by their tissue of origin: embryonic stem cells (ESCs) and non-embryonic, or postnatal, stem cells (Poliwoda et al., 2022; Singh et al., 2016) (Figure 1).

- **ESCs**, located in the inner cell mass of the blastocyst, are pluripotent cells that, if appropriately stimulated, can give rise to all tissues of the organism (Martin, 1981; Thomson et al., 1998). Their derivation, however, requires retrieval from early-stage embryos, thereby necessitating embryo destruction, and their high plasticity is associated with reduced biological controllability, including the risk of teratoma formation and other neoplastic lesions, as shown in several experimental models (Mountford, 2008; Porter et al., 2010).
- **Non-embryonic stem cells** include fetal stem cells (FSCs), which are present in developing organ rudiments during fetal life, and neonatal stem cells (NSCs), which can be isolated from sources such as umbilical cord blood and amniotic fluid, as well as ASCs populations (Abdulrazzak et al., 2010; De Coppi et al., 2007; Piscaglia, 2014). ASCs, which can be obtained from numerous sources (bone marrow, umbilical cord blood, placenta, connective tissues, and other organs), are typically multipotent or unipotent (Tarnowski and Sieron, 2006). Although they are less “flexible” than embryonic stem cells, they are biologically more controllable and therefore can be used with greater safety for potential clinical applications, as they are associated with a lower risk of neoplastic transformation and uncontrolled growth (Pittenger et al., 1999; Tarnowski and Sieron, 2006).

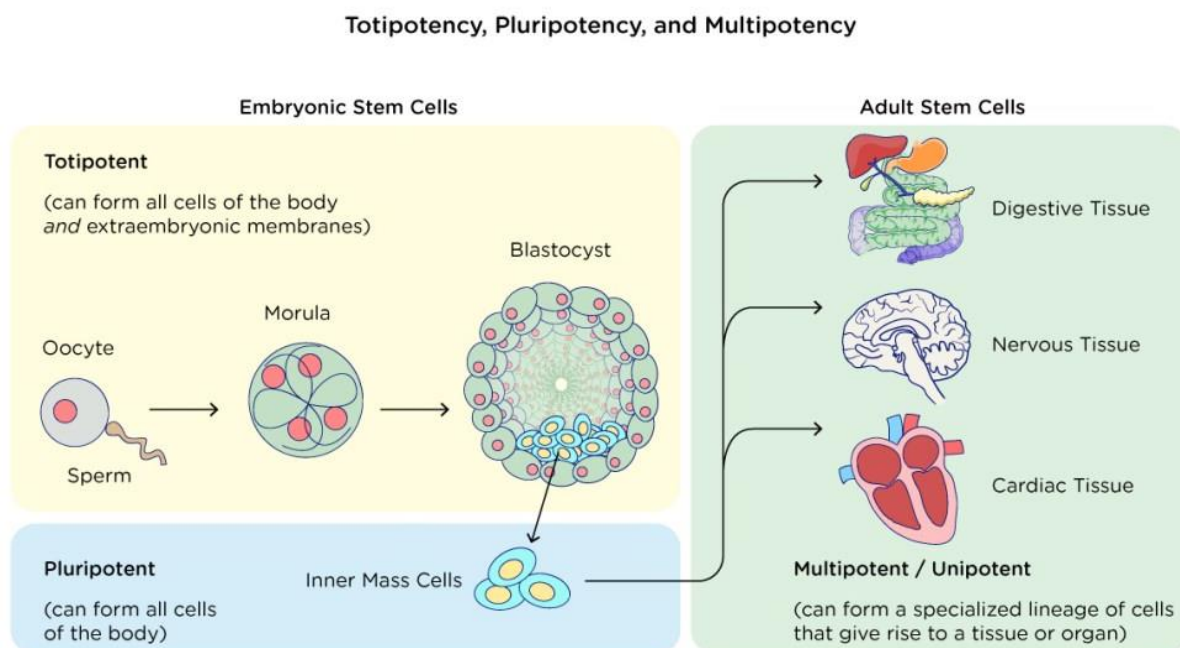


Figure 1 Schematic representation of stem cell classification based on differentiation potential (plasticity) [<https://jackwestin.com/>]

1.3 Adult stem cells (ASCs)

Adult stem cells are (ASCs) undifferentiated cells derived from mature organisms or organs. They possess self-renewal capacity, although more limited than that of embryonic stem cells, and are therefore multipotent or unipotent, with a comparatively restricted differentiation potential (Jackson et al., 2007). Their primary function is to maintain tissue homeostasis in their niche of residence and to participate in tissue repair following injury.

Adult stem cells were first described in the early 1960s, when two main populations were identified in the bone marrow:

- Hematopoietic stem cells (HSCs) give rise to the various blood cell lineages.
- Mesenchymal stem cells (MSCs), also known as bone marrow stromal cells, can differentiate into osteocytes, chondrocytes, adipocytes, and other connective tissue cells (Beltrami et al., 2003).

Further studies later demonstrated the presence of stem cells in organs traditionally considered to have little or no regenerative capacity, such as the brain and heart (Beltrami et al., 2003). Cardiac stem cells (CSCs) are multipotent cells that can differentiate into cardiomyocytes, endothelial cells, and smooth muscle cells, making them a potential tool for treating myocardial damage. Overall, adult stem cells have been identified in many organs, including the brain, bone marrow, blood vessels, skeleton, muscle, skin, liver and, to a lesser extent in the circulating blood.

Within their respective organs, adult stem cells reside in highly specialized microenvironments known as “niches”, where they remain in a quiescent state for prolonged periods and become activated when needed, initiating proliferation, migration out of the niche and differentiation (Urbanek et al., 2006). The niche is organized to meet the specific requirements of the stem cell. In contrast, each stem cell contributes to shaping and maintaining the architecture and composition of its own niche.

Early studies suggested that adult stem cells were strictly restricted to differentiating into cell types of their tissue of origin. Subsequent work has instead revealed a substantial plasticity. Unlike standard differentiation, where a stem cell produces specialized cells within its own lineage, *trans-differentiation* allows a cell to bypass its original developmental commitment (e.g., a mesodermal cell giving rise to ectodermal or endodermal lineages) without first returning to a pluripotent state. For example, bone marrow stem cells have been shown to differentiate into neurons, skeletal or cardiac muscle cells and hepatocytes (Condorelli et al.,

2001; Hombach-Klonisch et al., 2008; Xu et al., 2004). In contrast, neural stem cells can give rise to blood cells and skeletal muscle cells when exposed to appropriate growth and differentiation factors (Bjornson et al., 1999). These findings support the view that adult stem cells possess a remarkable degree of plasticity and, under specific conditions, can differentiate into cells with a different embryological origin from their native tissue. It is important to note that ASCs are not immortal. As we age, the effectiveness of the regenerative system declines due to two primary factors: intrinsic exhaustion, whereby stem cells accumulate DNA damage, and telomere shortening. This results in the stem cells entering a state of senescence, as well as niche degeneration caused by the inflammaging of the microenvironment surrounding them (X. Li et al., 2023; Ogrodnik and Gladyshev, 2023).

1.4 Mesenchymal stem cells (MSCs)

Mesenchymal stem cells are defined as “multipotent mesenchymal stromal cells” with high proliferative capacity, self-renewal properties, and the ability to differentiate into multiple mesenchymal-derived tissues (Figure 2). They originate from the mesoderm, the intermediate embryonic germ layer, and can therefore generate bone marrow stroma, adipose tissue, bone, cartilage, tendon, skeletal muscle and visceral mesoderm. Experimental evidence has also shown that, under appropriate conditions, MSCs can differentiate into non-mesodermal cell types, including neurons, epithelial cells, endothelial cells, hepatocytes, renal cells and lung cells (Bensaïd et al., 2003).

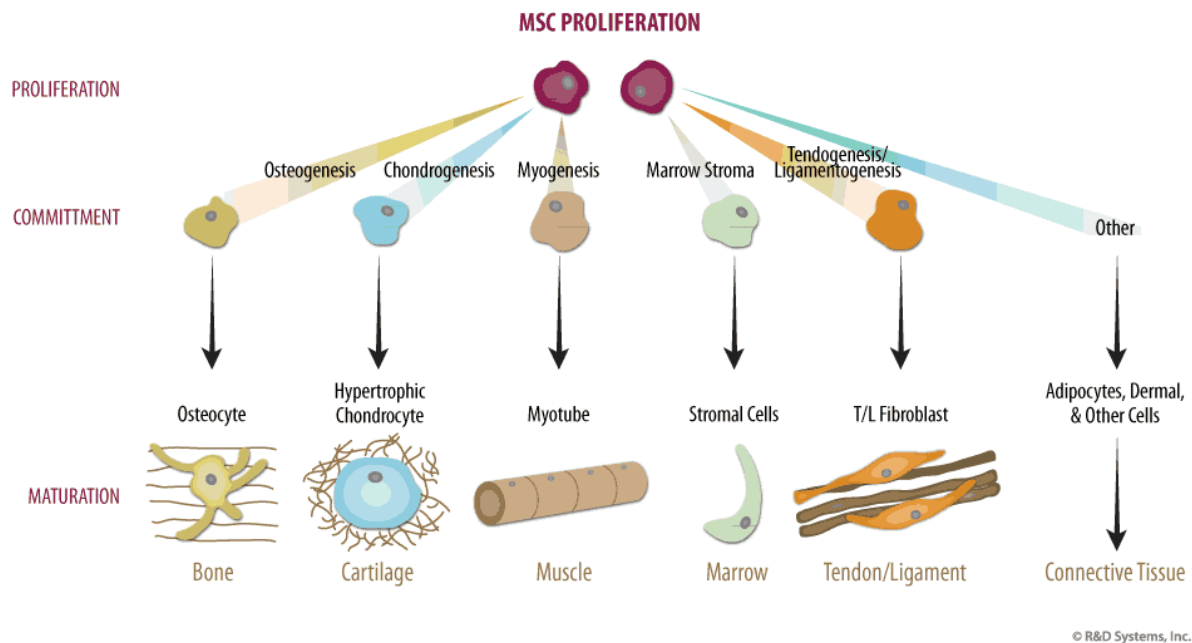


Figure 2 Differentiation potential of MSCs. [<https://parentsguidecordblood.org/en/news/not-all-umbilical-cord-tissue-banks-are-same-does-storage-method-compromise-parent-access>]

Although early studies focused on bone marrow, spleen, and thymus, it is now clear that many other tissues harbor MSCs, such as periosteum, synovial fluid, muscle, tendons and several connective tissues (Fajardo-Orduña et al., 2015). In addition to bone marrow, alternative MSC sources have been extensively investigated, including adipose tissue, peripheral blood, umbilical cord and fetal tissues.

The clinical use of bone marrow, derived MSCs is limited by several issues, including the invasive nature of bone marrow aspiration, the relatively low yield of stem cells per sample, and the age-dependent decline in cell recovery. These limitations have prompted the search for alternative autologous stem cell sources. Among these, adipose tissue has emerged as an excellent candidate due to its widespread distribution and easy accessibility. In vitro, adipose-derived cells share many features with bone marrow MSCs: adherence to plastic, fibroblast-like morphology, formation of fibroblast colony-forming units (CFU-F), expression of characteristic surface markers, and the capacity to differentiate into osteogenic, adipogenic and chondrogenic lineages under appropriate induction conditions (Dominici et al., 2006; Horwitz et al., 2005).

A distinctive immunophenotypic feature of MSCs, regardless of their tissue origin, is their consistent positivity (>95%) for a panel of surface markers (CD73, CD90, CD105 and CD44), and their negative expression (<2%) of hematopoietic and endothelial antigens (CD45, CD34, CD14, CD11b, CD19, CD79a and HLA-DR), as defined by the minimum criteria of the

International Society for Cell & Gene Therapy (ISCT) (Table 1). These markers, combined with trilineage differentiation potential and plastic adherence, enable the reliable identification of MSCs from other stromal populations (Bara et al., 2014).

Positive Stemness Markers	Negative Stemness Markers
CD73 (5'-nucleotidase)	CD45 (lymphocyte common antigen)
CD90 (Thy-1)	CD14 or CD11b
CD105 (endoglin)	CD19 or CD79a
STRO-1	CD15 (Sialyl-LewisX)
CD166 (activated leukocyte cell adhesion molecule protein)	HLA-DR (human leukocyte antigen subclass)
CD44	CD31 (platelet endothelial cell adhesion molecule 1)
CD29 (integrin β 1)	CD40
CD146 (melanoma cell adhesion molecule)	
CD271 (nerve growth factor receptor)	
CD54 (Intercellular Adhesion Molecule 1)	
CD106 (vascular cell adhesion molecule 1)	
SSEA-4 (stage-specific embryonic antigen-4)	
Nestin	

Table 1: Positive and negative stemness markers in mesenchymal stem cells (Crisan et al., 2008; Dominici et al., 2006; Gang et al., 2007; Sacchetti et al., 2007)

1.4.1 Mesenchymal Stem Cells (MSCs): Mechanism of Action and the dual role in regeneration

Mesenchymal stem cells (MSCs) play a crucial role in tissue regeneration through a dual mechanism of action: direct cell differentiation and indirect paracrine signaling (Bagno et al., 2022; Keating, 2012). This approach, mediated by the release of soluble factors and extracellular vesicles (EVs), mirrors the regenerative behavior observed for DPSCs in reference works.

In the direct mechanism, MSCs home to the injury site and integrate into tissues by differentiating into specialized cells. This includes the formation of pericytes, which stabilize

blood vessels via NG2 and α -SMA, as well as osteoblasts, a process stimulated by BMP-2 and dexamethasone, which activate Runx2 to produce collagen I and hydroxyapatite. Furthermore, MSCs can differentiate into chondrocytes (induced by TGF- β 3 in 3D environments to upregulate Sox9, collagen II, and aggrecan) and adipocytes (activated by insulin and PPAR γ , leading to the accumulation of structural lipids). While these cells physically reconstruct the tissue matrix, their long-term survival is frequently limited by anoikis and immune clearance (Chang et al., 2021; Leach and Whitehead, 2018; Maacha et al., 2020; Sagaradze et al., 2020; Samsonraj et al., 2017).

However, the therapeutic effect is predominantly dominated by the paracrine mechanism. MSCs secrete a wide range of bioactive factors, including vascular growth factors (VEGF, HGF), anti-inflammatory cytokines (IL-10, TGF- β), and EVs rich in microRNA. These factors reduce inflammation, promote angiogenesis, and activate endogenous progenitor cells without the need for extensive cell engraftment. Such paracrine signals are further amplified by conditions like hypoxia or inflammatory priming, which shape the regenerative microenvironment by promoting M2 macrophage polarization and mitochondrial transfer. This has been demonstrated in models of myocardial infarction, where the efficacy of the secretome alone matches or exceeds that of live cells (Gnecchi et al., 2016; Han et al., 2022; Maacha et al., 2020).

This duality, in which paracrine signaling contributes over 80% of the observed effects, explains the rising preference for cell-free strategies based on conditioned media or EVs. These strategies mitigate risks such as senescence and immunogenicity, opening new prospects for clinical applications in dental and vascular regeneration in synergy with DPSCs (C. Chang et al., 2021; Gnecchi et al., 2016; Mirotsoy et al., 2011). In summary, modern perspectives on MSCs emphasize their role as immunomodulatory sentinels. Rather than merely acting as building blocks for tissue replacement, they function as bioactive "factories" that secrete trophic factors and extracellular vesicles to orchestrate endogenous repair and dampen pathological inflammation.

1.5 Dental pulp stem cells (DPSCs)

DPSCs are MSCs residing within the dental pulp and display biological properties largely comparable to bone marrow-derived MSCs (BM-MSCs). They were first isolated and

characterized as a distinct MSC population by Gronthos and colleagues in 2000 (Gronthos et al., 2000). However, the presence of multipotent cells within the dental pulp had already been suggested by Yamamura in 1985 (Yamamura, 1985). Since those early observations, several MSC subpopulations have been identified in different tooth-associated niches and named according to their anatomical origin, including DPSCs, stem cells from human exfoliated deciduous teeth (SHEDs), periodontal ligament stem cells (PDLSCs), dental follicle stem cells (DFSCs), stem cells from the apical papilla (SCAPs), and gingival MSCs (GMSCs) (Huang et al., 2008; Miura et al., 2003; Seo et al., 2004; Zhang et al., 2012). DPSCs can be obtained from the pulp of both deciduous and permanent teeth, typically from teeth extracted for orthodontic reasons, impacted third molars, or supernumerary teeth, and are subsequently characterized by their surface antigen profile. Functionally, DPSCs are multipotent cells that fulfill all canonical MSC criteria, including adherence to plastic, expression of typical MSC markers, and the ability to differentiate along multiple mesenchymal lineages (Figure 3) (Aydin and Şahin, 2019; Delle Monache et al., 2022; Santilli et al., 2023).

Within healthy teeth, DPSCs are embedded in the central pulp chamber, a soft, highly vascularized connective tissue organized into four main zones: the peripheral odontogenic (odontoblast) layer, a cell-free zone, a cell-rich zone, and the central pulp core (Al Madhoun et al., 2021; Grawish, 2024). Developmentally, DPSCs originate from ectomesenchymal cells derived from migratory neural crest stem cells during early craniofacial morphogenesis (Gronthos et al., 2000; Y.-L. He et al., 2025). This neural crest origin underlies their pronounced neurotropic and neurogenic properties, which have been reported to exceed those of other MSC sources and have sparked considerable interest in their potential for treating neurodegenerative and neurovascular disorders (Candelise et al., 2023; Luo et al., 2018; Rahnama Sisakht et al., 2024; Solis-Castro et al., 2022). DPSCs represent an easily accessible source of mesenchymal stem cells, often derived from surgical waste such as wisdom teeth, with a unique characteristic: their neural crest origin. This origin provides them with a "competitive edge" in the regeneration of neural tissues compared to stem cells harvested from adipose tissue or bone marrow.

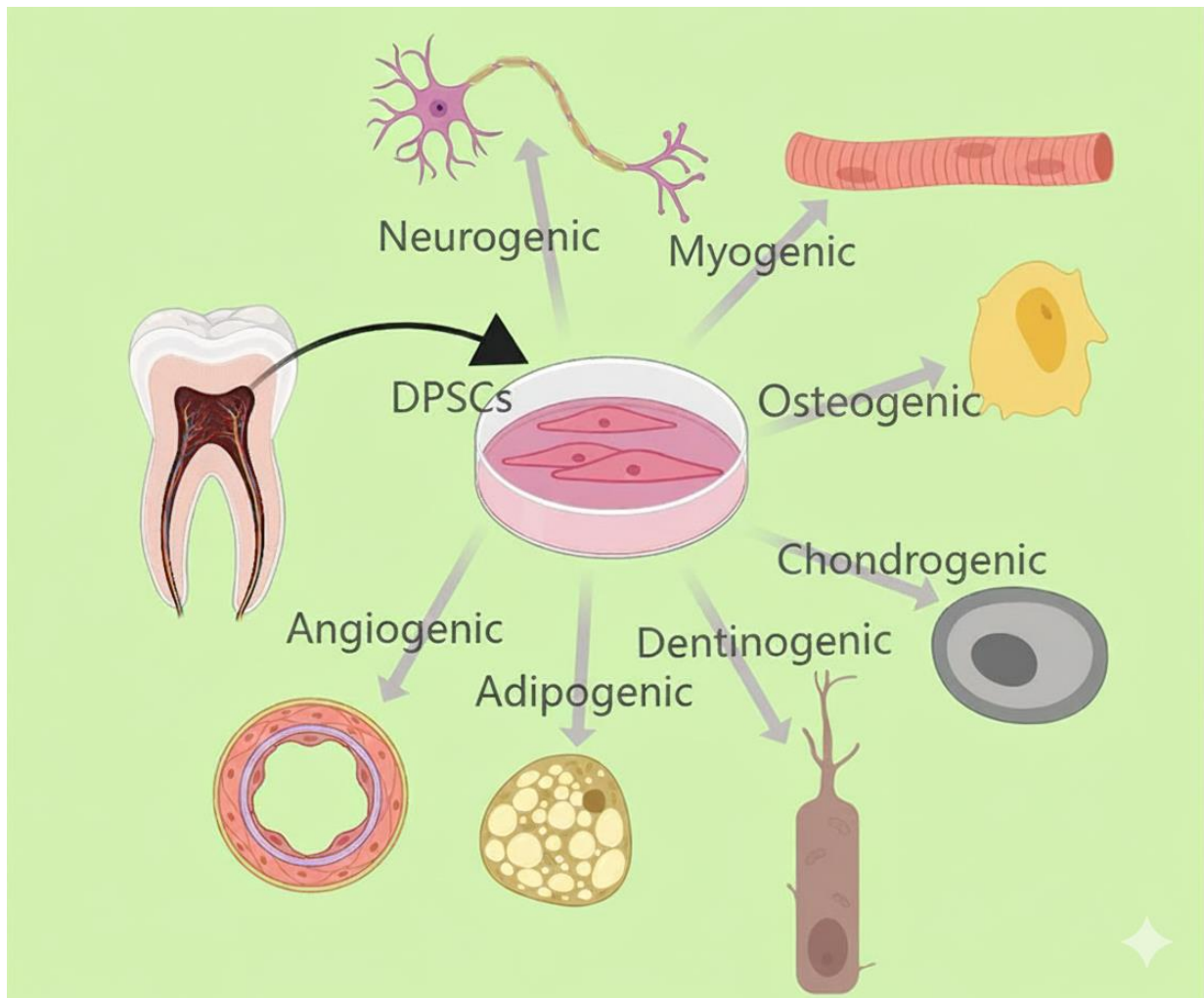


Figure 3 Localization and multipotent differentiation potential of DPSCs (F. Li et al., 2023).

1.5.1 DPSC isolation

Given the relevance of dental stem cells in regenerative medicine and tissue engineering, a variety of protocols for DPSC isolation, expansion, and banking have been established. Starting from the original method described by Gronthos and co-workers, subsequent studies have progressively optimized each step of dental pulp processing, ranging from tissue retrieval to characterization, differentiation assays, and cryopreservation, while also addressing minimally invasive approaches and ethical considerations (Ferro et al., 2012; Rodas-Junco and Villicaña, 2017; Tirino et al., 2012). Several pre-analytical variables can profoundly influence DPSC yield and quality, including tooth type (e.g., molar versus third molar), donor-related factors such as sex, age, and lifestyle, and conditions of tooth transport and short-term storage before processing. Perry et al. demonstrated that DPSCs remain viable for up to 5 days after extraction

when teeth are stored at 4 °C in phosphate-buffered saline (PBS). However, a marked decline in cell viability is evident after 24 hours of storage.

Three main strategies are commonly used for DPSC isolation: (i) outgrowth from tissue explants (OG); (ii) enzymatic digestion (ED) of pulp tissue; and (iii) mechanical dissociation, alone or in combination with enzymatic procedures (Ferrúa et al., 2017; Hilkens et al., 2013; Karamzadeh et al., 2012). In the OG approach, which is technically straightforward, small pulp fragments (approximately 1–3 mm³) are placed directly onto culture dishes, allowing mesenchymal/stromal cells to migrate out of the tissue and adhere to the plastic surface. Enzymatic digestion, on the other hand, aims to dissociate the extracellular matrix and efficiently release single viable cells; various enzyme cocktails have been proposed, mainly based on collagenases (types I, II, or IV), dispase, trypsin, or accutase. Among the available techniques, enzymatic digestion remains the most widely used for DPSC isolation and has been associated with higher proliferation and differentiation rates than explant outgrowth cultures (Huang et al., 2006; Karamzadeh et al., 2012; Lindroos et al., 2008; Scheller et al., 2008; Takeda et al., 2008).

In the widely used protocol derived from Gronthos' original method, pulp tissue is gently dissected from crown and root, then incubated for 1 hour at 37 °C in an enzymatic solution containing 3 mg/ml collagenase type I and 4 mg/ml dispase, which represents the most frequently employed enzyme combination. Numerous alternative digestion protocols for pulp dissociation have also been proposed and are summarized in Table 2.

Enzymatic solution	Conditions	teeth
3 mg/mL collagenase type I, 4 mg/mL dispase	30-60 min at 37 °C	third molars and molars
4 mg/mL collagenase type I, 4ml/mL dispase type II	24 h at 4 °C	deciduous teeth
4 mg/mL collagenase type I, 2 mg/mL dispase	1 h at 37 °C	incisors
0.2 mg/mL collagenase type I, 2 mg/mL dispase	70 min 37 °C	third molars
1 mg/mL collagenase type I, 2.4 mg/mL dispase	1 h at 37 °C	deciduous teeth, adult molar
3 mg/mL collagenase type I	1 h at 37 °C	third molars
2 mg/mL collagenase type I, dispase	30 min at 37 °C	deciduous teeth, third molar
0.2 % collagenase type I	1 h at 37 °C	incisors, canines, molars, deciduous teeth
0.2 % collagenase type II	30 min at 37 °C	third molar
1 mg/mL collagenase type I	30 min at 37 °C	third molar
0.1 % collagenase type IV	15-30 min at 37 °C	third molar
0.2 % trypsin	5 min at 37 °C	third molar
1 mg/mL accutase solution	30 min at 37 °C	third molar

Table 2: Enzymatic digestion protocols for DPSCs isolation (Mattei et al., 2025).

1.5.2 DPSC culture conditions

Efficient establishment of primary DPSC cultures requires robust cell attachment to the culture surface. This can be promoted by pre-coating plasticware with extracellular matrix (ECM) proteins, peptide-modified substrates, synthetic polycationic polymers, or commercially available culture-treated surfaces. Various ECM components, such as recombinant vitronectin, laminin-511, and laminin-521 combined with E-cadherin, as well as synthetic polymers including polyethyleneimine and poly[2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl) ammonium hydroxide] (PMED-SAH), have been shown to support long-term culture and maintenance of pluripotent stem cells (Rodin et al., 2014, 2010; Rowland et al., 2010; Vancha et al., 2004). Analogously, several studies suggest that ECM coatings can enhance DPSC adhesion, proliferation, and maintenance of stemness, in line with observations in other stem cell types. Kim et al. proposed that the ECM not only provides a structural scaffold for cell attachment but also delivers crucial biochemical and biomechanical cues that help preserve the native stem cell niche (Kim, 2005).

Following enzymatic digestion, DPSCs are commonly resuspended and seeded in basal media, such as α -MEM, high- or low-glucose DMEM, DMEM/F12, and DMEM-KO (Table 3). These media are typically supplemented with relatively high concentrations of fetal bovine serum (FBS, 10–20%), which supplies growth factors, hormones, attachment factors, and other components essential for cell metabolism, proliferation, and survival. Nevertheless, the use of FBS poses significant limitations in the context of clinical translation, including the risk of xenogeneic contamination, immune reactions, and variability in therapeutic efficacy (Bui et al., 2021; Schepici et al., 2022; Tekkatte et al., 2011). Consequently, there is growing interest in xeno-free or reduced-serum alternatives, such as media supplemented with human serum, umbilical cord blood serum, human platelet lysate (hPL), or fully chemically defined serum-free formulations (Eubanks et al., 2014; Govindasamy et al., 2011; Lee et al., 2011; Takeda-Kawaguchi et al., 2014; Tekkatte et al., 2011).

α -MEM	Human platelet lysate (hPL) 20% FBS + penicillin/streptomycin 10% FBS + 2mM L-Glutamine + 100 units/mL penicillin and 100 μ g/mL streptomycin 10% FBS + 2 mM L- Glutamine + 100 μ M ascorbic acid-2-phosphate + 100U/mL penicillin-G + 100 μ g/mL streptomycin solution 20% FBS + antibiotics (100 IU/mL Penicillin and 100 μ g/mL Streptomycin) 15% FBS + 100 U/mL penicillin/streptomycin
DMEM	10-20% FBS + penicillin/streptomycin
DMEM/F12	10% FBS+ penicillin/streptomycin
DMEM-LG	10% FBS + penicillin/streptomycin 20% FBS + 2 mM L-Glutamine + penicillin/streptomycin

	10% FBS+ 1% L-Glutamine + 100 U/mL penicillin + 100 µg/mL streptomycin
DMEM-KO	10-20% FBS + penicillin/streptomycin
Culture conditions	37 °C in a humid atmosphere with 5% CO ₂
Cryoconservation	10% DMSO in complete medium or FBS

Table 3: Culture media for DPSCs propagation (Mattei et al., 2025).

In standard culture practice, DPSCs are maintained at 37 °C in a humidified atmosphere with 5% CO₂, with medium changes usually performed every 2 days, and cells harvested at approximately 80% confluence to ensure optimal expansion and phenotype stability. During culture, several environmental parameters, including temperature, humidity, pH, and oxygen tension, critically influence DPSC behavior. Notably, exposure to atmospheric oxygen levels (21% O₂) has been reported to reduce proliferation and trigger antioxidant defense mechanisms in DPSCs compared with hypoxic conditions (3% O₂), underscoring the importance of reproducing physiologically relevant oxygen levels to preserve their regenerative potential better (Ahmed et al., 2016; El Alami et al., 2014; Mas-Bargues et al., 2019; Shi et al., 2019).

1.6 Dental Pulp Stem Cells as a Versatile Cell Source for Regenerative Medicine

Dental pulp stem cells (DPSCs) are a type of mesenchymal stem cell that exhibits the classic characteristics of MSCs, such as adhesion to plastic and an elongated, fibroblast-like morphology, when cultured in vitro (Al Madhoun et al., 2021). Pioneering studies have demonstrated that postnatal DPSCs can form clonogenic colonies with high proliferative capacity, thereby confirming their self-renewal potential in both in vitro and in vivo settings (Gronthos et al., 2002). Subsequent studies have demonstrated that, under the right molecular conditions, these cells can differentiate into various mesenchymal lineages, particularly osteo/odontogenic, but also chondrogenic and, to a lesser extent, adipogenic (Al Madhoun et al., 2021). Their marked propensity to form mineralized matrices and dentin- and bone-like structures has made DPSCs among the most widely studied candidates for tissue-engineering applications in dentistry and orthopedics (Bai et al., 2023).

In regenerative medicine, DPSCs have been used in numerous preclinical models to promote bone defect repair and dental tissue regeneration thanks to their combination of high proliferative capacity, marked osteogenic potential, and release of trophic and immunomodulatory factors (Aurrekoetxea et al., 2015). Several *in vitro* and *in vivo* studies have demonstrated that DPSCs can deposit an extracellular matrix rich in collagen and calcium phosphate when seeded on appropriate scaffolds or in combination with bioactive biomaterials. This promotes osseointegration and the filling of critical bone defects, as well as the neoformation of dentin-like tissue at the root or periapical level (Mealy and O’Kelly, 2015; Ravn Jakobsen et al., 2018). It has also been found that the secretome of DPSCs, particularly in the conditioned medium (DPSC CM) and extracellular vesicles, modulates local inflammation, stimulates the proliferation of resident progenitor cells, and enhances angiogenesis. This creates a microenvironment conducive to bone and dental pulp regeneration (Campos et al., 2023).

Overall, DPSCs' ability to differentiate along osteo/odontogenic lines and orchestrate processes of immunomodulation, neoangiogenesis and tissue remodelling through paracrine signals consolidates their role as a valuable stem cell resource for regenerative strategies aimed at recovering damaged dental and bone structures (Al Madhoun et al., 2021).

1.7 Organoids

Organoids represent a revolutionary advance in biomedical research: three-dimensional (3D) models derived from pluripotent stem cells (PSCs), induced pluripotent stem cells (iPSCs), embryonic stem cells (ESCs), adult stem cells (ASCs), or primary/cancer cells that recapitulate native human organs' architecture, cellular composition, function, and molecular characteristics (Lancaster and Knoblich, 2014; Yang et al., 2023). Their historical evolution traces back to 1907, when Henry Van Peters Wilson demonstrated dissociated sponge cells self-organizing into complete organisms, the first *in vitro* regeneration. Progress accelerated with embryonic stem cells (Thomson et al., 1998) and iPSCs (Takahashi and Yamanaka, 2006), culminating in 2009 when Sato et al. established Lgr5⁺ intestinal crypt organoids self-organizing into crypt-villus structures sans mesenchymal niche, pioneering single-ASC-derived 3D cultures (Sato et al., 2011). Eiraku (2008) simultaneously generated ESC-derived cerebral cortex via 3D aggregation (Eiraku et al., 2008) (Figure 4). Subsequent expansion encompassed mesendoderm

(stomach/liver/pancreas/lung/kidney) and neuroectoderm (brain/retina) organoids (Corrò et al., 2020).

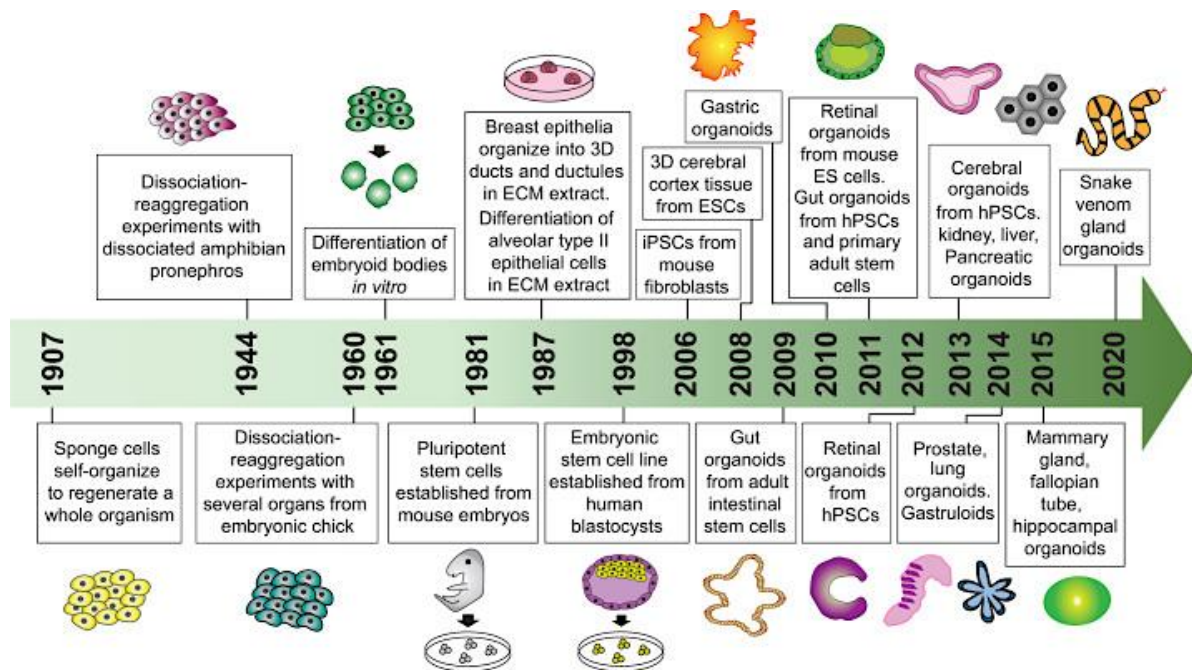


Figure 4 This is a timeline for the development of organoid cultures. A summary of the key landmark studies and breakthroughs that led to the development of various organoid technologies. Topics covered include extracellular matrix (ECM), embryonic stem cells (ESCs), human pluripotent stem cells (hPSCs) and induced pluripotent stem cells (iPSCs) (Corrò et al., 2020).

Unlike traditional two-dimensional (2D) monolayers, which constrain cells to unnatural planar growth and induce dedifferentiation, epithelial-mesenchymal transition (EMT) and loss of apico-basal polarity, as well as failing to capture tissue-specific stromal-vascular interactions, organoids embedded in ECM hydrogels, such as Matrigel, basement membrane extracts or synthetic peptide hydrogels, faithfully preserve the genomic stability, epigenetic landscape, self-renewal capacity and long-term proliferative potential of the parental tissue. They also enable longitudinal studies spanning months (Fatehullah et al., 2016; Garreta et al., 2021; Yang et al., 2023).

The lack of a universal standardized protocol for organoid generation highlights both the versatility of the technique and the nascent stage of the field. Protocols must be meticulously optimized for each cell source and target organ, drawing from an expanding methodological repertoire. This includes static embedding techniques such as suspended drop and U-bottom plates/ultra-low adhesion wells; dynamic suspension culture using orbital shakers, rotating flasks and rotating bioreactors; air-liquid interface (ALI) systems for epithelial polarization;

and sophisticated matrix-free approaches or next-generation organoids-on-chips that integrate microfluidic perfusion (Yang et al., 2023). These strategies systematically engineer the essential biomechanical, biochemical and topographical characteristics required to recapitulate the embryonic morphogenetic programs that guide crypt budding, lumen formation, branching morphogenesis, multicellular lineage commitment and progressive functional maturation towards adult-like physiology (Driehuis et al., 2020; Yang et al., 2023).

1.8 Organoids from Pluripotent Stem Cells vs Adult Stem Cells

Organoids derived from pluripotent stem cells (PSCs), embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) embody the foundational paradigm of organoid technology, involving stepwise directed differentiation that mimics embryonic organogenesis. These protocols progressively activate developmental signalling cascades (WNT/ β -catenin, BMP, FGF, Notch and SHH) to generate complex, multilineage structures that recapitulate all major organ compartments, including the epithelium (with apical-basal polarity and lumenogenesis), the mesenchyme (with fibroblasts and pericytes) and the endothelium (with vascular networks) (Figure 5) (C.-Y. Chang et al., 2021; Yin et al., 2016). PSCs excel at modelling early developmental stages, congenital malformations (e.g. microcephaly and polycystic kidney disease) and rare genetic diseases via isogenic CRISPR controls. However, they require extended timelines (6–12 weeks of maturation), intricate growth factor orchestration and exhibit variability in cyst formation efficiency (30–70%) and final cellular maturity between batches (Tang et al., 2022; Zhao et al., 2022).

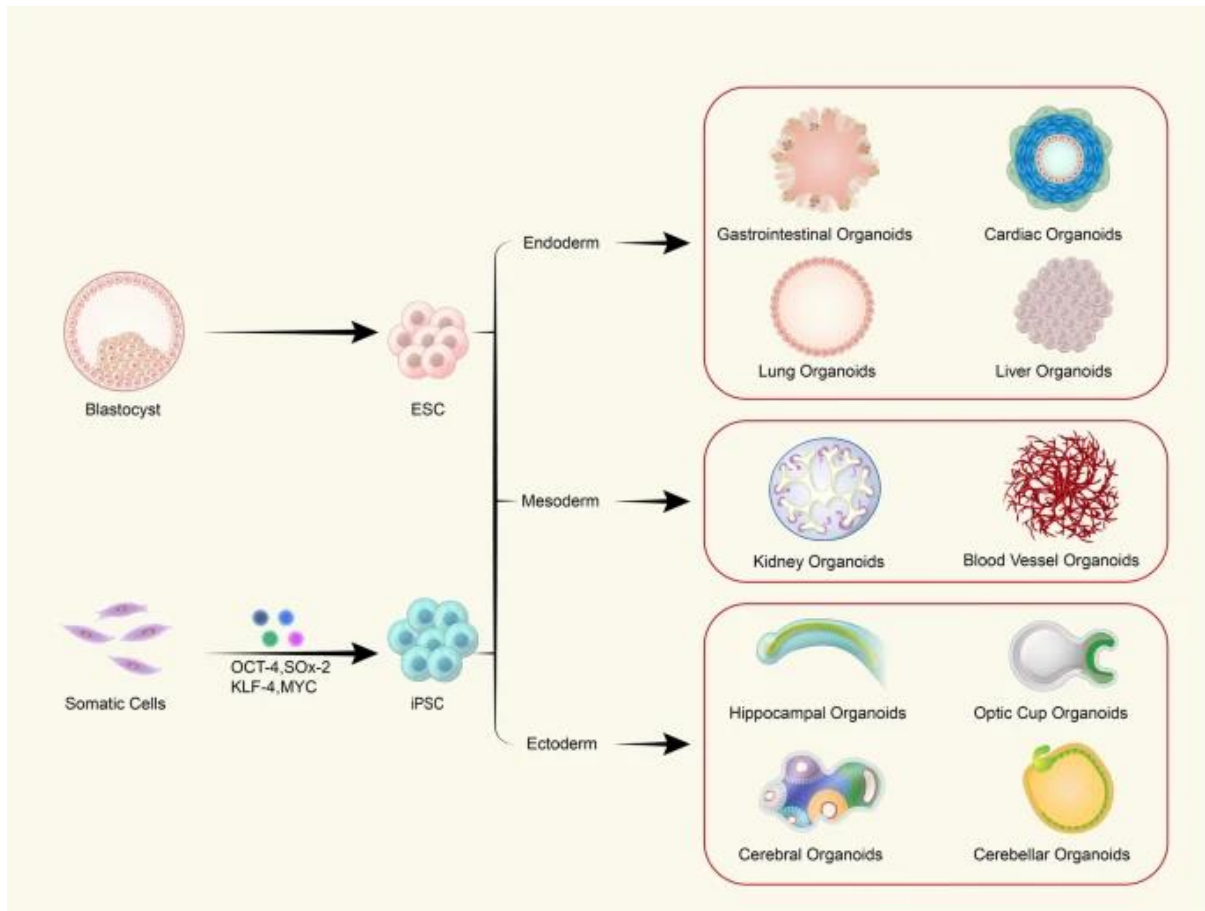


Figure 5 This diagram shows the main organoids that can be derived from iPSCs. ESCs and iPSCs are obtained by reprogramming somatic cells and can generate all three germ layers: the endoderm, the mesoderm and the ectoderm. These layers then develop into different organs, such as the liver, lungs, heart, kidneys and brain. Three-dimensional (3D) organoid technology allows many of the structural and functional characteristics of these tissues to be reproduced in vitro (Tang et al., 2022).

Conversely, organoids derived from ASCs and MSCs — lineage-restricted populations 'pre-committed' to tissue maintenance — leverage intrinsic self-organization, bypassing embryonic recapitulation. Minimal niche factors (WNT3a, EGF, Noggin and R-spondin1) are sufficient for the rapid formation of crypts, villi, glands, or alveoli within 7–14 days. ASC-derived organoids exhibit adult-like phenotypic and functional maturity, including mature epithelial polarization, specialized secretory lineages (Paneth/goblet cells for the intestine, Clara/ciliated cells for the airways, and hepatocytes/cholangiocytes for the liver), robust barrier function (TEER >1000 $\Omega \cdot \text{cm}^2$) and physiological responses to microbial and pathogenic challenges (Blutt et al., 2021; Boonekamp et al., 2020; Hu et al., 2024; Kim et al., 2020; Sun and Ding, 2017; Yang et al., 2025).

This maturity advantage makes ASC organoids more suitable than PSC models for studying adult tissue homeostasis (e.g. stem cell dynamics and circadian rhythms), regeneration (e.g. injury/repair cascades), chronic inflammation (e.g. IBD, COPD and NASH) and infectious diseases (e.g. SARS-CoV-2 tropism and bacterial dysbiosis). ASC organoids offer higher reproducibility, lower costs and greater physiological relevance than PSC models (Figure 6) (Hammerhøj et al., 2024; Heinzelmänn et al., 2024; Jin et al., 2024; Qian et al., 2022; Tang et al., 2022).

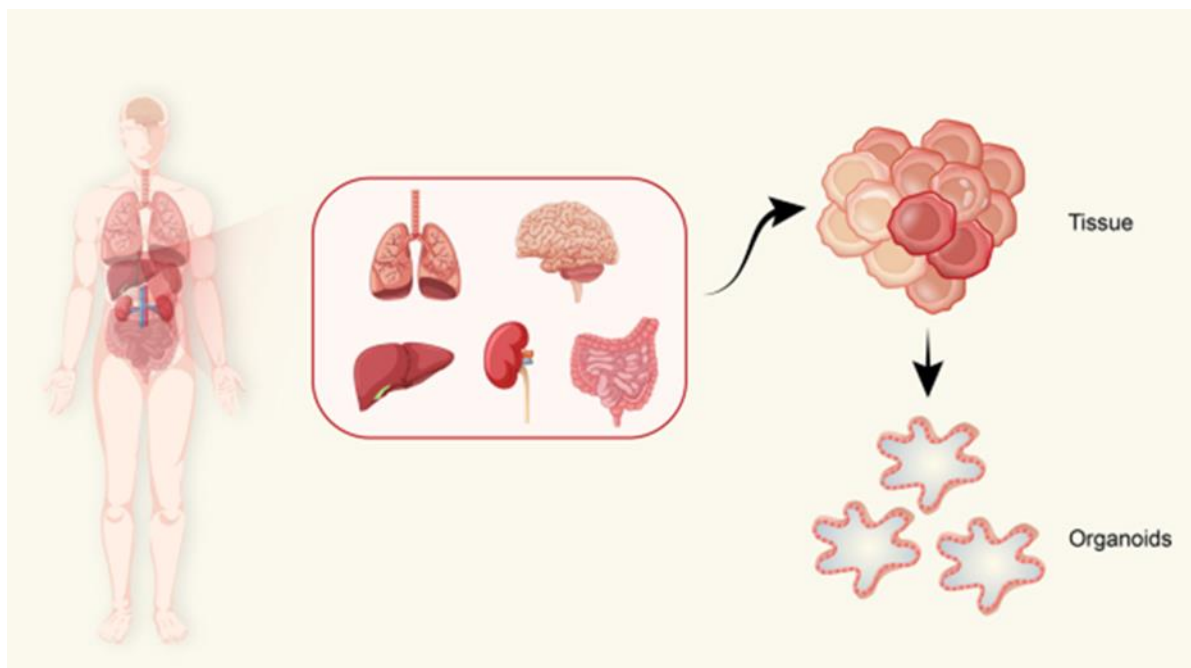


Figure 6 This schematic diagram illustrates the process of deriving organoids from ASCs, which are found in organs such as the lungs, brain, liver, intestines, and kidneys. Unlike ESCs, ASCs can generate organoids in vitro under appropriate culture conditions (Tang et al., 2022).

1.8.1 Organoid generation techniques

Organoid generation and culture techniques now encompass a wide range of approaches that differ in terms of cell aggregation methods, the degree of control over the microenvironment and potential for scalability. Despite this diversity, there is still not universally standardized protocol, and experimental conditions are primarily adapted to the type of tissue and cell source used. In general, organoids can be obtained through methods that promote cell-cell adhesion and three-dimensional self-organization. These methods minimize direct interaction with plastic culture surfaces and, as far as possible, recreate key characteristics of the in vivo stem cell niche (Zhao et al., 2022).

One group of strategies involves dynamic suspension culture systems, in which single cells or small aggregates are kept in constant motion using stirred-tank or spinner-flask bioreactors, rotating-wall vessels, or orbital shakers. Under these conditions, the cells remain in suspension and spontaneously form spheroidal aggregates, which benefit from improved medium mixing and more efficient nutrient and gas exchange. This reduces oxygen gradients compared to static cultures. These systems are particularly interesting for large-scale production as they enable the production of a high number of organoids. Still, they require large volumes of medium and careful control of fluid dynamic parameters to avoid excessive shear stress. They also require dedicated equipment, which is often expensive and not always available in academic laboratories (Unagolla and Jayasuriya, 2022).

As an alternative, stationary scaffold-free culture techniques offer a more straightforward, accessible solution. In this case, the cell suspension is seeded onto non-adherent surfaces, either by coating them with agarose or polyHEMA, or by using commercial 'ultra-low attachment' plates. These surfaces inhibit cell-substrate adhesion, thereby promoting aggregation through sedimentation and short-range cell-cell interactions. Using 96-well plates with U-shaped bottoms enables the generation of one organoid per well with relatively homogeneous dimensions. It allows independent monitoring and manipulation of individual constructs, which is particularly useful in cytotoxicity assays and drug screening. However, these platforms have some limitations, including moderate overall productivity, residual variability between wells and the need for careful handling to prevent fusion or loss of organoids during medium changes (Suarez-Martinez et al., 2022).

Among scaffold-free approaches, the hanging drop method is a classic, widely used technique for obtaining aggregates of a highly uniform size. Small volumes of cell suspension are deposited on the lid of a culture plate or on specially designed supports. After inverting the plate, surface tension retains the drops, and gravity causes cells to accumulate on the rounded bottom of each drop. This promotes rapid compaction into spheroids. The final size of the construct can be modulated by varying the initial cell number and the droplet volume. This makes the method particularly suitable for studies requiring precise size control. Conversely, the small volume limits the culture duration, medium changes are laborious and collecting individual organoids is time-consuming. This makes the technique unsuitable for large-scale production or long-term experiments (Figure 7) (Unagolla and Jayasuriya, 2022).

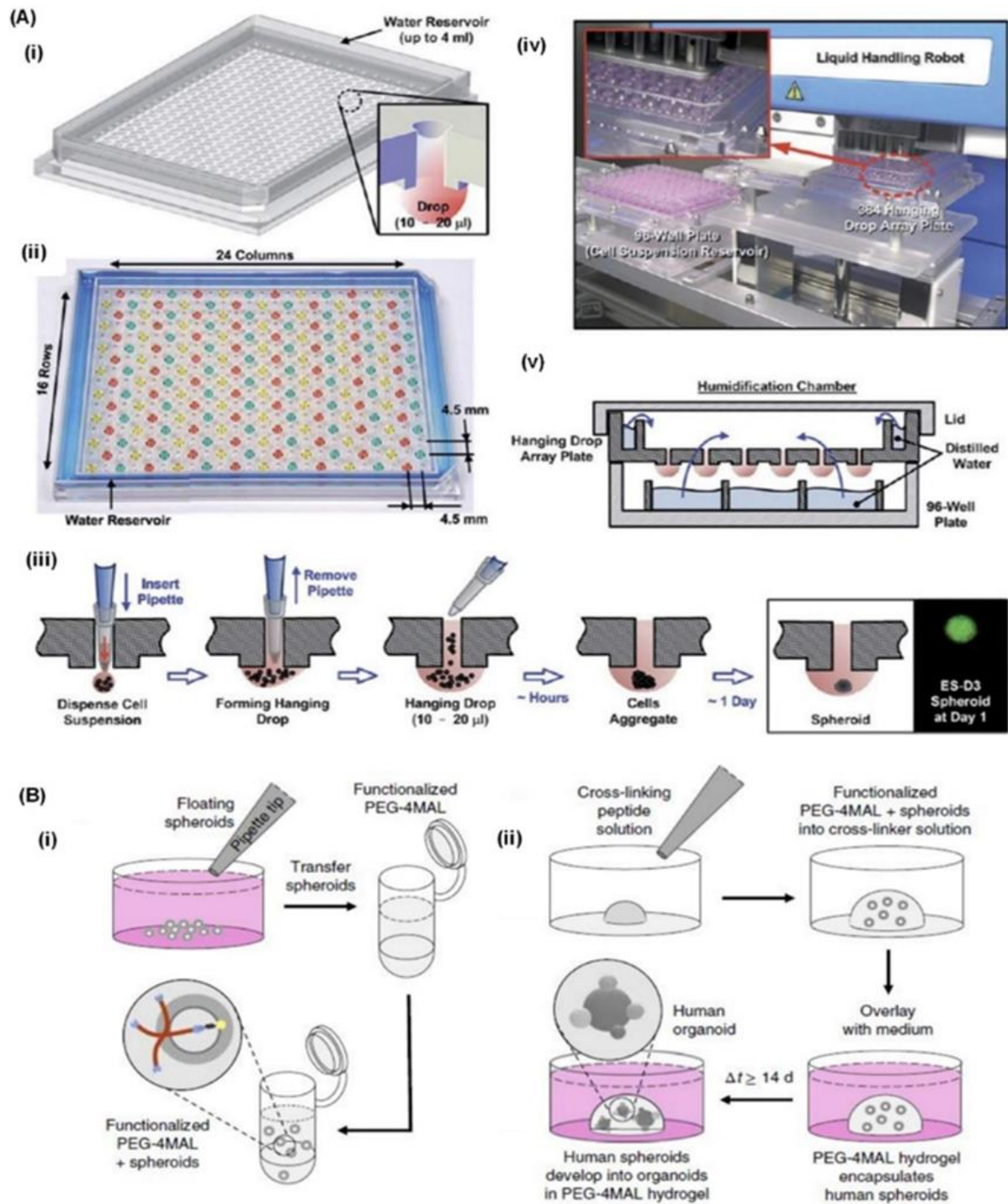


Figure 7 (A) The development and operation of the 384-drop hanging platform: (i) Technical illustration and morphological details of the designed plate. (ii) Technical illustration and morphological details of the designed plate. (iii) Dynamics of spheroid formation: individual cells aggregate to form a single three-dimensional structure within the first day of culture. (iv) Process scalability demonstration using robotics for liquid handling (96 simultaneous sites). (v) A diagram of the humidified chamber optimised for 3D growth. (B) Generation of organoids in a synthetic matrix: (i) The mixing phase of human (hPSC) spheroids and PEG-4MAL polymer precursors. (ii) The encapsulation and cross-linking process involves seeding the spheroids in the hydrogel, where they expand and transform into mature organoids (Unagolla and Jayasuriya, 2022).

In addition to support-free systems, organoid cultures immersed in ECMs are now the standard for many epithelial tissues and PSC-derived organoids and play a central role. In these protocols, stem cells or small tissue fragments are embedded in droplets or domes of Matrigel, collagen, or other natural hydrogels. These hydrogels act as biomimetic, three-dimensional scaffolds, providing adhesion, polarity, and mechanotransduction signals. This promotes the self-organization of cells into luminal structures and their differentiation into multiple cell lines (McCauley and Wells, 2017).

Matrigel-based approaches play a central role, both historically and currently, and remain the most common strategy for generating epithelial organoids and organoids derived from pluripotent and adult stem cells. In these protocols, cells (either individually or in small tissue fragments) are incorporated into small Matrigel domes or droplets, which are then deposited onto culture plates. After the Matrigel has polymerized at 37 °C, the droplets are covered with a culture medium that is enriched with a defined combination of growth factors and modulators of the WNT, BMP, TGF β and EGF pathways. If necessary, the medium also contains inhibitors of early differentiation signals. This configuration creates a three-dimensional micro-niche in which the Matrigel provides a rich laminin matrix that supports cell adhesion, apico-basal polarization, and lumen organization.

Meanwhile, the overlying medium precisely controls stem cell patterning and fate. This approach enables the production of relatively homogeneous organoids that can be expanded over long periods through repeated cycles of mechanically or enzymatically fragmenting the dome and re-embedding it in new Matrigel, whilst retaining the capacity to differentiate into various specialized cell types. However, the use of Matrigel raises several critical issues, including its incomplete definition, batch-to-batch variability and animal origin. These issues are driving the development of more controllable, 'Matrigel-like' synthetic hydrogels that are better compatible with future clinical applications (Daoud and Múnera, 2019).

To address these critical issues, materials engineering has developed synthetic hydrogels and 'designer' matrices with controlled chemical and physical characteristics, such as rigidity, degradability, RGD (Arg-Gly-Asp) ligand density and responsive cross-linking. These materials allow the microenvironment to be modulated in a controlled manner, enabling separation of mechanical and biochemical variables. The integration of bioengineering and organoid cultures in recent years has led to the development of microfabricated and microfluidic platforms that aim to improve uniformity, vascularization and dynamic control of

the microenvironment further. Microwell arrays and microstructured scaffolds enable the formation of organoids with highly standardized dimensions, which can be arranged in formats compatible with high-throughput screening. Meanwhile, 'organoid-on-a-chip' devices integrate controlled perfusion flows, gradients of soluble factors, and mechanical stimuli (e.g., shear stress and strain), bringing the system closer to physiological conditions *in vivo*.

Furthermore, combining 3D bioprinting with bioinks containing stem cells and natural or synthetic extracellular matrices enables the spatially controlled deposition of different cell types and matrices (Unagolla and Jayasuriya, 2022).

This wide range of technologies, including dynamic suspensions, non-adherent surfaces, hanging drops, and ECM matrices such as Matrigel or synthetic hydrogels illustrate the progress in the field. Of particular interest are organ-on-chip systems, which integrate organoids into dynamic, perfused environments to achieve functional vascularization, standardization, and reliable drug screening. Overall, these advancements demonstrate how the synergy between developmental biology principles and engineering strategies is progressively overcoming the limitations of traditional 3D models (Hoang and Ma, 2021).

Organ-on-chip platforms are the most advanced development in microfluidic technology for culturing organoids. They integrate stem cells into miniaturized devices that simulate dynamic physiological microenvironments through perfused flows, controlled chemical gradients, and mechanical and shear stress stimuli (Quintard et al., 2024). This overcomes the main limitations of static 3D models, such as central necrosis, hypoxia, structural heterogeneity, and poor reproducibility. Typically manufactured in PDMS (polydimethylsiloxane) or biocompatible hydrogels, these systems incorporate multiple chambers to house organoids derived from DPSCs or other stem cells. They also incorporate microfluidic channels for the continuous perfusion of nutrients, oxygen, and drugs; porous membranes for vascular co-cultures (e.g. HUVEC or iPSC-endothelium), which enable functional angiogenesis; and perfusable vascular networks. They also incorporate integrated sensors (e.g. electrical, optical, or biosensors) for the real-time monitoring of cell viability, protein secretions, metabolism, and drug responses (Papamichail et al., 2025). Furthermore, they have modular designs that can be used for multi-organ-on-chip systems to replicate systemic interactions. Compared to traditional organoids in Matrigel or dynamic suspensions, organ-on-chip platforms offer the following advantages: standardization (precise geometries and reduced volumes of less than 1 μ L); high-throughput scalability for drug screening in a 96-well format; accelerated cell maturation thanks to laminar flows that mimic microcirculation (speed 0.1–1 mm/s); prolonged cultures of up to 120 days without degradation; and greater pathophysiological relevance for modelling complex

pathologies such as pulp inflammation, dental caries, and endodontic regeneration. In the field of dental regeneration, 'tooth-on-a-chip' or 'pulp-on-a-chip' models use DPSCs on natural or synthetic dentin substrates to test biomaterials (e.g. mineral trioxide aggregate (MTA), bioceramics and fluorides) by evaluating tubular penetration, cytotoxicity, odontogenesis and dentin-like matrix deposition under dynamic conditions. Meanwhile, revascularized setups address the vascular challenge in regenerative scaffolds for vital pulp.

By merging developmental biology principles with microfluidic engineering, these technologies are driving organoid systems toward greater standardization and pathophysiological relevance. Such advancements are revolutionizing their application in studying disease mechanisms, conducting high-fidelity preclinical screenings, and developing personalized medicine from patient-specific biopsies (Artegiani and Hendriks, 2025; Hoang and Ma, 2021; Huang et al., 2023; Papamichail et al., 2025; Pierfelice et al., 2024).

1.9 Applications of Organoid Technology

Organoids, grounded in developmental biology principles, provide physiologically relevant 3D models that faithfully recapitulate human organs' architecture, cellular heterogeneity, and function, effectively bridging critical translational gaps between simplistic 2D cultures, animal models, and patient-derived xenografts (PDXs) (Tang et al., 2022; Yang et al., 2023) (Figure 8). This human-specificity positions organoids as transformative platforms across biomedical research, beginning with genetic disease modeling where genome-edited isogenic controls like CRISPR-corrected CFTR intestinal organoids replicate cystic fibrosis ion transport defects and mucus accumulation to enable rapid functional assays for diagnosis, drug screening, and personalized therapies (De Poel et al., 2020). In drug discovery and preclinical toxicity assessment, patient-derived colon organoids from familial adenomatous polyposis (FAP) patients test WNT pathway inhibitors while liver, heart, and kidney organoids predict organ-specific toxicities, substantially reducing costly clinical failures (Matsui and Shinozawa, 2021). Cancer research benefits immensely from patient-derived tumoroids that preserve intratumoral heterogeneity, tumor microenvironment interactions, and metastasis potential, with colorectal and pancreatic cancer biobanks achieving 90% accuracy in predicting clinical therapeutic responses (van de Wetering et al., 2015). These tumor organoid biobanks extend naturally into

personalized medicine across prostate, lung, colorectal, liver, pancreas, and gastric cancers, capturing inter- and intra-tumoral diversity to identify optimal drug sensitivities and minimize recurrence, including >85% predictive accuracy for PD-1/PD-L1 immunotherapy (Di Paola et al., 2025). Gene repair applications leverage CRISPR/Cas9 technologies to functionally correct patient-derived organoids, such as $\Delta F508$ CFTR mutations via homology-directed repair or multiplex editing achieving >70% efficiency in intestinal organoids for potential xenotransplantation (Schwank et al., 2013). Finally, transplantation and tissue regeneration address donor shortages and graft rejection through autologous organoids, exemplified by human intestinal organoids demonstrating marked expansion and functional maturation upon mouse engraftment, while DPSC-derived pulp organoids advance dental regenerative scaffolds despite remaining challenges in vascularization and full physiological maturity (Watson et al., 2014; Yang et al., 2023).

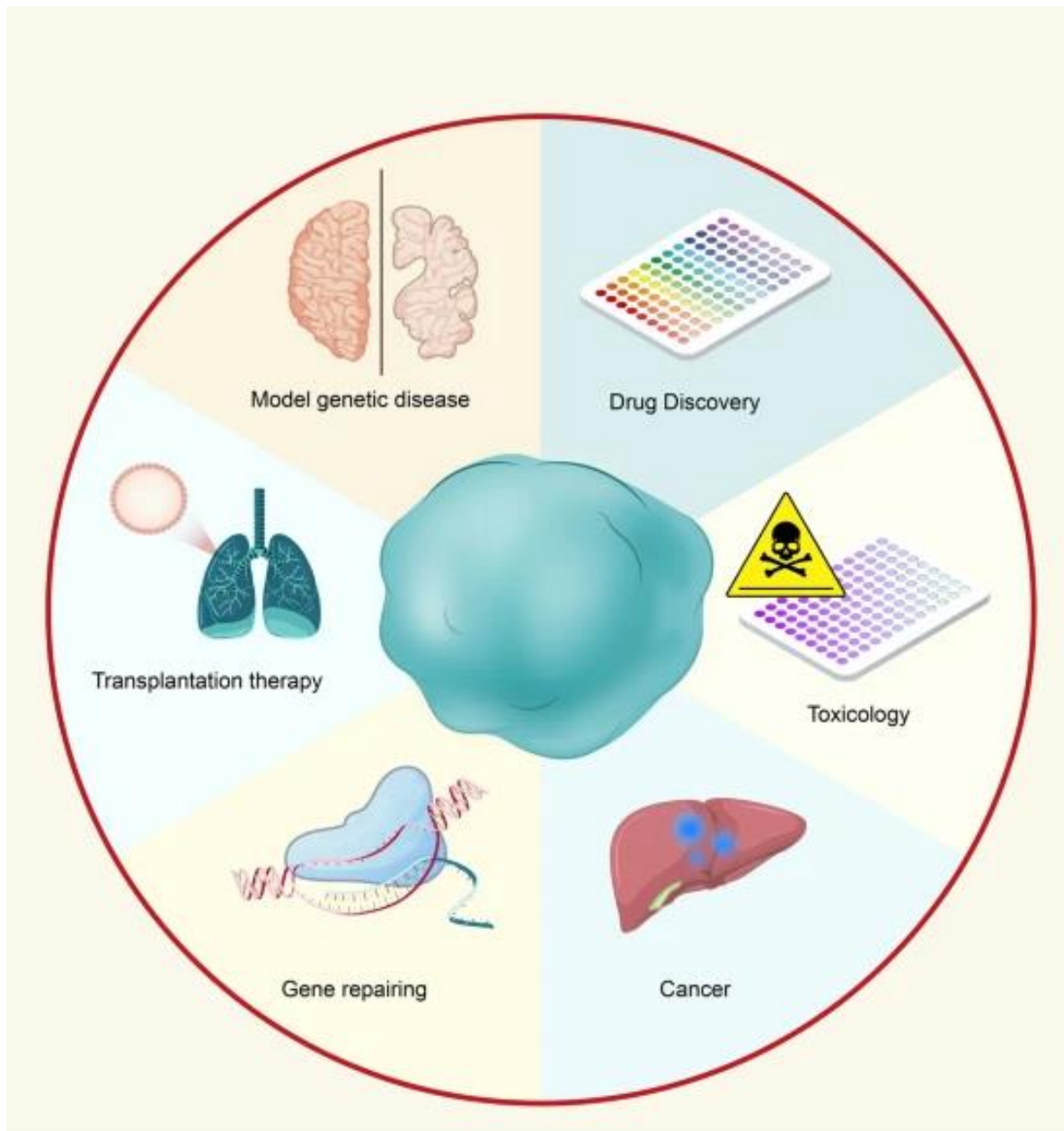


Figure 8 Schematic depiction of various applications of organoid technology (Tang et al., 2022).

1.9.1 Models of Genetic Diseases

One of the main applications of organoids is to accurately model human genetic diseases in order to clarify molecular pathogenesis, validate therapeutic hypotheses, and accelerate precision interventions (De Poel et al., 2020). Intestinal crypt organoids derived from Lgr5+ adult stem cells (ASCs) are a key example. CFTR mutant organoids from patients with cystic fibrosis (CF) show defective $\text{Cl}^-/\text{HCO}_3^-$ secretion, airway surface liquid (ASL) dehydration and forskolin-induced swelling (FIS) (Bulcaen et al., 2024). This swelling can be recovered by

more than 80% via CRISPR/Cas9 base editing or primary editing, which restores F508del trafficking/function (Muñoz et al., 2012; Schwank et al., 2013). In 2025, discoveries included prime editing, which corrected class II–V CFTR variants (e.g. G542X and W1282X) in primary airway organoids, achieving 60–90% functional recovery and paving the way for n-of-1 clinical trials (Bulcaen et al., 2024).

Beyond CF, organoids affected by polycystic kidney disease (PKD) and exhibiting PKD1/2 loss-of-function mutations can reproduce cystogenesis, mTOR hyperactivation and polycystin-1 trafficking defects that can be screened for using tolvaptan (Clevers and Nusse, 2012). Brain organoids exposed to the Zika virus model microcephaly by destroying cortical neurogenesis and inducing apoptosis (Garcez et al., 2016). iPSC liver organoids with α 1-antitrypsin deficiency (AATD) exhibit the accumulation of Z-allele polymers, endoplasmic reticulum (ER) stress and fibrosis, which can be alleviated by chemical chaperones (Kaserman et al., 2022). Cardiomyocytes with Barth syndrome exhibit tafazzin mutations that cause mitochondrial cardiolipin defects (Zhu et al., 2021).

1.9.2 Drug Discovery and Toxicity Assessment

Due to their human physiological fidelity and 3D microenvironment recapitulation, organoids represent transformative platforms for drug discovery, high-throughput screening, and preclinical toxicity assessment (Matsui and Shinozawa, 2021; Rae et al., 2021). Patient-derived colon organoids from patients with familial adenomatous polyposis (FAP), who harbour APC mutations that cause constitutive WNT/ β -catenin hyperactivation and crypt hyperplasia, can be used to screen compounds for inhibitors of proliferation, with tankyrase inhibitors (e.g. XAV939) reducing budding rate by over 70% and tumoroid viability (Baumann, 2017; Crespo et al., 2017).

This physiological relevance also applies to organ-specific toxicity: liver organoids express the full cytochrome P450 (CYP450) repertoire (3A4/2D6/1A2), metabolise over 90% of phase I drugs and can predict drug-induced liver injury (DILI) for troglitazone and acetaminophen with 87% accuracy via albumin and ATP readouts (Takasato et al., 2016). Cardiac organoids detect hERG channel liabilities and doxorubicin cardiotoxicity through sarcomere disruption and Ca^{2+} handling assays (Vlachogiannis et al., 2018). Kidney organoids model cisplatin/gentamicin nephrotoxicity via KIM-1/NGAL biomarkers, capturing proximal tubule injury that is absent in 2D models (Alnasser, 2025). Multi-organoid-on-chip systems integrate the liver and kidney axes to model idiosyncratic toxicity (e.g. that caused by terbinafine) (Yao et al., 2024). 2024

advances: 384-well DPSC dental organoids screen MTA/bioceramics for pulp regeneration cytotoxicity (Wu et al., 2024). Overall, organoids reduce clinical attrition by 30–50% compared to monolayers (Caipa Garcia et al., 2022; Matsui and Shinozawa, 2021).

1.9.3 Cancer Research

Patient-derived tumor organoids (PDTOs) excel in basic cancer modelling by accurately replicating the phenotypic, genomic, transcriptomic, and functional characteristics of primary tumors, including complex histoarchitecture, comprehensive mutation profiles, gene expression signatures, stromal interactions, and metastatic potential, while supporting long-term expansion and cryopreservation for extensive preclinical studies (S. Liu et al., 2025; van de Wetering et al., 2015). This remarkable physiological fidelity enables high-throughput drug screening with IC_{50} correlations $r=0.89–0.93$ versus patient clinical response, sophisticated co-culture tumor microenvironment (TME) studies incorporating cancer-associated fibroblasts and immune cells, and fundamental research elucidating oncogenesis mechanisms (Rae et al., 2021; Sachs et al., 2018; Wensink et al., 2021). Sato et al. pioneered culture conditions for APC-mutant colorectal adenomas/adenocarcinomas (CRC), achieving >85% establishment rates of pure epithelial cultures without exogenous Wnt ligand through RSPO1/Noggin/EGF niche factor supplementation (Clevers and Nusse, 2012; Sato et al., 2011). Preclinical biobanks validate predictive power: Hubrecht CRC organoid biobank ($n>1,000$) forecasts irinotecan/5-FU response AUC 0.93; Oncohemity Pancreatic Cancer Organoid Library ($n=78$) predicts gemcitabine/FOLFIRINOX efficacy 93% specificity. Applications span liver cholangiocarcinoma YAP1-mediated gemcitabine resistance, glioblastoma TMZ sensitivity with astrocyte co-culture invasion, breast cancer letrozole response (82% concordance), prostate PTEN-deficient enzalutamide contexts, and HNSCC DPSCs evaluating cisplatin/radiotherapy synergy capturing stromal invasion absent in 2D (Liu et al., 2021).

1.9.4 Personalized medicine

Clinically, PDO biobanks from patient-matched primary tumors (prostate, lung, colorectal, liver, pancreas, stomach) systematically capture individual inter- and intra-tumoral heterogeneity, evolutionary dynamics, rare subclones, and personalized tumor

microenvironment interactions that determine therapeutic response, overcoming traditional 2D culture limitations (Di Paola et al., 2025; Huang et al., 2024; Xie et al., 2023). Drug sensitivity testing (DST) identifies optimal patient-specific regimens minimizing off-target toxicities, resistance development, and recurrence while maximizing therapeutic (Tang et al., 2022; Zhou et al., 2021). Predictive organoids for PD-1/PD-L1 immunotherapy achieve >85% sensitivity/specificity across NSCLC/melanoma cohorts (Q. Wang et al., 2025). Princess Máxima Center pediatric biobank (n=2,231 cancers) forecasts chemotherapy response AUC 0.88; UCSF stomach/gastric PDOs predict 5-FU/oxaliplatin positive predictive value 91%; prostate PDOs stratify enzalutamide responders (CRPC, 80% concordance).

1.9.5 Gene Repair and Gene Therapy

CRISPR/Cas9 technologies have revolutionized gene correction in patient-derived organoids, enabling precise editing of disease-causing mutations while preserving 3D architecture and function for functional validation (Geurts et al., 2020; Schwank et al., 2013). Landmark cystic fibrosis (CF) studies demonstrate homology-directed repair (HDR) rescuing $\Delta F508$ -CFTR folding/trafficking in intestinal organoids, restoring >75% forskolin-induced swelling (FIS) and Cl^- currents comparable to healthy controls (Khatibi et al., 2021).

Recent advances leverage prime editing and base editing for higher precision/HDR-independent correction: 2024 iScience reports prime editing correcting class II L227R/N1303K CFTR variants in rectal organoids with 40-60% efficiency and full FIS rescue (Bulcaen et al., 2024). Multiplex editing strategies corrected >70% compound heterozygous CFTR mutations (e.g., $\Delta F508/G542X$) in human intestinal organoids, achieving mature CFTR apical localization and xenotransplantation potential (Fan et al., 2025).

Beyond CF, applications include TP53/RB1 dual knockout restoration in retinoblastoma organoids reversing tumorigenesis; DMD exon-skipping in Duchenne muscular dystrophy skeletal muscle organoids improving dystrophin expression >50%; and USP9X correction in DSCAM-mutant cortical organoids rescuing neuronal connectivity.

1.9.6 Transplantation and tissue regeneration

Although organ transplantation is the definitive treatment for end-stage organ failure, it faces profound limitations, including chronic global shortages of donors (>150,000 patients on waiting lists), acute and chronic allograft rejection necessitating lifelong immunosuppression,

HLA mismatch and ischaemia-reperfusion injury (X. Liu et al., 2025; Quintard et al., 2024). In vitro-derived organoids from patient-specific induced pluripotent stem cells (iPSCs) or adult stem cells (ASCs) are emerging as revolutionary autologous platforms, offering unlimited scalability, immunocompatibility and genetic personalization (Papamichail et al., 2025).

The seminal demonstration by Watson et al. involved the orthotopic transplantation of cryopreserved human small intestinal organoids into denuded mouse mucosa. This yielded an exponential 100-fold expansion of crypts and villi over four months, as well as spontaneous vascular anastomoses with the host circulation. The organoids underwent multilineage differentiation into Paneth cells, goblet cells and enteroendocrine cells, and exhibited mature, functional physiology, as evidenced by glucose and sodium cotransport and electrophysiological responses that rivaled those of native tissue (Watson et al., 2014). Oesophageal organoids can regenerate a stratified squamous epithelium after resection, dermal organoids can restore a multilayered epidermis and vascular networks for burn victims, and retinal organoids can engraft subretinally, restoring photoreceptor function and optogenetic responses in degeneration models.

Recent engineering advances have addressed engraftment barriers. Endothelial co-culture generates perfusable, vascularized assembloids that mitigate central necrosis, while microbiome priming enhances epithelial barrier maturation. Furthermore, suspension bioreactors can scale GMP production to 10^6 – 10^8 organoids per therapeutic dose (Papamichail et al., 2025).

Despite their promise, grafts exhibit relative immaturity, with limited mesenchymal and immune compartments, incomplete endocrine and vascular maturity, and challenges with orthotopic integration. This necessitates the use of hybrid constructs: decellularized scaffolds seeded with multi-lineage organoid sheets can restore 40% cardiac ejection fraction post-MI (Smith et al., 2022). Organoid transplantation heralds the dawn of donor-independent regenerative medicine.

1.10 Development of Organoids from DPSCs

Established protocols for generating organoids from DPSCs typically involve culturing the cells in specialized differentiation media for several weeks after embedding them in Matrigel droplets, allowing them to naturally self-assemble into three-dimensional structures. This

approach has proven effective in producing dental pulp-like organoids that exhibit mineralized deposits and key markers of bone or dental tissue. This has been demonstrated using standard validation techniques, including staining and imaging, as well as gene and protein expression analysis. More complex analyses, such as Raman spectroscopy, have also been employed (Rothermund et al., 2022).

To improve these models and make them more relevant to real-world applications, researchers have introduced complementary strategies that build on the basic self-assembly process (Calabrese et al., 2024). For instance, preconditioning DPSCs under low-oxygen conditions and co-culturing them with endothelial cells or pericytes promotes vascularization. This helps address issues such as poor nutrient diffusion, which can lead to central necrosis in larger organoids. It also encourages the formation of dentine-pulp complexes with functional pulp nuclei. Similarly, scaffold-free methods or those using ECM derived from natural dental pulp allow the formation of more complex structures, such as dental root organoids complete with pulp, dentine, cementum, and periodontal ligament, without the need for artificial support (J. He et al., 2025; Hermans et al., 2023; Ohlsson et al., 2022).

Further innovations incorporate advanced tools, such as 3D bioprinting and microfluidic devices, to improve the reproducibility and scalability of testing the toxicity or bioactivity of dental materials. These developments address the limitations of traditional two-dimensional cultures and open the door to clinically valid models for regenerative endodontics, bone tissue engineering and broader tissue repair applications such as skin and mucosal healing, thanks to the anti-inflammatory and angiogenic properties of DPSCs.

Organoids derived from dental pulp stem cells (DPSCs) have several applications, the most notable of which is their use in endodontic regeneration. They form scaffold-free dentin-pulp complexes that mimic the native structure. These complexes contain DSPP-positive odontoblasts at the periphery and central vascularized pulp that is rich in CD31+ endothelium. This has been demonstrated in root canal implants in mouse models. Vascularized DPSC constructs (E-CC) regenerate functional pulp by penetrating dentinal tubules. STRO-1+ stem-like cells are present in the core to enable continuous regeneration and overcome the limitations of traditional scaffolds. Treatments involving cannabidiol enhance organoid microspheres for bone regeneration in dental bone defects (Itoh et al., 2018; Katata et al., 2021; Liu et al., 2024; Rothermund et al., 2022).

3D organoids derived from dental pulp stem cells (DPSCs) promote dentin-like mineralisation and vascularisation when integrated into protocols for pulp and bone regeneration, which essential in maxillofacial reconstruction. These protocols include *E. coli*/P. gingivalis-resistant

dentin-pulp organoids. Advanced engineering enables the creation of organoids for pulp tissue regeneration, with applications in the treatment of bone trauma and congenital defects (Farshbaf et al., 2024; Fujii et al., 2023; Liu et al., 2024).

Furthermore, these organoids model oral pathologies such as caries and pulpitis for drug screening and testing of bacterial or inflammatory responses in spheroids and organoids-on-chip. They also accelerate personalized therapies via aggregational protocols. Extensions to musculoskeletal disease modelling support translational studies (Chen et al., 2025; Gouveia et al., 2025; Yao et al., 2024; Zhang et al., 2025).

DPSCs demonstrate neurogenic potential through their ability to differentiate into neuronal (MAP2+, β III-tubulin), glial and Schwann-like cells, primarily in 2D cultures (Gao et al., 2022).

SHED-DPSCs can generate brain organoid-like structures in 3D without the need for iPSC/hESC reprogramming, producing neural progenitors, partially stratified neurons, and neuronal differentiation (Gutierrez-Malacara et al., 2023).

Adult DPSCs form aggregational neural clusters with limited 3D homeostasis. However, these models lack the complete cortical organization (ventricular zone/cortical plate) of iPSC organoids, making them more suitable for dental nerve repair than for neurodegenerative diseases (Chen et al., 2025).

1.11 Methodological Challenges and Limitations of Standard Techniques for organoids development and analysis

Innovation in 3D model characterization strategies is crucial for more accurately assessing the degree to which native tissue and its microenvironment are reproduced in organoids (Lancia et al., 2025b). Progress has been remarkable, but significant methodological challenges remain, including experimental reproducibility, protocol standardization, high costs, and analyzing odontoblastic and osteogenic features in 3D (Lancia et al., 2025b).

While RT-qPCR and Western blotting are standard for gene and protein expression analysis, they require total tissue lysis, a task hindered by mineralized matrices. Additionally, the reliability of these methods is challenged by the instability of housekeeping genes, which exhibit a tendency toward modulation (Cherubini et al., 2021).

Similarly, traditional morphological techniques, such as histological staining or immunofluorescence, have inherent limitations and yield partial or distorted images of the cellular interior. This is particularly problematic in dense, mineralized tissues, such as those found in dental pulp organoids, where the penetration of dyes and antibodies is severely compromised in the deep layers. Moreover, reliance on xeno-derived components extends beyond matrices to culture media supplements like fetal bovine serum (FBS; 10-20% standard for DPSC expansion). Both Matrigel (murine/tumor origin: batch variability, autofluorescence, GMP incompatibility) and FBS (zoonotic risks, immunogenic reactions, accelerated senescence, reduced odontogenesis) preclude clinical translation. Xeno-/serum-free alternatives, human platelet lysate (hPL), recombinant ECM hydrogels, chemically defined media, enable GMP-compliant protocols while preserving 3D morphogenesis and odontogenic potential (Kozlowski et al., 2021; Wang et al., 2024). The presence of Matrigel further complicates analysis, especially for imaging techniques such as immunofluorescence, as it is autofluorescent, making it challenging to acquire sharp, background-free images (Lancia et al., 2025b; Xu et al., 2022).

Moreover, it is crucial to develop dental pulp organoids with high potential for bone tissue engineering and regenerative applications by implementing a guided protocol optimized for simplicity and reproducibility (J. Wang et al., 2025).

Raman microscopy has emerged as a powerful, non-invasive alternative for analyzing dental pulp organoids. By providing high spatial resolution without exogenous markers, it effectively complements conventional standard methods (Kim et al., 2021).

Integrating these methods improves the overall characterization of organoids, providing more detailed information about their architecture and functionality (Heinzelmann et al., 2024; Kamei and Wakamoto, 2025; Lancia et al., 2025b; Smaida et al., 2025; Tubbesing et al., 2022).

2 AIM OF THE WORK

Although traditional two-dimensional (2D) cell cultures are widely used, there is an urgent need for models that can accurately replicate the complex structure and functions of human tissues. This has made the development of organoids a key area of research in biomedicine. In this context, dental pulp stem cells (DPSCs) are an ideal cellular source, offering extraordinary multi-lineage plasticity and high regenerative potential. As well as being biologically versatile, DPSCs have a significant practical advantage in that they can be easily harvested from discarded tissue, such as that from third molar extractions during routine clinical procedures. Their neural crest origin, combined with this accessibility, makes DPSCs a preferred substrate for generating 3D organoid models aimed at advancing tissue engineering, disease modelling, and personalized regenerative medicine.

The primary objective of this work was to define and validate a standardized protocol for the generation of dental pulp organoids derived from DPSCs. Specifically, the work focused on the development of a three-dimensional (3D) model utilizing a scaffold-assisted stationary culture technique. This was achieved by seedings cell suspension into ultra-low attachment 96-well U-bottom plates, supplemented with Matrigel to provide a biomimetic ECM. This approach was designed to facilitate spontaneous cellular self-organization and to promote the structural and functional maturation of the organoids, overcoming the limitations of conventional scaffold-free systems.

Furthermore, another key objective of this study was to assess the functional plasticity and differentiation potential of these DPSC-derived organoids. Specifically, we focused on their ability to undergo osteo/odontogenic maturation when exposed to targeted biochemical stimuli. Pivotal challenge of this project was identifying and optimizing an analytical procedure specifically designed for 3D structures.

Due to the high density and structural complexity of organoids, conventional analytical methodologies had to be adapted to overcome critical limitations such as reagent penetration depth and imaging resolution. The resulting integrated approach includes:

- cryostatic micro-dissection techniques, optimized to preserve the structural and spatial integrity of the organoids during processing.
- colourimetric assays and targeted immunofluorescence to evaluate stemness and differentiation markers, optimized to minimize the confounding effect of Matrix.
- Raman microspectroscopy was implemented as a cutting-edge label-free and non-invasive tool to obtain a precise biochemical 'fingerprint' of the tissue maturation state.

In conclusion, this study presents a standardized protocol for producing dental pulp organoids and establishes a rigorous analytical methodology for their thorough characterization. This model could represent an innovative platform for future research into dental tissue regeneration and could be used as a reliable tool to study the biology and lineage commitment of DPSCs, as well as for in vitro pharmacological and toxicological screening.

3 MATERIALS AND METHODS

3.1 Dental pulp stem cells (DPSCs): Isolation, Culture and Characterization

DPSCs were isolated from impacted third molars of healthy young patients (aged 13–19 years) with dysodontiasis. These teeth were collected as discarded biological material during routine surgical extractions at the Department of Science, Dentistry, and Maxillofacial Surgery at Sapienza University in Rome with the informed consent of the patients, in accordance with ethical considerations (Declaration of Helsinki) and after they had signed the appropriate forms. Collection was approved by the ethics committee of the Umberto I Polyclinic of Rome (Project identification code: 4336).

3.1.1 DPSCs Isolation protocol

Immediately following extraction, the teeth were washed in phosphate-buffered saline (PBS) to remove blood and debris. A coronal cut was performed parallel to the roof of the pulp chamber. Within two hours, the samples were processed under sterile conditions. The dental pulp was mechanically recovered and minced into small fragments (approximately 1-3 mm) using a sterile scalpel and incubated in Hank's balanced salt solution (HBSS) supplemented with L-glutamine (Euroclone, Milan, Italy), 1% penicillin/streptomycin (Euroclone, Milan, Italy) and 0.1% amphotericin (Merck, Milan, Italy), at 37 °C in 5% CO₂ for 2 hours.

The solution was removed by centrifugation for 10 minutes at 1200 rpm. The resulting pellet was then resuspended in 0.1% type IV collagenase (Merck, Milan, Italy) in DMEM-Low Glucose (LG), after which it is incubated for 15 minutes at 37 °C in 5% CO₂. After a further 10 minutes of centrifugation at 1200 rpm, the supernatant was discarded and the cell pellet was resuspended in DMEM-LG supplemented with 10% fetal bovine serum (FBS) (Euroclone, Milan, Italy), L-glutamine (Euroclone, Milan, Italy) and 1% penicillin/streptomycin (Euroclone, Milan, Italy).

3.1.2 DPSCs Expansion and maintenance

DPSCs were maintained in a humidified incubator at 37 °C with 5% CO₂. The culture medium was replaced every 48h to ensure optimal nutrient availability. Then, we characterized them by flow cytometry for MSC expression, according to the minimal criteria defined by the International Society for Cellular Therapy (ISCT) and used in our previous studies (Delle Monache et al., 2022; Dominici et al., 2006; Mattei et al., 2025). For subsequent experiments, cells were used between the second and fifth passages.

3.1.3 DPSCs Characterization: Flow cytometry analysis

For each experiment, at least 1×10^5 cells were fixed with 4% paraformaldehyde (PFA) for 10 minutes at 4°C and then permeabilized with a permeabilizing solution (0.1% Triton X-100 in PBS) for an additional 10 minutes at the same temperature. After washing, the cells were incubated overnight at 4 °C with the following primary antibodies:

- Mouse anti-CD90 mAb (contained in a human MSC analysis kit, Merck Millipore, Milan, Italy)
- Positive MSCs markers: Mouse anti-CD90, anti-CD105, anti-CD73, anti-CD166, anti-CD271, and anti-CD146.
- Negative MSCs markers: Mouse anti-CD19 and anti-CD45.

Subsequently, cells were incubated with PE-conjugated anti-mouse IgG H&L or anti-rabbit IgG H&L (Cy5) (Abcam, Cambridge, MA, USA) for 30 minutes in the dark. All samples were analyzed with a FACScan cytometer (BD Accuri C6 Flow Cytometer) equipped with blue (488 nm) and red (640 nm) lasers (BD Biosciences, Milan, Italy). At least 40,000 events were acquired for each sample (Gan et al., 2020).

3.2 Set-up of a protocol for organoid development

A series of preliminary experiments was conducted to standardize the 3D culture protocol. The aim was to identify the optimal cell number and the optimal Matrigel® matrix support to ensure long-term viability and structural integrity.

3.2.1 Determination of Optimal Cell Density

To establish the ideal cell concentration for spontaneous aggregation, DPSCs were seeded at varying densities (25,000–50,000-75,000-100,000 cells per organoid) in ultra-low attachment (ULA) round-bottom plates.

3.2.2 Determination of Matrigel Concentration

To enhance the structural robustness and mimic the basement membrane of the dental pulp, a matrix was integrated into the 3D model. Further preliminary experiments were conducted to identify the optimal Matrigel® matrix concentration for maximizing cell aggregation. Various Matrigel® matrix solutions were tested (50%, 75%, and 100% Matrigel® matrix solutions) to allow the spheroids to withstand the mechanical stress of being transferred via micropipette or positioned onto experimental membranes without loss of spherical symmetry.

3.3 3D culture model: DPSCs organoids generation and analysis

3.3.1 3D structures formation via stationary technique

The organoids were generated using a stationary technique, both manually and automatically, with the help of Corning's BioInk Matribot (New York, USA), which enabled greater standardization of 3D structure formation and optimal reproducibility.

- Manual technique

The organoids were generated using a stationary technique involving culturing cell suspensions on non-adherent surfaces, specifically in 96-well plates with U-shaped bases. The DPSCs, which were maintained in two-dimensional culture until passage 3°–4°, were detached using trypsin and then centrifuged at 1,200 rpm for five minutes. The cells were counted, then placed in a centrifuge, and the resulting pellet was resuspended in Matrigel® matrix. It was essential to keep the Matrigel® solution and cells on ice to prevent the matrix from polymerizing prematurely.

Next, 10 µL drops containing 75,000 cells were dispensed onto sterilized parafilm. After 30 minutes' incubation at 37 °C to allow polymerization of the Matrigel® matrix, the drops were

transferred to 96-well U-bottom plates, each containing 200 μ L of DMEM-LG. The lack of an adhesive surface, combined with the geometry of the well, promoted spontaneous cell-to-cell aggregation through cadherin-mediated interactions (Lancia et al., 2025a). Growth and cell aggregation of the organoids were then monitored daily using the Incucyte® system under controlled culture conditions, with the medium changed every two. After three days of culture, adequate cell aggregation was observed, which was considered the initial time point (T0) (Figure 9). The transition from an initial cell cluster (Figure 9D) to a mature, compacted spheroid (Figure 9E) was defined by the following validated parameters:

Timeframe: A minimum of 48 hours was required for complete cadherin-mediated compaction.

Opacity: The development of a highly refractive, dark central core indicating high-density cell packing.

Boundary Definition: the formation of a sharp, smooth perimeter indicating the deposition of a rudimentary basal lamina facilitated by the Matrigel-supplemented medium.

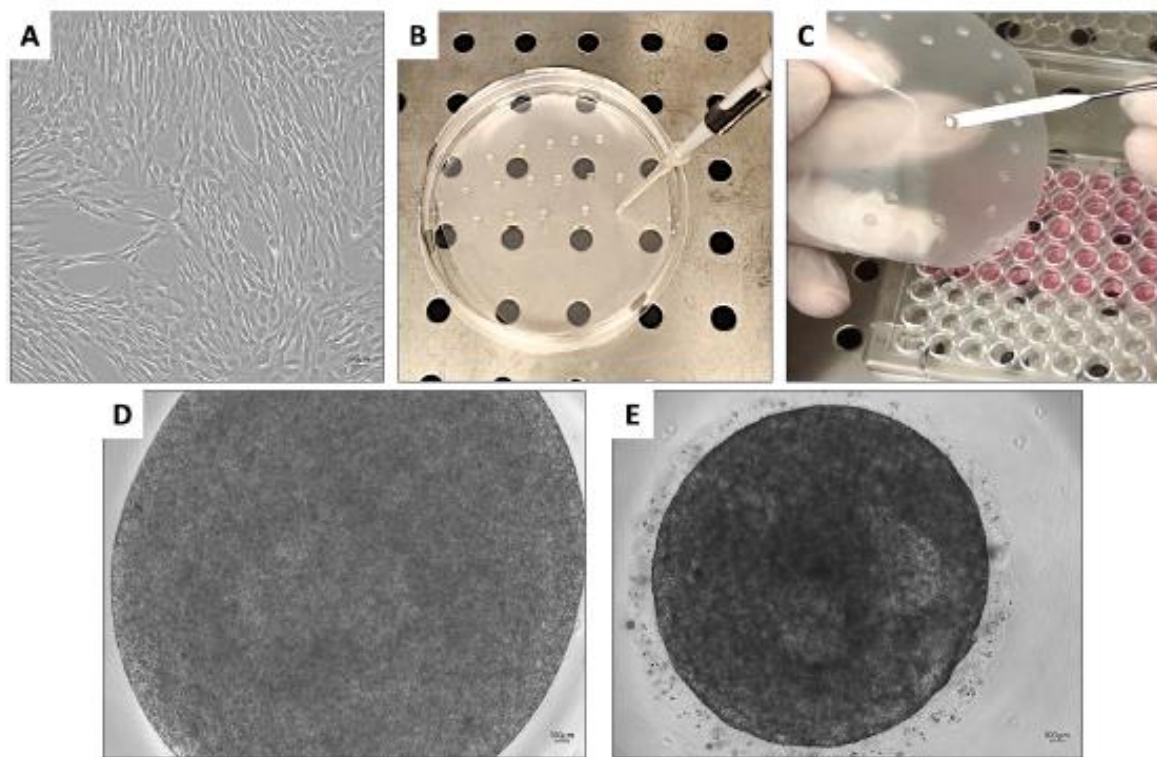


Figure 9 Steps for dental pulp organoid generation: from 2D to 3D culture. A) culture of DPSCs in 2D; B) formation of Matrigel® matrix droplets with cells; C) transfer of polymerized droplets into a 96-well U-bottom plate; D) observation of newly formed organoids (4X magnification); E) observation of organoids at T0 (4X magnification).

- *Automatic technique*

The MatriBot® system (Corning Incorporated, New York, USA) is an automated bioprinting system designed for the precise dispensing of bioinks and hydrogel matrices in 3D culture settings. It is controlled by Corning DNA Studio® software, which allows users to program patterns, volumes and positions via an intuitive interface (Figure 10).

In parallel, organoids were generated using Corning's MatriBot® system (New York, USA) with dedicated Corning DNA Studio® software. After suspending the DPSCs (passages 3–4) in Matrigel® and keeping them on ice to prevent premature polymerization, the cell-matrix mixture was loaded into a Luer Lok syringe (309658, Corning Incorporated, New York, NY, USA) and inserted into the MatriBot®. The instrument was programmed to dispense precise droplets (75,000 cells in 10 µL of Matrigel) onto a sterilized parafilm sheet placed on a 100-well plate, thereby replicating the steps of the manual method. After 30 minutes' incubation at 37 °C to allow Matrigel® to polymerize, the drops were transferred to 96-well U-bottom plates containing DMEM-LG. The growth and compaction of the organoids were monitored daily. T0 was defined as three days after adequate aggregation was observed. The organoids' development and growth were monitored as in the manual method until T26.



Figure 10 The image shows MatriBot® system (Corning Incorporated, New York, USA).

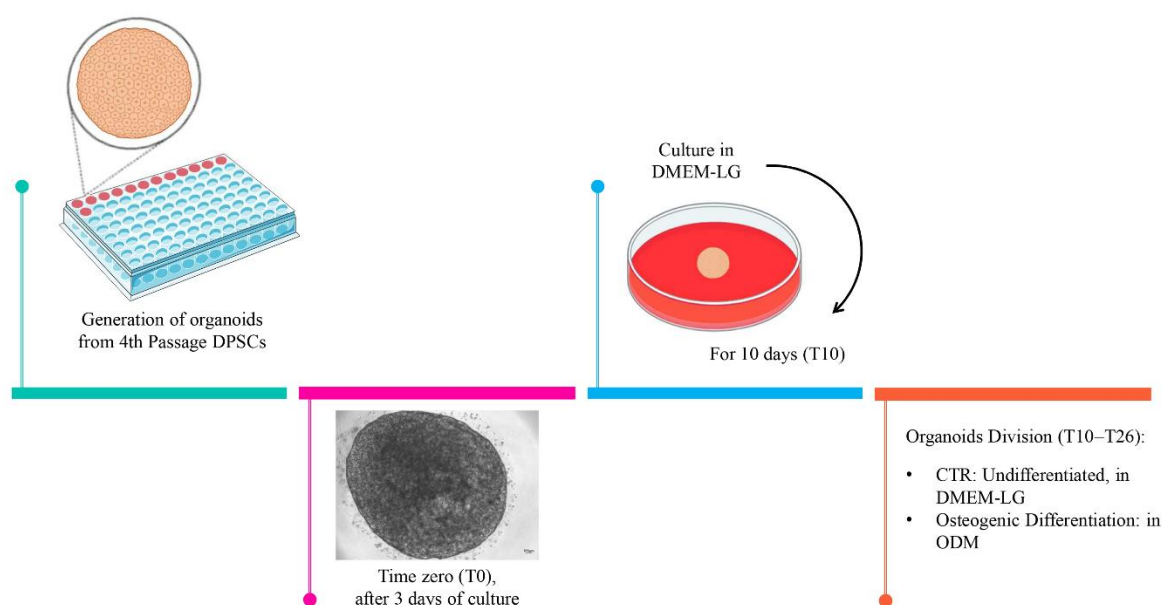
3.3.2 Odonto/Osteogenic differentiation organoids

After an initial 10-day expansion in basal medium (T10), the organoids were randomly assigned to the two experimental groups: a control group (CTR), which was maintained in basal DMEM-LG supplemented with 5% FBS and 1% Penicillin/Streptomycin, and an osteo/odontogenic differentiation group (ODM), which was cultured in a specific medium consisting of the basal medium DMEM-LG supplemented with specific osteogenic factors (Table 4).

DMEM LG	
FBS (fetal bovine serum)	5%
PES (Penicillin and Streptomycin)	1%
Ascorbic Acid	50 mg/ml
b-Glycerophosphate	10 nM
Dexamethasone	100 nM

Table 4 Osteogenic Differentiation Medium (ODM) Composition.

The cultures media were refreshed every 2–3 days (200 μ L/well) until the experimental endpoint at 26 days (T26), as shown in Scheme 1.



Scheme 1 Representative schematic steps for organoid growth. Following organoid generation, adequate cell aggregation was observed after three days, leading us to consider this as our time zero

(T0). After 10 days (T10) of culture, the organoids were divided into two groups: one group was maintained in DMEM-LG in a 96-well U-bottom plate, and the other group was subjected to osteogenic differentiation using a specific culture medium (ODM) from T10 to T26.

3.3.3 Organoids Viability Evaluation

The vitality and metabolic activity of the organoids were investigated using two distinct essays.

- Trypan Blue Exclusion Assay

Cell viability was determined using trypan blue, a vital dye that allows living cells to be distinguished from non-viable cells. Non-viable cells, with compromised membrane integrity, appear blue under an optical microscope (trypan blue positive), while viable cells maintain their integrity and remain colourless (trypan blue negative).

To perform the test, the culture medium was removed, and the organoids were enzymatically dissociated using accutase (Euroclone, Milan, Italy) at 37 °C for 20 minutes. To ensure complete dissociation of the dense organoid core, gentle mechanical pipetting was performed every 5 minutes. The dissociated cells were then collected by centrifugation at 1200 rpm for 10 minutes. The cell pellet was then resuspended in an appropriate amount of DMEM-LG medium, and the resulting cell suspension was mixed 1:1 with Trypan Blue dye and analyzed using a Bürker counting chamber. Non-viable cells were identified by blue nuclear staining.

- CCK-8 Assay

To quantify the metabolic activity and proliferation of the organoids, the Cell Counting Kit-8 (CCK-8) (Sigma-Aldrich, St. Louis, Missouri, USA) was used in accordance with the manufacturer's instructions.

This assay is based on the reduction of WST-8 (Water-Soluble Tetrazolium salt 8) tetrazolium salt by metabolic dehydrogenases in viable cells, which results in the formation of a water-soluble yellow-orange pigment called formazan. The resulting color intensity, measured in terms of absorbance, is directly proportional to the number of metabolically active cells and, consequently, to their viability.

Specifically, 10 µL of CCK-8 reagent was added to each well of a 96-well U-bottom plate containing organoids and 100 µL of culture medium. After incubating for four hours at 37 °C, the absorbance was measured at a wavelength of 450 nm using a microplate reader (model Infinite M200 Pro, Tecan, Nänikon, Switzerland).

3.4 Morphological and Histological Evaluation of Organoids

3.4.1 High-Resolution Stereomicroscopy

Macroscopic architecture evaluation and morphological analysis of the organoids were performed using a Zeiss Axio Zoom V6 stereomicroscope (Zeiss, Oberkochen, Germany).

This instrument permits the acquisition of high-resolution, three-dimensional images, particularly when using the Z-stacking technique. This feature enabled comprehensive visualization and in-depth study of the organoids' complex morphology.

Before image acquisition, the organoids were gently washed in PBS to remove any residual culture medium or debris, thus ensuring optimal optical clarity. The organoids were then placed on a plate and positioned under the Axio Zoom v6 for examination. High-quality images were acquired at a magnification of 63X.

3.4.2 Preparation of Frozen Sections

The organoids were washed in PBS and fixed in 4% PFA (Santa Cruz Biotechnology, Dallas, TX, USA) for 30 minutes at 4 °C. After washing in PBS, the organoids were placed into molds, embedded in OCT compound for frozen tissues (Scigen Scientific, Gardena, CA, USA), and rapidly frozen in liquid nitrogen with the addition of a cryoprotective solution (FBS: DMSO, at a ratio of 9:1). Cryosections were cut using a Leica CM1859 cryostat (Leica Biosystems, Milan, Italy) at -26°C to obtain sections of 14 µm thickness. These sections were mounted on Superfrost Plus slides (Menzel-Gläser, Braunschweig, Germany) and stored at -80 °C until required.

3.4.3 Hematoxylin and Eosin Staining

Organoid sections prepared as described in Section 3.4.2 were processed for Hematoxylin and Eosin staining.

In brief, the sections were equilibrated at room temperature (RT) for a few minutes, immersed in distilled water for 5 minutes, and incubated in hematoxylin solution for 7 minutes.

The slides were washed in water for 3 minutes to remove any excess of dye and subsequently incubated in eosin for 3 minutes. Eosin was removed by rinsing the slides in water. The slides were then dehydrated using a graded ethanol series (70% for 3 minutes, 95% for 3 minutes,

and 100% for 3 minutes). After dehydration, the samples were cleared by immersing them in xylene three times for 5 minutes each. Finally, the slides were mounted with coverslips using glycerol as the mounting medium. The samples were analyzed using a Leica Mateo TL microscope (Leica Biosystems, Milan, Italy).

3.4.4 Alizarin Red staining

The obtained sections, as described in Section “Preparation of Frozen Sections”, were stained using Alizarin Red.

First, the slides were washed in PBS, then fixed with 4% PFA (Santa Cruz Biotechnology, Dallas, TX, USA) at RT for 10 minutes. After three further washes in PBS, the samples were incubated with 2% Alizarin Red (Sigma-Aldrich, Milan, Italy) for 10 minutes after which they were rewashed with PBS to remove any excess stain. Dehydration of the samples was performed using a graded ethanol series (70% for 3 minutes, 95% for 3 minutes, and 100% for 3 minutes). After dehydration, the slides were mounted using glycerol. Staining images were captured using a Leica Mateo TL microscope (Leica Biosystems, Milan, Italy).

3.5 Gene and protein expression analysis

3.5.1 Matrigel® matrix removal

Matrigel® is a matrix derived from mouse sarcoma. It is widely used as a basement membrane substrate to support three-dimensional organoid culture thanks to its high concentration of ECM proteins and growth factors. However, it must be meticulously removed before molecular analysis. Due to the potential presence of confounding proteins and RNA derived from extracellular vesicles in Matrigel®, its digestion is a crucial step in ensuring data accuracy.

To effectively remove the Matrigel® matrix, an enzymatic degradation-based cell recovery solution (e.g., commercial Cell Recovery Solution) was used. At the end of the culture period, the protocol requires the organoids to be transferred to test tubes (eight organoids per tube) and quickly washed with cold PBS. After centrifugation at 5000 rpm for 10 minutes and removal of the PBS, the organoids were incubated with the Cell Recovery Solution (Corning, New York, USA) for 20 minutes at 4 °C. This process was repeated a second time to ensure complete dissolution of the matrix. After the second incubation, the sample was centrifuged again at

5000 rpm for 10 minutes, after which the cell pellet was collected for subsequent nucleic acid or protein extraction (Figure 11).

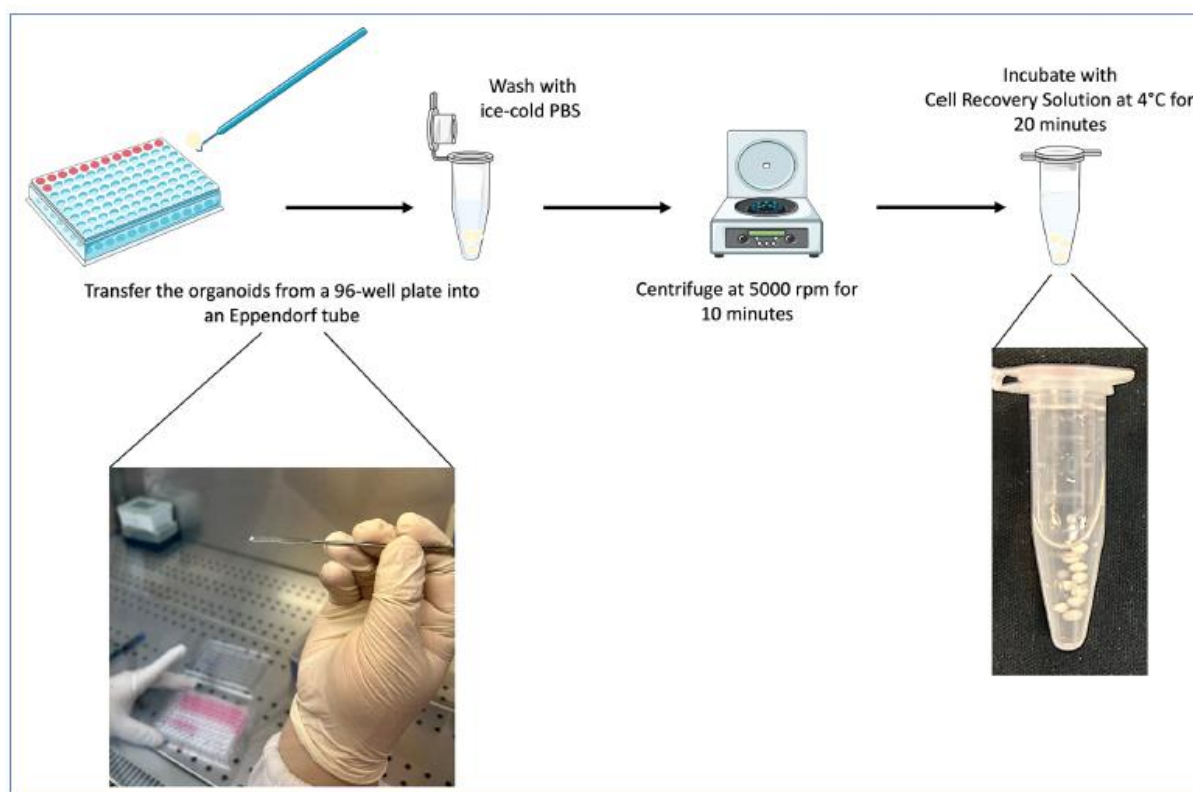


Figure 11 Illustrative diagram of the steps for Matrigel® matrix removal from organoids. Organoids were transferred into Eppendorf tubes, washed and centrifuged. The cell recovery solution treatment was repeated twice to minimize Matrigel® matrix contamination and ensure optimal nucleic acid or protein extraction.

3.5.2 Acid nucleic extraction

A Zymo-Research kit (ZYMO RESEARCH, California, USA) was used for nucleic acid extraction, in accordance with the manufacturer's instructions. After Matrigel® matrix digestion, the cell pellet was resuspended in TRIzol® reagent (Invitrogen™, Thermo Fisher Scientific, Waltham, USA). To optimize cell lysis and ensure complete homogenisation, the solution was vigorously pipetted to prevent clogging of the Zymo-Spin™ IICR columns. Sample preparation involved adding an equal volume of 95–100% ethanol to the TRIzol® lysate, mixing thoroughly, and transferring it to the Zymo-Spin™ IICR column. This was followed by treatment with DNase I to remove any genomic DNA (gDNA) contamination. A series of washes was then performed before eluting the purified RNA sample. The sample was

quantified by UV-visible spectrophotometry using a Nanophotometer (P330, IMPLEN, Monaco, Germany).

3.5.3 Real-Time PCR Analysis

For the RT-qPCR analysis, specific human sense and antisense primers were used. Real-time amplification was performed using the Luna Universal qPCR One-Step Kit (BioLabs, New England), according to the manufacturer's instructions. This kit allows the amplification of various genes directly from RNA. Additionally, amplification of the genes of interest (OSX and RUNX2) was performed alongside that of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a positive reference (Table 5). The amplification reaction was performed on a thermo fisher Real-Time PCR System 7500 (Waltham, Massachusetts, Stati Uniti), using the following program: an initial reverse transcription step at 55 °C for 10 min; a denaturation step at 95 °C for 1 min; 40 amplification cycles at 95 °C for 10 s and 60 °C for 30 s; and a melt curve at 60–95 °C for 15 s. The relative expression of the investigated genes was calculated using the comparative quantification method $2^{(-\Delta\Delta)} \text{Ct}$, with GAPDH serving as the reference gene.

Gene	Forward (5'→3')	Reverse (5'→3')
GAPDH	CTGCACCACCAACTGCTTAG	ACCTGGTGCTCAGTGTAGCC
OSX	ACGGGTCAGGTAGAGTGAGC	GGGATCCCCCTAATCAAGAG
RUNX2	CCGCTTCAGTGATTTAGGGC	GGGTCTGTAATCTGACTCTGTCC

Table 5 Primer sequences used in RT-PCR analysis.

3.5.4 Protein extraction and western blotting

Once the Matrigel® Matrix had been removed from the dental pulp organoids (according to the above-described protocol), a specific protocol was followed for the extraction of total proteins. Due to the potential difficulty of obtaining sufficient protein using only the lysis buffer, a different protein extraction protocol was employed for the organoids. First, the organoid was mechanically disrupted using a pestle to avoid excessive fragmentation, which would cause protein degradation. Next, lysis buffer 10X (Cell Signaling TECHNOLOGY®,

Danvers, USA) enriched with protease and phosphatase inhibitors was added, after which the organoid was incubated at 4 °C for 15 minutes to induce chemical lysis of the cells. The lysate was then subjected to five cycles of sonication (each consisting of one minute of sonication interspersed with five minutes of cooling in an ice bath) to ensure complete cell rupture and protein release.

Finally, to separate the soluble protein fraction, the samples were centrifuged at 14,000 rpm for 10 minutes at 4 °C. The resulting supernatant, containing the total proteins, was carefully collected for subsequent analysis. Protein quantification was performed using the Bradford assay, measuring the absorbance at 595 nm with a spectrophotometer Infinite® M Plex 30190085 (TECAN, Männedorf, Switzerland) (Figure 12).

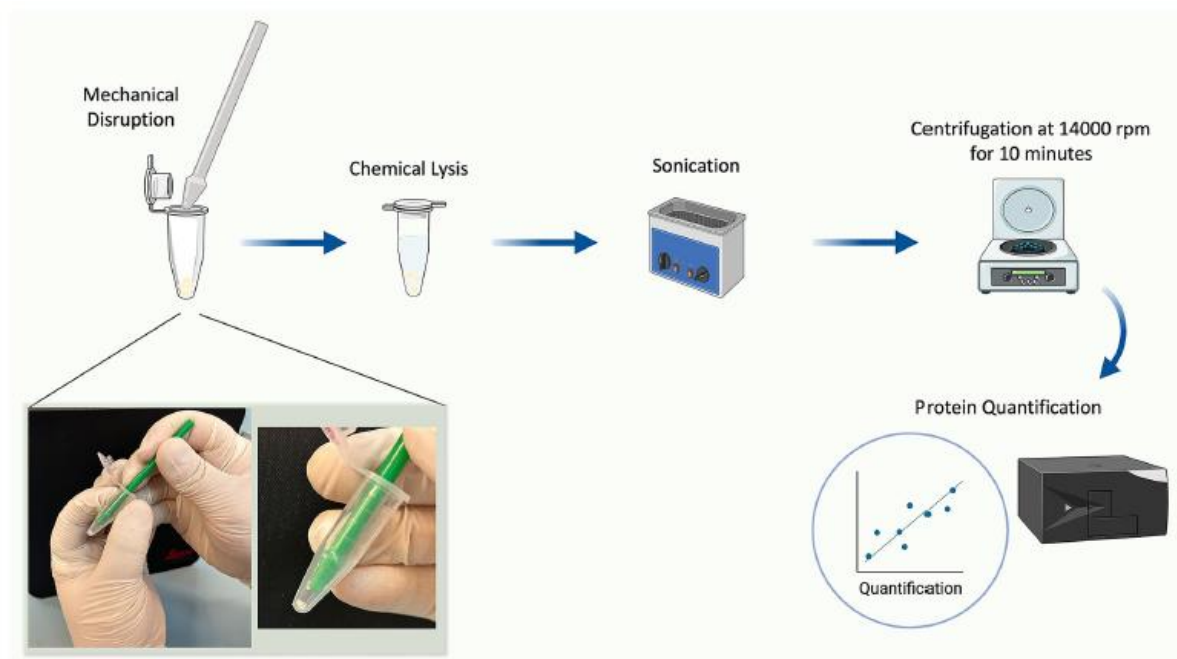


Figure 12 Protein extraction from organoids. The organoids are gently fragmented using a pestle and then subjected to five cycles of sonication (one minute on, five minutes on ice). The samples are then centrifuged, and the resulting supernatant is collected for protein quantification using the Bradford assay.

The extracted proteins were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then transferred electrophoretically to nitrocellulose membrane (Life Technologies, Monza, Italy), blocked with 5% bovine serum albumin (BSA) (Euroclone, Milan, Italy) in TBS containing 0.05% Tween 20 (Bio-Rad, Milan, Italy), and probed with rabbit anti-OSX, anti-Runx2 antibodies and anti-GAPDH (Santa Cruz Biotechnology). The antibodies were visualized using horseradish peroxidase (HRP)-

conjugated anti-rabbit IgG (Cell Signaling Technology, Danvers, MA, USA), and immunoreactivity was assessed via a chemiluminescence reaction with the ECL detection system (Thermo Fisher Scientific, Rockford, IL, USA) and a Chemidoc (Bio-Rad). Densitometric analysis was performed using Image Lab software (Bio-Rad).

3.5.5 Immunofluorescence analysis

Immunofluorescence was performed on cryosections to evaluate the spatial distribution of osteogenic markers and stemness maintenance. The sections, prepared as described in Section 3.4.2, underwent a specialized protocol to ensure optimal signal-to-noise ratios in a 3D context. The slides were washed twice for five minutes with PBS to remove OCT residue. To reduce autofluorescence, it is necessary to eliminate excess Matrigel® Matrix. To remove the matrix, the sections were incubated in a commercially available Cell Recovery Solution (Corning, New York, NY, USA) for 20 minutes at 4 °C.

The samples were then permeabilized with 0.05% Triton X-100 (Thermo Fisher Scientific, Waltham, MA, USA) in PBS for 10 minutes at 4 °C, followed by three 5-minute washes in PBS. Non-specific binding was blocked using a 3% BSA solution for 1 hour at RT. The samples were then incubated with the following primary antibodies:

- CD105 monoclonal mouse (Santa Cruz Biotechnology, Dallas, TX, USA) at a dilution of 1:50.
- OSX, monoclonal, mouse (Santa Cruz Biotechnology, Dallas, TX, USA), dilution 1:50.
- RUNX2, monoclonal rabbit (Cell Signaling Technology Inc., Danvers, MA, USA), dilution 1:250.
- FITC-Phalloidin, mouse (Merck Life Science, Sigma-Aldrich, Milan, Italy), dilution 1:1000.

All primary antibodies were diluted in a blocking buffer. This was to reduce any background. The antibodies were then incubated overnight at 4 °C. The slides were washed in PBS for 15 minutes (3 washes of five minutes each) and lightly dried at RT. The samples were treated with anti-mouse and anti-rabbit secondary antibodies, both labelled with Alexa Fluor 488 and 594 (ThermoFisher Scientific, Rockford, IL, USA) at a dilution of 1:1000 in PBS and incubated for

1 h at RT in the dark. The slides were then given a 15-minute wash in PBS (three washes of five minutes each) and dried lightly again.

For the nuclei staining, DAPI (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) was diluted 1:1000 in PBS and added to each slide.

Next, the samples were washed in PBS for 15 minutes. This was done in three washes of five minutes each. Then they were dried. Glycerol was used as a mounting medium. The A1 Zeiss Axiovert fluorescence microscope (Zeiss, Oberkochen, Germany) was used to analyse the samples and acquire images (Figure 13).

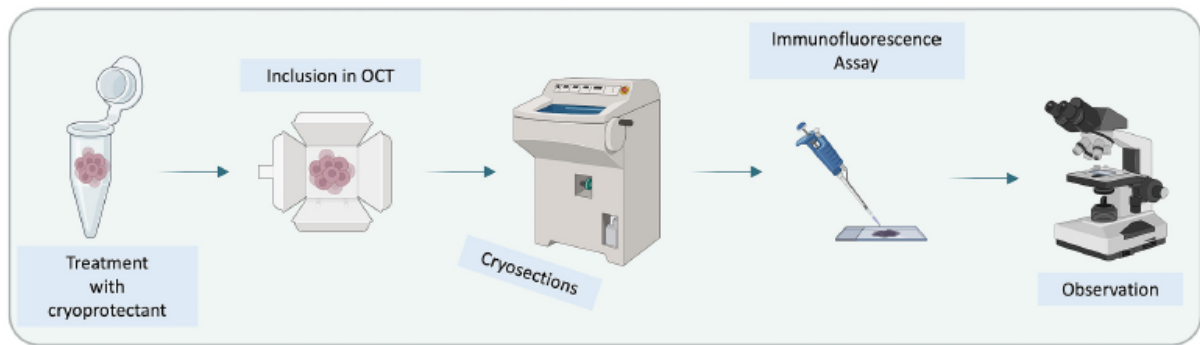


Figure 13 This diagram shows the steps in the organoid immunofluorescence protocol. The organoids were fixed in 4% PFA, cryoprotected, and embedded in OCT for cryosectioning. The sections were then stained with primary and secondary antibodies, mounted with glycerol, and observed using fluorescence microscopy.

3.6 Raman Microspectroscopy Analysis

3.6.1 Decellularization of organoids for Raman Analysis

Organoids were decellularized using a modified version of the Li et al protocol (Li et al., 2024). Briefly, the organoids were incubated in 0.025% (w/v) trypsin-EDTA at 37 °C for 90 min. They were then treated with 5% (v/v) Triton X-100 for 120 minutes at RT with agitation at 120 rpm. After PBS washes, samples were fixed in 4% PFA for 24 hours at 4 °C, washed again in PBS, and stored at 4 °C until Raman spectroscopy analysis.

3.6.2 Raman Microspectroscopy (RMS)

A Horiba Jobin-Yvon XploRA Nano Raman microspectrometer equipped with a 785 nm diode laser was used as a light source. All Raman microspectroscopy (RMS) measurements were performed using a $\times 100$ objective (Olympus, Tokyo, Japan). Before spectral acquisition, the spectrometer was calibrated to the 520.7 cm^{-1} line of silicon. A 1200 lines per mm grating was selected.

A $200\text{ }\mu\text{m}$ confocal pinhole was used for all measurements. The spectra were dispersed onto a Peltier-cooled CCD detector with a 16-bit dynamic range. For each experimental group, 10 Raman spectra were collected from three decellularized specimens.

All Raman spectra were corrected for the contribution of atmospheric carbon dioxide and water vapor using the atmospheric compensation routine in OPUS 7.5 software (Bruker Optics, Ettlinger, Germany), and vector normalized across the full spectral range using the normalization routine in OPUS 7.5 software. Specific area, intensity, and full width at half maximum (FWHM) values were then calculated to assess well-defined spectral markers (integration routines, OPUS 7.5 software).

3.7 Statistical Analysis

Data are reported as the mean $\pm$ standard deviation (SD) based on three independent experimental replicates ($n = 3$ per treatment and time point). Statistical analyses were performed using Prism 6 software (GraphPad Software, La Jolla, CA, USA). Group comparisons were conducted using Student's T-test or one-way ANOVA, as appropriate. A p -value of ≤ 0.05 was considered indicative of statistical significance.

4.Results

4.1 Cytofluorimetric Characterization of DPSCs

Following isolation, DPSCs were subjected to phenotypic characterization by flow cytometry. This analysis confirmed the widespread expression of the classic stem cell markers CD90 (99.9%), CD105 (94.6%), and CD73 (99.8%), which are commonly found in mesenchymal stromal cells (MSCs) (H. Li et al., 2023). Additionally, the cells exhibited partial positivity for CD146 and CD271. The presence of these latter markers is particularly noteworthy, as they are typically associated with the perivascular niche and are indicative of high odontogenic and neurogenic potential. Conversely cells were negative for CD19 and CD45, confirming the absence of any contamination from hematopoietic lineages (Table 6).

CD90	CD73	CD105	CD166	CD271	CD146	CD19	CD45
99,9%	99,8%	94,6%	100%	9%	9,5%	0,3%	0,2%

Table 6 Cytofluorimetric analysis of Immunophenotypic profile of isolated DPSCs.

4.2 Optimization of the Organoids development protocol

A series of preliminary experiments was conducted to standardize organoid generation. These tests indicated that using absolute (undiluted) Matrigel® matrix with an initial seeding density of 75,000 cells produced a single, well-compacted spheroid per well with a diameter of approximately 300–400 μm (Figure 14). Specifically, as shown in Figure 14A, cultures seeded with 25,000 cells resulted in small fragile clusters that were prone to mechanical dissociation, whereas high densities cultures (100,000 cells) led to the rapid formation of oversized spheroids, which exhibit poor cell distribution homogeneity with early signs of central core necrosis.

Moreover, diluted Matrigel® (50% and 75%) (Figure 14B) did not provide sufficient extracellular matrix support to sustain proper organoid formation.

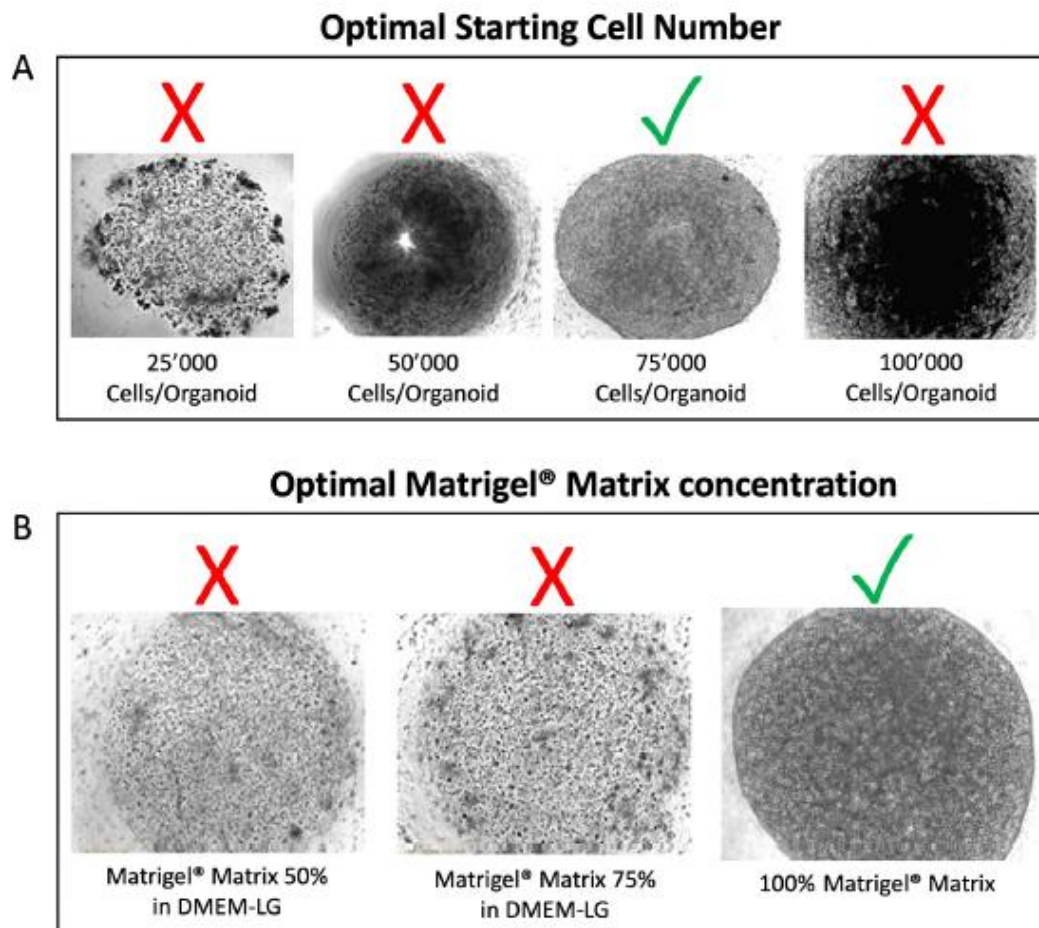


Figure 14 Representation of various organoids generated under different initial conditions: (A) determination of the optimal starting cell number; (B) determination of the Matrigel® matrix concentration. The images show failed (x) and successful (✓) organoid formation (4X magnification).

4.3 Standardization of the "Mature" Organoids generation

4.3.1 3D structures monitoring growth and the “switch” to differentiation

Once the protocol was standardized, organoid development and growth were monitored from the initial aggregation of 3D structures (T0) to the experimental endpoint (T26) as described in materials and methods (Scheme 1). Organoids were initially cultured in DMEM-LG medium,

and after three days, upon observation of stabilization of the 3D structures adequate cell aggregation, this timepoint was designated as T0.

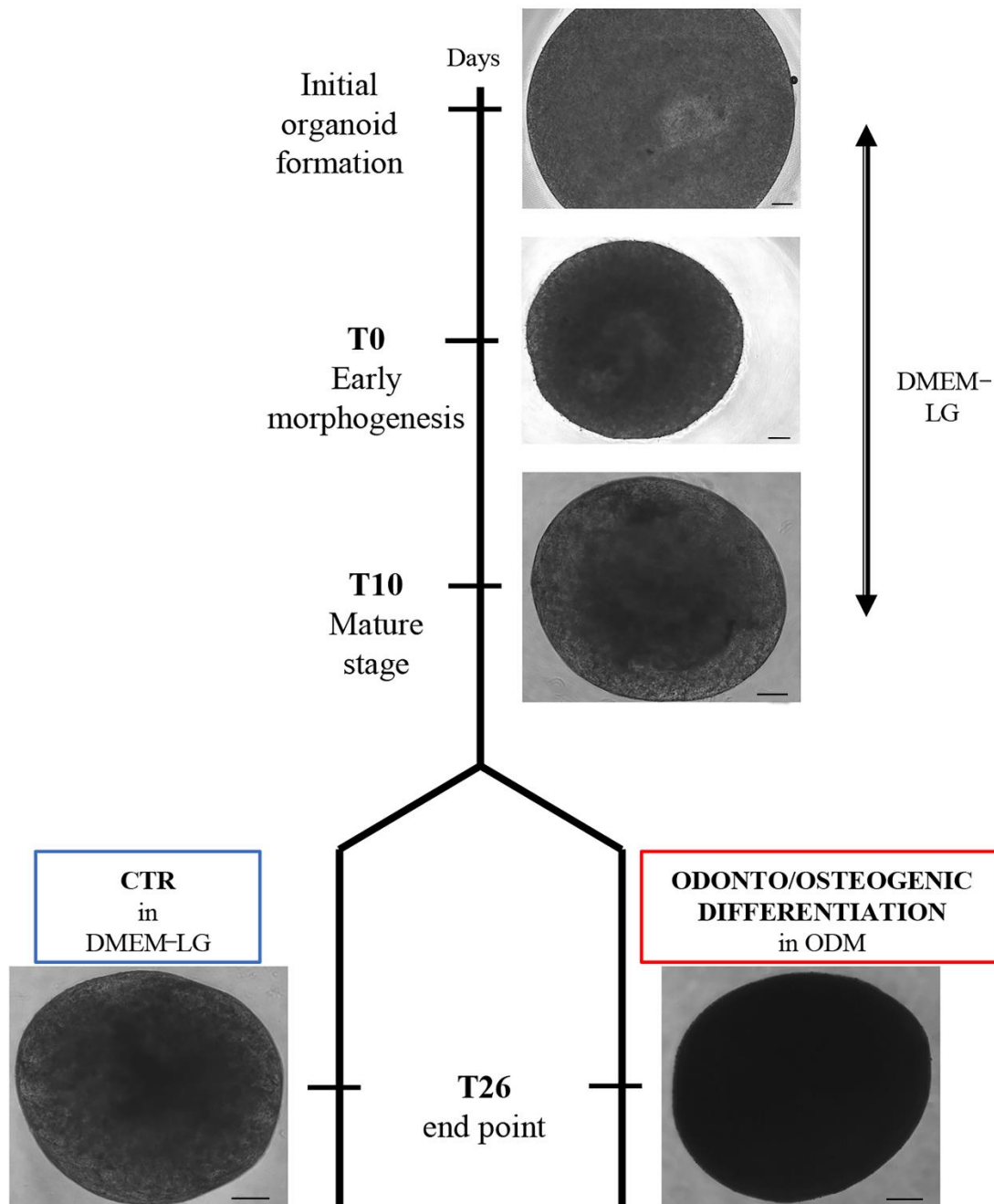


Figure 15 Generation of organoids: From T0 to the T26 endpoint. Representative images of organoid development, acquired using the optical Leica Mateo TL microscope (Magnify 4X), maintained in DMEM-LG (CTR) and undergoing odonto/osteogenic differentiation (ODONTO/OSTEO). The scales correspond to 200 μm.

Following the stabilization of the 3D structures at T10, we introduced the ODONTO/OSTEO differentiation medium. A visible divergence between the two experimental groups appeared and over the subsequent 16-days, the organoids were no more identical, developing distinct physical characteristics (Figure 15). The control organoids (CTR), maintained in basal medium, retained a regular, spherical morphology with a smooth, translucent, homogeneous surface and no macroscopic evidence of mineralized deposits, suggesting a stable state of undifferentiated maintenance. Conversely, the organoids exposed to the ODONTO/OSTEO induction medium underwent a progressive transformation. We observed a gradual loss of translucency as the structures became increasingly opaque. By the T26 endpoint, these organoids exhibited a characteristic "stony" white appearance when viewed under the stereomicroscope.

This change in optical density was not merely superficial; it was accompanied by an increasingly irregular and rugged surface texture as observed by High-Resolution Stereomicroscopy using a Zeiss Axio Zoom V6 stereomicroscope (Zeiss, Oberkochen, Germany). These macroscopic observations provided the first tangible evidence of an odontogenic commitment. The "stony" appearance and increased opacity strongly suggested that the cells had begun to modify their microenvironment, transitioning from a soft cellular aggregate into a dense, mineralized tissue. This morphological "darkening" could represent a real-time indicator of the deposition of a calcium-rich extracellular matrix, effectively marking the successful transition from a collection of stem cells to a functional, mineralizing 3D cell complex (Figure 16).

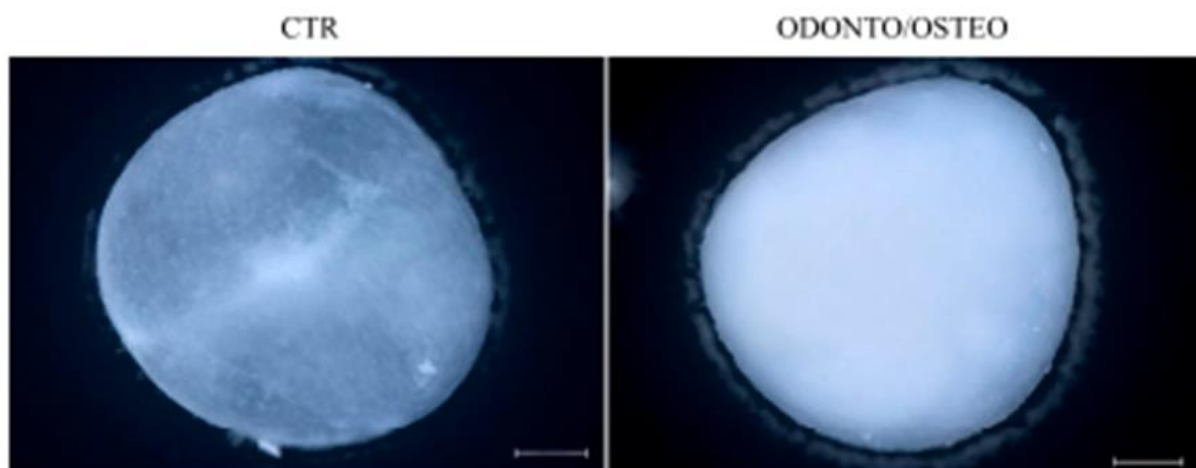


Figure 16 Stereomicroscopic analysis of DPSC-derived organoids cultured in control and differentiation conditions. Representative images of the organoids after 26 days in a standard culture medium (CTR) and an osteogenic differentiation medium (ODONTO/OSTEO) were acquired using a Zeiss Axio Zoom.VI6 stereo microscope with 63X magnification. The scales correspond to 200 μ m.

4.3.2 Automated Generation of Organoids from DPSCs using MatriBot®

Organoids generated using the MatriBot® system exhibited greater dimensional and morphological uniformity than those produced manually, demonstrating significantly higher reproducibility. The temperature-controlled print head eliminated complications arising from the premature polymerization of Matrigel®, thereby ensuring optimal thermal control.

Maturation followed the same pattern as the manual protocol, confirming functional equivalence (Figure 17).

However, the automated process required significantly higher quantities of Matrigel®, rendering it less cost-effective. Manual production therefore continued in order to optimize costs while maintaining acceptable quality and reproducibility.

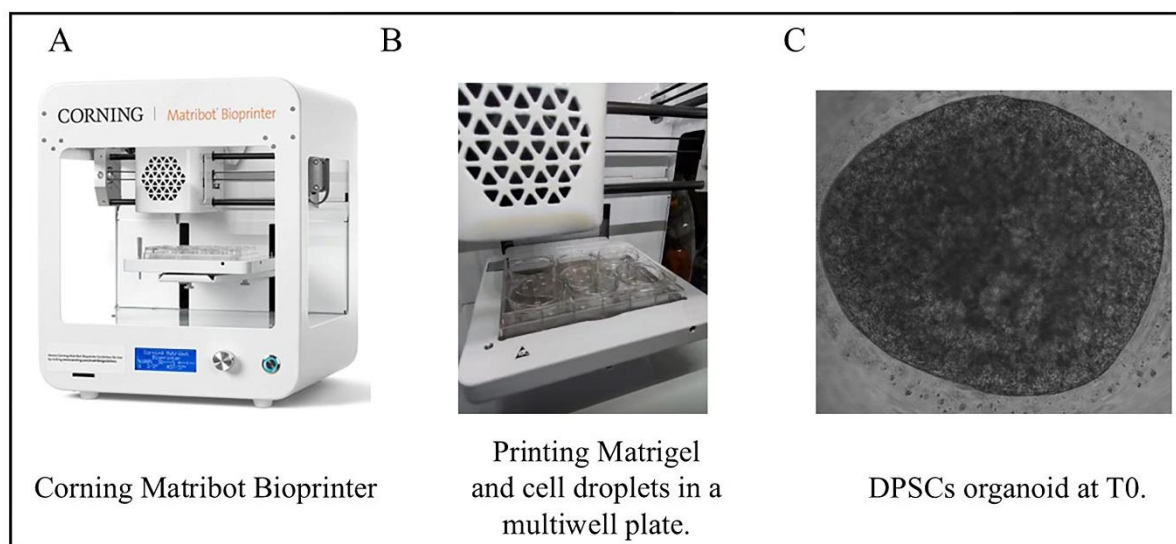


Figure 17 The image shows the protocol for producing organoids using a bioprinter. A) the Corning MatriBot bioprinter; B) printing Matrigel and cell droplets onto a sheet of parafilm placed in a 6-well plate; C) a DPSC organoid produced using the Corning MatriBot bioprinter after three days of culture (T0).

4.4 Assessment of Cell Viability and Metabolic Activity

4.4.1 Cell viability assessment by Trypan blue assay

To quantify this, we performed a Trypan Blue exclusion assay at the T26 endpoint to compare the total cellularity of the control (CTR) and differentiated (ODONTO/OSTEO) groups. Our results revealed low mortality in all experimental conditions, probably due to necrotic nuclei formed by hypoxic conditions, given the absence of vascular structures. As shown in Figure 18, the control organoids contained more cells (5,800,000) than the differentiated *ODONTO/OSTEO* organoids ($4,000,000 \pm 800,000$). This reduction in cell number was probably due to the differentiation medium, which slows cell growth and promotes differentiation of specific cell lines. Statistical analysis was performed using two-way ANOVA. Statistical significance was considered for * $p < 0.05$ and ** $p < 0.005$.

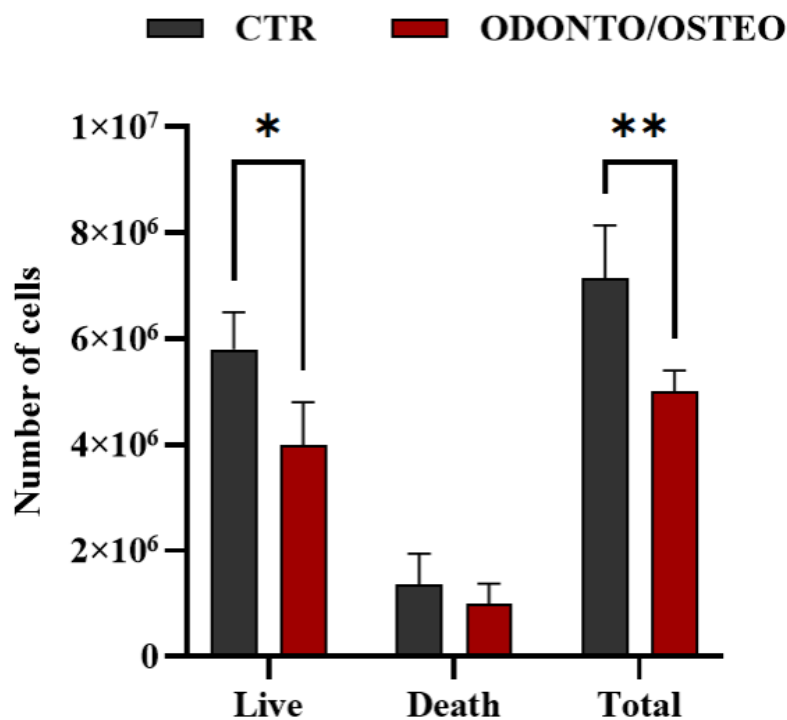


Figure 18 Representative histogram showing the number of live, dead, and total cells quantified using the trypan blue exclusion assay. Live and dead cells were measured in both control organoids (CTR) and differentiated organoids (ODONTO/OSTEO) at the end of the differentiation protocol (T26).

4.4.2 Metabolic activity determination by CCK-8 assay

To further investigate the health of the organoids, we employed the CCK-8 assay, which measures the metabolic activity of dehydrogenases associated with proliferation within viable cells. Although morphological observation showed an increase in the apparent density of differentiated organoids, the CCK-8 assay showed no statistically significant differences between the CTR and ODONTO/OSTEO groups (Figure 19). This suggests that the dark staining observed under the microscope is a product of high-density matrix packing rather than a simple increase in cell number given that cell density in the same-sized differentiated organoids was lower than in control organoids.

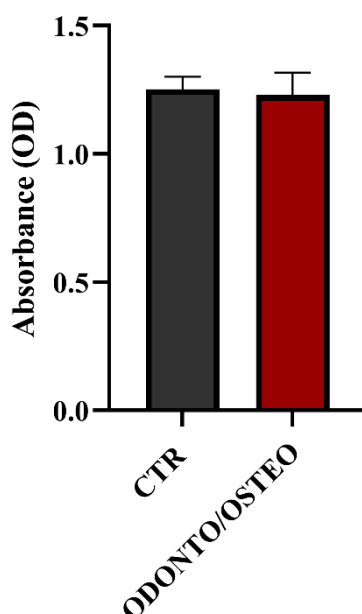


Figure 19 The histogram shows the results of cell proliferation assays conducted on organoids derived from dental pulp cells. Proliferation rates were measured in both CTR organoids (CTR) and differentiated organoids (ODONTO/OSTEO) at the end of the differentiation protocol (T26). Seven independent experimental replicates/treatments ($n = 7$) were performed.

4.5 Structural Validation and Mineralization Fingerprinting

To validate that the macroscopic "stony" appearance corresponded to true physiological mineralization, we employed a multi-disciplinary analytical approach.

4.5.1 Hematoxylin and Eosin staining

Organoid sections were examined to investigate their internal structure and morphology. Hematoxylin and Eosin (H&E) staining confirmed a stratified internal architecture. The outer layer thickness ranged between 30-50 μm in both differentiated and control organoids (Figure 19). At the end point (T26), a compact cellular area had formed in the outermost portion of the organoids. In contrast, the inner region appeared disorganized and sparse, with fewer cells (Figure 20). This observation is likely attributable to elevated cell mortality rates resulting from limited oxygen and nutrient supply to the organoid's internal structures.

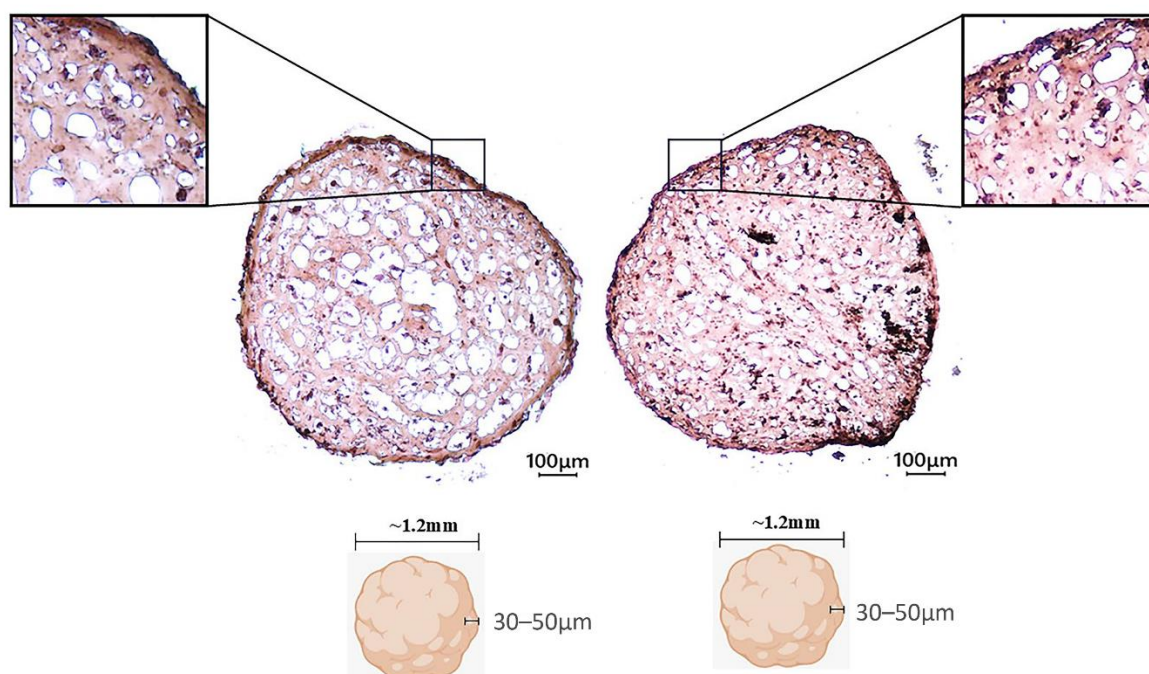


Figure 20 Representative images of the hematoxylin and eosin staining of the CTR and ODONTO/OSTEO organoids acquired using the optical Leica Mateo TL microscope (Magnify 10X). The scale corresponds to 100 μm .

4.5.2 Calcium Carbonate Deposition Assessment

Figure 21 shows that extensive and intense mineralization was observed in cryosections of ODONTO/OSTEO-differentiated organoids compared to CTR. The significant increase in

calcium salt deposition after Alizarin red staining demonstrates the formation of mineralized dental tissue, suggesting that the differentiated pulp cells (ODONTO/OSTEO) have acquired a mature odonto/osteogenic-like phenotype capable of effectively mineralizing the extracellular matrix.

In contrast, the CTR group showed no significant accumulation of calcium. These results revealed substantial calcium deposition in the differentiated organoids.

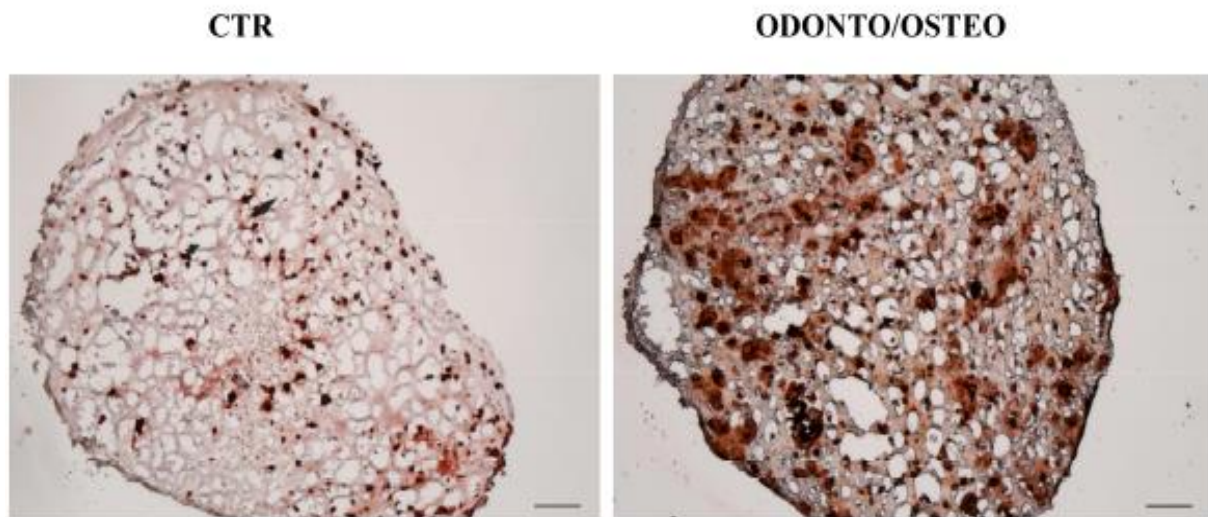


Figure 21 Calcium carbonate deposition in DPSCs organoids: Alizarin red staining of control (CTR) and differentiated (ODONTO/OSTEO) organoids. Image was acquired using the Leica Mateo TL optical microscope. Scale bar: 100 μ m (4X magnification).

4.5.3 Overcoming the Matrix Obstacle: Nucleic Acid and Protein Extraction

The presence of Matrigel® in the organoid culture initially presented an obstacle, as it interfered with the effective extraction of nucleic acids and proteins. To overcome this limitation, a protocol involving matrix degradation and removal using Cell Recovery Solution was optimized. Implementing this crucial step enabled us to obtain an adequate sample for subsequent analysis. The amount of RNA extracted was accurately measured using a spectrophotometer, while the Bradford assay was used to quantify the proteins. As shown in Figure 22, the developed protocol enabled the extraction of approximately 320 ng/ μ l of RNA and 190 μ g of protein from the matrix-depleted samples. In contrast, no nucleic acids or proteins could be extracted from samples in which Matrigel® was not removed.

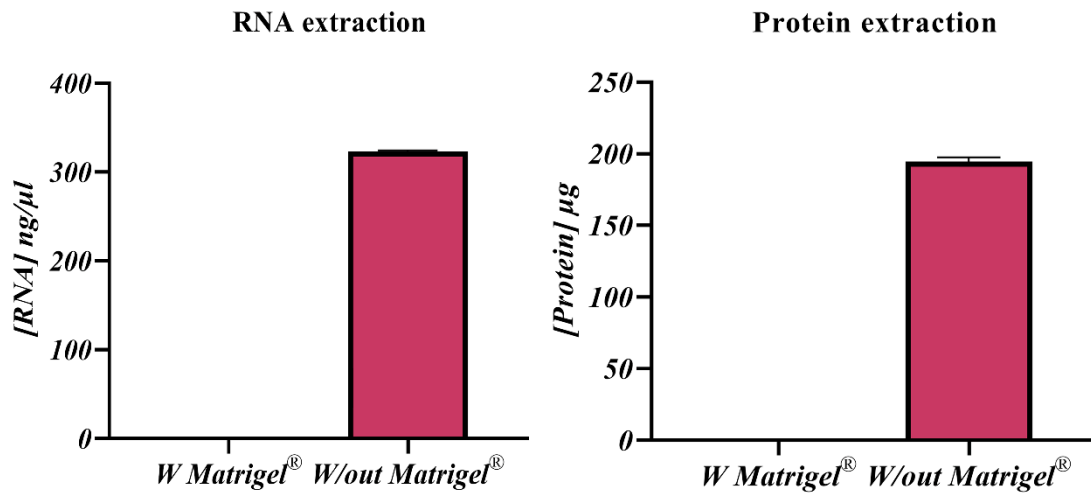


Figure 22 Comparison of RNA and protein extraction efficiency in organoid cultures. Quantification of (A) RNA concentration (ng/μl) measured by spectrophotometry and (B) total protein yield (μg) determined by Bradford assay. The graphs highlight the necessity of removing the Matrigel® matrix using Cell Recovery Solution (W/out Matrigel®) compared to untreated samples (W Matrigel®).

4.6 Validation of Physiological Mineralization

4.6.1 Molecular Approach Analysis of DPSCs Organoids by Real-Time PCR (RT-qPCR) and Western blotting

RT-qPCR and Western blotting were used for gene and protein analysis of organoids, respectively. However, a critical issue regarding the modulation of housekeeping genes between undifferentiated and differentiated organoids emerged in both methods (Figure 23). The non-specific variation in the expression of these genes, which were essential for normalization, compromised the reliability and validity of the obtained data. Consequently, the initial RT-qPCR and Western blotting results were not considered robust enough for conclusive molecular characterization. This outcome necessitated the search for alternative molecular methods for target analysis.

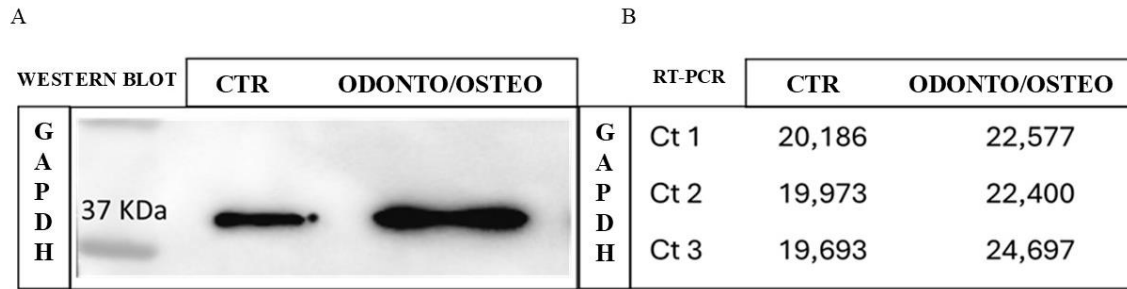


Figure 23 Analysis of GAPDH expressions in CTR and ODonTO/OSTEO samples. (A) Western blot membrane showing GAPDH protein bands (37kDa) for control (CTR) and differentiation (ODONTO/OSTEO) conditions. (B) Summary table of cycle threshold (Ct) values obtained by RT-PCR for the GAPDH gene in three independent experimental replicates (Ct 1, Ct 2, and Ct 3) under the same conditions.

4.6.2 Immunofluorescence Analysis of DPSCs Organoids

The proteins that show odonto/osteogenic differentiation (RUNX2 and OSX) and the marker of stemness (CD105) (see Figure 24) were studied in frozen sections of both groups of organoids using immunofluorescence analysis. The slices showed a solid fluorescent signal that had built up due to the different focal planes. Additionally, the inability to obtain high-quality images was due to the shallow depth of field of the high-magnification objectives. Despite this, we were able to observe the expression of the various markers of differentiation and stemness and perform a comparison between the undifferentiated and differentiated organoids.

Figure 24 A shows that, in comparison to the organoids that had become differentiated, the control organoids had higher levels of stem marker CD105, as revealed by images obtained under a fluorescence microscope. Furthermore, low expression of the odonto/osteogenic marker OSX was observed (green, Figure 24 B). In differentiated organoids, however, an apparent reduction in CD105 expression (Figure 24 A) and an increase in OSX and RUNX2 expressions, which is typically greater in DPSCs that have been differentiated than in undifferentiated samples (Figures 24 B and 24 C).

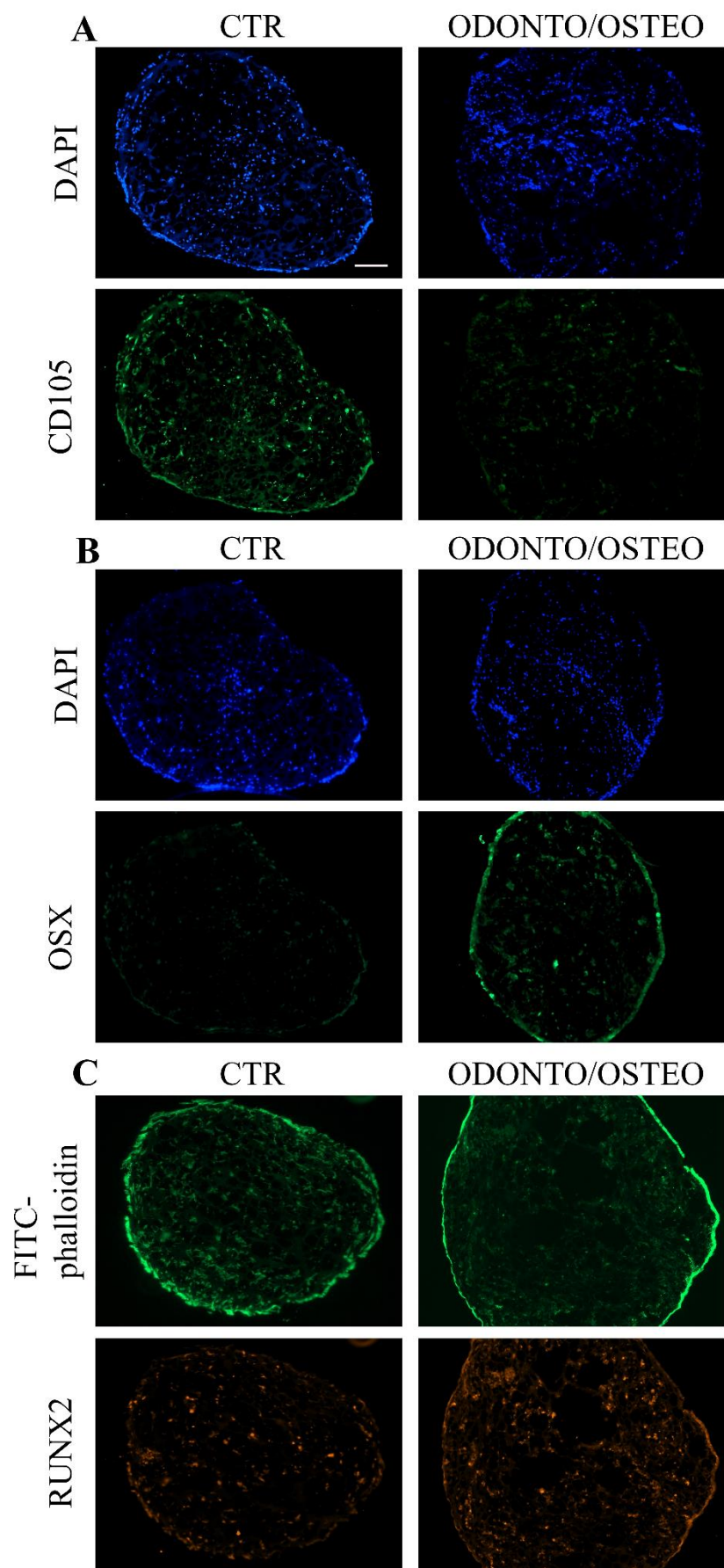


Figure 24 Representative images of immunofluorescence analysis of organoids for stemness and differentiation (ODONTO/OSTEO) markers. (A) Images show expression of CD105 in the CTR and differentiated organoids. (B) Images show expression of specific ODNTO/OSTEO markers (OSX) in CTR and differentiated organoids. (C) Images represent RUNX2 expression in CTR and differentiated samples. The samples were analyzed, and images were acquired with the A1 Zeiss Axiovert fluorescence microscope (Zeiss, Oberkochen, Germany). The scales correspond to 200 μm (10X magnification).

4.6.3 Raman microspectroscopy analysis

Figure 25 A shows the Raman spectra of the CTR and ODNTO/OSTEO experimental groups. Figure 25 B highlights the behaviour of the most significant peaks: proteins (band centred at 1660 cm^{-1} , Amide I peak of proteins) (Ramakrishnaiah et al., 2015; Torres et al., 2018); lipids (band centred at 1450 cm^{-1} , bending of CH_2 groups mainly from aliphatic chains) (Notarstefano et al., 2022; Orilisi et al., 2021); type A carbonates (band centred at 1100 cm^{-1} , type A CO_3^{2-} groups) (Ramakrishnaiah et al., 2015); type B carbonates (band centred at 1070 cm^{-1} , type B CO_3^{2-} groups) (Orilisi et al., 2021; Torres et al., 2018); hydroxyapatite (HA). - The band intensity is centred at 960 cm^{-1} for the first stretching mode (ν_1) of the PO_4^{3-} groups of hydroxyapatite (HA) (Notarstefano et al., 2023, 2022).

The intensity of the band is centred at 1030 cm^{-1} for the third stretching mode (ν_3) of the PO_4^{3-} groups of HA (Notarstefano et al., 2022; Orilisi et al., 2021). Analysis of these peaks showed that the Raman spectra acquired the typical characteristics of bioapatite (HA) after differentiation, displaying all the characteristic peaks, including those related to carbonates and phosphates. Significant variations were found between the CTR and ODNTO/OSTEO groups for all the selected spectral markers. The significantly reduced protein and lipid content is clearly attributable to an increase in the relative content of mineral deposits, probably in the form of HA. Generally, a comparison of the Raman spectra of the two experimental groups shows a shift from almost all type A CO_3^{2-} fractions in CTR to almost all type B CO_3^{2-} in ODNTO/OSTEO.

The three most widely used spectral markers were also calculated and compared between the CTR and ODNTO/OSTEO groups: mineral/matrix (A_{960}/A_{1660}), crystallinity ($1/\text{FWHM}_{960}$), and C/P (carbonates/phosphates, I_{1070}/I_{960}) (Figure 26).

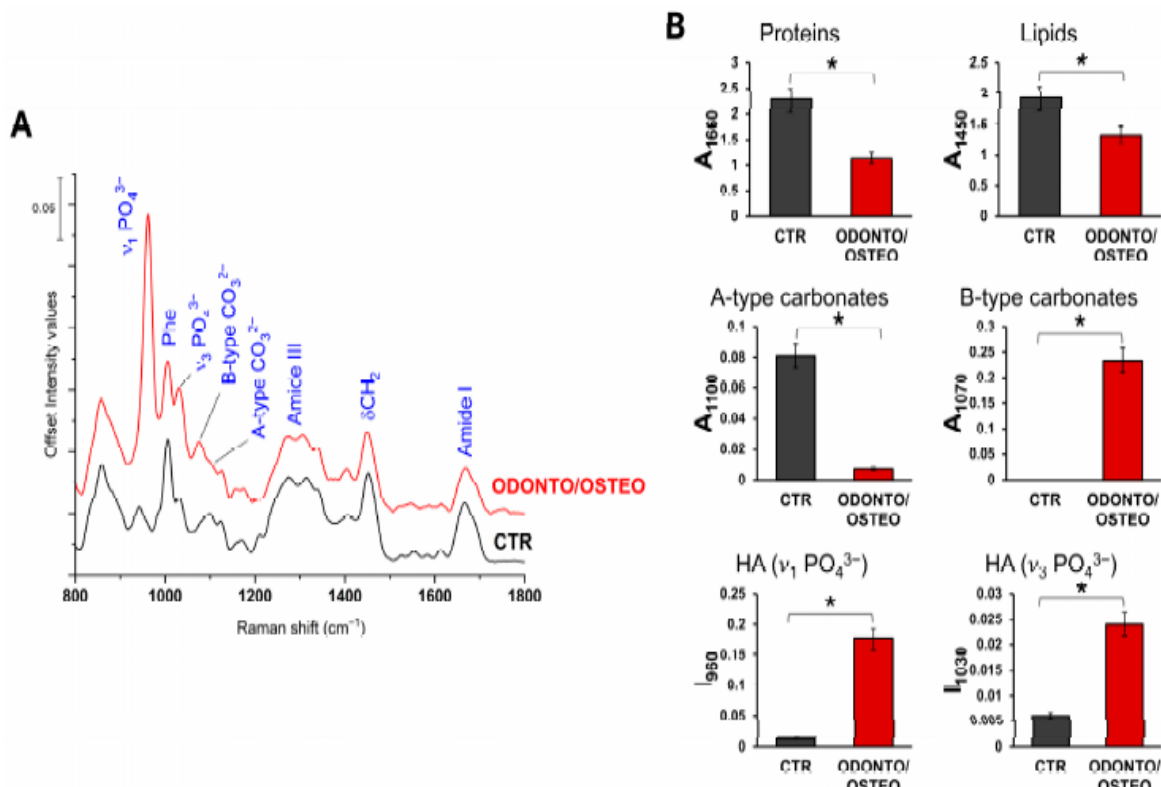


Figure 25 Raman microspectroscopy analysis of CTR and ODONTO/OSTEO organoids: (A) Average Raman spectra representative of the CTR and ODONTO/OSTEO experimental groups. The labels highlight the peaks of interest. For better viewing, the spectra are offset along the y-axis. (B) Statistical analysis of the numerical variation in proteins, lipids, A-type and B-type carbonates, and HA (ν₁ and ν₃) calculated values. Three specimens (n = 3) were analysed from each group, with ten spectra obtained from each specimen. Data are presented as mean ± SD. * Statistically significant value of ODONTO/OSTEO box charts with respect to CTR (p < 0.05; Student's t-test).

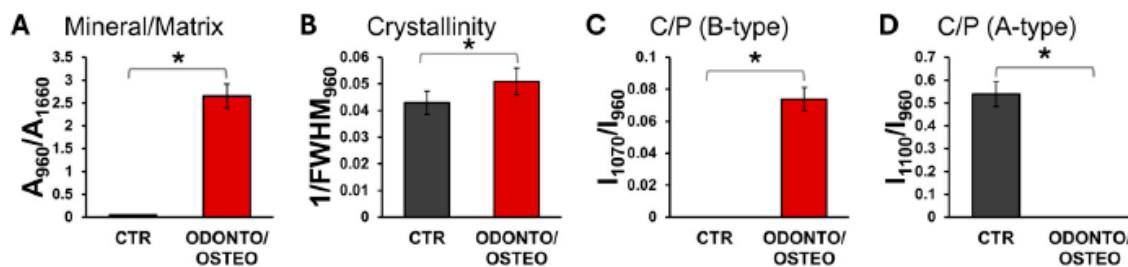


Figure 26 Raman spectral markers of differentiation. Statistical analysis of the numerical variation in the following spectral markers: (A) mineral/matrix, (B) crystallinity, (C) C/P (B-type), (D) C/P (A-type). Three specimens (n = 3) were analysed from each group, with ten spectra obtained from each specimen. Data are presented as mean ± SD. * Statistically significant values of ODONTO/OSTEO box charts with respect to CTR. (p < 0.05; Student's t-test).

5 Discussion

The results obtained in this project contribute to a broad research context that, in recent years, had increasingly focused on the regenerative potential of MSCs and the development of sophisticated three-dimensional in vitro models (Hodge et al., 2023). In particular, DPSCs have attracted growing interest due to their unique biological properties and their promising applications in both biotechnology and clinical settings (Fujii et al., 2023; Hsiao et al., 2021). In this project, we successfully established a standardized protocol for generating organoids from mesenchymal stem cells, particularly from DPSCs, moving beyond traditional 2D cultures to better replicate the complex spatial organization and cell-matrix interactions of the dental pulp niche and on establishing and validating experimental procedures for their structural and functional characterization. Our goal was twofold: to evaluate the ability to generate odonto/osteogenic organoids from DPSCs, and to explore alternative methods to real-time PCR and western blotting, specifically 3D immunofluorescence and Raman microspectroscopy the latter of which is a relatively new technique capable of providing an organoid's 'fingerprint' to indicate its state of differentiation (Ramakrishnaiah et al., 2015). The combination of these techniques is useful because it provides a combined functional and structural perspective on organoid development in terms of matrix composition and mineral deposition.

In particular, our findings demonstrated that the calcium phosphate deposition was elevated in differentiated organoids (following 16 days of differentiation) in comparison to the control group. In parallel, Raman microspectroscopy revealed spectral profiles consistent with those expected for a hydroxyapatite-based biomaterial. Furthermore, immunofluorescence analysis of the differentiated organoids revealed enhanced expression of odonto/osteogenic markers (RUNX2 and OSX) and reduced expression of the stemness marker CD105, confirming the observed morphological changes. using a relatively simple guided protocol (Lancia et al., 2025).

MSCs derived from dental pulp were first identified by Gronthos et al. in 2000 (Gronthos et al., 2000). These cells were able to self-renew and differentiate into various cell types, respond to specific environmental stimulus, generate new stem cells, and activate differentiation mechanisms (Gronthos et al., 2002). DPSCs were considered excellent candidates for regenerative medicine and the development of organoids. This suitability stems from their combination of favorable characteristics: they were highly accessible, highly versatile in

differentiation, and highly immunologically compatible. For these reasons, DPSCs represented an auspicious choice for the development of innovative regenerative therapies.

Specifically, dental pulp cells were isolated from young donors aged 13-19 years. These cells were analyzed to confirm their mesenchymal properties using flow cytometry, which showed high expression of CD90, CD73, CD166, and CD105 markers (90%), and low expression of CD19 and CD45 (less than 0.5%), as well as CD271 and CD146 (less than 10%) as established from (Mattei et al., 2025). Numerous studies had shown that dental pulp stem cells could differentiate into the odonto/osteoblast lineage (Zhou et al., 2023). For example, Baldión et al. demonstrated that DPSCs maintained under osteo-odontoblastic differentiation conditions showed time-dependent changes not only in growth but also in morphology (Baldión et al., 2018). Furthermore, it was widely recognized that fully developed osteoblasts played a crucial role in matrix formation and its subsequent mineralization (Capulli et al., 2014). Matrix mineralization was a critical indicator of osteoblast differentiation in bone research and was commonly assessed through the presence of calcium phosphate, particularly hydroxyapatite (Moester et al., 2014; Puchtler et al., 1969).

Three-dimensional (3D) cell cultures were considered one of the most innovative and promising tools in cell biology and experimental medicine (Fang and Eglen, 2017; Xie et al., 2024). Compared to traditional two-dimensional models, 3D systems were able to more accurately reproduce in vitro the spatial organization, cell–cell and cell–matrix interactions, and, more broadly, the microarchitecture and physiology of human tissues (Fang and Eglen, 2017; Xie et al., 2024). This increased structural complexity enabled a more realistic simulation of physiological conditions in vivo, thus opening new perspectives for studying the molecular mechanisms underlying cell proliferation, differentiation, and responses to external stimuli (Duval et al., 2017; Zhao et al., 2022).

Owing to these characteristics, 3D models were playing an increasingly central role in both basic research and translational applications, such as modeling neoplastic and degenerative diseases, drug screening, and tissue engineering (Fang and Eglen, 2017; Xie et al., 2024; Yao et al., 2024). However, despite remarkable advancements in recent years, there was still no universally standardized 3D culture model that ensured reproducibility and comparability across laboratories. This represented a significant challenge for the scientific community and emphasized the need for reliable, well-characterized experimental protocols.

One of the main results of this study was the optimization of the 3D model. Our preliminary experiments identified that a density of 75,000 cells in undiluted Matrigel® provided the optimal balance between structural integrity and nutrient diffusion. While 25,000 cells

produced fragile clusters and 100,000 cells led to premature central necrosis, the 75,000-cell model formed stable spheroids (300–400 μm) capable of undergoing a 26-day differentiation protocol. The use of U-bottom ultra-low attachment (ULA) plates facilitated cadherin-mediated compaction, resulting in the "mature" spheroid observed after 3 days (T0).

After 26 days of total culture, our protocol produced organoids with an average diameter of approximately 1.5 mm. This result was consistent with findings from other researchers who employed different and/or more sophisticated techniques for generating DPSC-derived organoids (Jeong et al., 2020).

The results of the CCK-8 test, which measures cell metabolic activity, showed no statistically significant differences between the CTR and ODONTO/OSTEO organoid groups. However, as we recently demonstrated using trypan blue staining, the number of viable cells in ODONTO/OSTEO organoids was lower than in CTR organoids (Lancia et al., 2025).

As reported, the metabolic activity of cells during differentiation was not always linearly correlated with cell proliferation. This reduction should not be interpreted as a failure of the culture, but rather as a physiological hallmark of successful differentiation (Lancia et al., 2025). In experimental medicine, it is well-established that as stem cells commit to a specific lineage, such as the odonto/osteogenic pathway, they undergo a metabolic "switch" (Yin et al., 2016). The energy previously allocated to rapid mitotic expansion is redirected toward the biosynthesis of a complex extracellular matrix and the expression of specialized proteins. Consequently, the lower cell count in the differentiated group directly reflects the cells exiting the cell cycle to adopt a mature, secretory phenotype (Lancia et al., 2025). Additionally, the trypan blue exclusion assay was performed to evaluate both cell viability and mortality in the examined samples. The mortality index was moderately relevant across all conditions, likely attributable to the formation of a necrotic core in the organoid's central region. This hypoxic necrosis results from the absence of vascular structures, which limited oxygen and nutrient diffusion to the innermost cells—a phenomenon consistent with well-established literature on 3D organoid cultures (Lancaster and Knoblich, 2014; Yin et al., 2016).

Histological analysis on cryosectioned samples stained with hematoxylin and eosin revealed a more organized cellular structure in the outer layer and a more disorganized internal structure, particularly in the central region. This spatial heterogeneity is a well-documented phenomenon in 3D cell culture systems, particularly in structures over 500 μm in diameter (Mulaudzi et al., 2024). In such cases, the innermost cells receive a reduced supply of oxygen and nutrients from the culture medium (Lancaster and Knoblich, 2014; Yin et al., 2016).

These preliminary results were corroborated by macroscopic morphological analysis using the Zeiss Axio Zoom V6 stereomicroscope that revealed distinct differences between undifferentiated and differentiated organoids. Specifically, the undifferentiated organoids appeared smooth and translucent, while the differentiated organoids exhibited an opaque surface. These observations led us to hypothesize the presence of mineralized matrix and differentiation-induced phenotypic changes. Indeed, functional calcium salt deposition analysis via Alizarin Red staining confirmed substantial calcium phosphate deposition in the differentiated organoids.

These results reinforce the established capacity of DPSCs to adopt an odontogenic/osteogenic phenotype *in vitro*, consistent with prior studies demonstrating their ability to generate mineralised tissue (Anwar Alebrahim et al., 2014; Dawood and Mahdee, 2025; Gronthos et al., 2000; Mortada and Mortada, 2018; Riccio et al., 2010; Tubbesing et al., 2022).

The capacity of DPSCs to generate mineralized tissue was confirmed with molecular precision using Raman microspectroscopy. This technique provides precise, accurate spectral information on the present minerals, examining the characteristic energies of their vibrational modes in an unmarked manner (Anwar Alebrahim et al., 2014). Spectral marker analysis of the ODONTO/OSTEO samples, in particular, showed all the characteristics of biological apatite, including intense and well-defined phosphate (PO_4^{3-}) bands at 960 cm^{-1} (ν_1) and 1030 cm^{-1} (ν_3), accompanied by signals corresponding to carbonate substitutions. As previously mentioned, two types of carbonate groups were identified in dental mineral material: type A (1100 cm^{-1}) and type B (1070 cm^{-1}). Notably, the latter represents the symmetric stretching mode 1 of CO_3^{2-} ions replacing PO_4^{3-} in the apatite lattice (Anwar Alebrahim et al., 2014; Notarstefano et al., 2022; Tubbesing et al., 2022), while type A carbonates replace OH^- groups (Notarstefano et al., 2022). This carbonate substitution is critical for HA solubility; in fact, variations in the intensity of these bands have indeed been observed in spectroscopic analyses of carious lesions (Tubbesing et al., 2022).

Moreover, to quantitatively assess the mineralization process, the mineral/matrix (A_{960}/A_{1660}), crystallinity ($1/\text{FWHM}_{960}$), and C/P (carbonates/phosphates, I_{1070}/I_{960}) spectral markers were calculated and compared.

The mineral-to-matrix ratio reflects the relative amounts of mineral and organic components. It is calculated using the PO_4^{3-} symmetric stretching mode at 960 cm^{-1} , which is the most intense hydroxyapatite (HA) mineral band, and Amide I at 1660 cm^{-1} , which represents all proteins (Notarstefano et al., 2022). The significantly higher value found in the ODONTO/OSTEO samples may indicate the deposition of more mature dental minerals.

Mineral crystallinity, which reflects the structural order of the hydroxyapatite crystals, was assessed using the inverse of the Full Width at Half Maximum (FWHM) to evaluate the crystallinity of HA; narrower band widths are therefore correlated with higher mineral crystallinity, and viceversa (Anwar Alebrahim et al., 2014). As shown by the histograms in Figure 26B, the difference in crystallinity between CTR and ODONTO/OSTEO, although significant, is not as evident as that observed for other parameters studied. This difference is clearly attributable to the profoundly different shape and height of the examined peak in the two sets of spectra: the CTR peak is very low and therefore poorly comparable with the ODONTO/OSTEO peak.

The C/P ratio is calculated by dividing the intensity of the peak at 1070 or 1100 cm^{-1} (type B or A) by the intensity of the peak at 960 cm^{-1} , which is assigned to the carbonate and phosphate groups in HA, respectively. In the literature, an increase in this ratio (particularly in type B carbonates) is associated with demineralization, as observed in caries (Enrich-Essvein et al., 2021). However, in this case, the behaviour of the C/P ratio appears to differ significantly when considering type A and type B carbonate groups. Here, the results suggest that the only difference is in the crystal structure between CTR and ODONTO/OSTEO.

Furthermore, the successful differentiation of DPSC-derived organoids into the odontogenic/osteogenic lineage was confirmed through immunofluorescence analysis. CTR organoids showed high expression of the stem cell marker CD105 and low levels of the differentiation markers RUNX2 and OSX. In contrast to previous studies, ODONTO/OSTEO organoids showed reduced CD105 expression and increased RUNX2 and OSX expression. Despite image quality being partially limited by section thickness, microscope depth of field, and autofluorescence issues arising from the matrix, differences in marker expression were clearly visible (La Noce et al., 2014).

Despite these advances, the study encountered inherent limitations. The necrotic core observed in histological analysis (H&E) is a direct result of the absence of vascular structures, which restricts the diffusion of oxygen and nutrients in structures larger than 500 μm (Lancaster and Knoblich, 2014). To overcome the limitations regarding the necrotic core formation and the low throughput of manual production, future research should move toward microfluidic systems (Organ-on-a-Chip). Unlike static cultures, microfluidic platforms provide a continuous supply of oxygen and nutrients via perfusion, effectively mimicking the physiological blood flow. This dynamic environment has been shown to significantly reduce internal hypoxia in large 3D structures (Guo et al., 2018).

A further intrinsic limitation is the use of Matrigel as extracellular matrix. Although essential for organoid formation, it exhibits autofluorescence that compromises imaging, challenges in nucleic acid/protein extraction, undefined composition with batch-to-batch variability, and xeno-origin (murine sarcoma) incompatible with translational applications (Aisenbrey and Murphy, 2020; Kozlowski et al., 2021; Wolff and Hendrix, 2025). Optimized removal protocols partially mitigate these issues, underscoring the need for xeno-free or synthetic alternatives in future developments.

Another limitation of this project was the small number of samples examined. This is because producing a large number of organoids by hand is difficult. Conversely, standardizing organoid production using innovative automated approaches requires expensive equipment.

Furthermore, the relatively short culture time of 26 days could be a limitation. We propose overcoming this in future studies by culturing organoids for 1–2 months. However, this approach could exacerbate the limitations of stationary culture techniques, leading to necrotic cores as organoids grow over time.

Moreover, the number of osteogenic and stemness markers that were examined was restricted. In future studies, we intend to expand the range of markers used to include both early and late markers. This will improve our understanding of the temporal dynamics of osteogenic differentiation. This will be carried out in combination with Raman microspectroscopy.

In conclusion, while further studies with extended time windows and intermediate time intervals could be conducted to evaluate the potential to obtain mature dental or bone mineral material, these results, along with others, validate our differentiation protocol and highlight the potential of 3D organoid models for studying DPSC biology and cell line commitment.

6 Conclusion

Dental pulp stem cells (DPSCs) are multipotent mesenchymal cells characterized by high differentiation plasticity, accessibility, and regenerative capacity. These properties make them ideal candidates for generating organoids and developing three-dimensional experimental models of dental tissues.

This doctoral thesis concludes by validating a guided, simplified and reproducible protocol for producing and characterising 3D organoids from DPSCs. This protocol demonstrates the cells' ability to acquire an odonto/osteogenic phenotype in the presence of a specific differentiation medium. After 26 days of culture, the resulting structures (average diameter ~1.5 mm) exhibited significantly greater calcium phosphate deposition in the differentiated groups than in the controls. This was confirmed by Alizarin Red staining and Raman microspectroscopy, which revealed spectral profiles characteristic of biological hydroxyapatite (PO_4^{3-} bands at $960/1030\text{ cm}^{-1}$). Three-dimensional immunofluorescence further corroborated these findings, highlighting the upregulation of RUNX2 and OSX and the downregulation of CD105 in the differentiated organoids.

The integration of these advanced techniques overcame the limitations of conventional, destructive methods, such as RT-qPCR and Western blot, which are invalidated by the instability of housekeeping genes (e.g., GAPDH). It also addressed critical issues with Matrigel, including autofluorescence, which compromises imaging, and the difficulty of extracting nucleic acids and proteins. This was overcome with optimized removal protocols. Additionally, Matrigel has a xeno origin from murine sarcoma, which is incompatible with translational applications requiring xeno-free matrices.

Despite limitations in small sample size, manual production, a short culture duration, and a restricted marker panel, this study confirms the robustness of the protocol and the potential of DPSC-derived organoids as a biomimetic platform for studying cell biology, lineage commitment, and dental and bone regenerative strategies. Potential applications include the use of bioinks to standardise and automate processes, the use of xeno-free matrices and the use of endothelial co-cultures to create vascularized models, thus enhancing scalability and clinical relevance.

7 References

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